Signalling Pathways Activated by TRAIL: Implications for Apoptosis

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by

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Abstract

Death receptors are a subfamily of the TNF (tumour necrosis factor) receptor (TNFR) family. They are characterised by a death domain (DD) motif within their intracellular domain (ICD), which is required for the induction of apoptosis. Engagement by the cognate ligand results in activation of DED (Death Effector Domain)-containing initiator caspases through a series of adaptor proteins. TRAIL (TNF-Related Apoptosis-Inducing Ligand) and its receptors represent a relatively new and poorly characterised subset of the TNF family. The transcription factor NF-κB is an important cellular survival pathway and its potential activation by TRAIL was assessed. TRAIL activates NF-κB only in TRAIL resistant cells whereas activation was only observed in sensitive cells in the presence of a caspase inhibitor suggesting a caspase-sensitive component. NF-κB activation could regulate TRAIL cytotoxicity as its inhibition in resistant cells led to them becoming sensitive to TRAIL while activation in sensitive cells made these cells refractory to TRAIL. The native TRAIL DISC (Death-Inducing Signalling Complex) was characterised and found to contain FADD (Fas-Associated Death Domain Protein) and the initiator caspases-8 and -10. FADD- and caspase-8-deficient cells were refractory to TRAIL-induced apoptosis indicating an obligatory role for these components in TRAIL signalling. DISCs were formed irrespective of the TRAIL sensitivity of the cell. Consistent with TRAIL activating NF-κB, RIP (Receptor Interacting Protein), a protein required for TNF-mediated NF-κB activation, was present within the DISC. Analysis of the native TNF-R1 signalling complex revealed the presence of proteins required for NF-κB activation, RIP and TRAF2 (TNF-Associated Factor 2) however the apoptotic mediators, namely FADD and caspase-8 were absent despite being required for apoptosis induction. Finally, others have reported that protein kinase C (PKC) activation can protect cells from death receptor apoptosis. PMA (Phorbol Myristate Acetate)-mediated PKC activation protected HeLa cells from TRAIL-induced apoptosis. DISC analysis revealed that PKC activation modulated apoptosis by inhibiting FADD recruitment.

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Abbreviations

APAF-1	apoptotic protease activating-factor 1
ATP	adenosine triphosphate
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	di-methyl sulphoxide
ELISA	enzyme-linked immunosorbent assay
FADD	Fas-associated death domain-containing protein
FITC	fluorescein isothiocyanate
FLICE	FADD-like ICE
FLIP	FLICE-like inhibitory protein
IAP	inhibitor of apoptosis
ICE	interleukin-1-converting enzyme
IPTG	β-D-thiogalactopyranoside
PBS	phosphate-buffered saline
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
TBS	TRIS-buffered saline
TBST	TBS containing 0.1% Tween-20
TBSMT	TBST containing 5% (w/v) Marvel™
TEMED	N,N,N' ,N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
TRADD	TNF receptor associated protein with death domain
TRAF	TNF receptor associated factor
TRAIL	TNF-related apotosis-inducing ligand

CHAPTER 1: INTRODUCTION

1.1 Apoptosis

Apoptosis plays a critical role in both development and tissue homeostasis in metazoans. Cells undergoing apoptosis are subject to a series of distinct morphological changes including cell shrinkage, nuclear condensation, plasma membrane blebbing and DNA fragmentation (reviewed in (Green *et al.*, 2002)). At the biochemical level these changes are associated with cell shrinkage, activation of endonucleases that function to cleave genomic DNA, protein fragmentation, and translocation of phosphatidylserine to the outer leaflet of the plasma membrane. The end result is a careful, controlled dismantling of the cell which is packaged into "apoptotic bodies" which are then phagocytosed by neighbouring cells. Because the contents of the cell are not released into the extracellular milieu there is not the damaging inflammatory response that is associated with other uncontrolled forms of cell death such as necrosis.

Apoptosis plays a central role in development helping to sculpt the body, shape the organs and remove evolutionary vestiges such as the interdigital webs (Meier *et al.*, 2000). It plays a crucial role in tissue homeostasis and is responsible for the deletion of cells where these are overproduced. For example those that fail to make synaptic connections in the nervous system or immune cells that fail to produce productive antigen specificities(Yuan *et al.*, 2000). In the immune system apoptosis is also necessary for the removal of virus/pathogen-infected cells and termination of an immune response thereby maintaining immune homeostasis (Siegel *et al.*, 2000). Another critical role is removal of damaged or non-functional cells which could potentially cause harm and lead to tumorigenesis (Green & Evan. 2002).

Inappropriate apoptosis, either excessive or insufficient, is therefore implicated in many pathological processes including neurodegenerative diseases such as Alzheimer's and Huntingdon's disease, ischaemic damage, autoimmune disorders and the pathogenesis of many cancers.

1.2 Apoptosis in *Caenorhabditis elegans*

The unique nature of the cells during development of the nematode worm *C. Elegans* led to the identification of specific genes which were responsible for the regulation and execution of programmed cell death. Out of a total of 1090 somatic cells, 131 are known to undergo cell death during development. Two genes, *ced-3* and *ced-4*, were initially identified which were sufficient to trigger the death of target cells during development. Later a further two genes, *ced-9* and *egl-1*, provided the mechanism of

control of this process such that a simple model was proposed for the regulation and execution of cell death in those 131 cells (Figure 1.1). Mammalian orthologues of these cell death genes were subsequently identified (reviewed in (Meier *et al.*, 2000)).

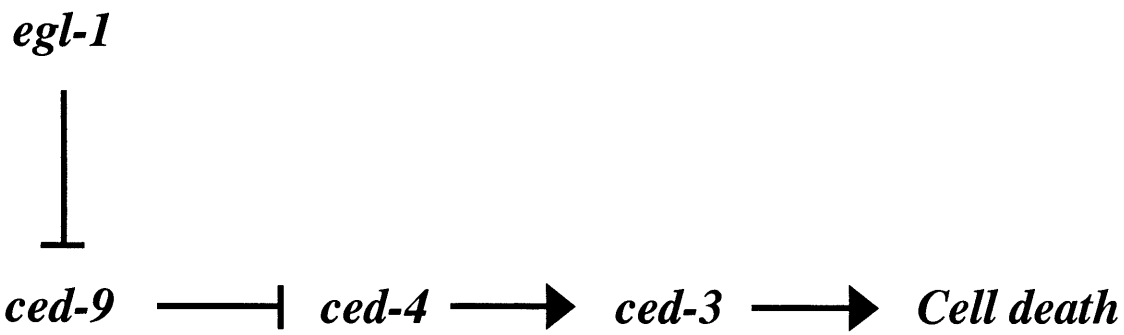


Figure 1.1 The apoptotic pathway in *C. elegans*.

ced-3 related genes were found to encode for a family of cysteine proteases with similarity to mammalian interleukin-1-convering enzyme (ICE). This family would later be renamed the 'caspases' (section 1.3.1). *ced-4* binds to and promotes the proteolytic activation of *ced-3*. The human *ced-4* homolog, APAF-1 (Apoptotic Protease Activating Factor-1), was identified biochemically as it shares limited homology to *ced-4* itself (Zou *et al.*, 1997). The fact that some APAF-$1^{-/-}$ mice survive to adulthood without any apparent developmental defects together with experiments in cells derived from APAF-1 null mice and in transgenics have suggested that APAF-1 may not be the true/only *ced-4* homolog in the mammalian system (Marsden *et al.*, 2002). *ced-9* mutation was found to lead to massive ectopic apoptosis leading to developmental cell death and thereby implicating it as a survival gene. *ced-9* was subsequently found to act upstream of *ced-3* and *ced-4* acting to inhibit cell death and represented the mammalian Bcl-2 family (reviewed in (Meier *et al.*, 2000)). The relatively large numbers of mammalian Bcl-2 homologs probably reflects the difference in complexity between the worm and mammalian systems. In this respect they also encode for the mammalian orthologs of *egl-1* which act upstream of *ced-9* to promote cell death and represent proapoptotic members of the Bcl-2 family such as Bax and Bid (section 1.5).

The complexity and importance of the apoptotic process obviously increases with the complexity of the organism. Apoptosis-deficient worms are, in general, developmentally and functionally normal and only differ from normal worms by the fact

that they contain the whole complement (1090) of somatic cells as none die during development. In contrast, mice which have been genetically engineered to be deficient in worm orthologues of *ced-3* (e.g. caspases-3 and -9), *ced-4* (APAf-1) and *egl-1* (e.g. Bax and Bid) display severe developmental defects and are embryonically lethal (Horvitz. 1999).

1.3 Apoptosis in the mammalian system

Apoptotic cells exhibit a number of key morphological changes regardless of the initiating stimulus (section 1.1). In mammals there are two pathways for initiation of apoptosis termed the 'intrinsic' and the 'extrinsic' pathways. DNA-damaging agents, kinase inhibitors and ionising radiation all induce cell death through the intrinsic apoptosis pathway. The cellular sensors responsible for reacting to these chemical insults are not well characterised but all these signals appear to converge at the mitochondria (section 1.4). The extrinsic pathway is activated by members of the TNF-R (Tumour Necrosis Factor-Receptor) family - the death receptors. Ligation of a death receptor by its cognate ligand leads to caspase activation that occurs at the intracellular domain of the receptor (section 1.6). The protein family that is central to both of these pathways comprises of the human *ced-3* homologs - the caspases.

1.3.1 Caspases

Caspases are the mammalian orthologues of the *C. elegans* death protein *ced-3*. The mammalian interleukin-1β-converting enzyme (ICE) was the first *ced-3* homolog to be identified and to date there are currently 14 mammalian caspases. They are synthesised as inactive precursors (zymogens) containing an N-terminal prodomain, a large subunit which contains the active site cysteine contained within in a conserved QACXG active site motif (X can be G, Q or R) and a C-terminal small subunit (reviewed in (Cohen. 1997)). Caspases have a distinct substrate specificity which is determined by the four residues amino-terminal to the cleavage site (P_1-P_4). They all share an absolute requirement for aspartic acid (Asp) in the P_1 position but the P_2-P_4 residues differ and provide distinct substrate specificities (Thornberry *et al.*, 1997). The structure of several caspases has been determined by X-ray crystallography and has revealed that the mature caspase is a heterotetramer consisting of two large/small (p20/p10) subunit heterodimers (reviewed in (Earnshaw *et al.*, 1999)). Caspase zymogen activation requires a minimum of two cleavages all of which involve Asp-X bonds. The first cleavage is between the small (p10)

and large (p20) subunits followed by a second cleavage between the p20 subunit and the prodomain. The finding that all of these cleavage sites appeared to follow an aspartic acid residue suggested that caspases may be capable of processing themselves and/or other caspase zymogens to generate mature enzymes.

The caspases can be split into two groups based on their physiological functions. The ICE-related caspases (-1, -4, -5, -11, -12, and -13) are now considered to function as mediators of inflammation and cytokine maturation (Martinon *et al.*, 2002). The remaining caspases function as true *ced-3* homologs and are involved in mediating the excecution phase of apoptosis. Active caspases cleave critical cellular proteins leading to the organised dismantling of the cell. Caspase cleavage of a protein substrate does not result in wholesale degradation of the target protein but is usually restricted to one or two cleavages which usually functions to inactivate, activate or compromise the substrate (reviewed in (Earnshaw *et al.*, 1999; Nicholson. 1999)). Caspases involved in apoptosis can therefore be further subdivided into those involved in the initiation of apoptosis - the 'initiator' caspases and those involved in the execution/demolition phase - the 'executioner/effector' caspases.

The initiator caspases (-2, -8, -9 and -10) are responsible for processing and activation of effector caspases. They contain long prodomains that interact with adaptor proteins and larger protein complexes whose function is to regulate their activation. Studies in cell-free systems have demonstrated that initiator caspases are capable of efficient activation of other caspases. Caspase-8 can cleave and activate procaspases -3, -7 and -9 *in vitro*, caspase-10 can cleave procaspases -3, -7 and -8 while caspase-9 can lead to activation of procaspases -3, -6 and -7. This suggested that limited activation of initiator caspases would result in a cascade of effector caspase activation.

1.3.2 Caspase activation

Three basic mechanisms for caspase activation have so far been described. The first is activation through cleavage by another caspase. As mentioned above, the finding that cleavage between a the prodomain and large and small subunit appeared to occur at a candidate caspase substrate site, Asp-X, suggested that caspases they would be capable of autocatalytic activation. The second mechanism of activation is known as the 'induced proximity' model (section 1.6.4). Some caspase zymogens contain low intrinsic proteolytic activity. However, when brought into close proximity, for example by oligomerisation on a protein scaffold, then it is purported that this low activity is sufficient

to allow the caspase zymogens to process and activate each other. The third mechanism appears to be that used by caspase-9. Unlike other caspase zymogens, processing of procaspase-9 results in an enzyme with very low activity. Instead caspase-9 requires a protein co-factor known as APAF-1 to attain full activity. APAF-1 is not therefore just seen as a caspase-9 activator but also as a regulator of caspase-9 activity, and is a key subunit of the APAF-1/caspase-9 holoenzyme complex (Rodriguez *et al.*, 1999).

1.3.3 Regulation of caspase activation

As the consequences of accidental caspase activation is potentially devastating, initiator caspase and effector caspase activation is tightly regulated. Activation of caspases-8 and -10 at the DISC (Death-Inducing Signalling Complex) by death receptor ligation can be inhibited by recruitment of the caspase-8 homolog, c-FLIP (section 1.6.5). Similarly, mitochondrial release of cytochrome *c* which is essential for caspase-9 activation through the APAF-1/caspase-9 holoenzyme pathway is regulated by Bcl-2 family members (section 1.5). Caspase activation and effector caspase activity can also be regulated by interaction with the inhibitor of apoptosis (IAP) proteins. These proteins were first identified in baculoviruses and contain conserved tandem repeat of ~70 amino acids termed baculovirus repeats (BIR domains). XIAP (X-Linked IAP), ML-IAP (MeLanoma IAP) and c-IAP-1 and -2 (cellular-IAP) are members that function by directly interacting and inhibiting active effector caspases (reviewed in (Salvesen *et al.*, 2002)). The presence of a ubiquitin ligase domain in most IAPs suggests that they may function to inhibit and mediate the removal of active caspases via the ubiquitin-proteasome system (Yang *et al.*, 2000). IAP inhibition of caspases can be antagonised by a number of recently described IAP antagonists. These proteins are not members of a specific family but contain a conserved motif of 5 amino acids which functions to disrupt IAP-caspase interactions. Two of the most recently described are SMAC (Second Mitochondrial Activator of Caspases) and Htr2A/Omi both of which are mitochondrial proteins that are released into the cytoplasm of a cell under an apoptotic stimulus (Hegde *et al.*, ; Martins *et al.*, 2002).

1.3.4 Role of caspases in apoptosis

Caspases are responsible for all the stereotypical morphological changes during apoptosis and the gradual discovery of caspase substrates has led to some insight into how these changes can occur. In most instances, the executioner/effector caspases (-3 -6 and -7) are responsible for cleavage of these substrates. For example, caspase-3-mediated cleavage of

ICAD (Inhibitor of Caspase-Activated DNase) prompts the release of CAD (Caspase-Activated DNase) which is then able to cleave DNA, leading to chromatin condensation and one of the classical hallmarks of apoptosis. Nuclear shrinkage and dissolution may also be a result of cleavage of nuclear structural proteins such as members of the lamin family (Takahashi *et al.*, 1996). Cleavage of the cells major structural proteins such as actin, fodrin and members of the keratin family may compromise cytoskeletal integrity, disrupt cell to cell contacts, contribute to shrinkage of the cell and ultimately facilitate packaging of cellular components (reviewed in (Wolf *et al.*, 1999)). As well as disrupting vital cellular processes caspases can also act to promote apoptosis by cleaving anti-apoptotic proteins possibly enhancing apoptotic signalling. Caspase-3-mediated cleavage of Bcl-2 and Bcl-$_{X}$L abrogates their antiapoptotic function and results in the generation of pro-apoptotic C-terminal fragments. Cleavage of the endogenous inhibitors of caspases, the IAPs also leads to their inactivation and results in the generation of pro-apoptotic fragments (Clem *et al.*, 2001). Importantly, both extrinsic and intrinsic pathways can ultimately converge through caspase-8-mediated cleavage of the proapoptotic Bcl-2 family member, Bid (section 1.5.1).

1.4 Mitochondria: central to the intrinsic apoptosis pathway

The intrinsic apoptosis pathway is activated by stimuli such as chemical insults, DNA damage, kinase inhibitors and ionising radiation. The intrinsic sensors for damage caused by these agents are not completely understood but members of the proapoptotic BH3-domain containing protein such as Bid and Bim have been implicated (section 1.5.1). Ultimately these proapoptotic signals converge at the mitochondria and generally result in the rupture of the outer mitochondrial membrane thereby leading to release of cytochrome *c* into the cytoplasm (Fig. 1.2) Binding of cytochrome *c* together with dATP (or ATP) to APAF-1 causes a controlled oligomerisation and the formation of a large (~700 kD) caspase-activating complex known as the 'apoptosome' (Cain *et al.*, 1999; Zou *et al.*, 1999). The apoptosome than acts as a platform allowing the autocatalytic activation of the initiator caspase-9 which in turn recruits and activates the effector caspases-3 and -7. As the release of cytochrome *c* from the mitochondria is such an important step during apoptosis it is tightly regulated by anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-$_{XL}$ (section 1.5). The apoptosome can also be activated by cytochrome *c* release mediated via the extrinsic-death receptor pathway acting on the proapoptotic Bcl-2 homolog, Bid (section 1.5.1).

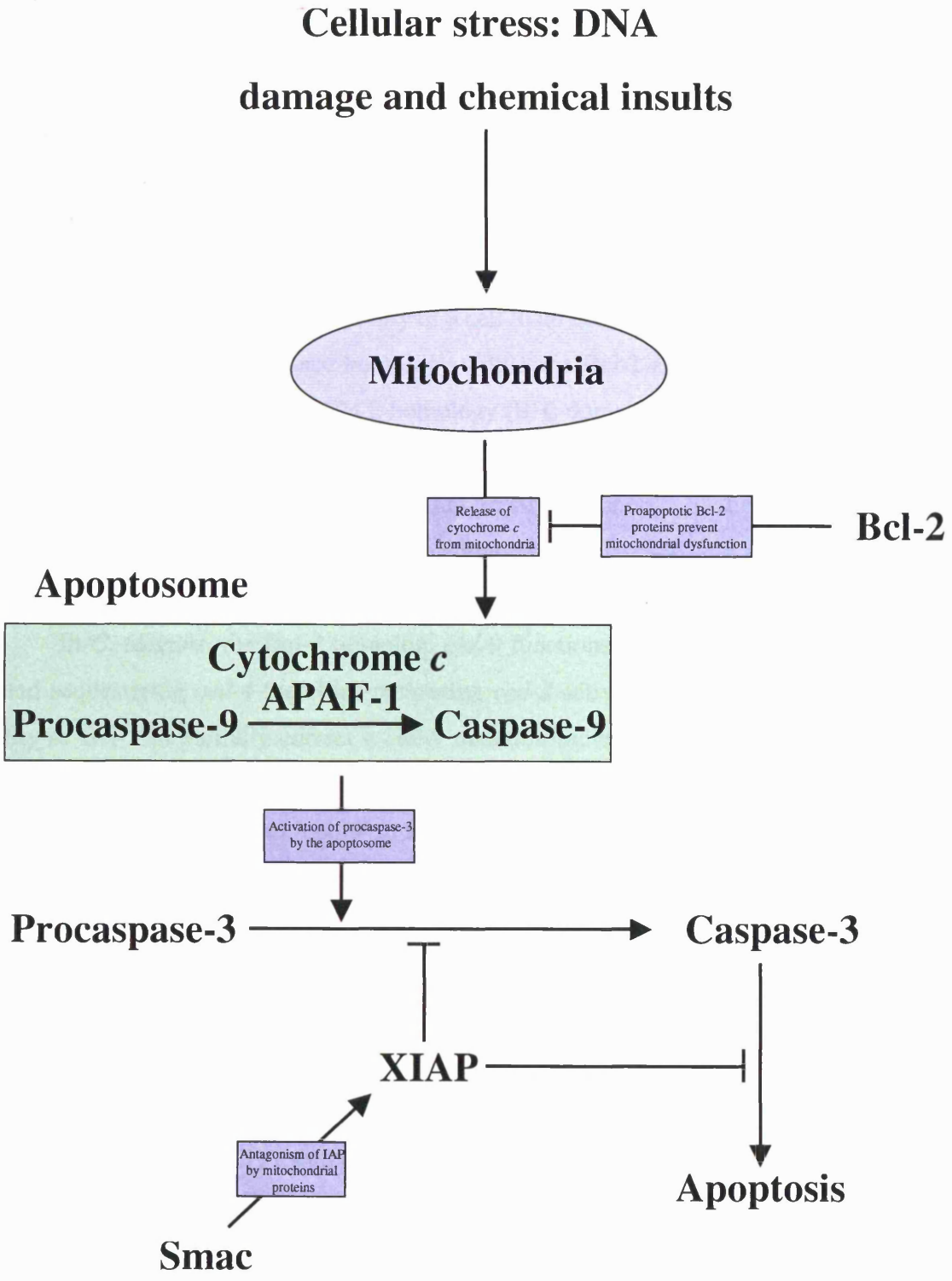


Figure 1.2 The intrinsic apoptosis pathway

(see text for details)

1.5 The Bcl-2 family

Bcl-2 was first identified as a proto-oncogene in follicular cell B-lymphoma at the chromosomal breakpoint between chromosome 18 and chromosome 14 (Tsujimoto *et al.*, 1985). The product of the Bcl-2 gene was subsequently found to promote resistance to apoptosis in lymphocytes (Vaux *et al.*, 1988). The Bcl-2 family can be subdivided into three groups; the antiapoptotic Bcl-2 subfamily (Bcl-2, Bcl-$_{XL}$, Bcl-$_{W}$, A1 and Mcl-1), and the proapoptotic Bax (Bax, Bak, Bok) and BH3 domain-containing (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma) subfamilies. The ratio between the pro and anti apoptotic groups may, in part, determine the susceptibility of a cell to an apoptotic stimulus.

The amino acid sequence homology within the Bcl-2 family is low and is mainly confined to four regions termed Bcl-2 homology (BH) domains. These domains have been shown to mediate protein-protein interactions and Bcl-2 family members act by either forming homo- or hetero- dimers with other family members. In this way proapoptotic family members can bind to and inactivate antiapoptotic members and vice-versa (Gross *et al.*, 1999).

In *C. elegans*, the Bcl-2 homolog, *ced-9* functions to inhibit apoptosis by binding to and sequestering *ced-4* therefore preventing *ced-3* activation (Vaux *et al.*, 1992). The ability of Bcl-2 to partially correct a *ced-9* mutation indicated that it too may function in mammalian cells to sequester the *ced-4* homolog APAF-1, thereby preventing activation of procaspase-9. Despite some reports to the contrary, it is now accepted that APAF-1 does not bind any Bcl-2 family members which must instead act to inhibit apoptosis elsewhere (Moriishi *et al.*, 1999). The structural similarity of some Bcl-2 family members to the pore-forming unit of diptheria toxin has led to the suggestion that they may function as pore forming agents within the outer mitochondrial membrane (Muchmore *et al.*, 1996). Upon mitochondrial binding of pro-apoptotic Bcl-2 family members such as Bax which allows the passage of cytochrome *c* and other apoptotic mediators (reviewed in (Zamzami *et al.*, 2001)). Bcl-2 family members therefore function to promote or prevent apoptosis by regulating the mitochondrial driven APAF-1/cytochrome *c*/caspase-9 activation pathway.

The anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-$_{X}$L do not block translocation of the BH3 domain-containing pro-apoptotic molecules to the mitochondria. Rather, they function by binding to Bax and/or Bak to inhibit their oligomerisation which ultimately leads to perturbation of the mitochondrial membrane (Antonsson *et al.*, 2001; Michhailov *et al.*, 2001; Nechushtan *et al.*, 2001).

1.5.1 The BH3-containing subfamily: the cellular sensors of cell death

The BH3 family or proteins appear to act as intracellular sensors which are capable of inducing apoptosis in response to developmental cues or intracellular damage (Huang *et al.*, 2000). In *C. elegans* the developmentally-driven death of the 131 somatic cells is regulated by the BH3-domain containing Bcl-2 family member, *egl-1*. In contrast, mammalian cells contain eight members of this subfamily and together they may provide more of a fine control over cell death by acting as sensors for different cellular organelles or for a wide variety of insults (Gross *et al.*, 1999; Huang & Strasser. 2000). BH3 proteins are usually sequestered within different cellular compartments and translocate to the mitochondria or interact with other Bcl-2 family members in response to an apoptotic stimulus. Sequestration and/or modification are generally the mechanisms that keep these proteins, including Bim and Bmf in check. (Puthalakath *et al.*, 2001; Puthalakath *et al.*, 1999). Bid, in contrast, is regulated by proteolytic cleavage. Triggering of death receptors and activation of caspase-8 leads to cleavage of Bid (Li *et al.*, 1998; Luo *et al.*, 1998). Truncated Bid (tBid) then translocates to the mitochondria, a process which is aided by myristoylation of the N-terminal glycine of tBid (Zha *et al.*, 2000). Bid can be processed by a number of other proteases including caspase-3, granzyme B and lysosomal proteases such as the cathepsins (Heibein *et al.*, 2000; Stoka *et al.*, 2001; Sutton *et al.*, 2000). It may thus play a role in other cell death initiation pathways apart from those induced by death receptor ligation. Processing of Bid by caspase-3 may also act as an amplifier of the initial apoptotic signal. These BH3 proteins could function to promote apoptosis by two mechanisms, the first by binding to and inactivating anti-apoptotic Bcl-2 family members and the second by binding to proapoptotic members such as Bax or Bak. However, the finding that they cannot kill in the absence of Bax and Bak suggests that their proapoptotic activity lies upstream of these components but within the same pathway (Wei *et al.*, 2001; Zhong *et al.*, 2001).

1.6 Apoptosis induced by death receptor ligation: the extrinsic apoptosis pathway

The extrinsic apoptosis pathway is initiated by members of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. Cytotoxic members of this family such as TNF-R1, CD95 (Fas), DR3 (APO3/TRAMP, LARD, wsl-1), TRAIL-R1 and TRAIL-R2 (section 1.9) are characterised by the presence of a death domain (DD) motif within their intracellular domains. The death domain (DD) is a region of ~80 amino acids that was originally identified in the intracellular domain of TNF-R1 and was

demonstrated to contain residues critical for TNF-mediated cytotoxicity (Tartaglia *et al.*, 1993b). The DD enables death receptors to engage the cell's apoptotic machinery. A model for apoptosis induction by death receptors is shown in figure 1.3.

1.6.1 The death-inducing signalling complex (DISC)

Perhaps the most well characterised death receptor pathway is that mediated by CD95/Fas. Initial studies observed that ligation of CD95 with an anti-CD95 agonistic antibody led to the recruitment of a number of proteins to the intracellular death domain. These proteins were initially designated cytotoxicity-dependent APO-1-associated proteins (CAP1-4) and were shown to be in a complex associated with CD95. These components were subsequently identified by 2-dimensional gel electrophoresis and mass-spectrometry. CAP-1 and CAP-2 were found to be differentially serine-phosphorylated forms of the same protein which was subsequently named FADD (Fas-Associated Death Domain containing protein)/MORT1 (Mediator Of Receptor-induced Toxicity 1) (Boldin *et al.*, 1996b; Chinnaiyan *et al.*, 1995). CAP-4 was identified as a member of the member of the caspase family and was initially termed FLICE (FADD Like ICE)/MACH (Mort 1 Associated CED-3 Homolog), but has subsequently been renamed caspase-8 (Boldin *et al.*, 1996a; Muzio *et al.*, 1996). CAP-3 has proven difficult to obtain sufficient quantities to identify however, a recent report has suggested that it bears homology to the prodomain of caspase-8 (Peter *et al.*, 2003a). This complex of proteins was collectively termed the 'death-inducing signalling complex' or DISC (Kischkel *et al.*, 1995).

1.6.2 Adaptor proteins: mediators of cell death

FADD is a bipartite molecule containing a C-terminal DD motif and an N-terminal motif termed the death-effector domain (DED). It functions as a molecular bridge between the DD of the respective receptor and the prodomain of the initiator caspase(s) involved in death receptor signalling, namely caspases-8 and -10. FADD mutants which blocked CD95-mediated apoptosis also inhibited TNF-mediated apoptosis suggesting that these death pathways converged at FADD. Later, gene knockout studies demonstrated that FADD is absolutely required for CD95 and TNF-mediated apoptosis. The phenotype of the $FADD^{-/-}$ mice also revealed a deficiency in T cell proliferation indicating that FADD may also play a role in T cell activation (Walsh *et al.*, 1998; Zhang *et al.*, 1998). While FADD directly interacts with the DD of CD95 its interaction with TNF-R1 is indirect and requires another adaptor protein TRADD (TNF Receptor Associated Death Domain

protein). TNF-R1 signalling appears to diverge at TRADD which acts as a platform for recruitment of other TNF intermediates which are responsible for mediating the different TNF signalling pathways such as NF-κB or activation of c-Jun N-terminal Kinase (JNK) (sections 1.8.1 and 1.8.4).

1.6.3 Initiator caspases involved in death receptor signalling

Caspase-8 was identified by several groups as part of the CD95 DISC, by EST database searching and by its interactions with FADD (Boldin *et al.*, 1996a; Muzio *et al.*, 1996; Srinivasula *et al.*, 1996). Procaspase-8 contains a long prodomain containing tandem DED repeats which mediate binding to FADD. Two splice-variants of 55 and 53 kD exist and the only difference between the two forms is a section of amino acids within the prodomain adjacent to the two DEDs (Medema *et al.*, 1997). The reason for the presence of two isoforms is unknown as they are both equally recruited and processed. It has also very recently been suggested that a third smaller caspase-8 splice variant may also exist (Peter *et al.*, 2003b). Cells derived from caspase-$8^{-/-}$ mice are refractory to CD95 and TNF-mediated apoptosis suggesting that it plays an obligatory role in death receptor-mediated apoptosis (Varfolomeev *et al.*, 1998). Caspase-10 is the only other caspase that contain DED repeats within its prodomain and was identified by searching EST databases using the DEDs of procaspase-8 (Fernandes-Alnemri *et al.*, 1996; Vincenz *et al.*, 1997). Like caspase-8, two splice forms of 56 and 54 kD exist and although not initially identified as a DISC component it has subsequently been demonstrated that caspase-10 is recruited to CD95 (Kischkel *et al.*, 2001; Sprick *et al.*, 2002; Wang *et al.*, 2001b). Whether it can functionally substitute for caspase-8 during death receptor apoptosis is unclear. The lack of a caspase-10 homolog in the mouse complicates the identification of its potential role although it may have different substrates to caspase-8 (Wang *et al.*, 2001b). The only other initiator caspase that has been proposed to play a role in death receptor signalling is caspase-2. It has been reported that caspase-2 can be recruited to TNF-R1 through the TNF signalling intermediate RIP (Receptor Interacting Protein), a protein involved in TNF-mediated NF-κB activation (section 1.8.2). RIP has been purported to bind another adaptor protein RAIDD (RIP-Associated ICH-1 Death Domain protein) which has an N-terminus that bears similarities to the prodomain of caspase-2 (Duan *et al.*, 1997). It was therefore suggested that there may be a TNF-R1-RIP-caspase-2 apoptosis activation pathway. However, as CD95 and TNF-mediated apoptosis are unaffected in caspase-$2^{-/-}$ cells the existence of this pathway is now questionable (Bergeron *et al.*, 1998).

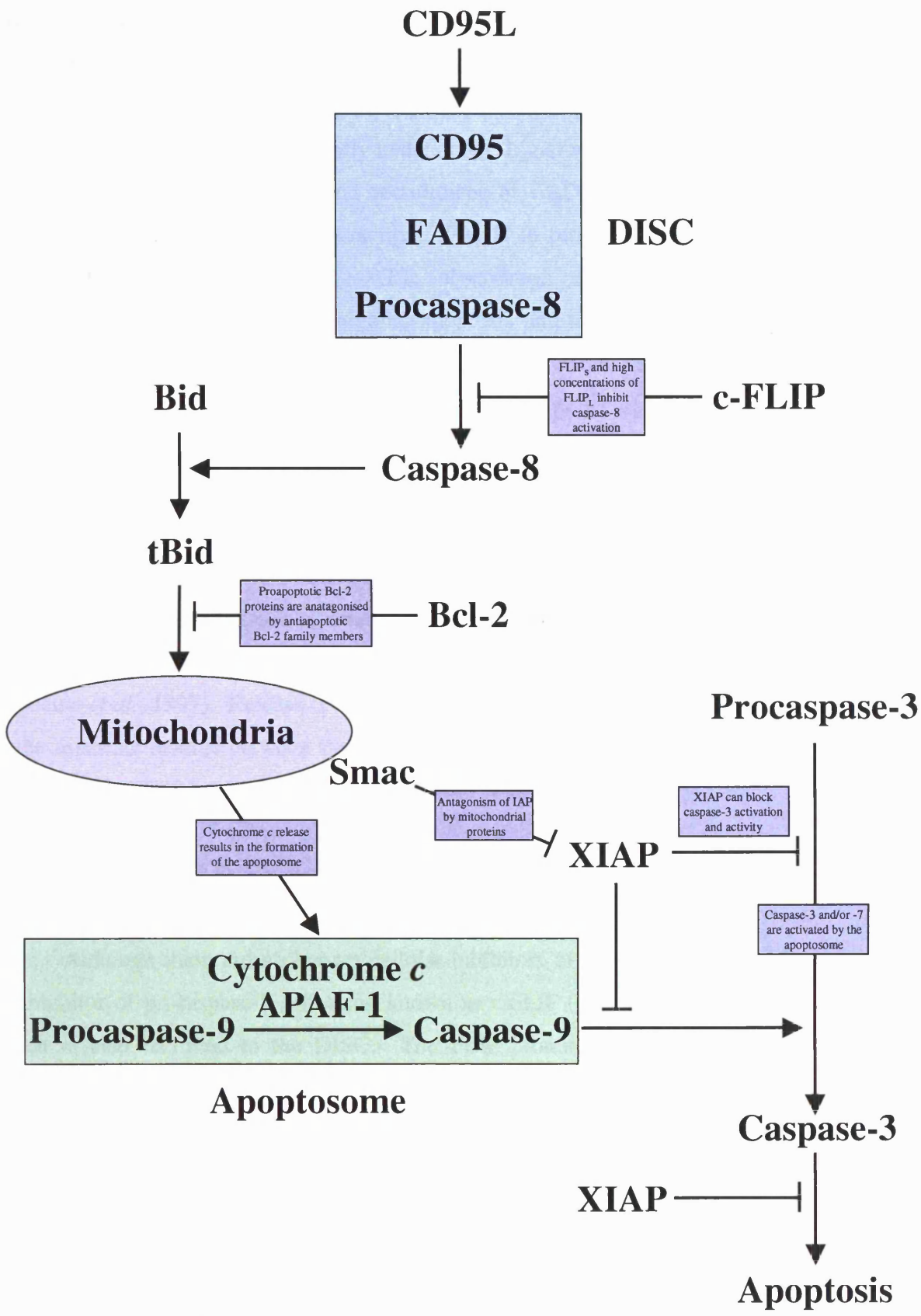


Figure 1.3 The extrinsic apoptosis pathway: induction of cell death by ligation of CD95

(see text for details)

1.6.4 Death receptor ligation: activation of procaspase-8 at the DISC

Although many of the main molecules involved in death receptor-mediated apoptosis were discovered some years ago the mechanism and regulation of caspase-8 activation within the DISC was poorly understood. Ligation of CD95 by CD95L results in oligomerisation of the receptor and recruitment of FADD to the DD of the receptor through a homophillic DD-DD interaction. FADD in turn recruits procaspase-8 (or-10) through a DED-DED interaction. The observation that enforced dimerisation of procaspase-8 resulted in cells undergoing apoptosis and that initiator caspase zymogens (such as caspases-8 and -10) contained low but detectable protease activity led to a rationale for initiator procaspase activation termed the "induced-proximity" model (Muzio *et al.*, 1998). This model suggests that when two initiator procaspases are brought into close proximity in a caspase-activating complex such as the DISC the low activity in the respective zymogens is sufficient to activate the other procaspase. Therefore, recruitment of procaspase-8 (-10) to the DISC results in its autocatalytic activation which proceeds in two steps. After removal of the small (p10) subunit, the large subunit (p18) is separated from the prodomain and the active heterotetramer $p18_2p10_2$ is released from the DISC (Medema *et al.*, 1997). Release is apparently required for apoptosis to proceed as mutation of the aspartate residue between the p18 subunit and the prodomain results in cells which are resistant to CD95-mediated apoptosis (Martin *et al.*, 1997).

1.6.5 Modulation of Death Receptor-Mediated Apoptosis by FLICE-Like Inhibitory Proteins (FLIPs)

Although there are no known cellular inhibitors of caspase-8 activity, there exists an inhibitor of procaspase-8 activation known as c-FLIP (FLICE-Like Inhibitory Protein) which is also recruited to the DISC. The FLIP proteins were identified by database searching using the DED motifs of caspase-8. Initially they were identified in the genome of gamma herpesviruses which led to the term v-FLIP (viral-FLIP). v-FLIPs, like caspase-8, contained two DEDs and were found to inhibit death receptor-mediated apoptosis (Thome *et al.*, 1997). Human cellular homologs of v-FLIP were termed cellular-FLIPs (c-FLIP) and were identified by a large number of groups (also termed Casper, MRIT, CLARP, usurpin, FLAME-1, CASH and I-FLICE) (Goltsev *et al.*, 1997; Hu *et al.*, 1997; Inohara *et al.*, 1997; Irmler *et al.*, 1997; Rasper *et al.*, 1998; Shu *et al.*, 1997; Srinivasula *et al.*, 1997). Multiple splice variants of c-FLIP were reported to exist but only two, $c\text{-}FLIP_L$ and $c\text{-}FLIP_S$, have been demonstrated to be translated. $c\text{-}FLIP_L$ resembles full length

caspase-8, and contains tandem DEDs and a caspase-like domain, however it lacks several conserved active site residues most notably the critical catalytic cysteine residue. c-$FLIP_S$ most closely resembles v-FLIP and consists of only the tandem DED motif but there is also a small C-terminal extension which is not present in v-FLIP or c-$FLIP_L$. The absence of residues critical for catalytic activity initially suggested that both c-$FLIP_L$ and c-$FLIP_S$ may act as a dominant-negative inhibitors of caspase-8 activation. It has recently been demonstrated that both FLIP isoforms in fact block procaspase-8 activation at the CD95 DISC but at different stages. c-$FLIP_L$ is recruited and processed by caspase-8 at the DISC resulting in the removal of the p12 subunit, in a manner similar to the initial activation of procaspase-8 itself. In the presence of c-$FLIP_L$ procaspase-8 is also subjected to this initial processing to the p43/p41 intermediates but no further processing occurs. In contrast, c-$FLIP_S$ recruitment results in no processing of procaspase-8 at the CD95 DISC (Krueger *et al.*, 2001).

Rasper and colleagues observed that levels of FLIP expression were reduced in infarcted cardiac tissue, with cardiac myocytes undergoing apoptosis upon ischemia and reperfusion being almost devoid of c-FLIP expression (Rasper *et al.*, 1998). Consistent with this, mice deficient in c-FLIP were found to be embryonically lethal and died most likely because of cardiac defects (Yeh *et al.*, 2000). These data clearly provided a role for c-FLIP in cardiac development. It was noticed at this time that the cardiac phenotype of the c-$FLIP^{-/-}$ mice bore a strong resemblance to that of the caspase-$8^{-/-}$ and $FADD^{-/-}$ mice. This suggested that c-FLIP, caspase-8 and FADD all play a crucial role in cardiac development although the exact signalling pathway(s) involved in cardiac myocytes is unknown (Varfolomeev *et al.*, 1998; Yeh *et al.*, 2000; Yeh *et al.*, 1998). Another physiological role of FLIP is during activation-induced cell death (AICD) in primary T cells. Activation of T cells results in downregulation of c-$FLIP_L$ and upregulation of CD95L leading to autocrine suicide (Irmler *et al.*, 1997). AICD can also been demonstrated to be enhanced by IL-2 due to upregulation of CD95L and downregulation of c-$FLIP_L$ (Refaeli *et al.*, 1996). c-$FLIP_S$ has also been demonstrated to play a role in the resistance of T cells to restimulation following triggering of the T cell receptor (TCR) (Kirchoff *et al.*, 2000).

1.6.6 c-FLIP$_L$ as an activator of procaspase-8

There is now growing evidence to suggest that c-FLIP$_L$ may actually function to facilitate procaspase-8 activation at the DISC when c-FLIP$_L$ recruited in small amounts. Some of the original studies describing the identification of c-FLIP observed that transient overexpression of c-FLIP$_L$ actually induced apoptosis (Goltsev *et al.*, 1997; Han *et al.*, 1997; Inohara *et al.*, 1997; Shu *et al.*, 1997). The reason behind this was not initially clear but more recently has led to the proposal that c-FLIP$_L$ may have a trifunctional effect on death receptor-mediated procaspase-8 activation. The induced proximity model proposes that caspase-8 is activated as a dimer at the DISC. Activation occurs in two steps: the first, removal of the small subunit is autocatalytic, and the second, separation of the prodomain and large subunit is transcatalytic. While the latter requires two catalytically active caspase-8 molecules as part of a dimer to process each other, the first step is only proposed to require one catalytically active procaspase-8 molecule. The other can instead be a c-FLIP$_L$ molecule which provides a caspase domain to achieve the correct active site conformation of the procaspase-8 molecule. c-FLIP$_S$, by contrast lacks the caspase domain and has only been demonstrated to be capable of inhibiting procaspase-8 activation. Therefore at low concentrations/levels of recruitment, c-FLIP$_L$ acts as an activator of procaspase-8 and thereby promotes apoptosis. It's second effect is as a dominant-negative inhibitor of procaspase-8 activation at the DISC thereby inhibiting full maturation and release of caspase-8. The third effect is observed when c-FLIP$_L$ is expressed at a high level and leads to the induction of apoptosis.

1.7 Inhibition of death receptor signalling through activation of protein kinase C (PKC)

Another pathway that has been implicated in providing protection from death receptor signalling is that mediated by activation of members of the protein kinase C (PKC) family of serine/threonine protein kinases. PKCs are central regulators of cell growth, transformation and cell death. PKC is a multigene family consisting of at least twelve distinct isoforms. The "conventional" PKCs (cPKC), α, β and γ, need calcium for maximal activity, the "novel" PKCs (nPKC), δ, ε and θ, are calcium-independent and the "atypical" PKCs (aPKC), ξ, ι and λ, in contrast to the cPKCs and nPKCs are not activated by phorbol esters such as PMA or diacylglycerols (DAG). Receptor-ligand interactions at the cell surface can lead to the activation of phospholipases which in turn catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate. This leads to the production of the

secondary messengers diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP3). IP3 mobilizes Ca^{2+} from intracellular stores, while DAG activates PKC by enhancing its affinity for Ca^{2+}.

Many years ago it was discovered that plant-derived tumour-promoting phorbol esters such as PMA (phorbol-myristate acetate) could substitute for DAG and directly activate DAG-sensitive PKCs, cPKC and nPKC (Castagna *et al.*, 1982). Phorbol esters have been observed to inhibit apoptosis induced by ligation of all death receptors. This in itself is unsurprising as PKC activation is a major promoter of cellular survival and can influence also the survival of tumour cells treated with chemotherapeutic agents. A number of studies have investigated the mechanism of inhibition of death receptor-induced apoptosis and, although exact mechanisms of inhibition are unknown, several potential targets have been suggested.

It is well documented that PKCs can activate the MAPK (Mitogen Activated Protein Kinase) pathway and a number of studies reports demonstrated that MAPK kinase inhibitors abrogate phorbol ester-mediated protection of CD95-mediated apoptosis (Holmstrom *et al.*, 2000; Holmstrom *et al.*, 1999; Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001). There is also evidence that PKC may protect against receptor, specifically CD95-mediated apoptosis by disrupting receptor aggregation at the cell membrane (Ruiz-Ruiz *et al.*, 1999). One possible explanation for this inhibition is provided by the effects of PKC on cytoskeletal rearrangements. CD95 has been demonstrated to interact with ezrin, an actin binding protein (Parlato *et al.*, 2000) and PKC activation has in turn been demonstrated to induce reorganisation of actin filaments through inhibition of an actin cross-linking protein, MARCKS, which is directly regulated by PKC (Hartwig *et al.*, 1991). Therefore, one potential explanation for the protective effect of PKC on CD95-mediated apoptosis is via reduced receptor mobility and aggregation mediated by the action of PKC on the actin cytoskeleton. Whether aggregation of other death receptors is similarly affected by PKC activation is currently unknown.

A critical role for PKC in the regulation of death receptor-mediated apoptosis has also been demonstrated using PKC inhibitors such as the bisindolylmaleimides. These staurosporine homologs have been demonstrated more potently inhibit cPKC isoforms (reviewed in (Gescher. 2000)). In certain cells types, these agents can sensitise cells to CD95-mediated apoptosis or abrogate the protective effect if PMA (Gomez-Angelats *et al.*, 2000). It has been suggested that PKC inhibition by certain bisindolylmaleimides leads to downregulation of c-FLIP and that this is therefore a potential mechanism whereby these

agents may sensitise some cells to death receptor stimuli (Willems *et al.*, 2000; Zhou *et al.*, 1999).

1.8 Other signalling pathways activated by TNF family members

As well as activating caspases, members of the death receptor family have been demonstrated to be capable of activating a number of other cellular signalling pathways (Fig. 1.4). Two of the most important appear to be activation of the transcription factor, NF-κB which plays an important role in promoting cellular survival, and activation of the stress activated protein kinase (SAPK)/c-Jun N-terminal Kinase (JNK) pathway. Inhibition of these pathways has in many cases been demonstrated to modulate death receptor signalling resulting in either enhancement or abrogation of death receptor signalling.

1.8.1 Activation of the transcription factor NF-κB

The transcription factor NF-κB plays a crucial role in the activation and regulation of the adaptive and innate immune response (Ghosh *et al.*, 2002). The NF-κB signalling pathway is mediated by members of the Rel family of transcription factors. Mammals express five Rel proteins which can be split into two groups. The first includes RelA (p65), RelB and c-Rel, which are synthesised as mature products. The second consists of NF-κB1 (p105) and NF-κB2 (p100), which are proteolytically processed to their p50 and p52 mature forms respectively. The NF-κB proteins share a conserved domain known as the Rel homology domain (RHD), which is responsible for DNA binding, dimerisation and association with the inhibitory IκB proteins.

NF-κB/Rel dimers are sequestered in the cytoplasm by interaction with a group of specific inhibitors, namely the IκBs. These proteins are characterised by the presence of multiple ankyrin repeats which interact with NF-κB/Rel dimers through the RHD. Ubiquitin-mediated degradation of the IκBs results in the release and nuclear translocation of the bound NF-κB/Rel dimers. Phosphorylation of IκBs precedes ubiquitination and is mediated by the IκB kinase (IKK) complex, also known as the signalosome. The IKK signalosome is a large multisubunit kinase complex consisting of the catalytic subunits IKKα and IKKβ and the regulatory and scaffold subunit IKKγ (NEMO). Genetic knockout studies have shown that the IKKβ and IKKγ subunits are required for NF-κB activation by all known proinflammatory stimuli including bacterial lipopolysaccharide, TNF-α, IL-1β, and double-stranded RNA (reviewed in (Ghosh & Karin. 2002)). The NF-κB activation

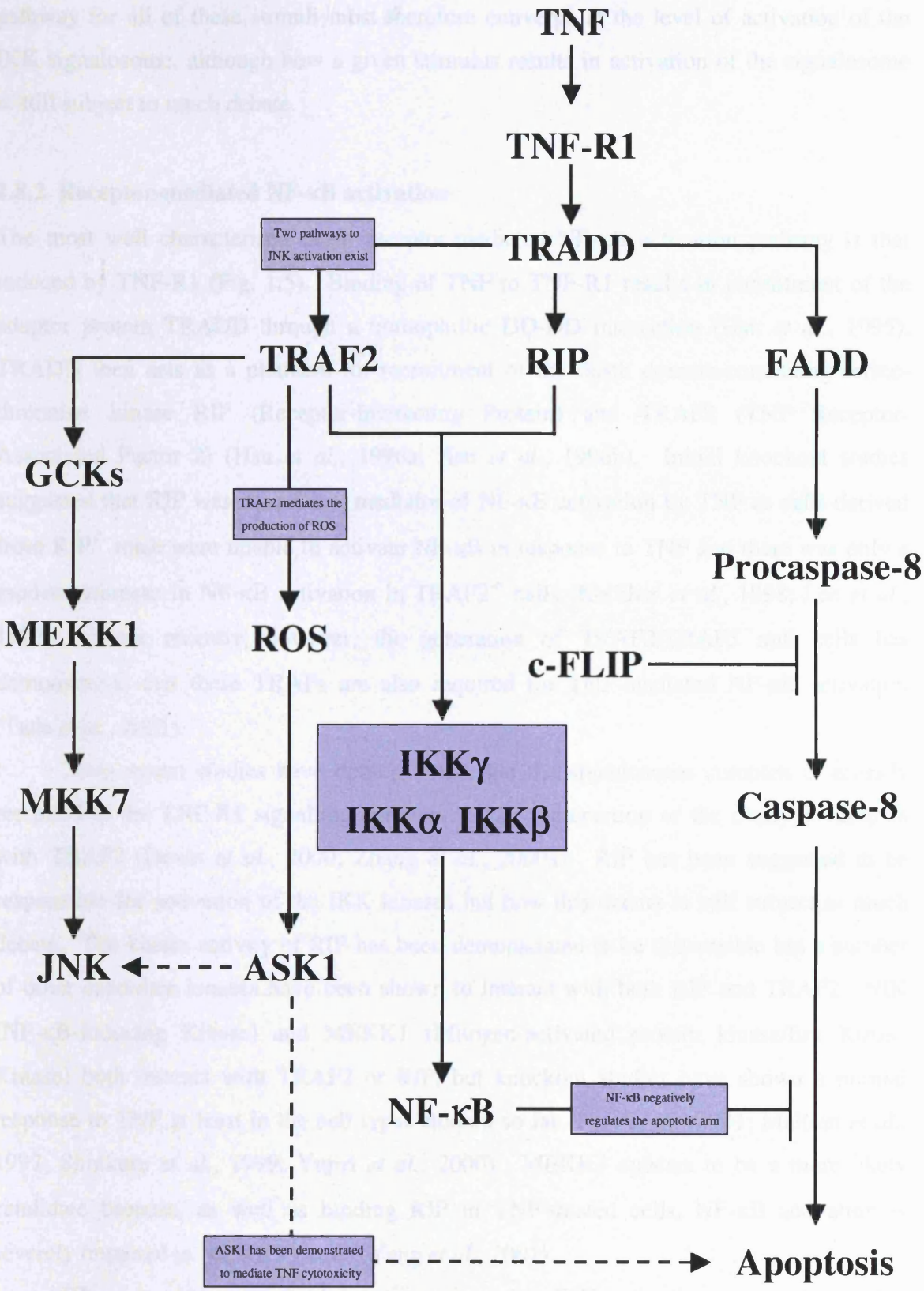


Figure 1.4 TNF-R1 activated signalling pathways (see text for details)
Hatched lines represent pathways were no direct interaction between the two components has been demonstrated

pathway for all of these stimuli must therefore converge at the level of activation of the IKK signalosome, although how a given stimulus results in activation of the signalosome is still subject to much debate.

1.8.2 Receptor-mediated NF-κB activation

The most well characterised death receptor-mediated NF-κB activation pathway is that induced by TNF-R1 (Fig. 1.5). Binding of TNF to TNF-R1 results in recruitment of the adaptor protein TRADD through a homophillic DD-DD interaction (Hsu *et al.*, 1995). TRADD then acts as a platform for recruitment of the death domain-containing serine-threonine kinase RIP (Receptor-Interacting Protein) and TRAF2 (TNF Receptor-Associated Factor 2) (Hsu *et al.*, 1996a; Hsu *et al.*, 1996b). Initial knockout studies suggested that RIP was the critical mediator of NF-κB activation by TNF as cells derived from $RIP^{-/-}$ mice were unable to activate NF-κB in response to TNF and there was only a modest decrease in NF-κB activation in $TRAF2^{-/-}$ cells (Kelliher *et al.*, 1998; Lee *et al.*, 1997). More recently, however, the generation of TRAF2/TRAF5 null cells has demonstrated that these TRAFs are also required for TNF-mediated NF-κB activation (Tada *et al.*, 2001).

Two recent studies have demonstrated that the signalosome complex is actually recruited to the TNF-R1 signalling complex through interaction of the catalytic subunits with TRAF2 (Devin *et al.*, 2000; Zhang *et al.*, 2000a). RIP has been suggested to be responsible for activation of the IKK kinases but how this occurs is still subject to much debate. The kinase activity of RIP has been demonstrated to be dispensible but a number of other candidate kinases have been shown to interact with both RIP and TRAF2. NIK (NF-κB-Inducing Kinase) and MEKK1 (Mitogen-activated protein kinase/Erk Kinase Kinase) both interact with TRAF2 or RIP, but knockout studies have shown a normal response to TNF at least in the cell types studied so far (Kim *et al.*, 2001; Malinin *et al.*, 1997; Shinkura *et al.*, 1999; Yujiri *et al.*, 2000). MEKK3 appears to be a more likely candidate because, as well as binding RIP in TNF-treated cells, NF-κB activation is severely impaired in $MEKK3^{-/-}$ cells (Yang *et al.*, 2001).

There is also some evidence to suggest that IKK activation may occur in the absence of an upstream kinase. Activation of the two catalytic IKK subunits IKK-α or β requires phosphorylation of two conserved residues located within their respective activation loops (Delhase *et al.*, 1999; Ling *et al.*, 1998). This phosphorylation can be

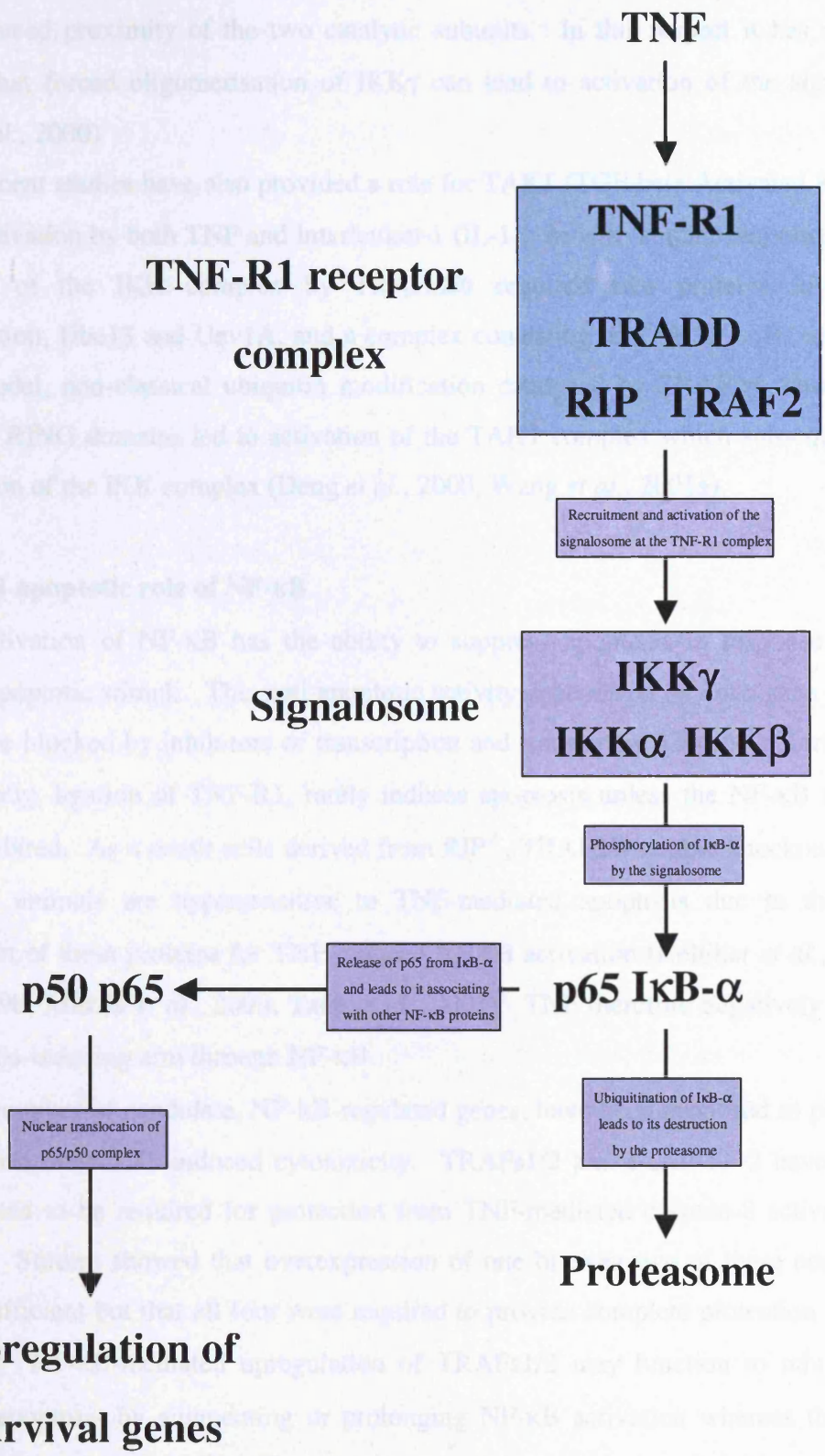


Figure 1.5 TNF-induced NF-κB activation pathway
(see text for details)

mediated by an upstream kinase but may also occur as a result of autophosphorylation due to the induced proximity of the two catalytic subunits. In this respect it has also been reported that forced oligomerisation of IKKγ can lead to activation of the signalosome (Poyet *et al.*, 2000).

Recent studies have also provided a role for TAK1 (TGF-beta Activated Kinase) in NF-κB activation by both TNF and Interleukin-1 (IL-1). *In vitro* studies demonstrated that activation of the IKK complex by TRAFs2/6 required two proteins involved in ubiquitination, Ubc13 and Uev1A, and a complex consisting of TAK1, TAB1 and TAB2. In this model, non-classical ubiquitin modification catalysed by TRAF2/6 through their respective RING domains led to activation of the TAK1 complex which subsequently led to activation of the IKK complex (Deng *et al.*, 2000; Wang *et al.*, 2001a).

1.8.3 Anti-apoptotic role of NF-κB

Activation of NF-κB has the ability to suppress apoptosis in response to many different apoptotic stimuli. This anti-apoptotic activity depends on *de novo* gene induction as it can be blocked by inhibitors of transcription and translation (Ghosh & Karin. 2002). Consequently, ligation of TNF-R1, rarely induces apoptosis unless the NF-κB activation arm is inhibited. As a result cells derived from $RIP^{-/-}$, TRAF2/5 double knockout, $IKK\beta^{-/-}$ or $IKK\gamma^{-/-}$ animals are hypersensitive to TNF-mediated apoptosis due to the critical requirement of these proteins for TNF-induced NF-κB activation (Kelliher *et al.*, 1998; Li *et al.*, 1999b; Makris *et al.*, 2000; Tada *et al.*, 2001). TNF therefore negatively regulates its apoptosis-inducing arm through NF-κB.

A number of candidate, NF-κB-regulated genes, have been proposed to play a role in protection from TNF-induced cytotoxicity. TRAFs1/2 and c-IAP-1, -2 have all been demonstrated to be required for protection from TNF-mediated caspase-8 activation and apoptosis. Studies showed that overexpression of one or even two of these components was not sufficient but that all four were required to provide complete protection (Wang *et al.*, 1998). NF-κB-mediated upregulation of TRAFs1/2 may function to inhibit TNF-mediated apoptosis by augmenting or prolonging NF-κB activation whereas the c-IAPs may function to directly inhibit effector caspases such as caspase-3 and -7 (Deveraux *et al.*, 1998). XIAP is another IAP family member regulated by NF-κB. As well as being capable of suppressing TNF-mediated apoptosis in cells expressing an IκB mutant, XIAP can inhibit apoptosis induced by a variety of stimuli and may function as a much broader

survival gene (Tamm *et al.*, 1998). The Bcl-2 homologs, A1 and Bcl-$_X$L are also subject to upregulation by NF-κB activation by a number of agents including TNF (Koshnan *et al.*, 1999; Zong *et al.*, 1999). Both of these anti-apoptotic Bcl-2 homologs (section 1.5) have been demonstrated to protect cells from TNF by preventing mitochondrial depolarisation and can rescue cells expressing a mutant form of IκB-α.

High or constitutive NF-κB activation has also been associated with the resistance of some tumours to anticancer treatments (Baeuerle *et al.*, 1994). Many chemotherapeutic agents have been demonstrated to activate NF-κB, a response which significantly reduces their effectiveness (Wang *et al.*, 1999a). Inhibition of the NF-κB pathway has therefore become a potential approach to enhance the apoptotic potential of tumour therapies. Initial approaches have used IκB mutants to completely suppress NF-κB activation within the target cells (Wang *et al.*, 1999a). Whether individual targeting of NF-κB-regulated anti-apoptotic genes provides a more specific approach is at present uncertain.

1.8.4 Activation of the stress activated protein kinase 1 (SAPK1)/c-Jun N-terminal kinase (JNK) kinase cascade

The SAPK/JNK pathway induces adaptive responses from a variety of stress signals. These responses are primarily as a result of changes in gene expression mediated by enhancing the function of transcription factors such as AP-1 (Activator Protein-1) (reviewed in (Shaulian *et al.*, 2002)). JNK modulates transcription through phosphorylation of various transcription factors including c-Jun and ATF2 and there is some suggestion that JNK activation may act in concert with NF-κB to prevent TNF-mediated apoptosis (Tang *et al.*, 2001). Prolonged JNK activation can however lead to apoptosis although the mechanism of this is unclear and its has also recently been proposed that JNK may play an important role in TNF-mediated cytotoxicity. TNF-induced activation of JNK occurs in a TRADD and TRAF2-dependent manner but is independent of both FADD and caspase-8 (Fig. 1.4) as it is not inhibited by DN-FADD (dominant-negative FADD) (Hsu *et al.*, 1996b). The pathway from TRAF2 to JNK involves the MAP (Mitogen-Activated Protein) kinase pathway member MEKK1 (MAP/Erk Kinase Kinase-1). Although it has not been shown to bind to TRAF2, kinase inactive MEKK1 mutants have been demonstrated to abrogate TNF-mediated JNK activation (Chadee *et al.*, 2002). Members of the GCK (Germinal Center Kinase) family are also implicated as they are activated by TNF, DN-GCK mutants block JNK activation and several members have been

shown to bind to TRAF2 (Shi *et al.*, 1999). GCKs can phosphorylate and cause oligomerisation of MEKK1 and may represent the bridge between TRAF2 and MEKK1 (Chadee *et al.*, 2002). MEKK1 has been demonstrated to activate MKK7 which is then able to directly phosphorylate JNK. Knockout studies have demonstrated that MKK7 is also required for TNF-mediated JNK activation (Tournier C. 2001). More than one method for TNF-mediated activation of JNK may exist. Stimulation of TNF-R1 leads to a TRAF2-dependent increase in ROS (Reactive Oxygen Species) (Chandel *et al.*, 2001b). This ROS generation has been shown to be mitochondrial in origin and results in the activation of ASK1 (Apoptosis Signalling Kinase-1) which in turn leads to JNK activation (Ichijo *et al.*, 1997). ASK1 activation has also been associated with TNF-mediated cytotoxicity, which is inhibited by a kinase dead ASK1 mutant (Ichijo *et al.*, 1997). While activation of JNK by TNF-R1 is a caspase-independent process, activation by CD95 and TRAIL-R1 and -R2 probably occurs as a result of caspase activation (MacFarlane *et al.*, 2000). In this regard, JNK activation can also be activated during apoptosis by caspases through cleavage of substrates such as MEKK1 (Deak *et al.*, 1998).

1.9 The TRAIL receptor family of death and decoy receptors

TRAIL/Apo-2 ligand (TNF Receptor Apoptosis Inducing Ligand) was identified by searching EST (Expressed Sequence Tag) databases for sequences bearing homology to conserved regions within other TNFR family members (Pitti *et al.*, 1996; Wiley *et al.*, 1995). Like other TNF family members, TRAIL is a type II transmembrane protein with an intracellular amino-terminus and the carboxy-terminus present outside the cell. The extracellular domain of TRAIL shares the highest amino acid homology with CD95L (28%) followed by TNF-α (23%) and lymphotoxin-β (23%). Unlike other members of the TNF family, which are subject to a restricted tissue distribution, TRAIL was found to have a relatively broad tissue distribution being expressed in spleen, thymus, placenta, prostate, ovary and peripheral blood lymphocytes but not testis, liver or brain. One of the most interesting observations noted with TRAIL was that it appeared to display a selective cytotoxicity, inducing apoptosis in tumour and transformed but not normal cells (Pitti *et al.*, 1996; Wiley *et al.*, 1995).

1.9.1 TRAIL Receptors

The broad tissue expression of TRAIL initially suggested that the regulation of TRAIL-induced apoptosis would be through restricted TRAIL receptor expression. There

was therefore some surprise when four independent TRAIL receptors were subsequently identified. TRAIL-R1 (DR4) (Pan *et al.*, 1997c) and TRAIL-R2 (DR5/TRICK/Killer) (MacFarlane *et al.*, 2000; Pan *et al.*, 1997a; Schneider *et al.*, 1997a; Screaton *et al.*, 1997; Sheridan *et al.*, 1997; Walczak *et al.*, 1997; Wu *et al.*, 2000) both carry the DD motif within their intracellular domains and are responsible for mediating TRAIL-induced apoptosis (Fig. 1.6). The reason for the existence of two TRAIL death receptors is not clear, although they have been demonstrated to be capable of forming homo- and hetero-trimers which may provide a mechanism to regulate TRAIL signalling (Schneider *et al.*, 1997b). A further two receptors were also identified; TRAIL-R3 (DcR1/LIT/TRID) lacks any form of transmembrane or intracellular domain and is linked to the cell surface by a glycophosphatidylinositol (GPI) anchor (MacFarlane *et al.*, 2000; Mongkolsapaya *et al.*, 1998; Pan *et al.*, 1997a; Schneider *et al.*, 1997a; Sheridan *et al.*, 1997). By contrast, TRAIL-R4 (DcR2, TRUNDD) does contain a transmembrane and intracellular domain but the death domain is truncated and lacks a number of key residues critical for engagement of apoptosis (Marsters *et al.*, 1997b; Pan *et al.*, 1998). As these latter two receptors lacked a functional death domain they were unable to induce apoptosis upon overexpression and were therefore proposed to act as "decoy" receptors functioning to sequester TRAIL from TRAIL-R1 and -R2 (Sheridan *et al.*, 1997). Some studies also demonstrated that TRAIL-R4, which still contained a substantial intracellular domain, was capable of activating NF-κB and therefore could also act to provide protection from TRAIL by inducing expression of anti-apoptotic genes (Degli-Esposti *et al.*, 1997). There also remains the possibility of a fifth TRAIL receptor. Osteoprotegerin (OPG) is a soluble TNF receptor homolog that increases bone density in vivo and inhibits osteoclastogenesis (Simonet *et al.*, 1997). OPG has been shown to bind TRAIL and inhibit TRAIL-induced apoptosis most likely by functioning as a decoy receptor (Emery *et al.*, 1998). TRAIL was also shown to block the anti-osteoclastogenic activity of OPG possibly by binding to OPG and acting as a "decoy ligand". Further evidence is however required to assess whether OPG and TRAIL do in fact share a physiological role. Like other TNF receptor family members all of the TRAIL receptors, with the exception of TRAIL-R3, are type I transmembrane proteins containing an extracellular carboxy-terminus and an intracellular DD-containing amino-terminus. The genes for the four TRAIL receptors are clustered on chromosome 8p21-22 suggesting that they evolved recently, most likely as a result of gene duplication (Ashkenazi *et al.*, 1998).

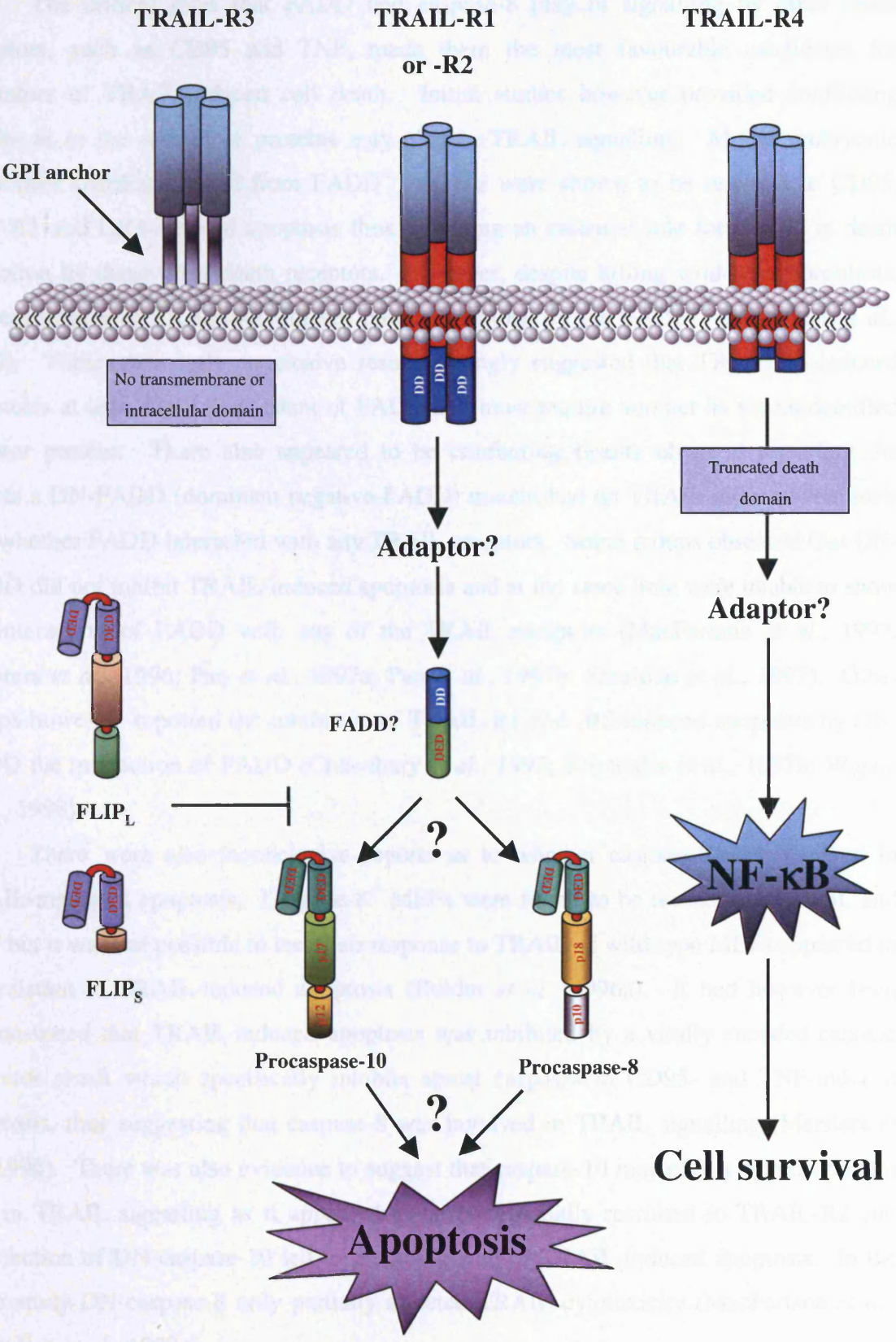


Figure 1.6 The TRAIL receptor family
(see text for details)

1.9.2 TRAIL signalling

The critical roles that FADD and caspase-8 play in signalling by other death receptors, such as CD95 and TNF, made them the most favourable candidates for mediators of TRAIL-induced cell death. Initial studies however provided conflicting results as to the role these proteins may play in TRAIL signalling. Mouse embryonic fibroblasts (MEFs) derived from $FADD^{-/-}$ animals were shown to be resistant to CD95, TNF-R1 and DR3-induced apoptosis thus providing an essential role for FADD in death induction by these three death receptors. However, despite killing wild-type fibroblasts, overexpression of TRAIL-R1 did not induce apoptosis in the $FADD^{-/-}$ cells (Yeh *et al.*, 1998). These apparently conclusive results strongly suggested that TRAIL-R1-induced apoptosis at least was independent of FADD and must require another as yet unidentified adaptor protein. There also appeared to be conflicting results obtained regarding the effects a DN-FADD (dominant negative-FADD) mutant had on TRAIL-induced apoptosis and whether FADD interacted with any TRAIL receptors. Some groups observed that DN-FADD did not inhibit TRAIL-induced apoptosis and at the same time were unable to show the interaction of FADD with any of the TRAIL receptors (MacFarlane *et al.*, 1997; Marsters *et al.*, 1996; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Sheridan *et al.*, 1997). Other groups however, reported the inhibition of TRAIL-R1 and -R2-induced apoptosis by DN-FADD the interaction of FADD (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997b; Wajant *et al.*, 1998).

There were also inconclusive reports as to whether caspase-8 was involved in TRAIL-mediated apoptosis. Caspase-$8^{-/-}$ MEFs were found to be resistant to CD95L and TNF but it was not possible to test their response to TRAIL as wild-type MEFs appeared to be resistant to TRAIL-induced apoptosis (Boldin *et al.*, 1996a). It had however been demonstrated that TRAIL-induced apoptosis was inhibited by a virally encoded caspase inhibitor crmA which specifically inhibits apical caspases in CD95- and TNF-induced apoptosis, thus suggesting that caspase-8 was involved in TRAIL signalling (Marsters *et al.*, 1996). There was also evidence to suggest that caspase-10 may play a more important role in TRAIL signalling as it appeared to be preferentially recruited to TRAIL-R2 and transfection of DN-caspase-10 led to the abrogation of TRAIL-induced apoptosis. In the same study DN-caspase-8 only partially affected TRAIL cytotoxicity (MacFarlane *et al.*, 2000; Pan *et al.*, 1997a).

More recently, a number of studies have addressed the issue of the role of FADD and caspase-8 in TRAIL signalling. In one study, the authors used $FADD^{-/-}$ MEFs that had

been stably transfected with TRAIL-R1 and -R2. Wild-type transfected MEFs were sensitive to TRAIL-induced apoptosis but the $FADD^{-/-}$ transfectants were not. The authors therefore concluded that TRAIL-induced apoptosis must require FADD (Kuang *et al.*, 2000). The native TRAIL DISC has also been isolated and analysed by several groups. Both FADD and caspase-8 are reported to be recruited and required for TRAIL-induced apoptosis (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000) (see also Chapter 4). Using TRAIL receptor blocking antibodies it was demonstrated that DISCs consisting solely of TRAIL-R1 or -R2 recruited FADD and caspase-8 and could independently induce apoptosis (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). No evidence of other death receptor associated proteins such as RIP, TRADD or TRAF2 was observed suggesting that these proteins do not play a role in TRAIL signalling. The reason for the conflicting results obtained by so many groups regarding the roles of FADD and caspase-8 in TRAIL signalling is still not clear but it is now accepted that both molecules are critically required for TRAIL-mediated cytotoxicity making them universal components of the death receptor machinery.

The precise role of caspase-10 in TRAIL signalling is however unclear. Initially caspase-10 was not identified as a TRAIL DISC component but was subsequently demonstrated to be recruited and activated by TRAIL receptor ligation (Sprick *et al.*, 2002). There is some suggestion that it may functionally substitute for caspase-8 in response to some death receptor pathways but other studies have failed to corroborate this (Sprick *et al.*, 2002). The absence of any caspase-10 in murine cells would also strongly suggest that it may be dispensable (Varfolomeev *et al.*, 1998).

Whether TRAIL and its receptors are capable of activating other signalling pathways such as JNK or NF-κB in a manner similar to TNF and the role that these pathways may play in TRAIL signalling has not yet been fully characterised. TRAIL has been demonstrated to be capable of activating JNK but this was found to be inhibited by a caspase inhibitor suggesting that this was more likely the result of caspase activation within the cell rather than as a direct result of TRAIL receptor ligation (MacFarlane *et al.*, 2000). In this respect, no direct interaction has been shown between TRAFs1/2, critical mediators of TNF-mediated JNK activation, and any TRAIL receptors (Ashkenazi & Dixit. 1998). As already outlined above NF-κB plays a critical role in modulating TNF cytotoxicity (section 1.8.3), however its precise role in TRAIL signalling is poorly characterised. In this respect, TNF induces a lethal NF-κB-mediated inflammatory response when used *in vivo* which has precluded its use as a tumour therapeutic. The fact

that TRAIL appears to be tolerated when administered *in vivo* would suggest that NF-κB activation by TRAIL would be significantly less potent than that induced by TNF. Despite this, activation, of NF-κB by TRAIL or by overexpression of TRAIL-R1 and -R2 has been observed in some studies but not others (Chaudhary *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Schneider *et al.*, 1997b; Sheridan *et al.*, 1997). Although it is clear that high constitutive NF-κB activation can provide protection against many forms of apoptosis including TRAIL the question still remains as to but NF-κB activation by TRAIL actually functions to antagonise TRAIL-induced apoptosis as it does with TNF.

The decoy receptor TRAIL-R4, despite containing an incomplete death domain, was reported to activate NF-κB in some, but not all studies suggesting that it may protect cells from TRAIL-induced cytotoxicity by one of two mechanisms (Degli-Esposti *et al.*, 1997; Hu *et al.*, 1999). Firstly, by sequestering TRAIL from the death receptors TRAIL-R1 and -R2, and secondly by activating an NF-κB-mediated antiapoptotic signal. Although some studies initially observed an interaction of the TRAIL receptors with TRADD and RIP (Schneider *et al.*, 1997b), critical mediators of TNF-mediated NFκB activation, these proteins were not reported to be components of the native TRAIL DISC (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Activation of NF-κB by TRAIL must therefore either be cell type- dependent or not require recruitment of RIP or TRADD to the DISC.

1.9.3 Modulation of TRAIL signalling: factors affecting TRAIL resistance/sensitivity

The existence of two decoy receptors initially provided a possible mechanism for the differential sensitivity to TRAIL-induced apoptosis observed in tumour and transformed cells when compared to normal cells (Pitti *et al.*, 1996; Wiley *et al.*, 1995). It was initially speculated that normal cells would express greater levels of the TRAIL decoy receptors than tumour cells and this would help to explain their differential sensitivity. However, studies in melanoma cells have demonstrated that expression of decoy receptors appears to play a very minor role in determining their susceptibility to TRAIL (Griffith *et al.*, 1998b; Zhang *et al.*, 1999). The TRAIL death and decoy receptors have also been demonstrated to exhibit very different subcellular localisations. In one study in melanoma cells, TRAIL-R1 and -R2 were found to be predominately expressed at the cell surface and were internalised into endosomes upon TRAIL binding. In the same cells, TRAIL-R3 and -R4 were not present at the cell surface but instead had a peri-nuclear distribution in unstimulated cells. TRAIL treatment resulted in trafficking of these 'decoy' receptors

from the nucleus to the cell surface (Zhang *et al.*, 2000b). The absence of cell surface TRAIL 'decoy' receptors at the instance of TRAIL binding is confusing as is their subsequent relocation, which given the rapid formation of the DISC in response to TRAIL would be too late to act to sequester TRAIL from the death receptors (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Another study which attempted to prove the decoy receptor hypothesis generated cells expressing a GPI-linked form of CD95. The authors obtained several clones that expressed varying amounts of the CD95 variant on the cell surface. However, rather than observing an increase in protection with increasing cell surface expression of GPI-CD95 the opposite was found. Clones expressing high levels of GPI-CD95 were the most sensitive to CD95L (Legembre *et al.*, 2002). As a result of this study the exact role of the GPI-linked TRAIL-R3 and the truncated TRAIL-R4 is still subject to debate. Taken together with the broad tissue distribution of TRAIL and the complex receptor system outlined above this instead suggested the existence of intracellular mechanisms for protection against TRAIL-induced apoptosis.

Intracellular mechanisms of regulation of TRAIL cytotoxicity appear to be similar to those of CD95 and TNF-R1. Cells overexpressing c-FLIP isoforms are similarly refractory to all death receptor stimuli including TRAIL ((Thome *et al.*, 1997) and 1.6.5). Modulation of endogenous FLIP levels using inhibitors of protein synthesis and transcription are therefore strategies that have been used to sensitise cells to TRAIL (Griffith *et al.*, 1998a; MacFarlane *et al.*, 2002). However, although with the recent evidence that c-$FLIP_L$ can act as an activator of procaspase-8, reducing endogenous c-$FLIP_L$ levels may in some instances actually function to promote TRAIL-induced apoptosis (section 1.6.6). As with other apoptotic stimuli endogenous levels of IAPs are important and strategies based on IAP antagonisation such as using Smac-like peptides has led to sensitisation and eradication of TRAIL resistant tumours *in vivo* (Beltinger *et al.*, 1999). The NF-κB pathway has also been implicated in TRAIL resistance. NF-κB-regulated anti-apoptotic genes such as the IAPs and anti-apoptotic Bcl-2 family members have all been implicated in providing resistance to TRAIL (section 1.5). In this respect it has been shown that IL-1, a potent activator of NF-κB, can protect cells from TRAIL-induced apoptosis (Kothny-Wilkes *et al.*, 1998). TRAIL has also displayed synergism with many chemotherapeutic agents, especially *in vivo* although this may just be a result of concurrent activation of intrinsic and extrinsic cell death pathways. It should be noted that all of these mechanisms of inhibition would be predicted to equally protect cells from

CD95, TNF and TRAIL-mediated cytotoxicity and that other TRAIL-specific pathways may await further identification.

1.9.4 The physiological role of TRAIL

The unique tumoricidal activity of TRAIL in the absence of cytotoxicity to normal cells has led to the suggestion that TRAIL may function as a natural tumour suppressor. The board expression of TRAIL mRNA and the complex receptor family system however, coupled with a lack of specific reagents such as antibodies for the determination of expression at the protein level initially made progress on determining the exact physiological role of TRAIL and its receptor system relatively slow.

Generation of a murine TRAIL neutralising antibody has been used to demonstrate freshly isolated T cells, natural killer (NK)-T cells, B cells, dendritic cells (DC) or monocytes do not express detectable levels of TRAIL on their cell surface (Fanger *et al.*, 1999; Griffith *et al.*, 1999b; Kayagaki *et al.*, 1999a; Kayagaki *et al.*, 1999b; Smyth *et al.*, 2001; Takeda *et al.*, 2001). However, inducible upregulation of cell surface TRAIL expression in these cell types by cytokines such as interferon-α and -γ has been demonstrated and has provided examples of a physiological role for TRAIL as a tumoricidal agent.

Activated monocyte macrophages have been demonstrated to have tumoricidal activity against a variety of tumor cell types and it has recently been suggested that TRAIL may play a role in this tumoricidal activity. Although they do not express detectable levels of TRAIL, activation of either with interferon-α or -γ was observed to lead to a rapid increase of cell surface TRAIL. This increase in cell surface TRAIL expression was found to coincide with the macrophages becoming resistant to TRAIL-induced apoptosis possibly due to a decrease in cell surface TRAIL-R2 which would act to protect the cell from autocrine suicide (Griffith *et al.*, 1999b). The increase in TRAIL and tumoricidal activity observed in IFN-treated cells appeared to be specific to IFNs as stimulation with IL-1, IL-2, IL-4, GM-CSF or a number of other cytokines failed to result in an increase in TRAIL expression. A similar observation has been noted in DCs where IFN-induced increase in TRAIL leads to the ability to kill TRAIL sensitive tumor cell lines but not normal cells. Killing mediated by IFN treatment was also blocked by soluble TRAIL receptor, again demonstrating a critical role for TRAIL in this process (Fanger *et al.*, 1999).

TRAIL is only expressed on monocyte-macrophages or DCs when stimulated with IFNs. By contrast, one of the few cells that has been demonstrated to produce TRAIL

constitutively is an NK subtype in the liver (Takeda *et al.*, 2001). The cytotoxicity of liver-derived NK cells against TRAIL-sensitive tumor cells can be partially inhibited by an anti-TRAIL neutralising antibody implicating TRAIL in the tumoricidal activity of these cells. Complete inhibition was obtained by using TRAIL in combination with the perforin inhibitor concamycin A (CMA) which implicates an important role for TRAIL and perforin in liver-derived NK cell-mediated cytotoxicity. In contrast, the cytotoxicity of splenic-derived NK cells was blocked by CMA treatment alone or in cells derived from perforin$^{-/-}$ mice indicating that TRAIL was not required for cytotoxicity in these cells.

Mice which are genetically deficient in TRAIL (TRAIL$^{-/-}$) have only recently been reported. Only limited details are available with the authors noting only that they "display no obvious histological, haematological or reproductive defects"(Cretney *et al.*, 2002). The limited studies currently performed in these mice have at least confirmed the earlier studies utilising the anti-TRAIL neutralising antibodies in the liver-derived NK cells as mentioned above. Increased liver metastasis was also observed in TRAIL$^{-/-}$ mice in a renal carcinoma model system. Once again NK cell-mediated production of TRAIL was implicated as there was a similar increase in metastasis when NK cells were depleted (Cretney *et al.*, 2002). TRAIL$^{-/-}$ mice also appeared to be more sensitive to tumour initiation by the chemical carcinogen, methylcholanthrene (MCA). An earlier onset of fibrosarcoma was observed in the TRAIL$^{-/-}$ compared to wild-type mice implicating an important role for TRAIL in tumour initiation and development as a result of MCA treatment (Cretney *et al.*, 2002).

Further characterisation of the TRAIL$^{-/-}$ phenotype will no doubt reveal more roles for TRAIL in other tissues and cells. Clearly though one of the important functions of TRAIL, at least in the liver, is one of tumor surveillance. To date there have currently been no reports of any TRAIL receptor knockouts.

1.9.5 TRAIL as a therapeutic agent

Although both CD95L and TNF can also induce apoptosis in tumour cells the systemic toxicity observed when these ligands have been used *in vivo* have precluded their use as therapeutic agents. Triggering of CD95, more specifically infusion of an agonistic-anti-Fas antibody led to lethal liver damage due to extensive hepatocyte apoptosis (Ogasawara *et al.*, 1993). TNF was observed to cause a lethal inflammatory response primarily due to activation of the proinflammatory transcription factor NF-κB (Tracey *et al.*, 1986). The apparent selectivity of TRAIL, affecting only tumour and transformed cells

but not normal cells and together with the broad tissue distribution of TRAIL mRNA suggested a possible role for therapeutic role for TRAIL (Pitti *et al.*, 1996; Wiley *et al.*, 1995). TRAIL has also been demonstrated to be tumoricidal to cancers with p53 mutations which is of particular importance as, in general, chemotherapy and irradiation require the function of wild-type p53's tumour-supressor function in order for them to act as anti-tumour agents (Levine. 1997). Cancers displaying wild-type p53 are also sensitive to TRAIL possibly due to the fact that TRAIL-R2 is a p53-regulated gene (Sheikh *et al.*, 1998; Wu *et al.*, 2000).

There also appears to be a degree of synergism between TRAIL and certain chemotherapeutic agents with the cytotoxic activity of TRAIL being enhanced when used in combination with chemotherapeutic agents. Treatment with 5-fluorouracil (5-FU) or CPT-11, in combination with TRAIL, led to marked tumour regression or complete tumor remission respectively when used in an *in vivo* xenograft model based on a number of colon carcinoma cell lines (Ashkenazi *et al.*, 1999). The mechanism of this synergism between TRAIL and certain chemotherapeutic agents is currently unknown but may be due to concurrent activation of the intrinsic and extrinsic apoptosis cell death pathways. In addition 5-FU has been show to upregulate TRAIL-R2 again leading to increased TRAIL sensitivity (Keane *et al.*, 1999; Oka *et al.*, 1997). UV irradiation has also been demonstrated to enhance TRAIL-mediated tumoricidal activity in human breast cancer xenografts. This synergistic effect was shown to be p53-dependent and again may involve upregulation of TRAIL-R2 (Chinnaiyan *et al.*, 2000).

Another mechanism that has been shown to enhance TRAIL sensitivity is modulation of IAP function, specifically through the use of IAP agonistic peptides based on the N-terminal amino acids of Smac. Malignant glioma is currently refractory to most treatment approaches (DeAngelis. 2001) and cell lines derived from such tumours have been demonstrated to contain increased levels of Bcl-2 and XIAP thus making them insensitive to TRAIL (Wagenknecht *et al.*, 1999). TRAIL sensitivity could however be restored in these cells by transfection of Smac. Furthermore, in a human glioma xenograft model, combined treatment with TRAIL and peptides containing the first four amino acids of Smac led to complete eradication of established tumors without any detectable toxicity to normal tissue (Fulda *et al.*, 2002). Therefore, even in tumors which are TRAIL-resistant, methods exist to sensitise the tumors to TRAIL without causing systemic toxicity.

A potential problem with the use of TRAIL as a therapeutic agent was highlighted by two studies, one suggesting that primary human hepatocytes were sensitive to TRAIL-induced apoptosis even though hepatocytes derived from non-human primates and other species did not appear to be sensitive (Jo *et al.*, 2000). The other study found that primary human astrocytes were also sensitive to TRAIL. The apparent cytotoxicity of these two normal cell types initially suggested that TRAIL may not be wholly suitable for use *in vivo* despite its being significantly less toxic than CD95 or TNF. It was later shown that the toxicity observed in the afore mentioned studies was due to the form of TRAIL used. Most forms of TRAIL are fused to epitope tags to allow efficient purification or aggregation of the ligand. The TRAIL used in the afore mentioned studies contained a polyhistidine tag (Kelley *et al.*, 2001). A form of TRAIL, known as Apo-2L, that was not fused to any heterologous sequences was then developed, and as well as being non-toxic to both primary hepatocytes and astrocytes it was found to be well tolerated *in vivo* as it is much less likely to be immunogenic than the TRAIL-fusion proteins. An *in vivo* study using Apo-2L in both cynomolgus monkeys and chimpanzees has further supported these results as no *in vivo* toxicity was observed (Kelley *et al.*, 2001; Lawrence *et al.*, 2001).

1.10 Aims and objectives:

The aim of this thesis are to further investigate signalling pathways induced by TRAIL receptor ligation and their ultimate effects on cell survival. TRAIL appears to have anti-tumour properties as it is well tolerated when administered in vivo and appears to display selective toxicity to tumour and transformed but not normal cells. Examining survival pathways such as the transcription factor NF-κB which regulate other members of the TNF-R family may elucidate differences between TRAIL signalling in tumour and normal cells. Examination of sensitive and cells which are refractory to TRAIL cytotoxicity lead to the identification of differences between components required for formation of the TRAIL DISC. It is suggested that TRAIL-mediated cytotoxicity may be regulated intracellularly rather than by its "decoy" receptors and therefore further characterisation of signalling pathways known to inhibit TRAIL signalling such as activation of PKC may help to explain its selective toxicity. Many normal cells display a differential sensitivity to CD95 and TNF and TRAIL suggesting that despite using identical components for activating the apoptotic machinery such as FADD there must be crucial differences in the mechanism by which these death receptors induced apoptosis and its regulation. If TRAIL is to be used as a tumour therapeutic then a complete understanding of its signalling pathways and their regulation will be essential.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All general laboratory chemicals were supplied by Sigma (Poole, U.K.) and Fisher (Loughborough, UK) and were of analytical grade unless otherwise stated. The poly-caspase inhibitor, Benzyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethylketone (z-VAD.fmk) was from Enzyme Systems (Dublin, CA, U.S.A.). Recombinant human TNF and enzyme-linked immunosorbent assay (ELISA) reagents were obtained from R & D Systems Inc (MN, U.S.A.). Phorbol Myristate Acetate (PMA) was obtained from Sigma. Bisindolylmaleimide I (GF109203X) was from Calbiochem (Nottingham, UK). Fluorescein isothiocyanate (FITC)-conjugated Annexin V was purchased from Bender Medsystems (Vienna, Austria).

2.2 Cell Biology Techniques

2.2.1 Cell Culture

Medium, Foetal Bovine Serum and other cell culture-related chemicals were purchased from Life Technologies Inc. (Paisley, U.K.). Cell culture plastic ware was from Becton Dickinson (CA, U.S.A.). Jurkat E6.1, U937, Human Embryonic Kidney Fibroblasts (HEK293) and HeLa cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC; Porton Down, UK). Parental (A3), Caspase-8 null and FADD null Jurkat cells (Juo *et al.*, 1998; Juo *et al.*, 1999) were a gift from Dr. J. Blenis, Harvard Medical School, Boston, U.S.A. MCF-7 cells been stably transfected with caspase-3 or control vector (Janicke *et al.*, 1998a) were a gift from Dr. A. Porter (National University of Singapore, Singapore) were cultured in RPMI medium containing 10 % Foetal Bovine Serum (FBS) and 5 % Glutamax™.

Suspension cells were cultured in Roswell Park Memorial Institute (RPMI) medium containing 10 % Foetal Bovine Serum (FBS) and 5 % Glutamax™ (Life Technologies Inc.). Cells were kept at a density of between 0.8 and 1.0×10^6 cells per ml by routine passage every 3 days. Cells were counted using a CASY 1 cell counter (Scharfe Systems; Reutlingen, Germany), and cell viability was determined before experiments using Annexin V and propidium iodide staining (2.2.2).

Adherent cells were passaged at around 80 % confluency (approximately every 3 days). Cells were washed once with prewarmed Phosphate Buffered Saline (PBS) then incubated at 37 °C for 5 min with trypsin (0.05 %) and EDTA (0.02%) in PBS. Cells were then washed with medium to inactivate the trypsin, collected by centrifugation then resuspended in fresh medium and used to seed further flasks or 6-well culture dishes as required.

All cells were maintained at 37 °C in a humidified 5 % CO_2 incubator. For transfection experiments medium was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) to avoid bacterial growth.

2.2.2 Purification of B-cells from patients with chronic lymphocytic leukemia (CLL)

Blood samples were obtained from B-CLL patients, staged according to the Binet system, during routine diagnosis at the Leicester Royal Infirmary with patient consent and local ethical committee approval. Primary B-CLL cells were kindly purified by Mr Roger Snowden (MRC Toxicology Unit, Leicester, UK). Briefly, whole blood was layered over Histopaque (Sigma) and B-CLL cells purified as previously described {Almond, 2001 #85}. T-cells were then depleted using anti-CD3 Dynabeads (Dynal, Merseyside, UK) which resulted in B-cells with a mean average purity of over 95 % $CD19^+CD5^+$ B-CLL cells. The purified B cells were then resuspended and cultured in RPMI 1640 medium containing 10 % FCS.

2.2.3 Annexin V and Propidium Iodide Staining

Apoptotic cells were quantified by measuring externalised phosphatidylserine as assessed by Annexin V and propidium iodide (PI) labelling {Martin, 1995 #54}. Non-adherent cells were collected by centrifugation and resuspended in Annexin buffer (10 mM HEPES/NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). Cells were then stained for 10 min on ice with Annexin V-FITC (Bender Medsystems). Propidium iodide (10 μl, 2 μg/ml in PBS) was then added for a further 2 minutes after which cells were analysed on a Facscan flow cytometer using CELLQuest software (Becton-Dickinson). Adherent cells in 6-well plates were first washed with PBS (medium and washes were kept and centrifuged to collect dead "floating" cells) then removed from plates using a trypsin/EDTA solution. Cells were resuspended in fresh medium and

incubated at 37 °C for 10 min to recover, after which they were centrifuged, resuspended in Annexin buffer and stained as described above for non-adherent cells.

2.2.4 Assessment of Cell Surface TRAIL Receptor Expression.

Cells were treated as indicated then washed twice by centrifugation (300 g, 3 min) in ice-cold PBS. Non-specific antibody binding was blocked by incubating cells in blocking buffer (10 % normal goat serum in PBS) for 30 min on ice. Cells were then incubated with anti-TRAIL receptor antibodies (2.3.4) or an isotype-matched control antibody for 1 h on ice. After a further two washes with ice-cold PBS, cells were incubated with goat anti-mouse FITC-conjugated ($F(ab)_2$ fragment) (DAKO) diluted in blocking buffer for 1 h on ice. Cell were then washed twice with ice-cold PBS and analysed immediately by flow cytometry.

2.2.5 Production of Cytosolic Fractions

Cells were treated as indicated, then collected by centrifugation (300 g, 3 min) followed by two washes with ice-cold PBS. Cells were incubated with lysis buffer buffer (20 mM HEPES pH 7.4, 250 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, Complete™ protease inhibitors (Roche)) containing 0.25% digitonin on ice for 20 min. The samples were then centrifuged at 20,000 × *g* for 10 min, and the cytosolic fractions (supernatants) were collected. Cytosolic fractions were then separated by SDS-PAGE and immunoblotted for cytochrome *c* and the IAP antagonist, Smac.

2.3 Biochemical Techniques

2.3.1 Preparation of Samples for SDS-PAGE

For Western blot analysis of whole cells, treated cells were washed once with ice-cold phosphate buffered saline (PBS) and then solubilised with an appropriate volume of Laemmli sample buffer. To enhance solubilisation, samples were also sonicated using an MSE sonicator (5 sec/on, 2 sec off for 5 cycles). Samples were then boiled for 5 min and centrifuged at 13,000 rpm (1 min) prior to loading. Once in sample buffer, samples were stored at -80 °C until required.

Laemmli Sample Buffer

60 mM Tris/HCl (pH 6.8)

2 % (w/v) sodium dodecyl sulphate (SDS)

15 % (v/v) glycerol

0.05 % (w/v) Bromophenol Blue

5 % (v/v) β-2-mercaptoethanol

Resolving Gel Buffer

1.5 M Tris/HCl (pH 8.8)

0.4% (w/v) SDS

Stacking Gel Buffer

0.5 M Tris/HCl (pH 6.8)

0.4 % (w/v) SDS

	15%	*13% Resolving*	*12% Gel*	*10%*	*7%*	*4% Stacking Gel*
Gel Buffer	6.25	6.25	6.25	6.25	6.25	5
30% w/v Acrylamide/Bisacrylamide mix	12.5	10.83	10.0	8.33	5.83	2.7
Water	6.08	7.76	8.6	10.26	12.76	12.3
10% Ammonium Persulphate	150µl	150µl	150µl	150µl	150µl	150µl
TEMED[1]	20µl	20µl	20µl	20µl	20µl	20µl

[1]TEMED: N,N,N' ,N'-tetramethylethylenediamine

Table 2.1 Recipes for Resolving and Stacking polyacrylamide gels.

The Mini-Protean II gel system (Bio-Rad) was used for gel electrophoresis, and set up according to the manufacturer's instructions. Resolving and stacking gel solutions were made up as indicated in Table 2.1. The gel percentage used depended on the mass of the protein of interest.

2.3.2 Immunostaining of Proteins on Nitrocellulose Membranes (Western blotting)

After electrophoresis proteins were transferred to Nitrocellulose membranes: Hybond C (Amersham Pharmacia Biotech) essentially as described (Towbin *et al.*, 1992). After transfer membranes were washed briefly with TBST (20 mM Tris/HCL (pH 7.6), 150 mM NaCl containing 0.1 % Tween-20) then "blocked" for 1 h with TBSMT (TBST containing

5 % Marvel^{TM}). After a brief wash with TBST, the membranes were incubated with the relevant primary antibody (Table. 2.2) for 1 h followed by a secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h. Membranes were washed with TBSMT then TBST after incubations. Blots were then developed using the enhanced chemiluminescence system (ECL, Amersham) and proteins visualised on X-ray film (Kodak; NY, U.S.A.).

2.3.3 Commassie Blue staining of proteins on PAGE gels

After SDS-PAGE gels were stained with Coomassie blue stain (0.25 % Coomassie Blue R250, 45 % Methanol, 10 % Acetic Acid) for 1 h at room temperature. Gels were then destained in successive washes with destain solution (10 % glacial acetic acid and 50 % methanol).

2.3.4 2-Dimensional Analysis of Proteins using Isoelectric Focussing (IEF).

2-dimensional electrophoresis was carried out using the IPGphor system (Amersham-Pharmacia) essentially as described by the manufacturer. Briefly, DISC samples were resuspended in rehydration buffer (8M Urea, 2% CHAPS, 2% IPG buffer, pH4-7, trace bromophenol blue) and mixed at room temperature for 4 h. Samples were then applied to IPG (Immobilized pH Gradient) strips with a pH range of 4-7. Strips were then subjected to active rehydration at 50 V for 12 h followed by IEF (500 V, 30 min - 1000 V, 30 min - 8000 V, 1 h). After focussing, strips were equilibrated in SDS equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M Urea, 30% (w/v) glycerol, 2% SDS, trace bromophenol blue) for 15 min at room temperature. Second-dimension electrophoresis and Western blotting was carried out as described above (2.3.1 and 2.3.2).

2.3.4 Antibodies

Antibody	Species	Dilution	Source
Bid	rabbit (p)	1/2000	Dr. X. Wang, University of Texas, USA
Bid	rabbit (p)	1/1000	Cell Signalling (Beverley, MA,USA)
Caspase-2	rabbit (p)	1/500	Santa Cruz (Santa Cruz, CA, USA)
Caspase-3	rabbit (p)	1/2000	Dr. D. Nicholson (Merck Frosst, Canada)
Caspase-8	rabbit (p)	1/2000	Dr. X. Sun (Sun *et al.*, 1999)
Caspase-10	mouse (mAb)	1/1000	MBL (Nagoya, Japan)
Cytochrome c	mouse (mAb)	1/1000	BD Pharmingen (CA, U.S.A)
FADD	mouse (mAb)	1/500	BD Transduction Labs (CA, U.S.A)
FLIP/Usurpin	rabbit (p)	1/3000	Dr. D. Nicholson (Rasper *et al.*, 1998)
PARP (C-2-10)	mouse (mAb)	1/10,000	Dr. G. Poirier (Laval University, Quebec, Canada)
RIP	mouse (mAb)	1/500	BD Transduction Labs
Smac	rabbit (p)	1/2000	Dr. X. Sun (Sun *et al.*, 2002b)
TRADD	mouse (mAb)	1/500	BD Transduction Labs
TRAF-2	mouse (mAb)	1/500	BD Pharmingen
TRAIL-R1	mouse (mAb)	1/1000	Immunex Corp. (Griffith *et al.*, 1999a)
TRAIL-R2	mouse (mAb)	1/1000	Immunex Corp. (Griffith *et al.*, 1999a)
TRAIL-R3	mouse (mAb)	1/1000	Immunex Corp. (Griffith *et al.*, 1999a)
TRAIL-R4	mouse (mAb)	1/1000	Immunex Corp. (Griffith *et al.*, 1999a)
anti-mouse IgG-HRP conjugate	goat (p)	1/1500	Sigma
anti-rabbit IgG HRP conjugate	goat (p)	1/1500	Dako (Cambridge, UK)
Streptavidin-HRP conjugate	n/a	1/2000	Amersham

mAb - Monoclonal Antibody
p - Polyclonal Antibody

Table 2.2 Sources of Antibodies used in studies.

2.4 Molecular Biology Protocols

2.4.1 Plasmids

pcDNA3.1 was obtained from Invitrogen (Paisley, UK). TRAIL-R1, -R2, R3 and -R4 in pcDNA3 and pet28b-TRAIL (residues 95-281) and pRSC (a bicistronic vector containing a β-galactosidase reporter) were gifts from Dr Emad Alnemri (Kimmel Cancer Centre, Philadelphia, USA) and have been described elsewhere (MacFarlane *et al.*, 1997). p(NF-κB)$_4$-tk-sPAP (secreted Placental Alkaline Phosphatase), pRSV-lactamase and pcDNA3.1 wsl-1 were gifts from Glaxo Smithkline, Stevenage, Herts UK. pCMV2-I-κBM (S32A/S36A) was obtained from Clontech (Hants, UK). pGEX 4T.1 was obtained from Amersham Pharmacia Biotech.

2.4.2 Bacterial Strains and Culture Conditions.

Escherichia Coli strains DH5-α and BL-21 (DE3) were obtained from Life Technologies Ltd. and were routinely grown and maintained on Luria agar containing the required antibiotic. For liquid cultures Luria-Bertani (LB) medium (10 g bacto-tryptone, 5 g bacto yeast extract, 10 g NaCl in 1L sterile water) Anachem (Beds, UK) was used and bacteria were grown at 37 °C with shaking.

2.4.3 Transformation of *E.Coli*

DH5α subcloning efficiency cells (1×10^8 transformants/μg DNA) were used for routine transformations. For transformation of ligation mixes (2.4.11), library efficienct DH5α cells (1×10^8 transformants/μg DNA) (Life Technologies Ltd.) were used. All procedures were carried out under aseptic conditions. DNA (200-500 ng) was added to cells which were then incubated on ice for 30 min prior to heat shock (42 °C, 45 secs). After being allowed to recover on ice for 2 min, 1ml of SOC medium (Life Technologies Ltd.) was added and cells grown for 1 h at 37 °C. Cells were then plated onto the appropriate selective medium and grown overnight at 37 °C.

2.4.4 Preparation of Plasmid DNA

Plasmid DNA was prepared using kits supplied by Qiagen Ltd. (Surrey, U.K). Kits used depended on the quantity or quality of DNA required. Generally mini-preps kits provided sufficient quantities of DNA for everyday work (10-15 μg), and for general stocks,

however for transfection experiments larger quantities were required and hence maxi-preps were carried out. All kits are based on a standard protocol (Sambrook J *et al.*, (2002)). Bacteria are first subjected to alkaline lysis which denatures plasmid DNA, chromosomal DNA as well as cellular protein. Chromosomal DNA and proteins are then "salted out" and removed by centrifugation. Plasmid DNA is then collected from the lysate on a membrane and eluted using Milli-Q water.

2.4.5 Quantification of DNA

DNA was quantified by first diluting in Milli-Q water then measuring A_{260nm} using a DNA/RNA calculator (Amersham/Pharmacia Biotech). Double-stranded DNA (dsDNA) concentration was then calculated using the formula:

$$\text{dsDNA concentration } (\mu g/ml) = (A_{260} \times 100 \text{ (dilution)} \times 50)/1000$$

Purity of isolated DNA could be assessed using the A_{260nm}/A_{280nm} ratio. Plasmid DNA has an A_{260nm}/A_{280nm} ratio of 1.8.

2.4.6 DNA Electrophoresis on Agarose Gels

Purified and digested plasmid DNA was analysed using agarose gel electrophoresis essentially as described (Sambrook J *et al.*, (2002)). Briefly, agarose (0.5-2% w/v) was dissolved by heating in TAE buffer (40 mM Tris:Acetate (pH 8.5), 2 mM EDTA) and after cooling to ~50 °C ethidium bromide 0.5 μg/ml was added and the gel poured. DNA samples were prepared with 10× Orange G loading buffer (0.5% (w/v) Orange G, 25% (w/v) Ficoll-400, 20 mM EDTA) in Milli-Q water and applied directly to wells in the gel. Gels were electrophoresed in TAE buffer at 100 V for 30-60 min.

2.4.7 Construct Generation

pcDNA3 MutTRAIL-R2 (ΔSer324-Ser369) and pet28b-TNF (Val55-stop) were constructed using standard molecular biology protocols (Sambrook J *et al.*, (2002)). For generation of pet28b TNF (Val55-stop) an Expressed Sequence Tag (EST) containing the whole coding sequence of human TNF (Accession number AA767151) was obtained from the human genome mapping project (HGMP) (Cambridge, UK). The coding sequence for residues Val55-stop was amplified by Polymerase Chain Reaction (PCR) as described in

2.4.9. using the following primers: upstream (5' CGC GGA TCC GGT GAT CGG CCC CCA GAG G 3') and downstream (5' CCG CTC GAG CGG TCA CAG GGC AAT GAT CCC AAA G 3') obtained from the Protein and Nucleic Acid Laboratory (PNACL, University of Leicester, UK). To facilitate cloning the upstream primer contained a BamH1 restriction site and the downstream an Xho1 site.

2.4.8 Precipitation of Primers

Primers (100 µl) were precipitated by the addition of 1 ml butanol. Following vortex mixing precipitated DNA was collected by centrifugation (13,000 g, 10 min) and dried under a vacuum. Pellets were resuspended in Milli-Q water and DNA quantified by measuring A_{260nm} using a DNA/RNA calculator (Amersham-Pharmacia Biotech). Primer concentration was then determined using the following formula:

$$\text{Concentration (pmol/µl)} = A_{260} \times \text{dilution} \times 100/(1.5\,N_A + 0.71\,N_C + 1.2\,N_G + 0.84\,N_T)$$

(where N is the number of residues of base A, C, G or T.)

2.4.9 Polymerase Chain Reaction (PCR)

Amplification of DNA fragments by PCR was performed using *Pfu* Turbo polymerase (Stratagene, Amsterdam, The Netherlands) according to the manufacturers instructions with the appropriate primers in a Perkin Elmer GeneAmp 9700 thermal cycler (Perkin Elmer, CA USA). All PCR reactions were gel purified before further manipulation.

2.4.10 Restriction Digestion of Plasmid DNA/PCR Products

Restriction enzymes and buffers were obtained from Life Technologies Ltd. and used according to manufacturer's instructions. Digestions were generally set-up and left for up to 16 h (37 °C), depending on the amount of DNA and enzyme efficiency. Digested DNA was purified by gel electrophoresis (2.4.6) followed by gel extraction using a Qiaquick™ gel extraction kit (Qiagen).

2.4.11 Ligation of Digested DNA Fragments

Gel-purified-restriction digested DNA with cohesive ends was ligated using T7 DNA ligase (Life Technologies Ltd.) according to the manufacturers instructions. A number of

ligation reactions were generally set up with differing vector:insert ratios. Ligation reactions were then incubated at 16 °C overnight in a Perkin Elmer 480 DNA Thermal cylcer. Control ligations containing vector only and vector with ligase were also set-up to ensure that vector had been effectively linearised by both restriction digests. Ligation reactions were then used to transform *E.Coli* library efficiency DH5α cells as described above (2.4.3). All constructs were sequence verified before use (PNACL).

2.5 Reporter Gene Assays

2.5.1 Transfection of DNA into Mammalian Cells

To perform NF-κB reporter assays, cells were transfected with reporter constructs p(NF-κB)$_4$-tk-sPAP (secreted Placental Alkaline Phosphatase) and pRSV-lactamase using Effectene^{TM} (Qiagen) as per manufacturer's instructions. When using DNA for reporter assays an endotoxin-free plasmid DNA maxi-prep kit was utilised as bacterial lipopolysaccharide (LPS) is a strong inducer of NF-κB activity (Hawiger *et al.*, 1999).

2.5.2 Secreted Placental Alkaline Phosphatase Assay (sPAP):

Medium from p(NF-κB)4-tk-sPAP-transfected cells was heat-treated (65 °C, 30 min) to inactivate any endogenous alkaline phosphatase activity; placental alkaline phosphatase is less temperature sensitive. Medium (30 µl) was then assayed for alkaline phosphatase activity in duplicate in 96-well microtiter plates (Nunc). 150 µl of p-nitrophenyl phosphate (200 µg/ml) in 1 M diethanolamine containing 0.5 mM $MgCl_2$ was added to each well. Plates were then incubated for up to 2 h at room temperature and the absorbance at 405 nm measured every 30 min in a Labsystems iEMS plate reader (Labsystems Affinity Sensors, Cambridge, UK).

2.5.3 β-Lactamase Assay:

Conditioned medium (30 µl) from pRSV-lactamase-transfected cells was assayed for β-lactamase activity in duplicate 96-well plates. 150 µl of Nitrocefin (Glaxo Smithkline, Stevenage, Herts, UK) (200 ng/ml in 50 mM NaK_2PO_4 (pH 7.0) containing 0.1% Triton X-100 (v/v)) was added and plates incubated at room temperature. Absorbance at 492 nm was then measured every 30 min for 2 h. sPAP data was normalised using β-lactamase measurements.

2.5.4 Staining of LacZ-Transfected Cells for Apoptosis Assessment.

Apoptosis measurements in transfected cells was assessed by co-transfecting cells with 250 ng of a β-galactosidase-encoding plasmid, pRSC-LacZ. Medium was removed, and cells were fixed for 15 min with 0.25 % glutaraldehyde in PBS. Following two washes with PBS, X-Gal stain solution was added and cells were stained for 16 h at 37 °C. Apoptosis in transfected cells was assessed by counting the number of blue cells displaying the morphological features of apoptosis compared with the total number of blue cells.

X-Gal stain solution:

40µl	1M $MgCl_2$
1ml	100mM $K_4Fe(CN)_6$
1ml	100mM $K_3Fe(CN)_6$
100µl	4% X-Gal in DMF (40mg/ml)
18ml	PBS

2.6 Preparation of Recombinant proteins

2.6.1 Induction of TRAIL/TNF in *E.Coli*

E.Coli (BL21) were transformed with 200 ng pet28b-TRAIL/pet28b-TNF as described (2.4.3). Single colonies were picked and used to inoculate a 10 ml LB-Kan cultures. Overnight cultures were then sub-cultured into 400 ml of LB-Kan and grown for 3 h. Cultures were then induced with 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) for a further 3 h at 27 °C. Bacteria were then pelleted at 5000 rpm for 5 min washed once with ice-cold PBS and stored at -80 °C until required. In order to assess protein induction, 1 ml of culture, prior and post IPTG stimulation, was routinely removed and the resulting pellets analysed by SDS-PAGE and Coomassie blue staining (2.3.3).

2.6.2 Purification of recombinant TRAIL/TNF

E. Coli pellets representing 400 ml cultures were first resuspended in 10 ml of lysis buffer (30 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10% Glycerol (w/v) 1% Triton X-100 (v/v), 10 mM β-glycerophosphate, 1 mM Na_3VO_4 and 5 mM NaF containing Complete™-EDTA free protease inhibitors (Roche Biochemicals), then sonicated on ice. Lysates were then incubated on ice for 30 min and sonication was then repeated. Following centrifugation at

15, 000 g for 30 min at 4 °C, supernatants were supplemented with 500 mM imidazole to a final concentration of 20 mM. 400 µl of Ni-NTA Agarose beads (Qiagen), previously equilibrated with lysis buffer, were then added and lysates were incubated for 2 h on an end-to-end shaker "daisy wheel" at 4 °C. Beads were pelleted at 1000 rpm for 3 min and washed with lysis buffer (6 × 800 µl).

2.6.3 Elution of unlabeled recombinant TRAIL/TNF

Following washes, bound TRAIL was eluted with 150 mM EDTA in PBS (6× 400 µl). Fractions 1-5 were immediately pooled and further purified on a Sephacryl S-200 (16/60) gel filtration column (Amersham Pharmacia Biotech) which had been previously equilibrated with 30 mM Tris/HCl (pH 7.5) and 150 mM NaCl. After the void volume had eluted, 25 × 2 ml fractions were collected and analysed by SDS-PAGE and Coomassie stained fractions containing TRAIL/TNF were pooled and assayed for protein content (2.6.6), aliquotted and stored at -80 °C until required.

2.6.4 Labelling of TRAIL/TNF with biotin

Following washes with lysis buffer, beads were washed with PBS (4 × 600µl), resuspended in 1 ml PBS and then labelled with 25 µl of D-Biotinoyl-ε-amidocaproic acid-N-hydroxysuccinimide ester (20 mg/ml) (Roche) on an end-to-end shaker for 1 h at 4 °C. Beads were then washed with lysis buffer (4 × 800 µl) and eluted with 150 mM EDTA and then subjected to gel filtration chromatography. Fractions from S-200 columns were then subjected to Coomassie staining to assess purity. Based on staining, peak fractions were pooled, aliquoted and stored at -80 °C until required. Protein concentration was measured using a colourimetric protein assay kit (2.6.6).

2.6.5 Assessment of biotin incorporation

To ensure that TRAIL/TNF had been biotin labelled, gel filtration fractions were blotted onto nitrocellulose. Following blocking with TBSMT, membranes were probed with streptavidin-HRP conjugate (1/2000) and blots then developed with ECL. The streptavidin conjugate directly labels any biotin labelled proteins.

2.6.6 Measurement of Protein Concentration

The Bio-Rad protein assay kit (Bio-Rad), is based on the Bradford method for determination of protein content (Bradford. 1976), was used according to the manufacturer's instructions. A standard curve was constructed using known concentrations of a bovine serum albumin (BSA) standard and the A_{595nm} was measured for each sample and standard in triplicate. Unknown protein concentrations were then calculated from the plotted BSA standard curve.

2.7 DISC Analysis

2.7.1 Precipitation of the TRAIL/TNF DISC

TRAIL and TNF DISCs were prepared from the following numbers of cells per treatment , HeLa and 293 cells (3×10^7), Jurkats (6×10^7), BCLL (600×10^7) and U937 (6×10^7) cells per treatment. Generally cells were treated with bTRAIL (1 μg/ml) or bTNF (200 ng/ml) for the indicated time periods after which they were washed ×3 with ice-cold PBS. Cells were then lysed by the addition of 3 ml of lysis buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100 containing Complete^{TM} protease inhibitors) for 30 min on ice. Lysates were then cleared by centrifugation at 13, 000 g (30 min, 4 °C) and receptor complexes precipitated by the addition of streptavidin-agarose beads (Sigma) and overnight incubation on an end-to-end shaker at 4 °C. Beads were washed with lysis buffer (5 × 400 µl) and complexes eluted directly by the addition of SDS-PAGE sample buffer.

2.7.2 Receptor intracellular domain interactions

The intracellular domains (ICDs) of TRAIL-R1 and -R2 fused to GST (glutathione-*S*-transferase) were provided by Dr Michelle Hughes (MRC Toxicology Unit, Leicester, UK). HeLa cells were treated with PMA (20ng/ml) for 30 min (control cells were untreated), washed with PBS and then harvested by trypsinisation. Cell pellets were resuspended in 3ml of lysis buffer (30mM Tris.HCl pH 7.5; 150mM NaCl; 10% (v/v) Glycerol; 1% (v/v) Triton-X100; 10mM β-glycerophosphate; 1mM Na_3VO_4; 5mM NaF containing complete protease inhibitors) and incubated on ice for 45 min. Aliquots of the supernatant containing 5mg protein at 10mg/ml were incubated for 16 h at 4 °C with 10µg of purified GST-TRAIL receptors bound to Sepharose beads. Control pulldowns were

carried out with purified GST alone. Bound proteins were pelleted by centrifugation at 1000 rpm for 3 min, washed five times in PBS containing protease inhibitors, and released from the beads by boiling for 5 min in SDS sample buffer. The binding of FADD to GST-TRAIL-R1/-R2 was assessed by Western blotting (2.3.2).

2.8 Statistical analysis of Reporter Assays

2.8.1 Comparison of two data series

Reporter assays are expressed as the average +/- SEM of three independent experiments. T- tests were carried out on each data set using a Bonferroni correction for two tests: Control vs Trail; Control vs TNF. *P* values of $p < 0.025$ and < 0.005 were considered to be significant and highly significant respectively.

carried out with purified GST alone. Bound proteins were pelleted by centrifugation at 1000 rpm for 3 min, washed five times in PBS containing protease inhibitors, and released from the beads by boiling for 5 min in SDS sample buffer. The binding of FADD to GST-TRAIL-R1/-R2 was assessed by Western blotting (2.3.2).

CHAPTER 3: ACTIVATION OF THE TRANSCRIPTION FACTOR NF-κB: CONSEQUENCES FOR TRAIL-INDUCED CYTOTOXICITY

3.1 Introduction

The first indication that NF-κB functions as a major survival pathway came from studies in RelA$^{-/-}$/p65$^{-/-}$ mice. This phenotype is lethal at embryonic day 15, with the mice exhibiting massive liver apoptosis (Beg *et al.*, 1995). It was later found that cells derived from these mice were extremely sensitive to the cytotoxic effects of TNF-α. Disruption of other components of the NF-κB pathway such as IKK-β and IKK-γ/NEMO also result in increased sensitivity to TNF as well as embryonic lethality and a similar phenotype and to that seen in RelA $^{-/-}$ mice (Li *et al.*, 1999a; Li *et al.*, 1999b; Makris *et al.*, 2000; Schmidt-Supprian *et al.*, 2000). Deletion of either TNF-α or the predominant TNF receptor, TNF-R1, rescues the lethal IKK-β$^{-/-}$ phenotype (Alcamo *et al.*, 2001; Doi *et al.*, 1999) providing further evidence for the role of NF-κB in preventing TNF-induced cell death.

The major signalling pathway initiated by members of the TNF-R family such as TNF-R1, wsl-1 and CD40 is activation of NF-κB (Baud *et al.*, 2001). TNF-R1, therefore, negatively regulates its own cytotoxic ability through activation of NF-κB. Sensitisation of cells to TNF can be achieved by inhibiting various steps in the NF-κB activation pathway. Inhibition of IKK phosphorylation, IκB-α degradation by the proteasome or inhibiting nuclear translocation of Rel complexes (Karin. 1998; Rothwarf *et al.*, 1999) all lead to increased sensitivity to TNF-induced cytotoxicity.

The protective effect mediated by NF-κB requires *de novo* protein synthesis as cells can also be sensitized to TNF by inhibitors of protein synthesis or transcription, such as, cycloheximide and actinomycin D respectively (Begg *et al.*, 1996; Van Antwerp *et al.*, 1996). The exact NF-κB-regulated genes which are responsible for mediating resistance to apoptosis is unknown. Several candidates have been identified and include IAP family members and a number of anti-apoptotic Bcl-2 family members (Wang *et al.*, 1998). In general, cytotoxic members of the TNF-R family, such as, CD95 weakly activate NF-κB and therefore do not require sensitisation by such agents (Baud & Karin. 2001). TRAIL is similarly cytotoxic, at least to tumour cells, in the absence of inhibitors of protein synthesis or inhibitors of transcription and would, like CD95, be expected to only weakly activate NF-κB.

The effect of NF-κB activation on TRAIL signalling is less well characterised than that by TNF. TRAIL has been demonstrated to be able to activate NF-κB activate in some cellular models but not others (Chaudhary *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Schneider *et al.*, 1997b; Sheridan *et al.*, 1997). Activation has been reported by

overexpression TRAIL-R1 and -R2 but as this also resulted in apoptosis in the transfected cells, the significance of this is unclear (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997b). TRAIL-R4 has, in some studies, been demonstrated to activate NF-κB and this together with its incomplete death domain motif this is suggested to be part of its function as a "decoy" receptor protecting cells from TRAIL cytotoxicity (Degli-Esposti *et al.*, 1997). In other studies, however, no NF-κB activation was observed with TRAIL-R4 overexpression (Marsters *et al.*, 1997a). None of the TRAIL receptors have been demonstrated to interact with the adaptor proteins required for TNF-mediated NF-κB activation such as RIP and TRAF2, TRAIL may therefore use different signalling intermediates to induce NF-κB activation (MacFarlane *et al.*, 2002). Finally, it is unclear whether NF-κB is capable of providing cellular resistance to TRAIL and if TRAIL can, in some models, negatively regulate its apoptotic arm by activation of NF-κB.

Experiments described in this chapter were therefore aimed at assessing if TRAIL activates NF-κB and the role that it may play in TRAIL signalling. Data presented in this chapter will also determine the TRAIL receptors responsible for NF-κB activation and the role NF-κB activation plays in TRAIL signalling. In particular, the role played by one of the TRAIL "decoy" receptors, TRAIL-R4, will be considered. NF-κB activation negatively regulates TNF-mediated cytotoxicity and therefore the effect of activation or inhibition of NF-κB on TRAIL-induced cytotoxicity will also be addressed.

3.2 Results

3.2.1 TRAIL activates NF-κB but only in cells which are not sensitive to TRAIL-induced apoptosis

An NF-κB reporter system was employed to assess TRAIL-induced NF-κB activation in HeLa and HEK293 (293) cells. The system consists of 4× κB consensus sequences upstream of a secretable placental alkaline phosphatase reporter gene. Transfected cells were treated with TRAIL for 24 h after which time conditioned medium was removed and analysed for alkaline phosphatase activity. Results were normalised against a second, β-lactamase, reporter construct.

TRAIL was found to produce a small, but reproducible, induction of NF-κB in 293 but not HeLa cells. In contrast, TNF-α, which was used as a positive control for NF-κB activation, produced a marked increase in reporter activity in both cell lines (Fig. 3.1 A). Because of the modest increase in reporter activity observed with TRAIL treatment, medium from cells was also analysed for the presence of IL-8, a known NF-κB gene product. Production of this "physiological NF-κB reporter" was essentially consistent with the data obtained from the reporter assay. No basal IL-8 production was observed in control 293 cultures, however, both TNF and TRAIL produced a marked increase in IL-8 production in these cells (Fig. 3.1 B). Induction of IL-8 by TRAIL was ~7 fold less than that induced by TNF and was similar to that observed with the reporter assay (compare Fig. 3.1 A and B). In HeLa cells, which displayed higher basal levels of IL-8, production was increased by a small extent by TRAIL and more extensively by

TNF (Fig. 3.1 B). In order to investigate whether there was any correlation between NF-κB activation in 293 and HeLa cells and the sensitivity of these cells to TRAIL-induced apoptosis, cleavage of PARP, a caspase-3 and -7 substrate, was assessed. In apoptotic cells, PARP is cleaved at a DEVD↓G motif to yield a characteristic 85 kDa fragment (Lazebnik *et al.*, 1994). Following exposure to TRAIL, PARP was cleaved to an 85 kDa fragment in HeLa but not in 293 cells indicating that HeLa but not 293 cells were sensitive to TRAIL-induced apoptosis (Fig. 3.2). Taken together these data suggested that the resistance of 293 cells to TRAIL-induced apoptosis may, in part, be linked to their ability to activate NF-κB in response to TRAIL.

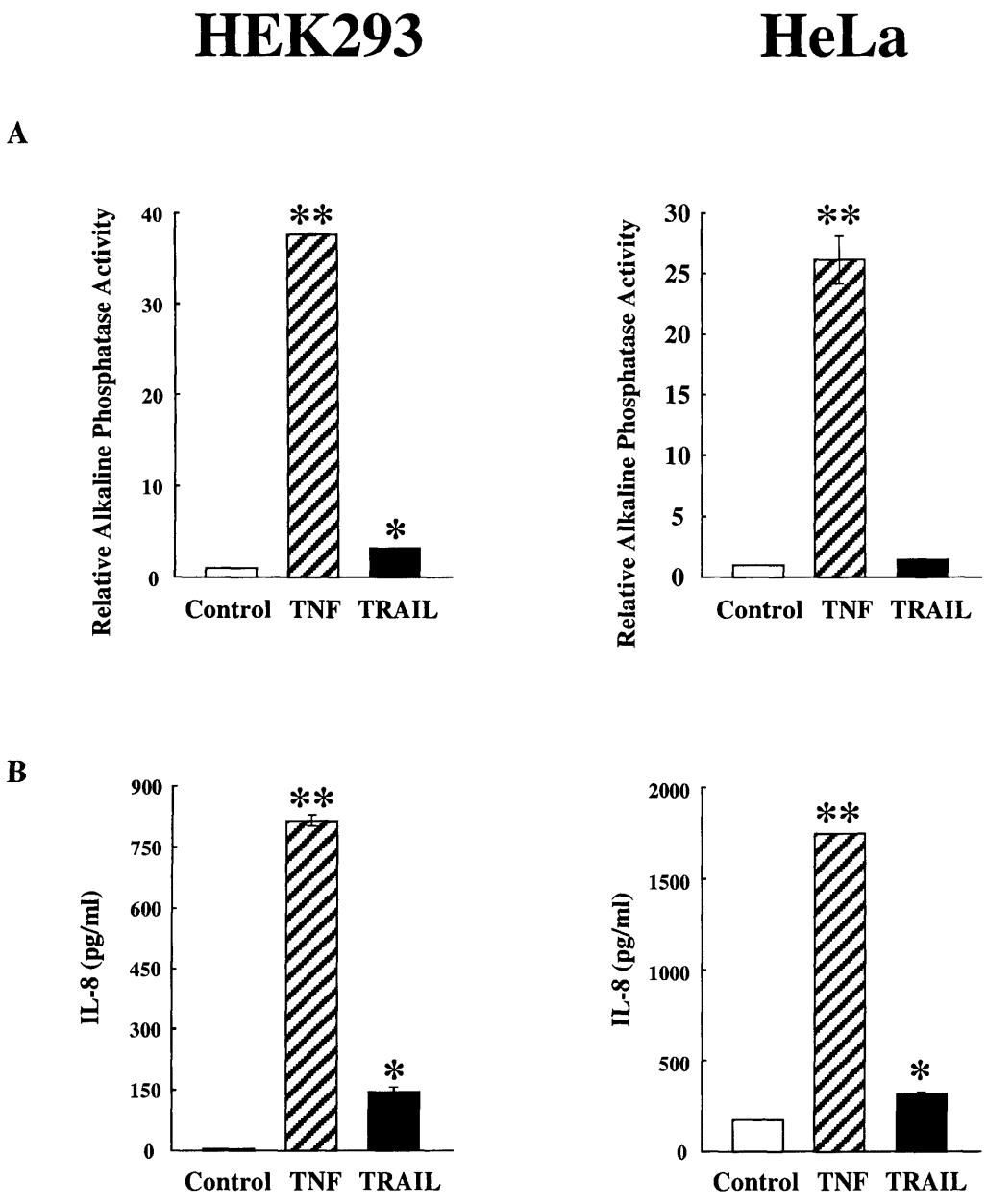


Figure 3.1 TRAIL activates NF-κB in HEK293 but not HeLa cells, activation is associated with an increased production of the NF-κB gene product interleukin-8 (IL-8). (A) HeLa and 293 cells were transfected with 0.1 μg of an NF-κB-alkaline phosphatase reporter construct and 0.1 μg β-lactamase reporter construct. Fresh medium was added 16 h after transfection and the cells treated with TNF-α (10 ng/ml) or TRAIL (1 μg/ml). Reporter gene activity was measured 24 h later and results were normalized using β-lactamase expression levels. (B) Medium was also assayed for production of IL-8 as described in *Materials and Methods*. Data are presented as fold-increase above control from three independent experiments and error bars represent the mean ± SEM. * p <0.025; ** p <0.005

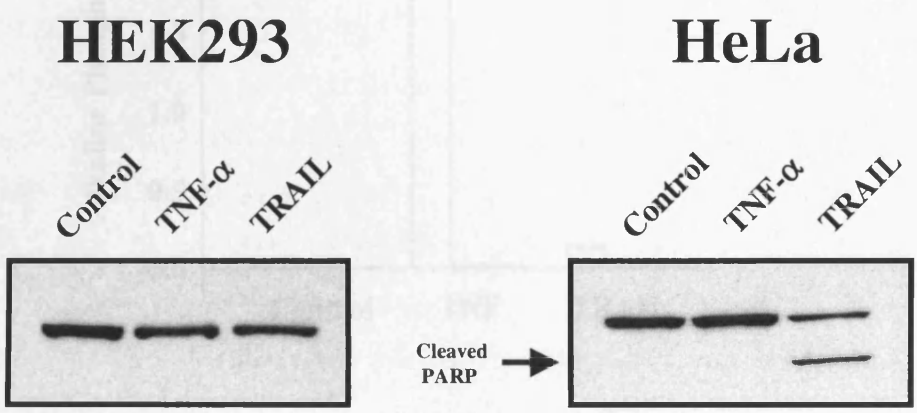


Figure 3.2 HeLa, but not HEK293, cells are sensitive to TRAIL-induced apoptosis. Cells from the experiment shown in Fig 3.1 were subjected to SDS-PAGE followed by Western blotting. Membranes were probed with a mouse monoclonal antibody against PARP. Arrows represent intact (118 kDa) or cleaved PARP (85 kDa).

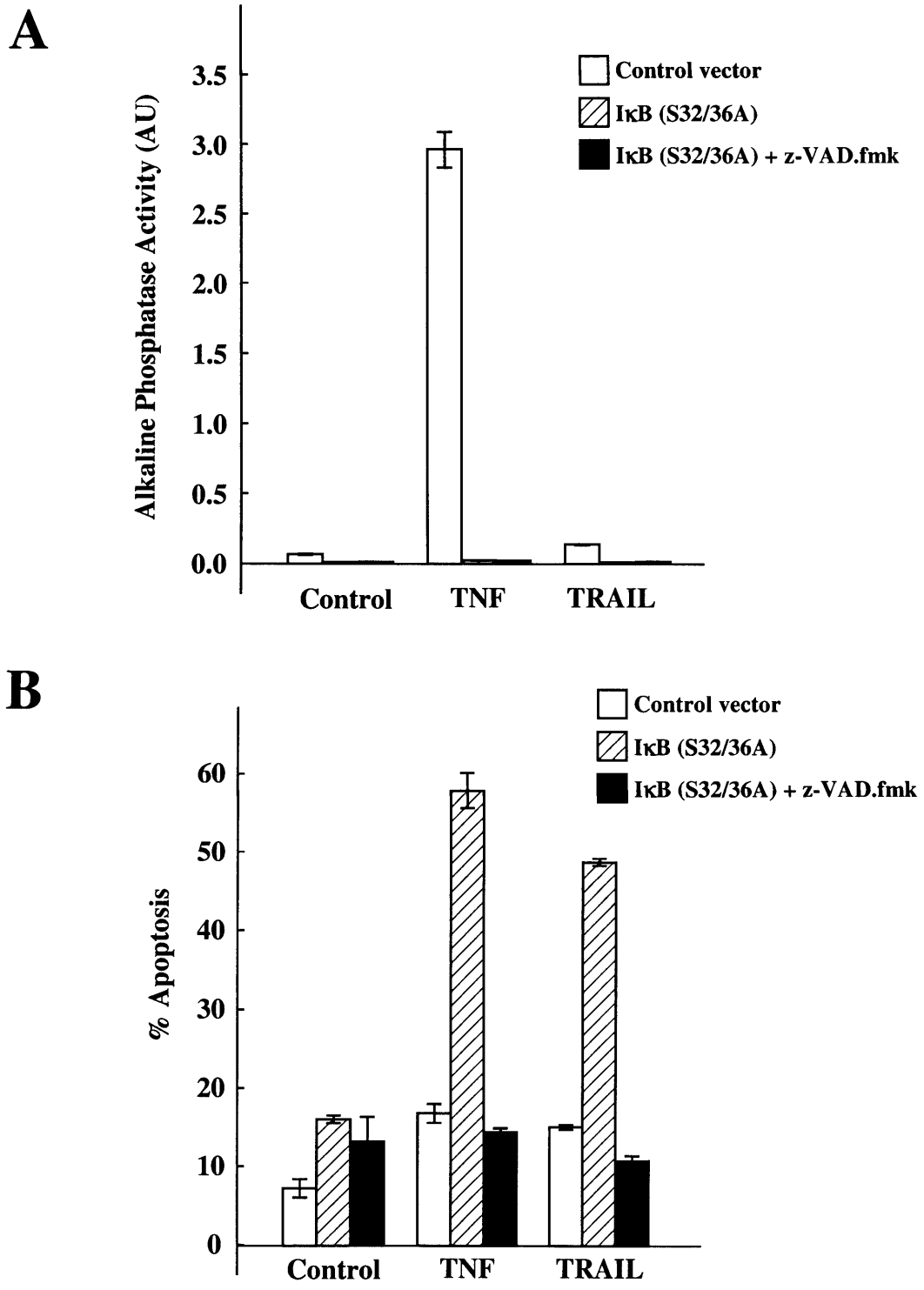


Figure 3.3 Inhibition of NF-κB sensitises 293 cells to TRAIL-induced apoptosis. (A) 293 cells were transfected with 0.1 μg NF-κB reporter, 50 ng of a β-galactosidase-containing construct, pRSC, together with 0.1 μg of pCMV-IκB-α (S32/36A). Medium was removed 16 h after transfection, fresh medium added, and the cells then treated with TNF (10 ng/ml) or TRAIL (1 μg/ml). Where indicated. z-VAD.fmk (20 μM) was used as a 1 h pre-treatment. After 24 h, medium was removed and assayed for alkaline phosphatase reporter activity. (B) Apoptosis in transfected cells was assessed by staining for β-galactosidase production using X-gal which produces a blue precipitate within transfected cells. Percentage apoptosis was assessed by comparing total blue cells in each well with the total number of blue cells displaying apoptotic morphology. All transfections were carried out in duplicate and the data presented represent three independent experiments. Error bars are ± the SEM.

3.2.2 Inhibition of NF-κB activation sensitises cells to TRAIL-induced apoptosis

In order to assess whether there was a relationship between the activation of NF-κB by TRAIL in 293 cells (Fig. 3.1 A) and their relative insensitivity to TRAIL-induced apoptosis (Fig. 3.2), NF-κB activation was blocked in these cells by overexpression of an IκB-α (S32/36A) mutant. This mutant cannot be phosphorylated by IKKs and thus blocks NF-κB activation by numerous stimuli including TNF (Brown *et al.*, 1995). Apoptosis was then assessed in transfected cells by co-expression of a β-galactosidase expression vector and morphological assessment of cells displaying apoptotic features as described in *Materials and Methods*. Overexpression of the IκB-α (S32/36A) mutant resulted in complete abrogation of both TNF and TRAIL-induced reporter activation (Fig. 3.3 A). In control vector-transfected cells, TNF and TRAIL induced a small amount of apoptosis, only ~5% above that observed in untreated control vector-transfected cells (Fig 3.3 B). However, in IκB-α (S32/36A)-transfected cells, both TNF and TRAIL induced marked apoptosis in 50-60% of transfected cells, which was completely abrogated by the poly caspase inhibitor z-VAD.fmk. These data demonstrate that blocking the NF-κB pathway in 293 cells can sensitise them to both TNF and TRAIL-induced apoptosis.

3.2.3 Activation of NF-κB by overexpression of the NF-κB-inducing kinase (NIK) protects TRAIL sensitive cells from TRAIL-induced apoptosis

In order to determine the potential role of NF-κB in ameliorating TRAIL-induced apoptosis, HeLa cells were transfected with the NF-κB-Inducing Kinase (NIK). Overexpression of NIK potently activates NF-κB (Malinin *et al.*, 1997) and catalytically inactive forms of NIK block NF-κB activation in response to a number of stimuli including TNF-α (Song *et al.*, 1997).

Overexpression of NIK led to activation of the reporter system which was abrogated in the presence of the IκB-α (S32/36A) mutant (Fig. 3.4 A). Only a small amount (10 ng) of the NIK vector was required to produce a large activation of the NF-κB reporter. When these cells were then exposed to TRAIL for 2 h, ~70% apoptosis was evident in control-transfected cells over this period. Although transfection of NIK alone induced some background apoptosis, the NIK-transfected cells were much less sensitive to TRAIL-induced apoptosis than control- transfected cells (Fig. 3.4 B). Co-expression of NIK with the IκB (S32/36A)

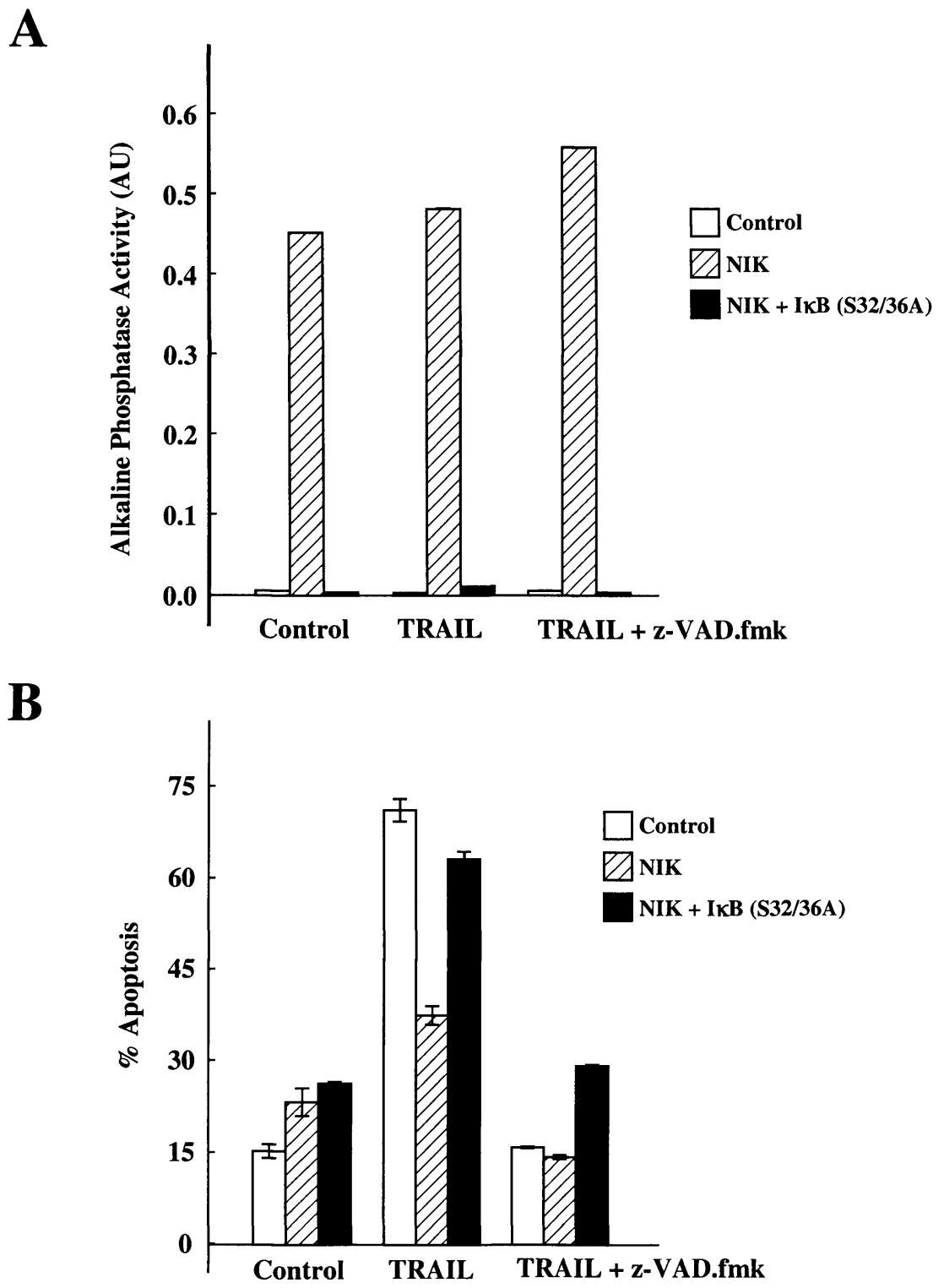
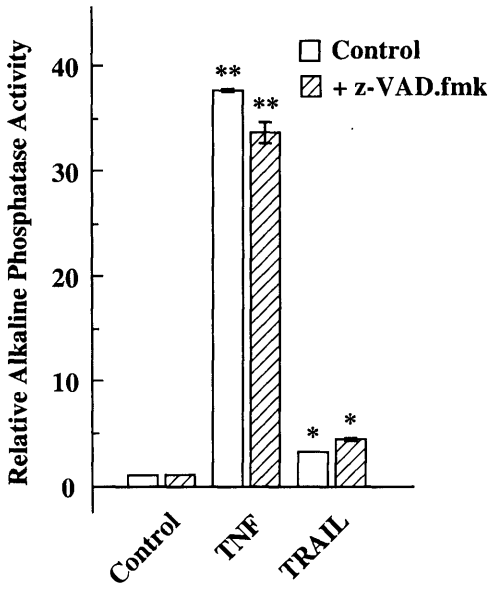


Figure 3.4 Upregulation of NF-κB by overexpression of the NF-κB-Inducing Kinase (NIK) protects HeLa cells from TRAIL-induced apoptosis. (A) HeLa cells were transfected with 0.1 μg of NF-κB reporter, 50 ng of a β-galactosidase-containing construct pRSC together with 5 ng of pcDNA3-NIK. Where indicated NIK-induced-NF-κB activation was blocked by co-transfection of pCMV-IκB (S32/36A). Medium was removed 16 h after transfection, and assayed for reporter activity. (B) Fresh medium was added and cells were further incubated for 2 h, either alone, or in the presence of TRAIL (1 μg/ml). Z-VAD.fmk (20 μM) was used where indicated as a pretreatment (30 min). Cells were then stained with X-gal, and apoptosis was assessed by comparing the total number of normal blue cells in each well to the number of blue cells displaying morphological features of apoptosis such as membrane blebbing and nuclear condensation. All transfections were carried out in duplicate and the data presented represent three independent experiments. Error bars are ± the SEM.

HEK293



HeLa

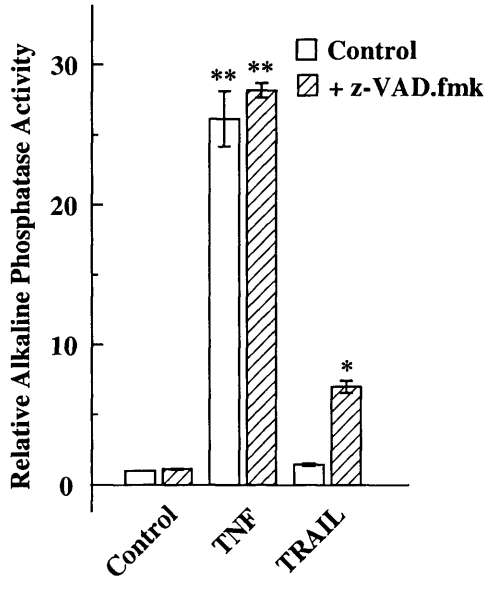


Figure 3.5 Effect of z-VAD.fmk on TRAIL-induced NF-κB Activation. 293 and HeLa cells were transfected with NF-κB and β-lactamase reporter constructs. 16 h after transfection medium was replaced and cells were treated with TNF (10 ng/ml) or TRAIL (1 μg/ml). z-VAD.fmk (20 μM) was included as a 1 h pretreatment where indicated and reporter gene activity was measured 24 h after treatment. All transfections were carried out in duplicate and the data presented represent three independent experiments. Error bars are ± the SEM. * p <0.025; ** p <0.005

mutant completely blocked NIK-induced NF-κB activation (Fig. 3.4A) and restored the TRAIL sensitivity of the cells (Fig. 3.4 B). Addition of z-VAD.fmk (20 μM) prior to TRAIL treatment abrogated TRAIL-induced apoptosis in control vector-transfected cells and those that had previously been re-sensitised to TRAIL by transfection of the IκB mutant. These data demonstrate that a NIK-mediated NF-κB activation can protect HeLa cells against TRAIL-induced apoptosis, and that inhibition of this activation with an IκB (S32/26A) mutant can abolish this protective effect.

3.2.4 Inhibition of TRAIL-induced apoptosis by the poly-caspase inhibitor z-VAD.fmk reveals a caspase-sensitive NF-κB signalling component in TRAIL sensitive cells

The lack of NF-κB activation by TRAIL in HeLa cells may have been related to the sensitivity of these cells to TRAIL-induced apoptosis. In order to test this hypothesis, cells were pre-incubated with the poly-caspase inhibitor, z-VAD.fmk which blocks TRAIL-induced apoptosis by inhibiting activation of the apical caspase, caspase-8. Pre-incubation of HeLa cells for 1 h prior to treatment with TRAIL resulted in a marked increase in both NF-κB activation (Fig. 3.5) and IL-8 production (Fig. 3.6). In contrast, similar treatment of 293 cells with z-VAD.fmk before TRAIL treatment resulted in only a small increase in NF-κB activation over untreated cells (Fig. 3.5). TNF-induced NF-κB activation and IL-8 production were not increased by z-VAD.fmk pretreatment in either cell type, which is consistent with the inability of TNF to induce apoptosis at this time point. Taken together these data clearly demonstrated that TRAIL was capable of activating NF-κB in HeLa cells but only in the presence of an inhibitor of caspases suggesting that there was a caspase-sensitive component of TRAIL-induced NF-κB activation in these cells.

3.2.5 TRAIL-induced NF-κB activation is mediated by TRAIL-R1 and TRAIL-R2 but not by TRAIL-R3 or TRAIL-R4. TRAIL-R1 and -R2-mediated NF-κB activation in HeLa cells requires the presence of z-VAD.fmk

To examine the contribution of individual TRAIL receptors to the TRAIL-induced NF-κB activation observed, each of the four TRAIL receptors were overexpressed in the presence of the NF-κB reporter system. Expression of the two "death-inducing" TRAIL receptors, TRAIL-R1 and -R2 caused NF-κB activation in 293 (Fig. 3.7 A), but not HeLa

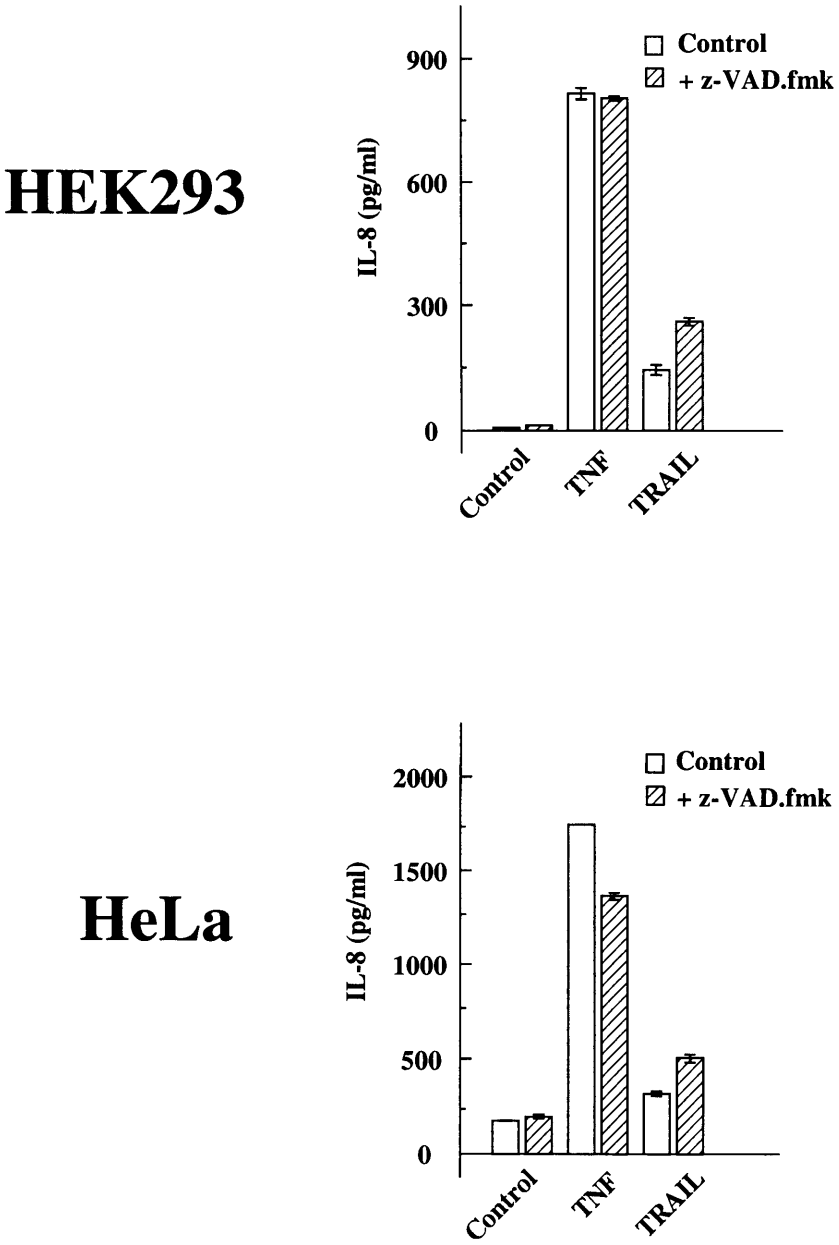


Figure 3.6 Effect of z-VAD.fmk on TRAIL-induced IL-8 Secretion. 293 and HeLa cells were transfected with NF-κB and β-lactamase reporter constructs. 16 h after transfection medium was replaced and cells were treated with TNF (10 ng/ml) or TRAIL (1 μg/ml). z-VAD.fmk (20 μM) was included as a 1 h pretreatment where indicated. 24 h after treatment medium was removed and IL-8 production measured by ELISA as described in *Materials and Methods*. All transfections were carried out in duplicate and the data presented represent three independent experiments. Error bars are ± the SEM.

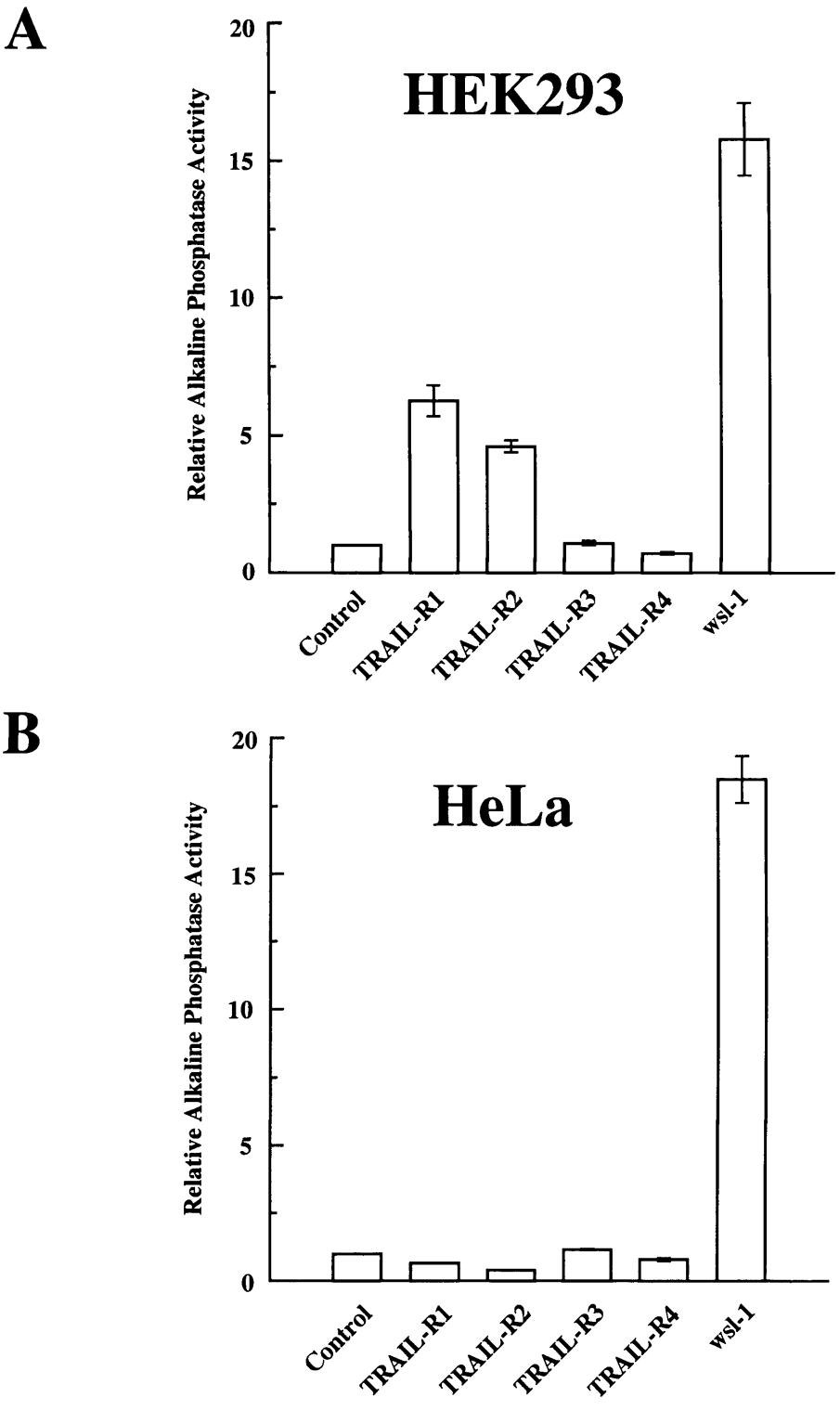


Figure 3.7 Overexpression of TRAIL-R1 and TRAIL-R2 activates NF-κB in 293, but not HeLa cells. In order to study the individual NF-κB signaling component of the individual TRAIL receptors, 293 (A) and HeLa (B) cells were transfected with the indicated TRAIL receptor construct (200 ng) in the presence of the NF-κB (100 ng) and β-lactamase (100 ng) reporters. A wsl-1-containing construct was used as a positive control for NF-κB activation and control transfections were supplemented with empty control vector. After 16 h the medium was changed and reporter assays performed after a further 24 h. Results were normalized using β-lactamase expression levels. Data presented represent three independent experiments and the error bars represent the mean ± SEM.

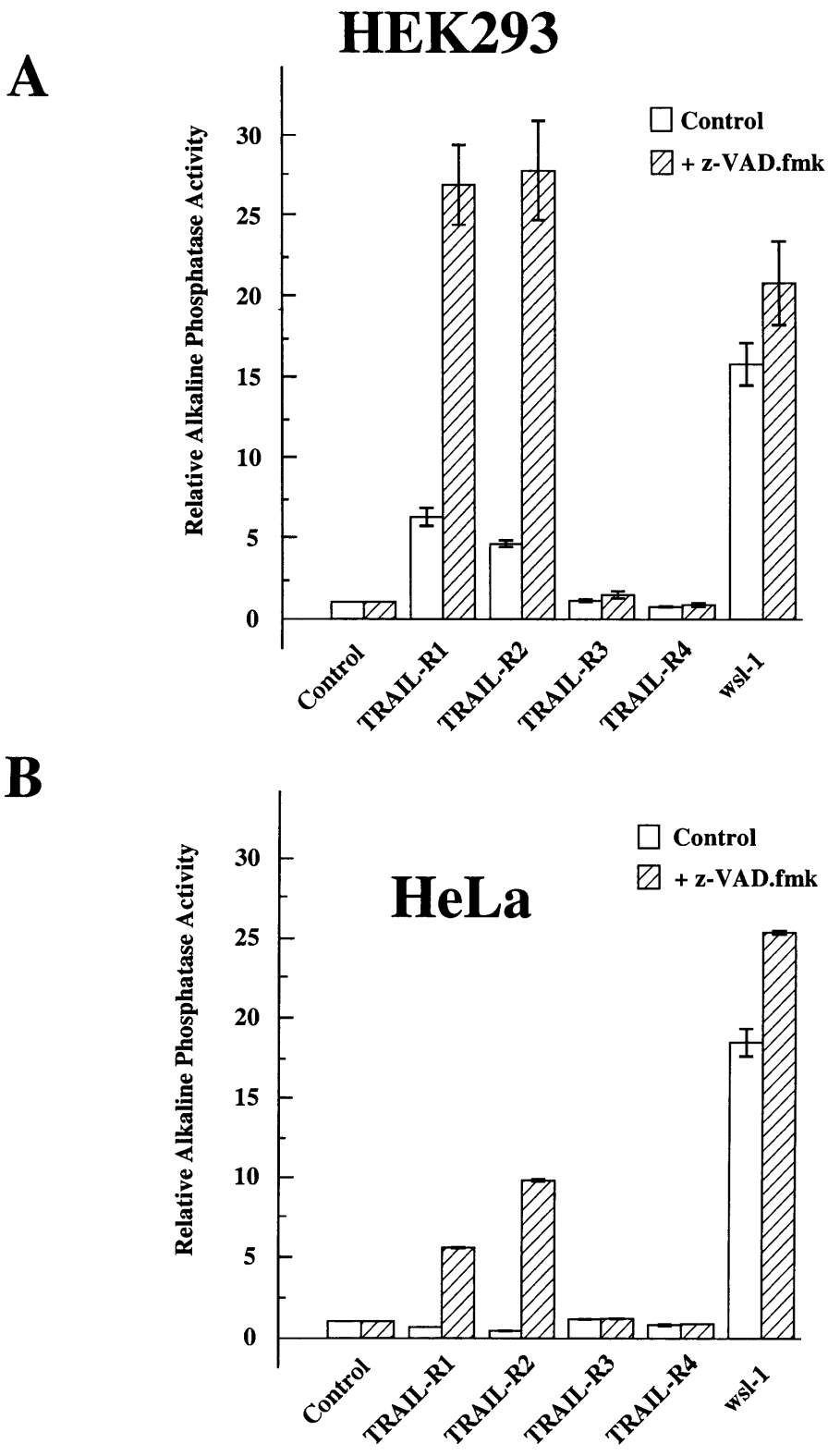


Figure 3.8 Requirement of caspase inhibition for TRAIL-R1 and -R2-mediated NF-κB activation in HeLa cells. 293 (A) and HeLa (B) cells were transfected as described in Figure 3.7. z-VAD.fmk (20 μM) was included where indicated at the time of transfection and also 16 h later when medium was changed. Reporter assays were performed after a further 24 h. Results were normalized using β-lactamase expression levels. Data presented represent three independent experiments and the error bars represent the mean ± SEM.

cells (Fig. 3.7 B). No NF-κB activation was observed in either cell type following overexpression of TRAIL-R3 or -R4. Activation of NF-κB by a related death receptor, wsl-1, occurred in both cell lines and was ~3-fold higher than that induced by TRAIL-R1 or -R2 in 293 cells. The lack of NF-κB activation in TRAIL-R3-transfected cells was unsurprising as this TRAIL receptor lacks a cytoplasmic domain and is therefore presumed to be incapable of engaging any intracellular signal transduction pathways (Degli-Esposti *et al.*, 1997). The lack of activation with TRAIL-R4 however, was surprising, and was in contrast to that reported in a number of other studies (Aicher *et al.*, 1999; Degli-Esposti *et al.*, 1997; Hu *et al.*, 1999).

In the presence of z-VAD.fmk, both TRAIL-R1 and -R2, which alone had no effect on NF-κB reporter activity (Fig 3.7 B), caused a significant increase in reporter gene activity in HeLa cells (Fig. 3.8 A). NF-κB activation by wsl-1 was also potentiated by z-VAD.fmk. Transfection of TRAIL-R1 and -R2 in 293 cells in the presence of z-VAD.fmk caused a large potentiation in reporter gene activity (Fig. 3.8A) when compared with untreated cells. NF-κB activation induced by wsl-1 was also potentiated but to a lesser extent. Although 293 cells are relatively resistant to TRAIL-induced apoptosis (Fig. 3.2), overexpression of death receptors such as Fas, TNF-R1 and TRAIL-R1 and -R2 results in ligand-independent receptor activation and thus extensive apoptosis (Boldin *et al.*, 1995), irrespective of the inherent sensitivity of these cells to the respective ligand. No NF-κB activation was evident in either cell line in response to TRAIL-R3 or -R4 overexpression in the presence or absence of z-VAD.fmk (Figs. 3.8 A and B). This inability of z-VAD.fmk to reveal an NF-κB component of TRAIL-R3 and -R4 signalling is presumably because these receptors are unable to either induce caspase-recruitment or activation. Taken together these data provide indirect evidence for TRAIL-R1 and -R2 -induced NF-κB activation being, in part, a caspase-sensitive process at least in cells that are sensitive to TRAIL-induced apoptosis. These findings are also consistent with these data obtained with TRAIL ligand in TRAIL-sensitive HeLa cells (Fig. 3.5).

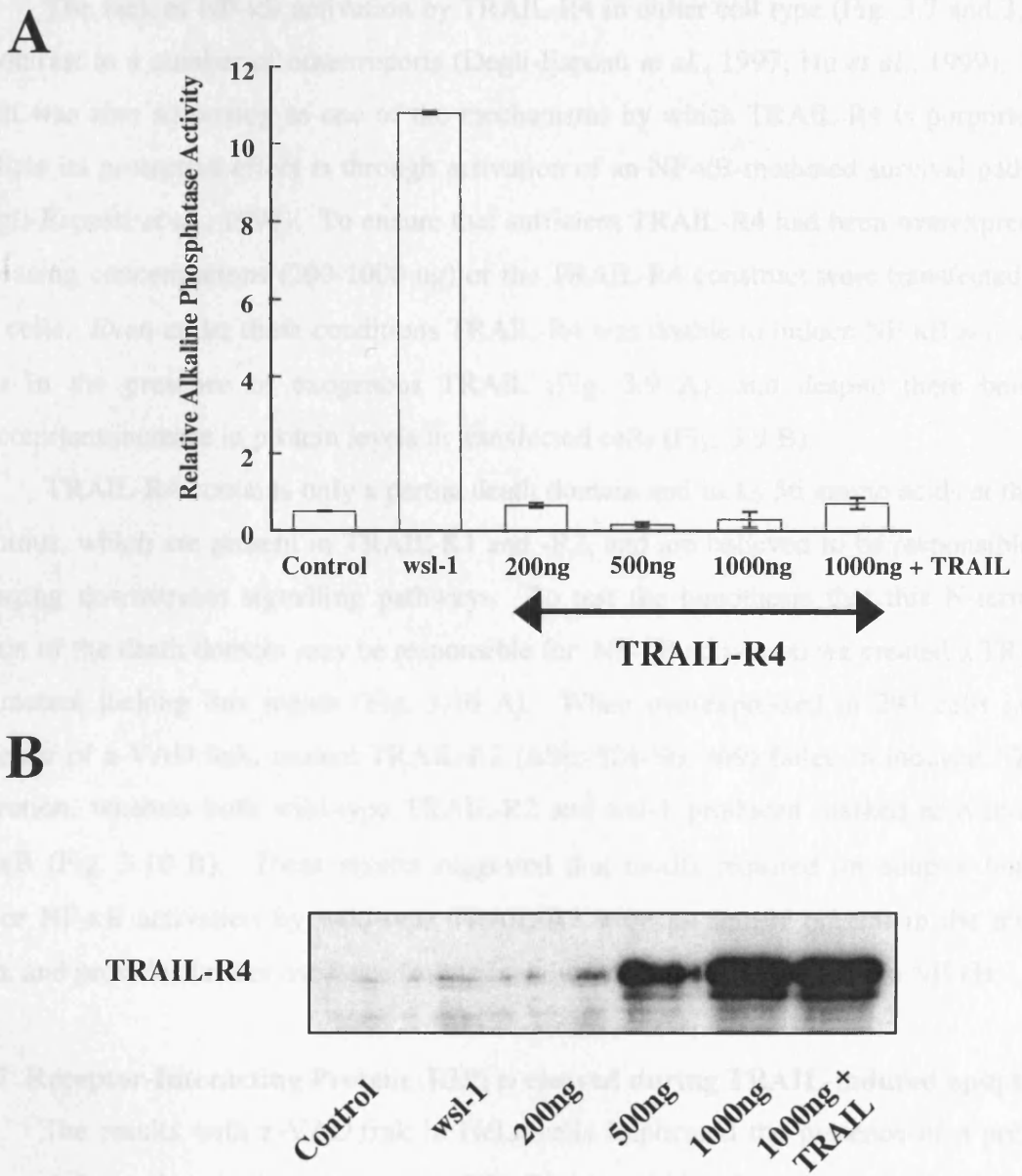


Figure 3.9 TRAIL-R4 overexpression does not activate NF-κB. (A) 293 cells were co-transfected with reporter constructs together with increasing amounts of a TRAIL-R4 encoding vector as indicated. wsl-1 was included as a positive control for NF-κB activation and control transfections were supplemented with empty vector. After 16 h, fresh medium was added and cells incubated for a further 24 h when reporter assays were performed. TRAIL (1 μg/ml) was included where indicated. (B) Transfected 293 cells were harvested at the time of reporter assay and subjected to Western blotting with an anti-TRAIL-R4 monoclonal antibody. Data presented represent three independent experiments, and the error bars represent the mean ± SEM.

3.2.6 TRAIL-R4 does not activate NF-κB

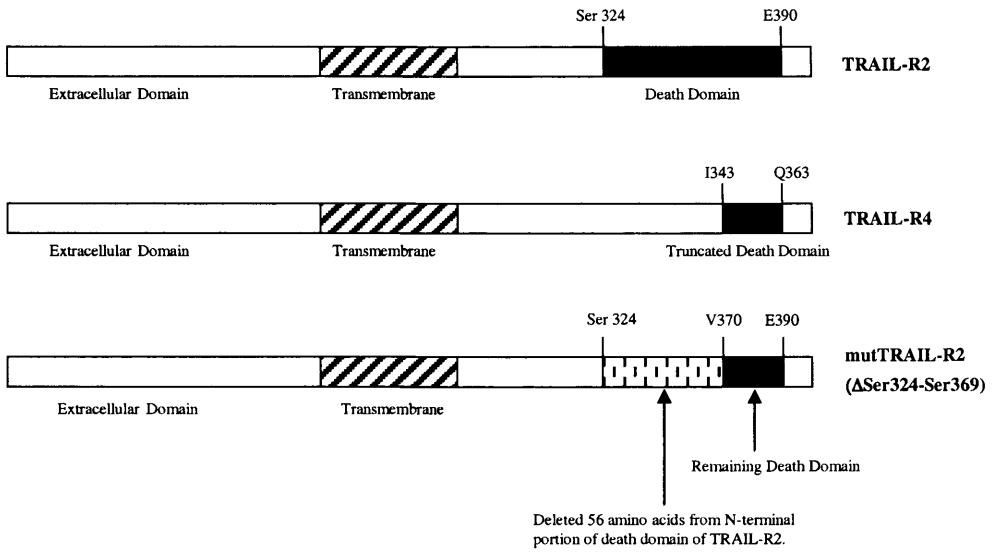
The lack of NF-κB activation by TRAIL-R4 in either cell type (Fig. 3.7 and 3.8) is in contrast to a number of other reports (Degli-Esposti *et al.*, 1997; Hu *et al.*, 1999). This result was also surprising as one of the mechanisms by which TRAIL-R4 is purported to mediate its protective effect is through activation of an NF-κB-mediated survival pathway (Degli-Esposti *et al.*, 1997). To ensure that sufficient TRAIL-R4 had been overexpressed, increasing concentrations (200-1000 ng) of the TRAIL-R4 construct were transfected into 293 cells. Even under these conditions TRAIL-R4 was unable to induce NF-κB activation even in the presence of exogenous TRAIL (Fig. 3.9 A), and despite there being a concomitant increase in protein levels in transfected cells (Fig. 3.9 B).

TRAIL-R4 contains only a partial death domain and lacks 56 amino acids at the N-terminus, which are present in TRAIL-R1 and -R2, and are believed to be responsible for engaging downstream signalling pathways. To test the hypothesis that this N-terminal region of the death domain may be responsible for NF-κB activation we created a TRAIL-R2 mutant lacking this region (Fig. 3.10 A). When overexpressed in 293 cells in the presence of z-VAD.fmk, mutant TRAIL-R2 (ΔSer-324-Ser-369) failed to induced NF-κB activation, whereas both wild-type TRAIL-R2 and wsl-1 produced marked activation of NF-κB (Fig. 3.10 B). These results suggested that motifs required for adaptor binding and/or NF-κB activation by wild-type TRAIL-R2 were no longer present in the mutant form, and provides further evidence for the inability of TRAIL-R4 to activate NF-κB.

3.2.7 Receptor-Interacting Protein (RIP) is cleaved during TRAIL-induced apoptosis

The results with z-VAD.fmk in HeLa cells implicated the presence of a protein, whose cleavage by caspases, prevented TRAIL-induced NF-κB activation (Fig. 3.5). In the TNF-R1 signalling pathway, Receptor Interacting Protein (RIP), a death domain-containing kinase has been implicated in TNF-mediated NF-κB activation (Hsu *et al.*, 1996a). Cells derived from RIP $^{-/-}$ mice are unable to activate NF-κB in response to TNF-α and as a consequence these mice are hypersensitive to the cytotoxic effects of TNF (Kelliher *et al.*, 1998). RIP has also been recently been shown to be cleaved during TNF-induced apoptosis. This cleavage was found to be mediated by caspase-8 and resulted in the generation of a dominant-negative fragment which inhibits TNF-induced NF-κB activation (Lin *et al.*, 1999; Martinon *et al.*, 2000). By analogy, cleavage of RIP during TRAIL-induced apoptosis could explain the observation that activation of NF-κB in

A



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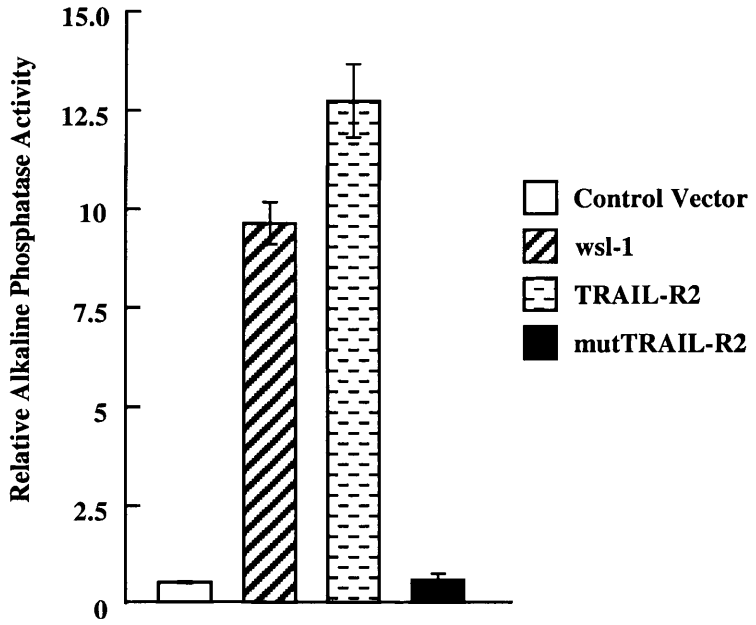


Figure 3.10 A TRAIL-R2 partial death domain mutant does not activate NF-κB. (A) Schematic illustration of wild-type TRAIL-R2, TRAIL-R4 and the TRAIL-R2 death domain mutant, mutTRAIL-R2 (ΔSer324-369). (B) 293 cells were co-transfected with reporter constructs together with 200 ng of the indicated receptor or mutTRAIL-R2 construct for 16 h in the presence of z-VAD.fmk (20 μM). Medium was then changed, fresh z-VAD.fmk added and cells incubated for a further 24 h before reporter assays were performed. Data presented represent three independent experiments, and the error bars represent the mean ± SEM.

TRAIL-sensitive HeLa cells only occurred when TRAIL-induced caspase activation was blocked (Fig. 3.5). Cleavage of RIP as well as the activation of caspase-8 was therefore studied by Western blotting. In HeLa cells, treatment with TRAIL resulted in the loss of the two pro-forms of caspase-8, p55/53, resulting in generation of the intermediate p43/41 and the large subunit, p18 (Fig. 3.11). TRAIL-treatment also resulted in the cleavage of RIP to a 42 kDa immunoreactive fragment, corresponding to the reported RIP cleavage product (Lin *et al.*, 1999), and cleavage of PARP to its well characterized 85 kDa product. Processing of caspase-8 and cleavage of RIP and PARP were completely inhibited by z-VAD.fmk, which is consistent with RIP cleavage being mediated by caspases (Fig. 3.11). No cleavage of caspase-8, PARP or RIP was observed in 293 cells under the same conditions, which is in agreement with the relative resistance of these cells to TRAIL-induced apoptosis (Fig. 3.11). These data demonstrated that a caspase-dependent cleavage of RIP was associated with TRAIL-induced apoptosis in HeLa cells and supports the hypothesis that caspase-mediated RIP cleavage could prevent TRAIL-induced NF-κB activation within these cells.

3.2.8 Contribution of effector caspases to RIP cleavage

In order to determine whether the cleavage of RIP was mediated entirely by caspase-8 as proposed (Lin *et al.*, 1999; Martinon *et al.*, 2000) or whether effector caspases such as caspase-3 also played a role, cleavage of RIP was studied in MCF-7 cells, which do not express functional caspase-3 (Janicke *et al.*, 1998b). TRAIL induced a time-dependent processing of caspase-8 to its p43/p41 and p18 fragments as well as cleavage of Bid (Fig. 3.12 lanes 1-7), a preferred caspase-8 substrate (Li *et al.*, 1998), both of which were completely inhibited by z-VAD.fmk (Fig. 3.12 lane 8). This was consistent with the ability of z-VAD.fmk to inhibit death receptor-induced apoptosis by inhibiting the processing of the apical caspase-8 to its active form. Interestingly, no cleavage of RIP or PARP was observed in MCF-7 cells (Fig. 3.12 lanes 2-7). These results support the hypothesis that caspase-8 was not solely responsible for the cleavage of RIP but rather that this cleavage was mediated, in part, by caspase-3.

In order to test this hypothesis, MCF7 cells that had been stably transfected with caspase-3 (Janicke *et al.*, 1998b) were used. As in the caspase-3 null cells, TRAIL induced a time-dependent processing of caspase-8 and its substrate Bid, however, generation of the active caspase-8 p18 subunit and truncated Bid was enhanced in the cells transfected with

caspase-3 (Fig. 3.12 lanes 9-15). Enhanced cleavage of these proteins could be due either to a direct effect of caspase-3 or due to the engagement of a positive feedback loop whereby active capase-3 directly or indirectly activates caspase-8, hence leading to the generation of increasing amounts of active caspase-8 and cleaved Bid. In the caspase-3-transfected MCF-7 cells, PARP also displayed a time-dependent processing to its 85 kDa product as a consequence of the presence of caspase-3 activity within these cells (Fig. 3.12 lanes 10-15).

Interestingly, RIP was also cleaved in these cells, and although no cleavage fragment was evident, there was a time-dependent loss of the full-length form, which was almost complete at 6 h (Fig. 3.12 lane 15). The lack of an observable RIP fragment in MCF-7 cells may have been due to its rapid degradation within these cells.

In agreement with RIP cleavage being a caspase-mediated event, no loss of intact RIP was observed in the presence of z-VAD.fmk (Fig. 3.12 lane 16). No loss of intact RIP was observed in caspase-3 null cells, although caspase-8 and Bid were cleaved. When caspase-3 was introduced, cleavage of these components was enhanced and there was also loss of intact RIP. This suggested that RIP cleavage in these cells was mediated directly by caspase-3 or indirectly through enhanced cleavage of caspase-8. These data support the hypothesis that caspase-8 is not solely responsible for cleavage of RIP, but that RIP cleavage is also mediated by caspase-3.

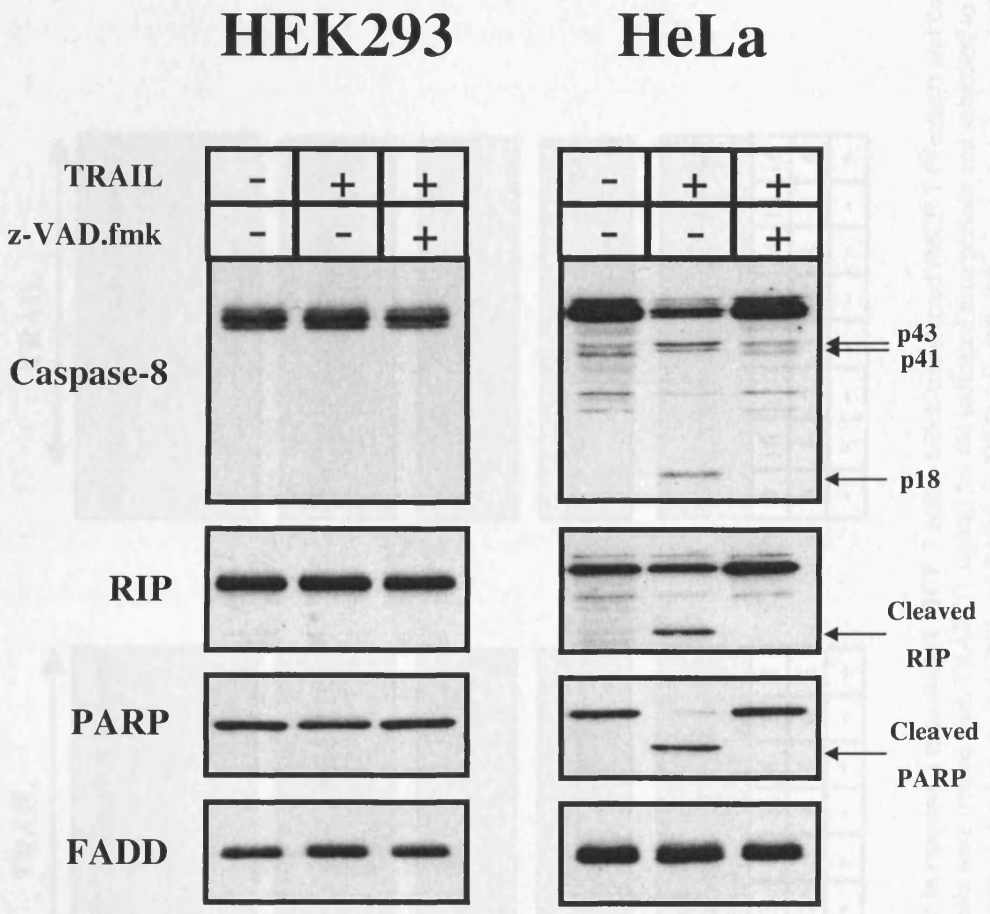


Figure 3.11 Caspase-8, RIP and PARP are cleaved after TRAIL treatment in HeLa but not 293 cells. 293 and HeLa cells were treated with TRAIL (1 μg/ml) for 6 h either in the presence or absence of z-VAD.fmk (20 μM). Cells were harvested and analysed by Western blotting using antibodies to caspase-8, RIP and PARP. The adaptor protein FADD was used as a protein loading control.

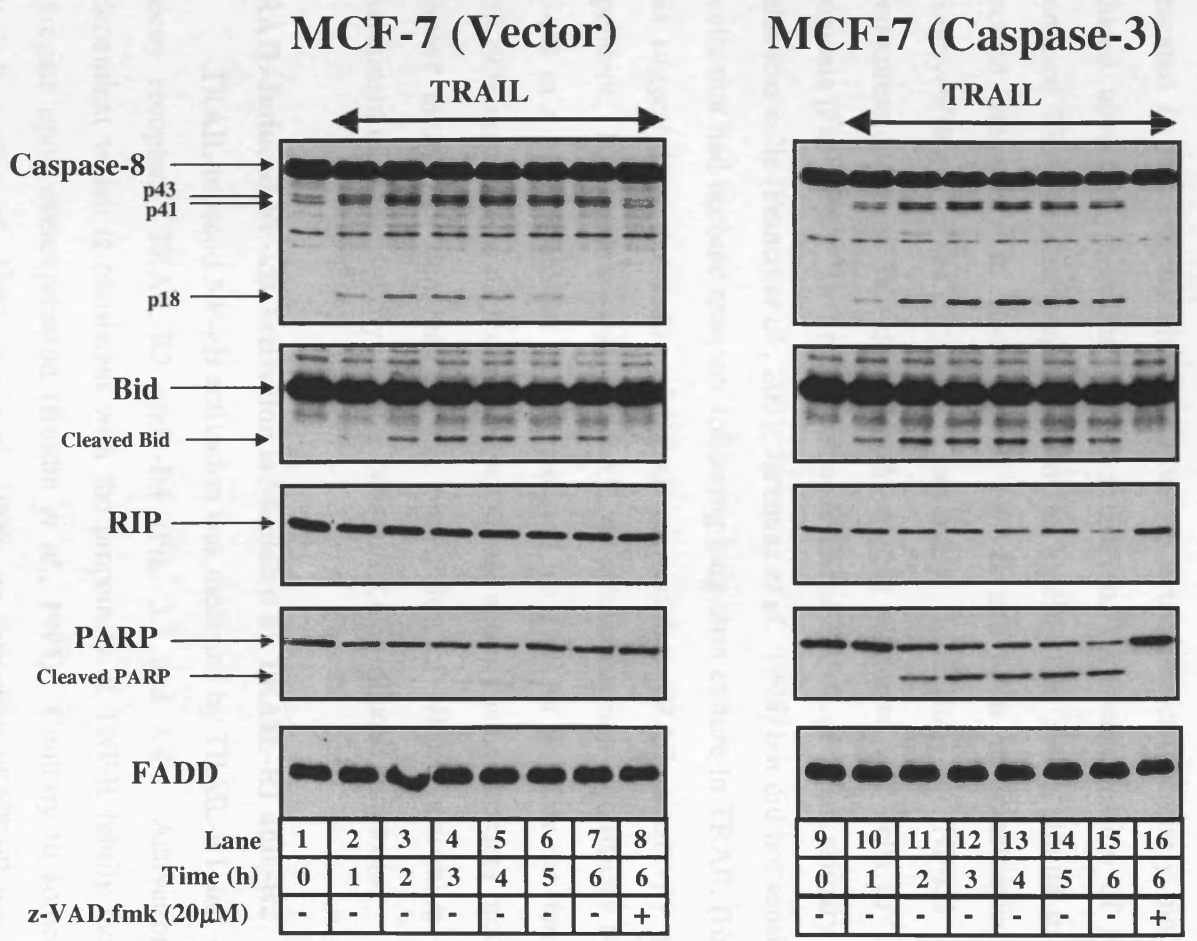


Figure 3.12 Enhanced processing of RIP in caspase-3 transfected MCF-7 cells. Mock-transfected (MCF-7 (Vector)) and caspase-3-transfected (MCF-7 (Caspase-3)) MCF-7 cells were treated with TRAIL (1 μg/ml) for the indicated time periods and subjected to Western blotting using antibodies to caspase-8, the caspase-8 substrate Bid, RIP and PARP. z-VAD.fmk (20 μM) was included where indicated as a 1 h pre-treatment. The adaptor protein FADD was used as a protein loading control.

3.3 Discussion

TRAIL-induced cytotoxicity can be antagonised by NF-κB activation

In most cell types the predominant downstream signalling event of TNF is not apoptosis but NF-κB activation. TNF negatively regulates its own cytotoxic ability through the upregulation of NF-κB-regulated anti-apoptotic genes (Wang *et al.*, 1998) and inhibition of NF-κB activation restores its cytotoxicity (Van Antwerp *et al.*, 1998). Data presented in this chapter show that NF-κB activation can similarly modulate TRAIL-induced apoptosis. Increased NF-κB activation, by overexpression of NIK, markedly decreased TRAIL-induced apoptosis in HeLa cells (Fig. 3.4B), similar to the protection reported previously in IL-1-induced NF-κB activation in transformed keratinocytes (Kothny-Wilkes *et al.*, 1998). Conversely, inhibition of NF-κB activation by overexpression of an IκB-α (S32/36A) mutant sensitised 293 cells to TRAIL-induced apoptosis (Fig. 3.3B). This mutant also sensitizes TRAIL-resistant primary leukaemic and melanoma cells (Franco *et al.*, 2001; Jeremias *et al.*, 1998) but did not sensitize HeLa-TL-R cells that had become resistant following long-term culture in TRAIL (Hu *et al.*, 1999). This suggests that the ability of NF-κB to modulate TRAIL sensitivity may be model-dependent. It is unclear whether TRAIL modulates its own cytotoxicity by activation of NF-κB in a manner similar to that reported for TNF, or whether resistant cells such as HEK293s may have a high constitutive NF-κB activity which confers protection. Taken together these data demonstrate that modulation of NF-κB activation can be a key determinant of the sensitivity of some cells to TRAIL-induced apoptosis.

TRAIL-Induced NF-κB Activation is Mediated by TRAIL-R1 and -R2

TRAIL-induced NF-κB activation was mediated by TRAIL-R1 and -R2 but not the "decoy receptors" TRAIL-R3 and -R4 (Fig. 3.7 and 3.8). Activation was ligand-independent which is consistent with the propensity of TNF-R family members to self-aggregate upon overexpression (Boldin *et al.*, 1995). Contrary to some other reports, (Degli-Esposti *et al.*, 1997; Hu *et al.*, 1999), no activation of NF-κB was observed with TRAIL-R4 despite gross overexpression and subsequent addition of TRAIL (Fig. 3.9B). Previous studies have proposed a model for the protection of cells by TRAIL-R4 via the activation of an NF-κB-mediated survival pathway (Degli-Esposti *et al.*, 1997). This model is not easily explained as TRAIL-R4 contains a truncated death domain, which

lacks a number of key residues conserved throughout the TNF-R family that have been implicated in cytotoxicity and survival signalling (Tartaglia *et al.*, 1993a; Tartaglia *et al.*, 1993b). A TRAIL-R2 mutant, containing a truncated death domain resembling that found in TRAIL-R4, was unable to activate NF-κB (Fig. 3.10B). This suggested that residues or motifs required for NF-κB activation were absent in this TRAIL-R2 mutant. When one such residue, Ile-225, is mutated to Asn in CD95, it is responsible for the lymphoproliferative (lpr) phenotype in mice (Eberstadt *et al.*, 1997). This residue is conserved in both TNF-R1 and TRAIL-R2 (Leu-351 and Leu-334, respectively) and, when similarly mutated, results in loss of receptor cytotoxicity (Chaudhary *et al.*, 1997; Tartaglia *et al.*, 1993b). Interestingly, this mutation has also been demonstrated to abolish TRAIL-R2-mediated NF-κB activation (Chaudhary *et al.*, 1997). The lack of any NF-κB activation by TRAIL-R4 observed in this study is in agreement with an earlier study (Marsters *et al.*, 1997a) and a very recent study (Meng *et al.*, 2000), which demonstrated that TRAIL-R4 is capable of protecting colon carcinoma cells from TRAIL-R2- and p53-mediated apoptosis. This protective effect was localized to the first 43 amino acids of the cytoplasmic domain and not within the remaining portion of the death domain. Thus TRAIL-R4 may mediate as yet unknown signalling pathways, which protect against TRAIL-induced apoptosis and are independent of NF-κB activation.

Caspase inhibition significantly potentiates TRAIL-induced NF-κB activation in TRAIL-sensitive cells

TRAIL-induced NF-κB activation, appeared to require a molecule(s), which was inactivated following caspase cleavage as no NF-κB activation was apparent in HeLa cells with TRAIL or TRAIL-R1 or -R2 overexpression unless z-VAD.fmk was present (Figs. 3.5 and 3.8). RIP is absolutely required for TNF-mediated NF-κB activation and its loss in cells results in them becoming hypersensitive to the cytotoxic effects of TNF (Barcena *et al.*, 1996). RIP has been implicated in receptor-mediated NF-κB activation through direct interaction with the IKK signalosome component, NEMO/IKK-γ (Zhang *et al.*, 2000a). A recent study has also provided evidence that RIP may be absolutely required for TRAIL-mediated NF-κB activation as no activation was observed in TRAIL-treated $RIP^{-/-}$ cells (Lin *et al.*, 2000). RIP is therefore clearly a candidate molecule, based on the observations that TRAIL induced a caspase-dependent cleavage of RIP in HeLa cells (Fig. 3.11), which, when prevented by z-VAD.fmk, led to a marked increase in NF-κB activation (Figs. 3.5).

Taken together, these data provided a potential mechanism for the potentiation of TRAIL-induced NF-κB signalling observed in the presence of z-VAD.fmk. The lack any of NF-κB activation in HeLa cells in the absence of z-VAD.fmk appears to support this hypothesis.

In summary, TRAIL is capable of activating NF-κB but this activation is much less pronounced than that observed with TNF-α. Activation is mediated by TRAIL-R1 and -R2 and appears to occur only in TRAIL-resistant cells or in TRAIL-sensitive cells which have been pre-treated with a caspase inhibitor. RIP, the serine/threonine kinase associated with TNF-R1 and obligatory for NF-κB activation by TNF was found to be cleaved during TRAIL-induced apoptosis implicating it as the caspase-sensitive component in TRAIL-induced NF-κB activation. Caspases have been demonstrated to be able to cleave a number of key components of the NF-κB pathway such as IKK-β, IκB-α and p65 (Barkett *et al.*, 1997; Levkau *et al.*, 1999; Reuther *et al.*, 1999). RIP however is upstream of all these other substrates at least in the TNF signalling pathway and may represent a more immediate caspase target. Although RIP was originally shown to be processed solely by caspase-8 in this pathway data presented in this chapter show, at least in TRAIL-induced apoptosis, it can also be processed by caspase-3 (Fig. 12). NF-κB activation was also demonstrated to be able to modulate TRAIL-induced cytotoxicity similar to that previously observed with TNF. NF-κB activation protected a TRAIL sensitive cell line and inhibition of NF-κB sensitised a TRAIL resistant cell. It however, seems unlikely that TRAIL actually negatively regulates its own cytotoxicity in a similar manner to TNF as TRAIL-induced NF-κB activation is much less pronounced than that induced by TNF. Rather, one would propose that cells with high constitutive NF-κB activation or which are receiving external survival prompts are protected from TRAIL cytotoxicity. TRAIL also induced a physiological NF-κB reporter, IL-8, whose production mimicked the transfected reporter (Figs. 3.1 and 3.5). TRAIL, therefore, like TNF, can also be regarded as a pro-inflammatory cytokine especially in TRAIL-resistant cells. This may be of importance when consideration of TRAIL as a chemotherapeutic agent as most normal cells are TRAIL-resistant.

CHAPTER 4: ANALYSIS OF THE NATIVE TRAIL DEATH-INDUCING SIGNALLING COMPLEX (DISC).

4.1 INTRODUCTION

Death receptors belong to the TNF/NGF receptor superfamily. They contain a number of conserved cysteine-rich repeats within the extracellular domain and a conserved region (~68 amino acids) within the intracellular domain which contains residues required for engagement of the cell death pathway and is thus termed the death domain (DD) (Tartaglia *et al.*, 1993b). The most extensively studied death receptor has been the Fas/CD95 system. Engagement of Fas/CD95 by FasL/CD95L results in trimerisation and clustering at the cell surface (Boldin *et al.*, 1995). This clustering leads to recruitment of the adaptor protein, FADD, a bipartite molecule containing an N-terminal DD and C-terminal death-effector domain (DED). FADD interacts with the Fas receptor through a homophillic DD-DD interaction and with procaspase-8 through DED and as such it acts as an adaptor protein bridging the gap between the intracellular domains of the death receptors and the DED-containing prodomain of the initiator caspases implicated in death receptor signalling such as caspase-8. Activation of procaspase-8 is thought to occur by trans-catalysis when neighbouring procaspase-8 molecules are brought into close proximity by cell surface receptor clustering (Muzio *et al.*, 1998). The resulting complex is known as the death-inducing signalling complex or DISC.

There is some controversy as to the components of the TRAIL DISC. Most of the TRAIL receptor interaction data was obtained from overexpression experiments and results obtained are contradictory, with some groups demonstrating a role for FADD in TRAIL signalling while others speculated that other, unspecified, proteins and not FADD were involved (Chaudhary *et al.*, 1997; MacFarlane *et al.*, 1997; Pan *et al.*, 1997b; Schneider *et al.*, 1997b). Data obtained from gene ablation studies has also proved inconclusive. Mouse embryonic fibroblasts (MEFs) are, in general, insensitive to the cytotoxic effects of TRAIL or lack TRAIL receptors and therefore do not undergo apoptosis when treated with TRAIL. As a result of this, the effects of TRAIL on caspase-8 null MEFs was impossible to test (Varfolomeev *et al.*, 1998). In a paper describing the phenotype of FADD-null mice and MEFs derived from these animals the authors circumvented this problem by overexpressing DR4 (TRAIL-R1), and showed that apoptosis induced by TRAIL-R1 overexpression was FADD-dependent (Yeh *et al.*, 1998). No data was provided on TRAIL-R2 and thus it was suggested that it may require an additional adaptor.

In order to try to address these inconsistencies concerning TRAIL receptor signalling this chapter describes the isolation and characterisation of the native TRAIL DISC. Data presented show that FADD, caspase-8 and c-FLIP are all components of the

TRAIL DISC. DISC formation was observed in both TRAIL resistant and sensitive cell lines, indicating that TRAIL resistance was not necessarily due to the inability to form a DISC. Isolates from resistant cells did however appear to have less capacity to recruit FADD and procaspase-8 despite containing equivalent amounts of death-inducing TRAIL receptors. RIP, the adaptor molecule implicated in TNF-R1-mediated NF-κB activation, was also found to be recruited and cleaved at the DISC providing further evidence of a role for NF-κB in TRAIL signalling. DISC formation was also observed in TRAIL-resistant primary B-cell Chronic Lymphocytic Leukaemia (B-CLL) cells. Furthermore, DISCs isolated from both TRAIL sensitive and resistant cells were shown to be functionally active as they were capable of processing exogenously added procaspase-8.

4.2 RESULTS

4.2.1 The adaptor protein FADD and caspase-8 are components of the TRAIL DISC.

To facilitate isolation of the TRAIL DISC recombinant TRAIL was labelled with a biotin ester as described in *Materials and Methods* (2.6.4). Biotinylated TRAIL (bTRAIL) had similar apoptosis-inducing properties to unlabelled TRAIL and had the added advantage that it could be precipitated by exploiting the high affinity and specific binding which biotin has for the protein avidin. To further extend the observations made in Chapter 3, HeLa and 293 cells were initially used as model cell lines in order to try to elucidate the mechanism(s) of TRAIL sensitivity and resistance.

Treatment of HeLa cells with bTRAIL resulted in a time-dependent recruitment of FADD and procaspase-8 to the DISC (Fig. 4.1). Both the p55 and p53 zymogen forms of procaspase-8 were present in the DISC and their activation resulted in partial processing to the intermediate p43 and p41 forms, which arise following removal of the small p12 subunit (Srinivasula *et al.*, 1996). FADD and caspase-8 recruitment appeared to peak at 30 min with levels being maintained up to 60 min. The addition of z-VAD.fmk did not affect recruitment levels and appeared to have no significant inhibitory effect on the generation of p43 and p41 fragments.

Interestingly, both FADD and procaspase-8 were also recruited in DISCs precipitated from 293 cells despite these cells being resistant to TRAIL-induced apoptosis (Fig. 3.2). Levels of recruitment were, however, considerably lower than in the HeLa DISCs despite the cells displaying similar expression levels as judged by cell lysate comparisons of FADD and caspase-8. Caspase-8 levels appeared to peak at 30 min similar to that seen in HeLas, but then were clearly reduced at 60 min and could only be maintained by the addition of z-VAD.fmk which again failed to block initial procaspase-8 processing within the DISC (Fig. 4.1).

The two described splice forms of TRAIL-R2 were present and precipitated in both HeLa and 293 cells (Screaton *et al.*, 1997). The larger isoform was predominant although there appeared to be no preferential recruitment of either form. Surprisingly, the level of TRAIL-R2 precipitated was similar in both cell lines which was in contrast to the different levels of FADD and caspase-8 recruited. Only TRAIL-R2 was present in the unstimulated receptor

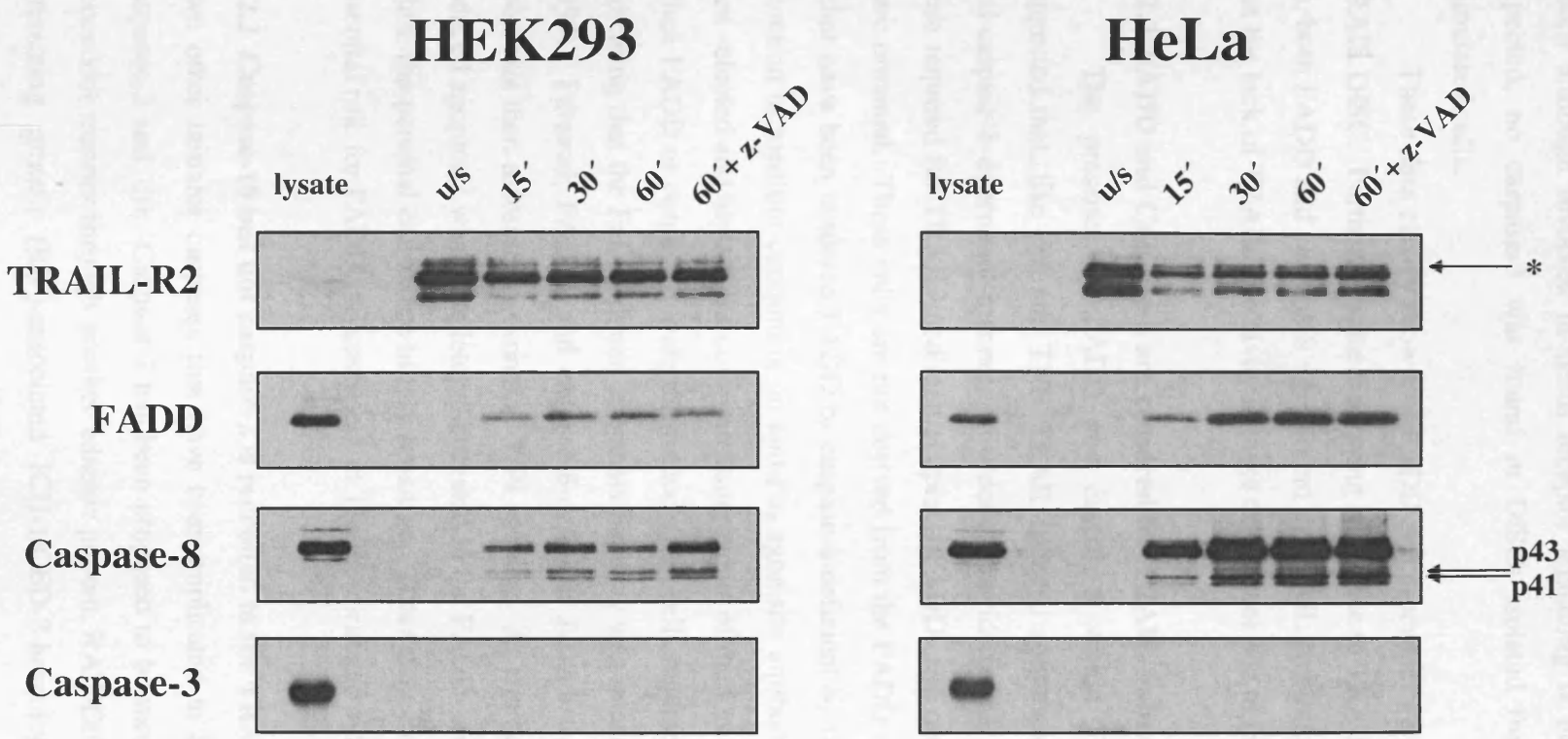


Figure. 4.1 FADD and caspase-8 are components of the TRAIL DISC. 293 and HeLa cells (3×10^7) were treated with biotinylated TRAIL (bTRAIL) for up to 60 min and, where indicated, cells were pre-treated for 60 min with z-VAD.fmk (20 μM). After treatment, DISCs were isolated as described in *Material and Methods*. Unstimulated receptor controls (u/s) represent addition of bTRAIL to an equivalent volume of lysate isolated from unstimulated cells. TRAIL receptor complexes were immunoblotted for the presence of TRAIL-R2, FADD and caspase-8 using specific antibodies. Lysates isolated from unstimulated control cells were included as a positive control for the expression of all these proteins in both 293 and HeLa cells. Procaspase-3, which has never been implicated as a DISC component, was included as a negative control. To enable comparison of the relative amounts of each component recruited to the DISC equivalent exposures are shown. * indicates a minor non-specific band detected by the TRAIL-R2 antibody.

controls demonstrating that FADD and caspase-8 recruitment required the presence of TRAIL. As an additional control precipitates were analysed for the presence of caspase-3 which although involved in death receptor signalling is not a DISC component. As expected, no caspase-3 was found in DISCs isolated from unstimulated or TRAIL stimulated cells.

These data clearly show that FADD and procaspase-8 are components of the native TRAIL DISC. Further, despite displaying resistance to TRAIL-induced apoptosis (Fig. 3.1 B), both FADD and caspase-8 were found in TRAIL precipitates from 293 cells indicating that the lack of TRAIL sensitivity in these cells is not due to the inability to form a DISC.

4.2.2 FADD and Caspase-8 are required for TRAIL-induced apoptosis.

The presence of FADD and caspase-8 within TRAIL precipitates strongly suggested that, like Fas and TNF, TRAIL-induced apoptosis would proceed in a FADD and caspase-8-dependent manner. In order to provide further proof that these components were required for TRAIL-mediated apoptosis FADD- and caspase-8-deficient Jurkat cells were obtained. These cells are not derived from the FADD or caspase-8 null animals but rather have been rendered FADD or caspase-8-deficient by random mutation followed by selection in medium containing an anti-Fas agonistic antibody. Fas resistant clones were then selected and blotted for common components of the Fas-mediated apoptosis pathway. When FADD or caspase-8 were reintroduced the cells regained their sensitivity to anti-Fas indicating that the Fas-mediated apoptosis pathway was intact (Juo *et al.*, 1998; Juo *et al.*, 1999). Parental, FADD- and caspase-8-deficient Jurkats were treated with TRAIL and apoptosis then assessed by Annexin V/PI staining. As can be seen in Figure 4.2, TRAIL-induced apoptosis was completely abrogated in the FADD- and caspase-8-deficient Jurkats while the parental cells were highly sensitive. These data clearly provide evidence for an essential role for FADD and caspase-8 in TRAIL-mediated cytotoxicity.

4.2.3 Caspase-10 but not caspase-2 is recruited to the TRAIL DISC

Two other initiator caspases that have been implicated in death receptor signalling are caspases-2 and -10. Caspase-2 has been proposed to be recruited to the DISC in a RIP-dependent manner through another adaptor protein, RAIDD/CRADD. RAIDD (receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain)/CRADD (caspase and RIP adapter with death domain) was identified as an unusual bipartite molecule. RAIDD contains a C-terminal DD which binds RIP and an

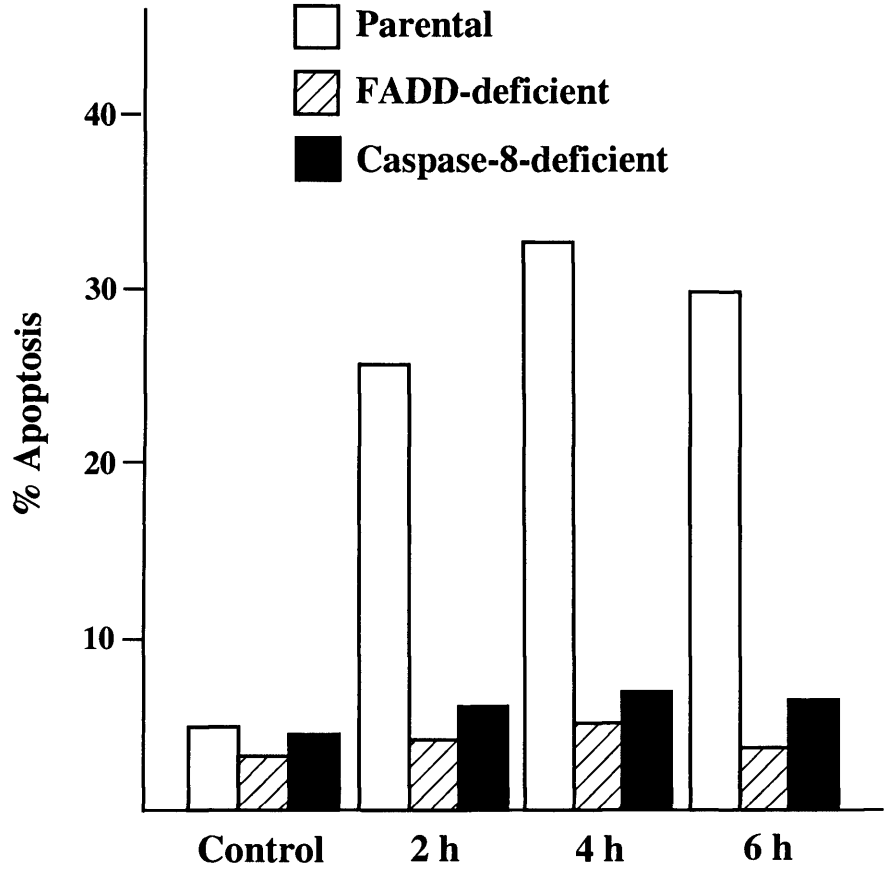


Figure 4.2 Requirement of FADD and Caspase-8 for TRAIL-induced apoptosis. Parental (A3), FADD-deficient and caspase-8-deficient Jurkat T cells were treated with TRAIL (1 μg/ml) for up to 6 h. Apoptosis was assessed by Annexin V/propidium iodide staining as described in *Materials and Methods (2.2.2).*

N-terminal domain which binds procaspase-2 (ICH-1). RAIDD was therefore purported to act in a similar manner to FADD bridging the intracellular domain of death receptors and the long pro-domain of the initiator caspases, in this case procaspase-2. Despite the presence of caspase-2 in the lysates, no caspase-2 was observed in TRAIL precipitates from 293 cells (Fig. 4.3 A) or HeLa cells (data not shown).

Caspase-10 (FLICE-2/Mch4) was identified by its high homology to caspase-8. It is the only other caspase to contain DED motifs in its prodomain which suggests it may play a role in death receptor signalling (Vincenz & Dixit. 1997). Interestingly HeLa cells do not contain any caspase-10 (data not shown). In contrast, 293 cells do express caspase-10 which is recruited to the TRAIL DISC. A doublet representing the ~59 and ~55 kDa proforms was observed in precipitates from TRAIL treated cells (Fig. 4.3 B). Lower molecular weight species were also present representing the ~47 and ~43 kDa intermediates minus the small subunit. The particular antibody used was raised against an epitope in the prodomain so no active subunits were observed but the prodomain itself was clearly visible at ~25 kDa. When DISCs were isolated in the presence of z-VAD.fmk, no prodomain was detected in the precipitates, while the ~47 and ~43 kDa forms were reduced and the corresponding proforms appeared to accumulate.

Two initiator caspases, caspases-8 and -10, are therefore implicated in TRAIL signalling and have shown to be recruited and processed at the DISC. Unlike caspase-8 however, caspase-10 appears to be dispensable for TRAIL-induced apoptosis as it was not expressed in the TRAIL-sensitive HeLa cells (data not shown).

4.2.4 The inactive caspase-8 homolog c-FLIP is recruited and cleaved at the DISC.

There are no described mammalian cellular inhibitors of caspase-8. Mammalian cells do however possess v-FLIP (viral-FLIP), and an inactive-caspase-8 homolog known as c-FLIP (cellular-FLIP). c-FLIP exists as a long (c-FLIP$_L$) and a short (c-FLIP$_S$) splice variant, both of which are capable of protecting cells from death receptor-induced apoptosis (Irmler *et al.*, 1997; Thome *et al.*, 1997). Because they lack residues required for caspase-8 activity, critically the active site cysteine, both FLIP isoforms are purported to act as dominant-negative inhibitors of caspase-8 activation (Thome *et al.*, 1997).

Both 293 and HeLa cells expressed significant levels of only the c-FLIP$_L$ isoform which following TRAIL stimulation was recruited to DISCs isolated from both cell types (Fig. 4.4 A). A ~43 kDa fragment was also observed in precipitates from stimulated cells

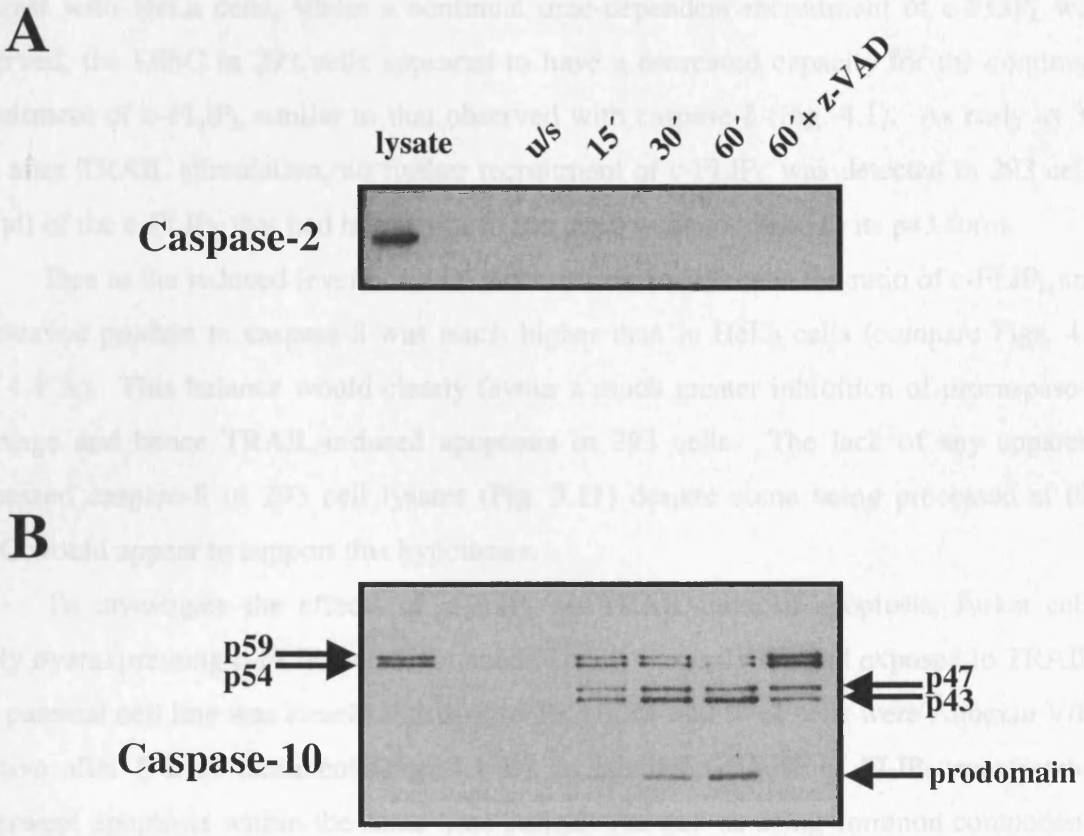


Figure. 4.3 Caspase-10 but not caspase-2 is recruited to the TRAIL DISC in 293 cells. TRAIL DISCs were precipitated from 293 cells as described in Figure 4.1. Precipitated complexes were analysed for the presence of caspases-2 (A) and -10 (B) the two other initiator caspases that have been implicated in death receptor signalling.

which represents the product obtained following removal of the C-terminal p12 subunit of c-FLIP$_L$. Like full-length c-FLIP$_L$, the p43 fragment can also inactivate the DISC by preventing further recruitment of procaspase-8 into the complex (Scaffidi *et al.*, 1999). In contrast with HeLa cells, where a continual time-dependent recruitment of c-FLIP$_L$ was observed, the DISC in 293 cells appeared to have a decreased capacity for the continual recruitment of c-FLIP$_L$ similar to that observed with caspase-8 (Fig. 4.1). As early as 30 min after TRAIL stimulation, no further recruitment of c-FLIP$_L$ was detected in 293 cells and all of the c-FLIP$_L$ that had initially been recruited was processed to its p43 form.

Due to the reduced level of FADD recruitment in 293 cells the ratio of c-FLIP$_L$ and its cleaved product to caspase-8 was much higher than in HeLa cells (compare Figs. 4.1 and 4.4 A). This balance would clearly favour a much greater inhibition of procaspase-8 cleavage and hence TRAIL-induced apoptosis in 293 cells. The lack of any apparent processed caspase-8 in 293 cell lysates (Fig. 3.11) despite some being processed at the DISC would appear to support this hypothesis.

To investigate the effects of c-FLIP$_L$ on TRAIL-induced apoptosis, Jurkat cells stably overexpressing c-FLIP$_L$ were obtained (Thome *et al.*, 1997) and exposed to TRAIL. The parental cell line was clearly sensitive to TRAIL as >40 % of cells were Annexin V/PI positive after 6 h of treatment (Fig. 4.4 B). In contrast, <15 % of FLIP$_L$-transfectants underwent apoptosis within the same time period. As well as using common components of the death receptor pathway, such as FADD and procaspase-8, the presence of FLIP within the TRAIL precipitates suggests that activation of procaspase-8 by TRAIL may share similarities with Fas.

4.2.5 RIP is recruited and cleaved at the DISC.

Data presented in Chapter 3 demonstrated that TRAIL is capable of activating NF-κB in both HeLas and 293s, although activation in HeLa cells required the presence of the caspase inhibitor z-VAD.fmk (Fig. 3.5). RIP has previously been shown to be absolutely required for TNF-mediated NF-κB activation (Ting *et al.*, 1996), however, its role in TRAIL signalling is somewhat confusing. Some studies have shown it to interact with TRAIL receptors (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997b) while others have shown no interaction (MacFarlane *et al.*, 1997; Pan *et al.*, 1997b). Like the confusion surrounding the role of FADD, all these studies were based on overexpression and not on endogenous levels of these proteins. Native TRAIL DISC precipitates were therefore analysed for the presence of RIP.

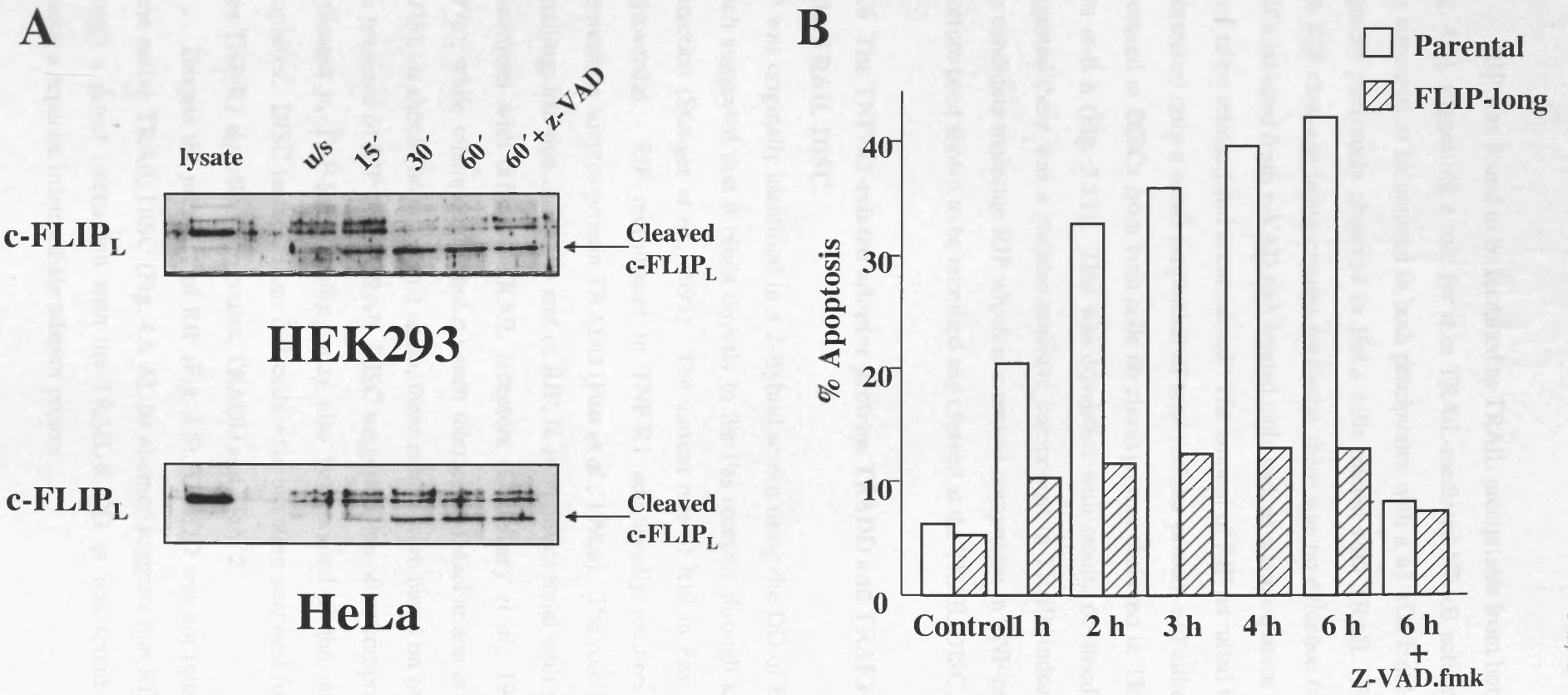


Figure. 4.4 The inactive caspase-8 homolog, c-FLIP$_L$ is recruited and cleaved at the TRAIL DISC: Effect of c-FLIP$_L$ on TRAIL-induced apoptosis. (A) TRAIL DISCs were precipitated from 293 and HeLa cells as described in Figure 4.1. Precipitated complexes were analysed for the presence of c-FLIP. (B) Parental and c-FLIP$_L$ overexpressing Jurkats were treated with TRAIL (1 μg/ml) for up to 6 h, where indicated cells were pretreated with z-VAD.fmk (20 μM, 30 min). Apoptosis was assessed by Annexin V/propidium iodide staining as described in *Materials and Methods*.

RIP was found to be recruited to TRAIL precipitates from both HeLa and 293 cells (Fig. 4.5), suggesting a role for it in TRAIL-mediated NF-κB activation. Interestingly, it also appeared to be cleaved in both precipitates with a 43 kDa band corresponding to the fragment previously observed in HeLa cells treated with TRAIL (Fig. 3.11). Consistent with RIP cleavage being caspase-mediated, there was no evidence of the p43 fragment in DISCs isolated from z-VAD.fmk-treated cells and, as a consequence of this intact RIP was found to be retained and accumulated. The amount of RIP recruited to DISCs in both cells represented only a small proportion of total cellular protein and although it was shown be processed in DISCs from both cells no cleavage was observed in TRAIL-treated 293 cells even at 6 h (Fig. 3.11). This was consistent with results obtained in Chapter 3, which suggested there was a caspase-sensitive component to TRAIL-induced NF-κB activation. The candidate molecule RIP which is a critical component in TNF-mediated apoptosis has therefore been shown to be recruited and cleaved at the TRAIL DISC.

4.2.6 The TNF-R1-related adaptor proteins TRADD and TRAF2 are not components of the TRAIL DISC.

RIP was originally identified in a 2-Hybrid screen using the DD of Fas as a "bait" protein which suggested that it binds directly to the Fas receptor through a homophillic DD/DD interaction (Stanger *et al.*, 1995). The current role of RIP in Fas signalling is however controversial. RIP recruitment to TNF-R1 additionally requires the presence of an intermediate adaptor protein TRADD (Hsu *et al.*, 1996a). The role of TRADD in TRAIL signalling, like that of FADD and of RIP, is also controversial with some studies showing interactions with different TRAIL receptors (Chaudhary *et al.*, 1997; Schneider *et al.*, 1997b), while others could find no such interaction (MacFarlane *et al.*, 1997; Pan *et al.*, 1997b). It should be noted that again, these results were based on overexpression studies. The presence of RIP in the TRAIL DISC suggested that other components previously only implicated in TNF-R1 signalling may also be recruited to the isolated native TRAIL complexes. DISC isolates from 293 cells were therefore analysed for the presence of two other TNF-R1 signalling molecules, TRADD and TRAF2.

Despite the presence of RIP (Fig. 4.5), TRADD was not found to be a component of the native TRAIL DISC (Fig. 4.6 A). Its absence suggests that RIP recruitment may be through a direct interaction with the TRAIL-R DD as was found with Fas rather than through a separate intermediate adaptor protein.

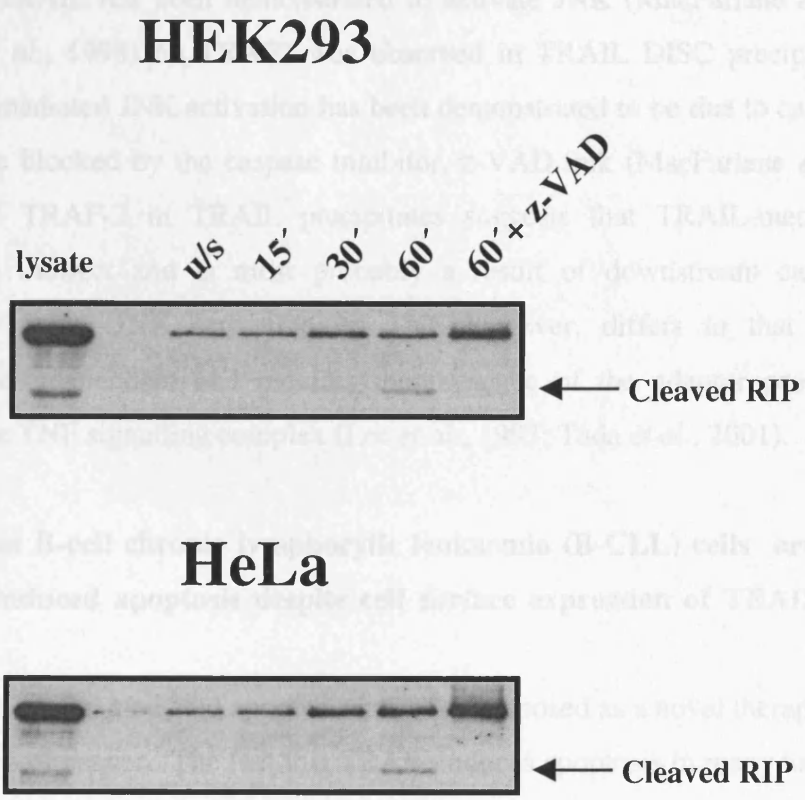


Figure. 4.5 Receptor Interacting Protein (RIP) is recruited and cleaved at the TRAIL DISC in both HeLa and 293 cells. TRAIL DISCs were precipitated from 293 and HeLa cells as described in Figure 4.1. Precipitated complexes were analysed for the presence of RIP and cleaved RIP.

TRADD also binds another protein, TRAF2, which has been demonstrated to be absolutely required for TNF-mediated activation of c-Jun N-terminal kinase (JNK) (Lee *et al.*, 1997) and also has a role in TNF-mediated NF-κB activation (Tada *et al.*, 2001). Despite the fact that TRAIL has been demonstrated to activate JNK (MacFarlane *et al.*, 2000; Muhlenbeck *et al.*, 1998) no TRAF2 was observed in TRAIL DISC precipitates (Fig. 4.6 B). TRAIL-mediated JNK activation has been demonstrated to be due to caspase activation as it can be blocked by the caspase inhibitor, z-VAD.fmk (MacFarlane *et al.*, 2000). The lack of TRAF-2 in TRAIL precipitates suggests that TRAIL-mediated activation of JNK is indirect and is most probably a result of downstream caspase activation within the cell. JNK activation by TNF however, differs in that it is predominantly caspase-independent and requires the presence of the adaptor proteins, TRAFs -2 and -5 in the TNF signalling complex (Lee *et al.*, 1997; Tada *et al.*, 2001).

4.2.7 Primary human B-cell chronic lymphocytic leukaemia (B-CLL) cells are not sensitive to TRAIL-induced apoptosis despite cell surface expression of TRAIL-R1 and -R2.

Exploitation of death receptor-mediated apoptosis has been proposed as a novel therapeutic strategy for many forms of cancer. The fact that TRAIL induces apoptosis in many tumour and transformed cell lines but not in normal cells has led to the possibility of using it as a therapeutic agent for the treatment of tumours. The high systemic toxicity observed with FasL/CD95L and TNF precludes their use as therapeutic agents. B-CLL is one of the most common haematological malignancies in the Western world. It is characterised by the accumulation of mature, non-proliferating B cells that co-express CD5 and CD23 (Rozman *et al.*, 1995). This accumulation represents the failure to undergo apoptosis rather than excessive cellular proliferation. B-CLL cells have previously been shown to be resistant to CD95 (Roue *et al.*, 2001). In order to assess sensitivity to TRAIL, primary B-CLL cells isolated from patients were treated with TRAIL for 24 h prior to apoptosis assessment by Annexin V/PI staining and flow cytometry. Apart from a degree of "spontaneous apoptosis" (~27%) which occurs when these cells are cultured *ex vivo* for long periods of time no apoptosis was observed in these cells in the presence of TRAIL (Fig. 4.7). Jurkat cells were used as a positive control and displayed over 70% apoptosis after only 6 h treatment with TRAIL (Fig. 4.7). The apoptotic machinery was intact within the primary B-CLL cells as they readily underwent apoptosis when treated with the proteasome inhibitor MG132 (Fig. 4.7). Apoptosis data in primary B-CLL cells is representative of

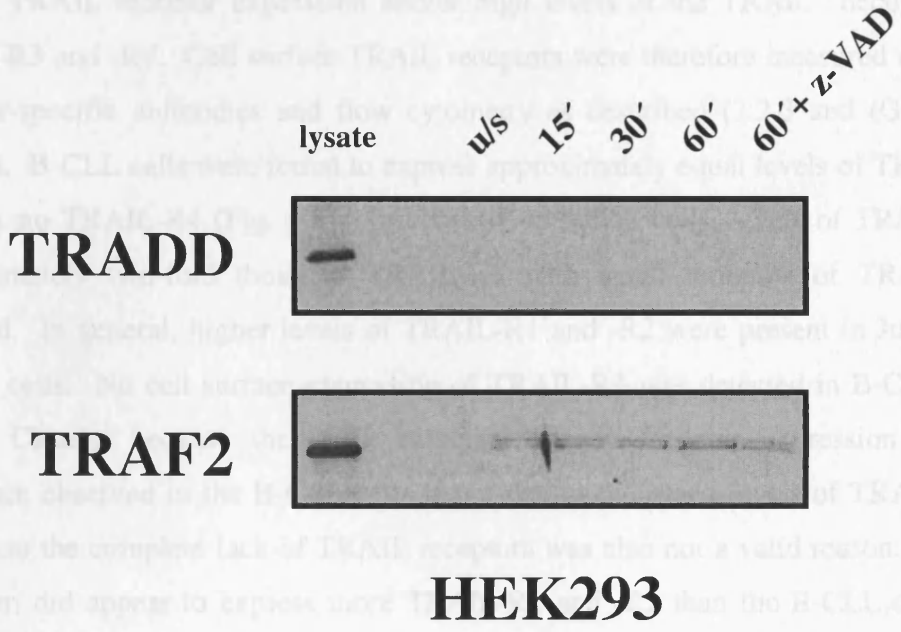


Figure. 4.6 The adaptor proteins TRADD and TRAF2 are not components of the TRAIL DISC. TRAIL DISCs were precipitated from 293 cells as described in Figure 4.1. Precipitated complexes were analysed for the presence of the adaptors (A) TRADD and (B) TRAF2.

that obtained in at least 30 B-CLL cases all of which were resistant to TRAIL (MacFarlane *et al.*, 2002).

The resistance of primary B-CLL cells could, in part, be due to a lack of cell surface TRAIL receptor expression and/or high levels of the TRAIL "decoy" receptors, TRAIL-R3 and -R4. Cell surface TRAIL receptors were therefore measured using TRAIL receptor-specific antibodies and flow cytometry as described (2.2.3 and (Griffith *et al.*, 1999a)). B-CLL cells were found to express approximately equal levels of TRAIL-R1 and -R2 but no TRAIL-R4 (Fig. 4.8). In contrast, in Jurkat cells, levels of TRAIL-R2 were approximately two-fold those of TRAIL-R1 with small amounts of TRAIL-R4 also detected. In general, higher levels of TRAIL-R1 and -R2 were present in Jurkats than in B-CLL cells. No cell surface expression of TRAIL-R3 was detected in B-CLL or Jurkat cells. Clearly, because they lack detectable decoy receptor expression the TRAIL resistance observed in the B-CLL cells is not due to increased levels of TRAIL-R3 and -R4. Also the complete lack of TRAIL receptors was also not a valid reason. Jurkat cells however, did appear to express more TRAIL-R1 and -R2 than the B-CLL cells and this increased expression correlated with them being more sensitive to TRAIL.

4.2.8 TRAIL DISC formation in TRAIL-resistant primary B-CLL cells.

The absence of procaspase-8 processing in TRAIL-treated B-CLL cells (MacFarlane *et al.*, 2002) suggested that an apical event in TRAIL signalling was blocked in these cells. Results obtained on cell surface TRAIL receptor analysis failed to provide explanation for the apparent lack of sensitivity of the B-CLL cells to TRAIL. Another explanation was the possibility was that B-CLL cells were unable to form a functional DISC in response to TRAIL, although it should be noted that 293 cells which were also TRAIL-resistant (Fig. 3.11) still formed a DISC (Fig. 4.1). TRAIL induced DISC formation in the TRAIL sensitive Jurkat T cells (Fig. 4.9, lane 3). In Jurkat DISC precipitates FADD was present and caspase-8 was processed to its p43 and p41 forms with the p18 large subunit also detected within DISC precipitates.

Consistent with the data obtained in the 293 cells, TRAIL also induced DISC formation in B-CLL cells as judged by FADD and caspase-8 recruitment (Fig. 4.9) although approximately 10-fold as many cells were required compared to that used for Jurkats. Interestingly, although caspase-8 was recruited and there was evidence of the presence of partially processed p43 and p41 forms, there was no evidence of the p18 large

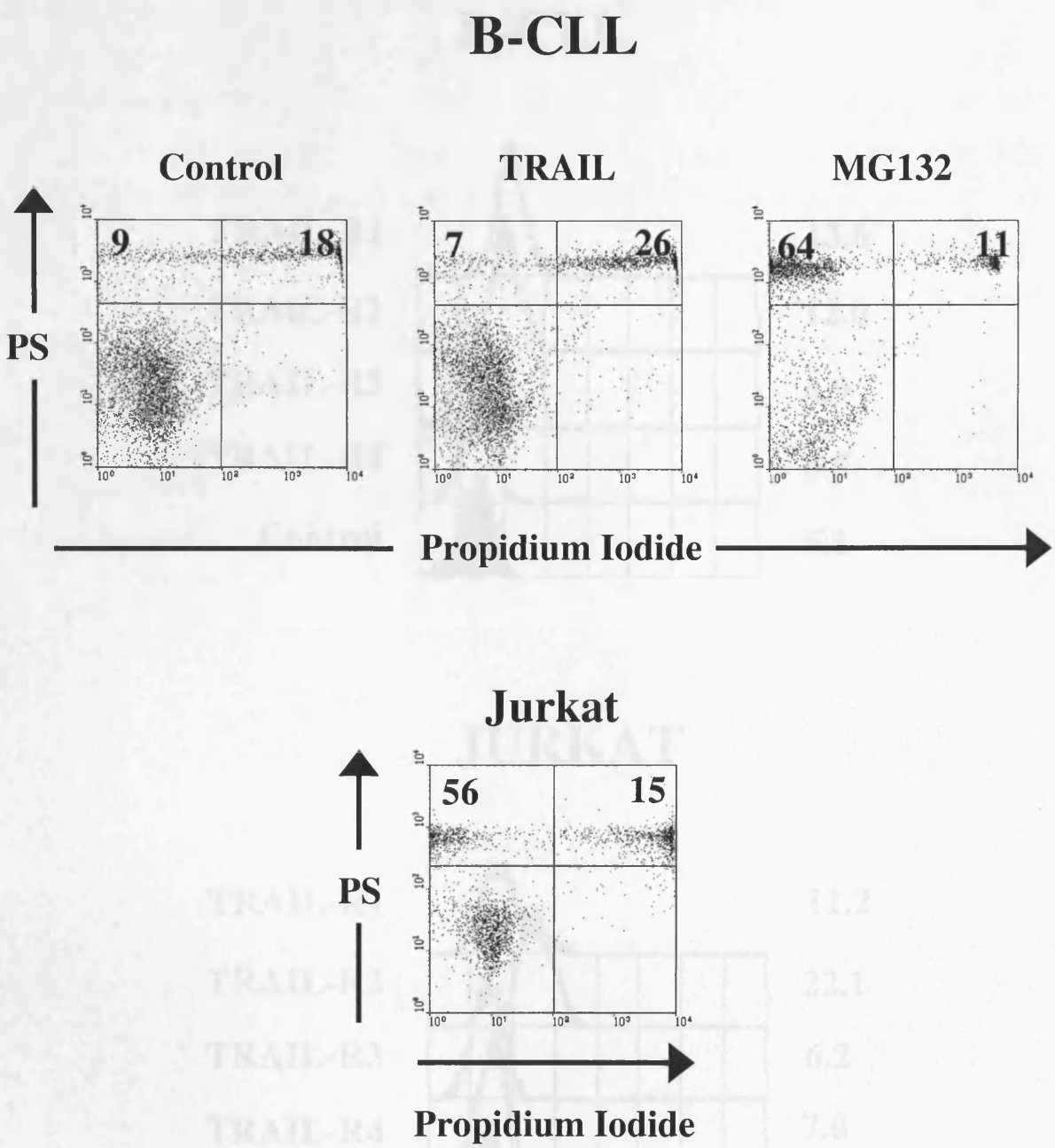


Figure. 4.7 Primary B-cell Chronic Lymphocytic Leukaemia cells are resistant to TRAIL-induced apoptosis despite expressing cell surface TRAIL receptors. Freshly isolated B-CLL cells were isolated and incubated with TRAIL for 24 h. Jurkat cells were incubated for 6 h. Apoptosis was assessed by Annexin V/PI staining. The numbers in the upper left quadrant represent the percentage of cells that are Annexin V positive and PI negative (apoptotic). The upper right represents cells which are Annexin V and PI positive (cells undergoing secondary necrosis).

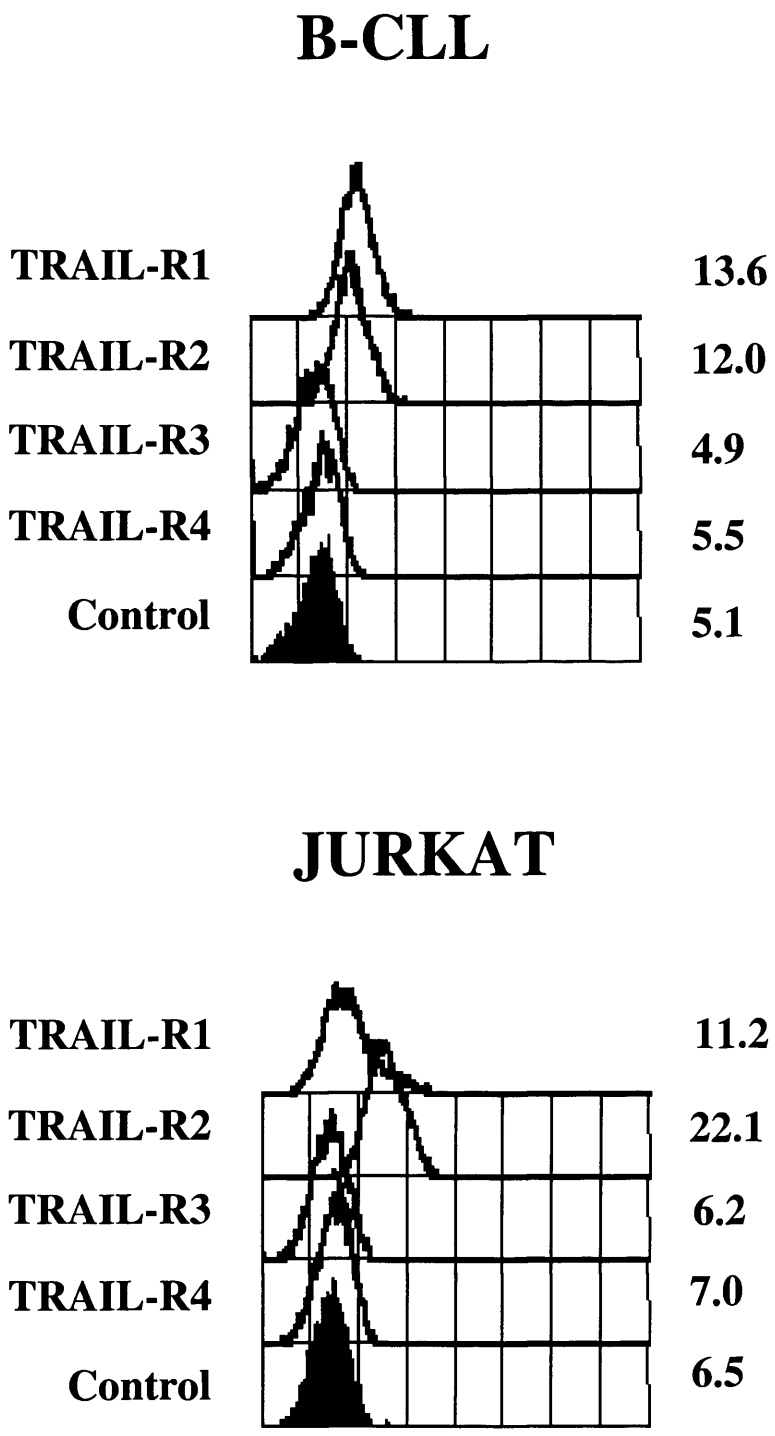


Figure. 4.8 TRAIL Resistance of BCLL cells is not due to a lack of cell surface TRAIL receptors. Cell surface TRAIL receptors were measured by flow-cytometry using TRAIL receptor-specific monoclonal antibodies followed by a FITC-conjugated secondary antibody as described in *Materials and Methods*. Values shown are mean fluorescence intensity.

subunit which was clearly present in the Jurkat DISC (Fig. 4.9). Less TRAIL-R2 was precipitated in Jurkat DISC precipitates which is consistent with the finding of lower surface expression of TRAIL-R2 on B-CLL cells (Fig. 4.8).

As demonstrated previously with 293 and HeLa cells (Fig. 4.4), c-FLIP$_L$ was both recruited and cleaved in DISCs isolated from both Jurkat and B-CLL cells (Fig. 4.4 A). Surprisingly, however, although both Jurkat and B-CLL cells also expressed the short splice-variant, c-FLIP$_S$, only the long variant was recruited to the DISC. The reasons behind this are not clear as the short form still contains the two DED motifs present in procaspase-8 which are required for its recruitment to the DISC, and it is still capable of protecting against Fas and TRAIL-induced apoptosis (Thome *et al.*, 1997).

Thus, formation of low levels of DISC, rather than the inability to form a DISC, may in part be the basis for the lack of TRAIL-induced apoptosis in primary B-CLL cells when compared to Jurkat cells.

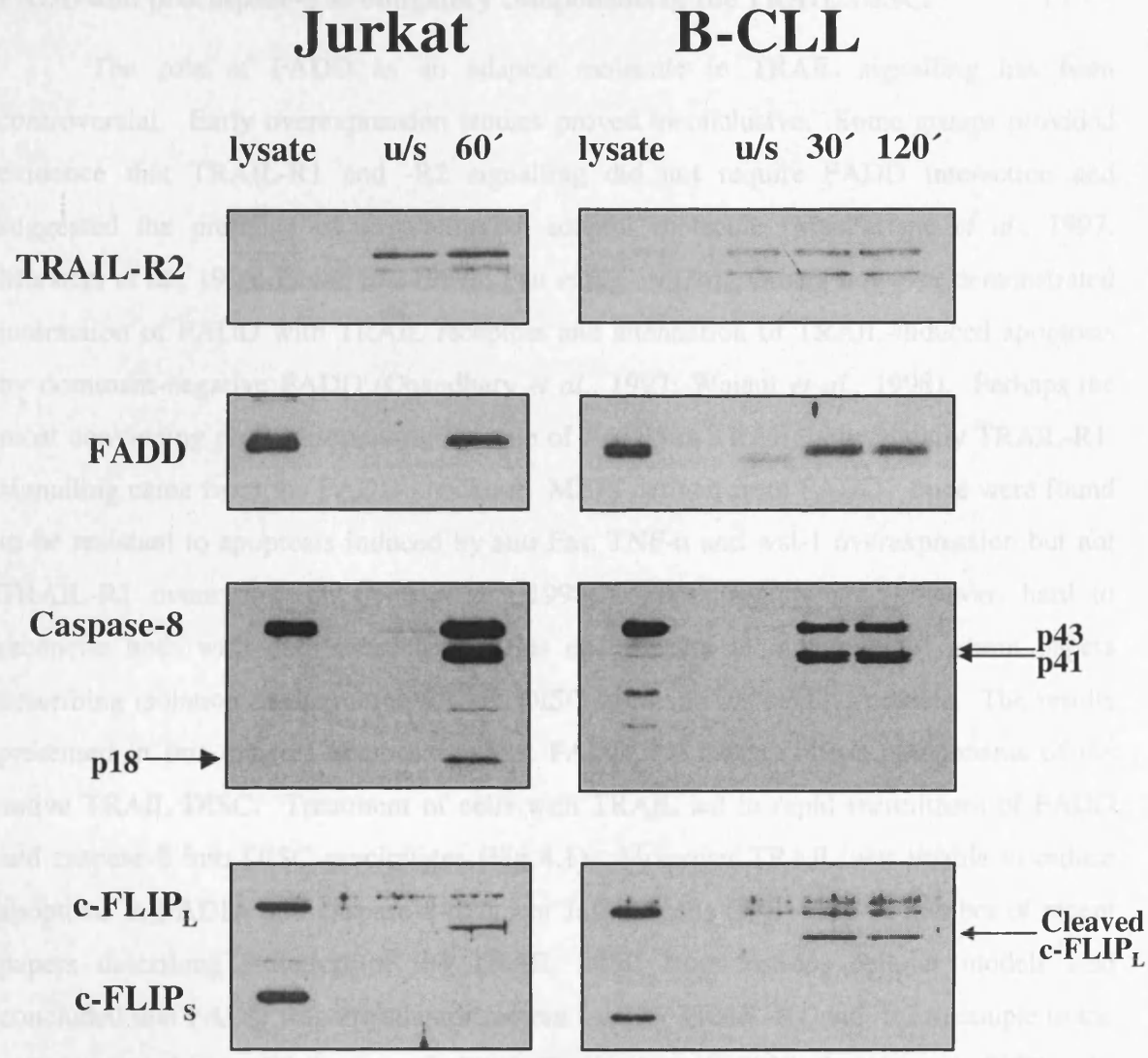


Figure. 4.9 TRAIL-DISC formation occurs in primary B-CLL cells despite their resistance to TRAIL. TRAIL DISCs were isolated from primary B-CLL (3×10^8) cells essentially as described in *Materials and Methods* (2.7.1). Jurkat cells (2.5×10^7) were used as a positive control for DISC formation. DISCs were analysed for FADD, procaspase-8 and c-FLIP. To enable a comparison the same concentrations of antibodies and similar exposure times were used. It should be noted that ~10-fold more B-CLL cells were required for DISC analysis compared to Jurkat cells.

4.3 DISCUSSION

FADD and procaspase-8 as obligatory components of the TRAIL DISC.

The role of FADD as an adaptor molecule in TRAIL signalling has been controversial. Early overexpression studies proved inconclusive. Some groups provided evidence that TRAIL-R1 and -R2 signalling did not require FADD interaction and suggested the presence of an alternative adaptor molecule (MacFarlane *et al.*, 1997; Marsters *et al.*, 1996; Pan *et al.*, 1997a; Pan *et al.*, 1997b). Others however demonstrated interaction of FADD with TRAIL receptors and attenuation of TRAIL-induced apoptosis by dominant-negative FADD (Chaudhary *et al.*, 1997; Wajant *et al.*, 1998). Perhaps the most convincing proof discounting the role of FADD in TRAIL, specifically TRAIL-R1, signalling came from the FADD knockout. MEFs derived from $FADD^{-/-}$ mice were found to be resistant to apoptosis induced by anti-Fas, TNF-α and wsl-1 overexpression but not TRAIL-R1 overexpression (Yeh *et al.*, 1998). These reports are, however, hard to reconcile both with data presented in this chapter and in a number of recent papers describing isolation of the native TRAIL DISC from various cellular models. The results presented in this chapter demonstrate that FADD and caspase-8 are components of the native TRAIL DISC. Treatment of cells with TRAIL led to rapid recruitment of FADD and caspase-8 into DISC precipitates (Fig 4.1). Moreover TRAIL was unable to induce apoptosis in FADD- and caspase-8-deficient Jurkats cells (Fig. 4.2). A number of recent papers describing isolation of the TRAIL DISC from various cellular models also concluded that FADD was the adaptor protein used by TRAIL-R1 and -R2 to couple to the apoptotic machinery (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). As none of the cell lines used in this study express TRAIL-R1 exclusively there is still a possibility that TRAIL-R1 uses an adaptor distinct to FADD as implied in the $FADD^{-/-}$ MEFs. This question has subsequently been addressed by using specific TRAIL receptor blocking antibodies which have demonstrated that both TRAIL-R1 and -R2 independently recruit FADD (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). A further study made use of the previously described $FADD^{-/-}$ MEFs. MEFs do not express TRAIL receptors (Varfolomeev *et al.*, 1998) and it was therefore impossible to treat them with TRAIL ligand. To circumvent this problem and to avoid transient receptor overexpression studies, another group addressed this issue by stably introducing TRAIL-R1 and -R2 into wild-type and $FADD^{-/-}$ MEFs. While wild-type transfectants were sensitive to TRAIL the FADD

null transfectants were not (Kuang *et al.*, 2000). Taken together the overwhelming evidence is now that FADD is a component of the TRAIL signalling pathway and appears to act as a universal adaptor linking the death receptors of the TNF family to the DED-containing long prodomain caspases, caspases-8 and -10. Specificity within the TNF-R family would therefore be thought to be primarily through differential expression of the various receptors and ligands and not through the use of specific adaptors for different subfamily members.

Recruitment and cleavage of RIP at the DISC.

Data presented in Chapter 3 demonstrated that TRAIL was able to activate NF-κB in HeLa (TRAIL-sensitive) cells only in the presence of the poly-caspase inhibitor z-VAD.fmk (Fig. 3.5). This suggested a caspase-sensitive component to TRAIL-induced NF-κB activation in these cells. RIP was clearly a candidate molecule as it was cleaved during TRAIL-induced apoptosis (Fig. 3.11) and has been shown to be critically required for TNF-mediated NF-κB activation (Kelliher *et al.*, 1998). Caspase-mediated cleavage of RIP has also been demonstrated to produce an C-terminal fragment which appears to act as a dominant-negative inhibitor of TNF-induced NF-κB activation and therefore functions to promote TNF-mediated cytotoxicity (Lin *et al.*, 1999). RIP however has never been previously been shown to be recruited to the native TRAIL DISC (Kischkel *et al.*, 2000; Sprick *et al.*, 2000).

Data presented in this chapter now demonstrate that RIP is a component of the TRAIL DISC and also appears to be processed within the DISC (Fig. 4.5). In the presence of z-VAD.fmk full length RIP clearly accumulated in DISC precipitates. Importantly, previous studies have not used z-VAD.fmk which may explain their inability to identify RIP as a component. These data, together with a recent paper describing the attenuation of TRAIL-induced NF-κB activation in RIP null cells (Lin *et al.*, 2000) and the sensitisation of cells to TRAIL by overexpression of a dominant/negative RIP fragment clearly provide a role for RIP in TRAIL-mediated apoptosis.

Role of other caspases in TRAIL receptor signalling.

The role of caspase-10 in TRAIL-induced apoptosis is unclear. Although, like caspase-8, it is the only other caspase to contain DEDs it was not initially found to be a DISC component (Sprick *et al.*, 2000). Data presented in this chapter, however, shows that

caspase-10 is a component of the TRAIL DISC at least in 293 cells and displays similar recruitment and processing to caspase-8 (Fig. 4.3). A requirement for caspase-10 in TRAIL-induced apoptosis is not entirely clear as HeLa cells which are highly sensitive to TRAIL (Fig. 3.1B) completely lacked its expression. Caspase-10, therefore, would appear to be dispensable for TRAIL-induced apoptosis at least in this cell type. Although one recent report states that caspase-10 can functionally substitute for caspase-8 in death receptor-mediated apoptosis (Wang *et al.*, 2001b), data presented in this chapter and a recent report (Kischkel *et al.*, 2001; Sprick *et al.*, 2002) would appear to contradict this. Firstly, as mentioned above, the absence of caspase-10 in HeLa cells in no way appears to modulate their sensitivity to TRAIL. Secondly caspase-8 null Jurkats, which have been shown to contain caspase-10 (Sprick *et al.*, 2002), were not sensitive to TRAIL (Fig. 4.2). The physiological role of caspase-10 is also unclear. Mutations in caspase-10 have been linked with patients suffering with autoimmune lymphoproliferative syndrome (ALPS), a disorder characterised by abnormal lymphocyte and dendritic cell homeostasis and immune tolerance (Wang *et al.*, 1999b). One of these mutations however was found to be present at high frequency in the Danish population (Gronbaek *et al.*, 2000) and as this particular polymorphism leads to a protein with reduced activity the significance of this observation now unclear.

The other caspase reportedly involved in death receptor signalling (through CD95 at least) is caspase-2. In this study, there was no evidence of caspase-2 recruitment to the TRAIL DISC despite the presence of its reported intermediate adaptor protein RIP (Fig. 4.5). Caspase-2 has been purported to be recruited via the adaptor molecule RAIDD/CRADD which is itself recruited via RIP (Ahmad *et al.*, 1997; Duan & Dixit. 1997). However, this interaction has only been demonstrated to occur in over-expressed systems and the exact physiological significance is questionable when considering the phenotype of caspase-2 null mice. Cell derived from these animals display no defects in either TNF or anti-CD95-induced apoptosis (Bergeron *et al.*, 1998).

Formation of a DISC in TRAIL resistant cells and the role of c-FLIP.

TRAIL DISC formation did not appear to be dependent on the TRAIL sensitivity of the cell as DISCs were formed in both TRAIL sensitive and resistant cells (Figs. 4.1 and 4.9). In HeLa and 293 cells there were a number of differences relating to the amounts of components recruited which may help to explain their very different sensitivities. Despite the levels of TRAIL-R2 precipitated from both cell lines being equivalent there was clearly

less FADD recruited to the DISC in 293 cells (Fig. 4.1). Procaspase-8 recruitment is dependent upon FADD and hence this resulted in less procaspase-8 being precipitated from 293 cells. The explanation for the reduced FADD recruitment observed is unclear but clearly the amount of procaspase-8 recruited to the DISC in HeLa cells compared to that in 293 cells could help to explain the different sensitivities of these cells. DISCs isolated from TRAIL-resistant primary B-CLL also contained FADD and partially processed procaspase-8 (Fig. 4.9).

One important observation was that the actual amount of procaspase-8 recruited to the TRAIL DISCs, even those isolated from sensitive cells, represented a very small proportion of the total cellular content. Whether this is the total amount of procaspase-8 which can be recruited and processed at the DISC or if additional procaspase-8 can be recruited and processed is unknown and would be difficult to study experimentally. Such a "cycling" action of procaspase-8 through the DISC would be a favourable hypothesis and may help to explain the role of c-FLIP$_L$ in inhibiting such processes. The failure to fully process FLIP may lead to its continued presence within the DISC thereby resulting in a complex with a decreased capacity for recruitment and activation of procaspase-8. Some support for such a hypothesis was seen in DISCs isolated from 293 cells where levels of procaspase-8 and c-FLIP recruitment clearly decreased with time (Figs. 4.1 and 4.4). In contrast no such decrease was observed in the TRAIL sensitive HeLa cells.

Although c-FLIP$_L$ was demonstrated to be recruited and processed at the TRAIL DISC (Fig. 4.4) in all of the cell lines studied, the lack of recruitment of c-FLIP$_S$ in both Jurkat and B-CLL cells which express c-FLIP$_S$ was surprising (Fig. 4.9). The DEDs of c-FLIP$_S$ are identical to those in c-FLIP$_L$ and they only differ in a short C-terminal section (Keuger *et al.*, 2001). Stable overexpression of c-FLIP$_S$ in cells attenuates Fas, TNF and TRAIL-induced apoptosis (Thome *et al.*, 1997) which would be thought to be as a result of competition with procaspase-8 for DISC recruitment. The significance of the failure to recruit c-FLIP$_S$ to the native TRAIL DISC would therefore argue against such a model existing *in vivo*.

Summary - TRAIL is like Fas.

Data presented in this chapter strongly suggests that TRAIL signalling has more in common with Fas than with other TNF family members such as TNF-R1 and wsl-1/DR3. Although it has been reported that both Fas and TRAIL can activate NF-κB (Ponton *et al.*, 1996) and Fig. 3.1) the predominant signalling event from these receptors is clearly the

activation of caspases. Caspase activation "masks" NF-κB activation in sensitive cells possibly through cleavage of RIP which has been demonstrated to be a component and substrate of the TRAIL DISC. To facilitate this both Fas and TRAIL-R1 and -R2 appear to recruit FADD directly rather than through an intermediate adaptor such as TRADD. Other proteins previously implicated in TNF signalling such as TRAF-2 are not recruited (Fig. 4.6), despite TRAIL being capable of activating JNK (MacFarlane *et al.*, 2000). Similar to Fas, TRAIL-mediated apoptosis can also be inhibited by c-FLIP although only the long form of c-FLIP appears to be recruited to the DISC (Fig. 4.4). Data in this chapter also demonstrates that a cellular resistance to TRAIL does not necessarily imply that a cell is unable to form a DISC, however, the caspase activation capacity of the DISC formed may be a major factor that determines cellular TRAIL sensitivity.

CHAPTER 5: ANALYSIS OF THE TUMOUR NECROSIS FACTOR SIGNALLING COMPLEX

5.1 INTRODUCTION

The term death domain was coined for a ~80 amino acid region within the intracellular domain of TNF-R1 which contained a number of residues which when mutated resulted in abrogation of TNF-induced cytotoxicity (Tartaglia *et al.*, 1991). This domain was subsequently found to be conserved in a number of other TNF-R family members and also in their associated adaptor molecules including FADD and RIP (Ashkenazi & Dixit. 1998; Wallach *et al.*, 1999).

Despite being originally identified in TNF-R1 the most well characterised DD containing pathway is that induced by CD95. CD95 ligation leads to recruitment of FADD through a direct DD interaction, which in turn leads to recruitment and processing of procaspase-8. Residues within the TNF-R1 DD that have been demonstrated to lead to abrogation of cytotoxicity also lead to abrogation of CD95-induced cytotoxicity through disruption of the DD-DD interaction (Chinnaiyan *et al.*, 1995; Eberstadt *et al.*, 1997; Tartaglia *et al.*, 1991; Wallach *et al.*, 1999). The TNF-R1 DD, however, was not observed to interact with FADD directly but was subsequently found to bind another DD-containing adaptor protein, TRADD and therefore recruit FADD indirectly (Cahill *et al.*, 1996; Hsu *et al.*, 1995). TRADD also acts as a platform for the recruitment of other signaling intermediates, such as RIP, a DD-containing kinase and TRAF2, a member of the TRAF family (TNF Receptor-Associated Factor), into the TNF-R1 signaling complex (Hsu *et al.*, 1996a). It is generally believed that TNF-induced signaling then diverges at this point; TRAF2/RIP recruitment leads to activation of downstream kinases in the NF-κB and JNK (c-Jun N-terminal kinase) pathways, while FADD recruitment leads to apoptosis (Hsu *et al.*, 1996a).

Unlike CD95 and TRAIL, the dominant signalling pathway activated by TNF is NF-κB, which functions to antagonise the apoptosis-inducing arm of TNF signalling. TNF therefore negatively regulates its own apoptotic pathway through activation of NF-κB and cannot mediate apoptosis unless this pathway is blocked (Van Antwerp *et al.*, 1996). As a consequence, in the majority of cell types, TNF-induced apoptosis requires the presence of inhibitors of transcription or translation, such as cycloheximide, which are believed to block induction of NF-κB-regulated survival genes (Boulares *et al.*, 1999). TNF-induced apoptosis of procaspase-8 would therefore be predicted to be induced more slowly than that by CD95L or TRAIL. As has been demonstrated in the previous chapter and other studies, DISC formation and caspase-8 activation induced by CD95 or TRAIL ligation is rapid (Figs. 4.1 and 4.9 and (Kischkel *et al.*, 2000; Medema *et al.*, 1997; Muzio *et al.*,

1996; Sprick *et al.*, 2000). In this respect the native TNF signalling complex has never been demonstrated to recruit caspase-8 and the majority of work done on characterising the TNF-R1 signalling complex has been carried out using overexpressed proteins.

This chapter therefore describes the isolation and characterisation of endogenous TNF signalling complexes. Treatment of HeLa cells with TNF led to the rapid recruitment of TRADD, RIP and TRAF2. Interestingly, proteins recruited to the TNF precipitates underwent some type of modification that was not apparent in whole cells lysates. Despite the presence of these core signalling components there was no evidence of the apoptotic mediators FADD or caspase-8. These mediators were, however, clearly required as TNF-mediated apoptosis was abrogated in FADD- and caspase-8-deficient Jurkat cells.

5.2 RESULTS

5.2.1 TRADD, TRAF2 and RIP but not FADD or caspase-8 are recruited to the native TNF signalling complex: TRADD, TRAF2 and RIP appear to be modified upon recruitment

As already mentioned above most of the early studies involving the TNF signalling were carried out using overexpression of the implicated signalling molecules, therefore in order to characterise the native TNF signalling complex experiments were carried out in a similar manner to those used to look at the TRAIL DISC in Chapter 4. The extracellular domain of human TNF-α was cloned and biotin labelled as described in *Materials and Methods*. HeLa cells were initially used as a model cell line to look at the TNF DISC and precipitates were then characterised for the presence of the TNF signalling intermediates TRADD, RIP and TRAF2. Furthermore, as HeLa cells do not express TNF-R2 so TNF-induced TNF-R1 signalling complexes could be studied exclusively (Chan *et al.*, 2000).

Treatment with biotinylated-TNF (bTNF) led to recruitment of TRADD, RIP and TRAF2 to the TNF precipitates (Fig. 5.1A). Surprisingly however, although these proteins were present as a single band in the lysates they were clearly subjected to some form of modification in the TNF precipitates. TRADD appeared to exhibit a "ladder like" appearance with several higher molecular weight forms visible above the monomeric 36 kDa form present in the lysate. TRADD has not previously been reported to form a dimer and, as the smaller of these species were clearly too small to be dimeric TRADD, this could not be the explanation for the higher molecular weight species observed. Modification of RIP and TRAF2 appeared to differ from that of TRADD in that they appeared as more of a "smear" of protein on the blot rather than a discreet series of higher molecular weight species. Again both proteins only appeared as single bands in cell lysates. The modifications observed were unlikely to artefactual due to non-specific antibody binding as the monoclonal antibodies used for Western blot analysis were clearly specific when used on whole cell lysates. Modification of TRADD and TRAF2 has not been previously reported in any of the early TNF studies however, a recent report looking at the molecular basis for TNF-mediated NF-κB signalling did report a similar pattern for RIP (Zhang *et al.*, 2000a), and although it was speculated that this appeared to be classic of a ubiquitin-modified protein this suggestion was not confirmed.

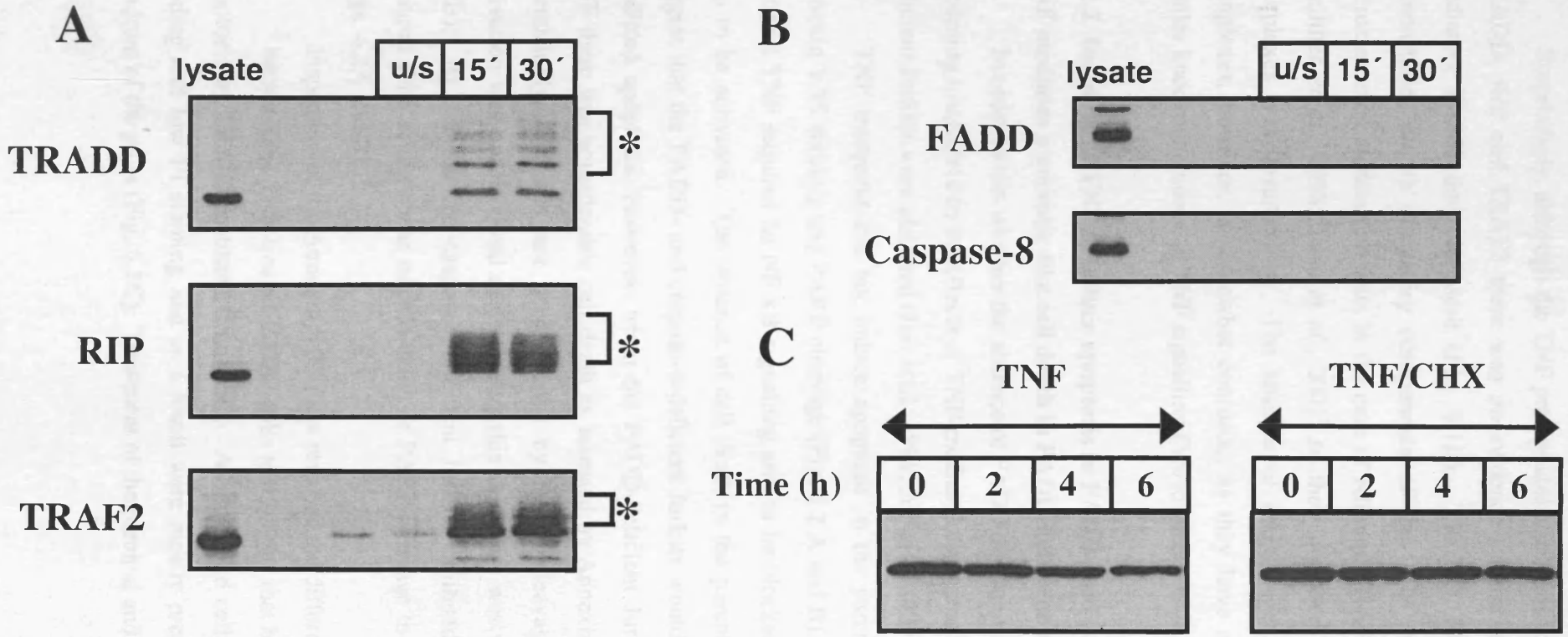


Figure 5.1 TRADD, TRAF2 and RIP are all components of the TNF signalling complex: FADD and caspase-8 are not recruited. HeLa cells (3×10^7/treatment) were treated with biotinylated TNF (bTNF) 200 ng/ml for indicated time periods. After treatment TNF complexes were precipitated essentially as described for TRAIL complexes (Fig. 4.1). Precipitated complexes were analysed for the presence of known TNF signalling components, (A) TRADD, RIP and TRAF2. * indicates modified species of TRADD, RIP and TRAF2 which only appear in precipitates after TNF stimulation. (B) TNF complexes were also analysed for the apoptotic mediators FADD and caspase-8. (C) HeLa cells were treated with TNF (200 ng/ml) alone or TNF in the presence of cycloheximide (1 μM) for 6 h after which PARP cleavage was assessed.

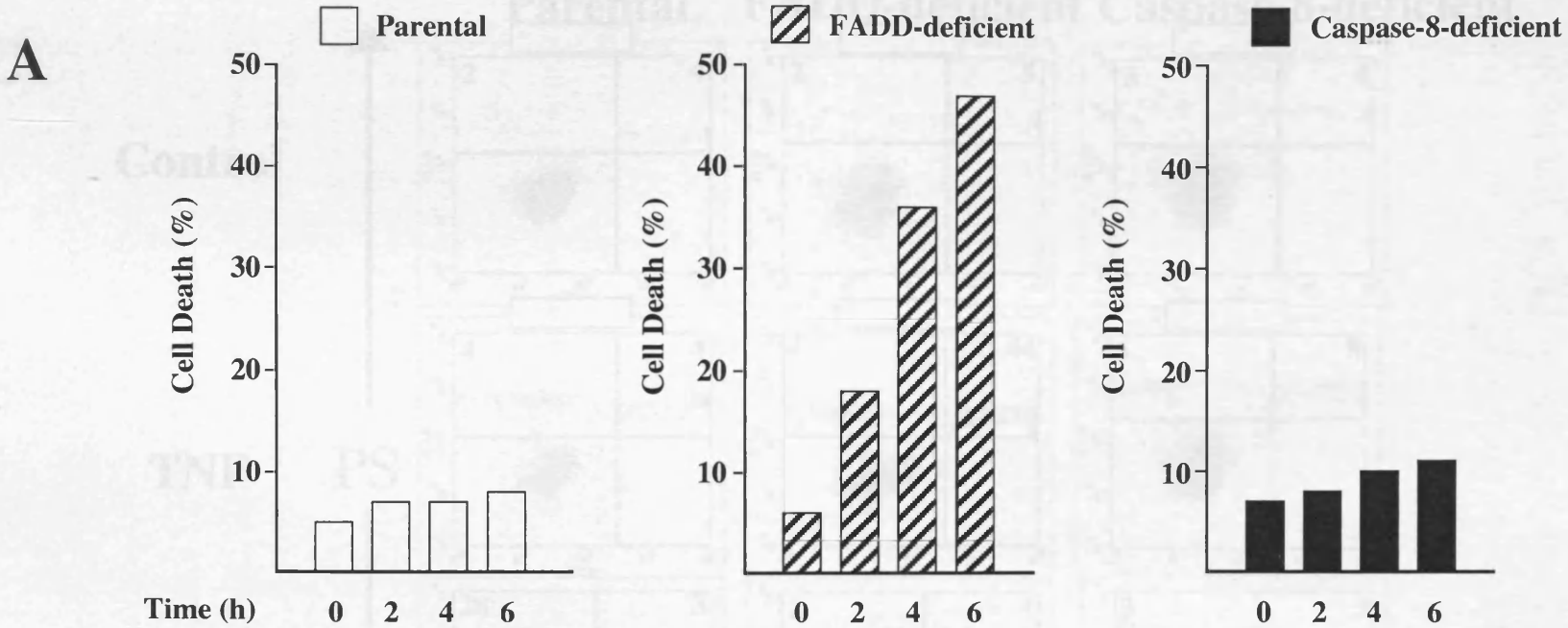
Surprisingly, although the TNF precipitates contained the TNFR1 adaptor proteins TRADD, RIP and TRAF2 there was no evidence for the recruitment of the apoptotic mediators FADD and caspase-8 (Fig. 5.1B). TRADD, RIP and TRAF2 have been demonstrated to be obligatory components of the TNF signalling complex both in overexpression studies and also, in the case of RIP and TRAF2, in gene knockout studies (Kelliher *et al.*, 1998; Tada *et al.*, 2001) so their presence in the native isolated TNF complexes was unsurprising. The absence of FADD and caspase-8 within the same complexes, however, is somewhat confusing as they have also been implicated, using similar knockout studies, in TNF signalling (Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998).

5.2.2 Inability of TNF to induce apoptosis in FADD- and caspase-8-deficient Jurkats: TNF mediates a necrotic-like cell death in FADD-deficient Jurkats

In order to test whether the absence of FADD and caspase-8 did in fact lead to cells becoming insensitive to the effects of TNF-mediated apoptosis FADD- and caspase-8-deficient Jurkats were obtained (Juo *et al.*, 1998; Juo *et al.*, 1999).

TNF treatment did not induce apoptosis in the parental Jurkats as assessed by Annexin V/PI staining and PARP cleavage (Fig. 5.2 A and B). This is consistent with the fact that TNF requires its NF-κB signalling arm to be blocked for the caspase activation arm to be activated. The absence of cell death in the parental cell line would strongly suggest that the FADD- and caspase-8-deficient Jurkats would also be refractory to TNF-mediated apoptosis. However, when the FADD-deficient Jurkat cells were treated with TNF there was considerable cell death as assessed by Annexin V/PI staining (Fig. 5.2A). Interestingly, this was not accompanied by PARP cleavage suggesting that caspase activation was not involved and therefore this cell death was not classical apoptosis (Fig. 5.2B). By contrast the caspase-8-deficient Jurkats exhibited a similar response to the parental cells as there was no cell death or PARP cleavage in response to TNF treatment (Figs. 4.2A and B).

Inspection of the Annexin V/PI plots revealed the differences in cell death observed between the TNF-treated FADD nulls and Jurkats that had been induced to undergo apoptosis by TRAIL treatment (Fig. 5.2C). All untreated cells exhibited low Annexin V binding and low PI staining and as a result were mostly present within the bottom left quadrant of the plots (Fig. 5.2C). Treatment of the parental and caspase-8-deficient cells


A
Parental
FADD-deficient
Caspase-8-deficient
Cell Death (%)
50
40
30
20
10
Time (h)
0 2 4 6
B
Time (h)
PARP
p85
Cell death (%)
0 2 4 6
5 7 7 8
6 18 36 47
7 8 10 11
w/t Jurkats
FADD deficient
Caspase-8 deficient

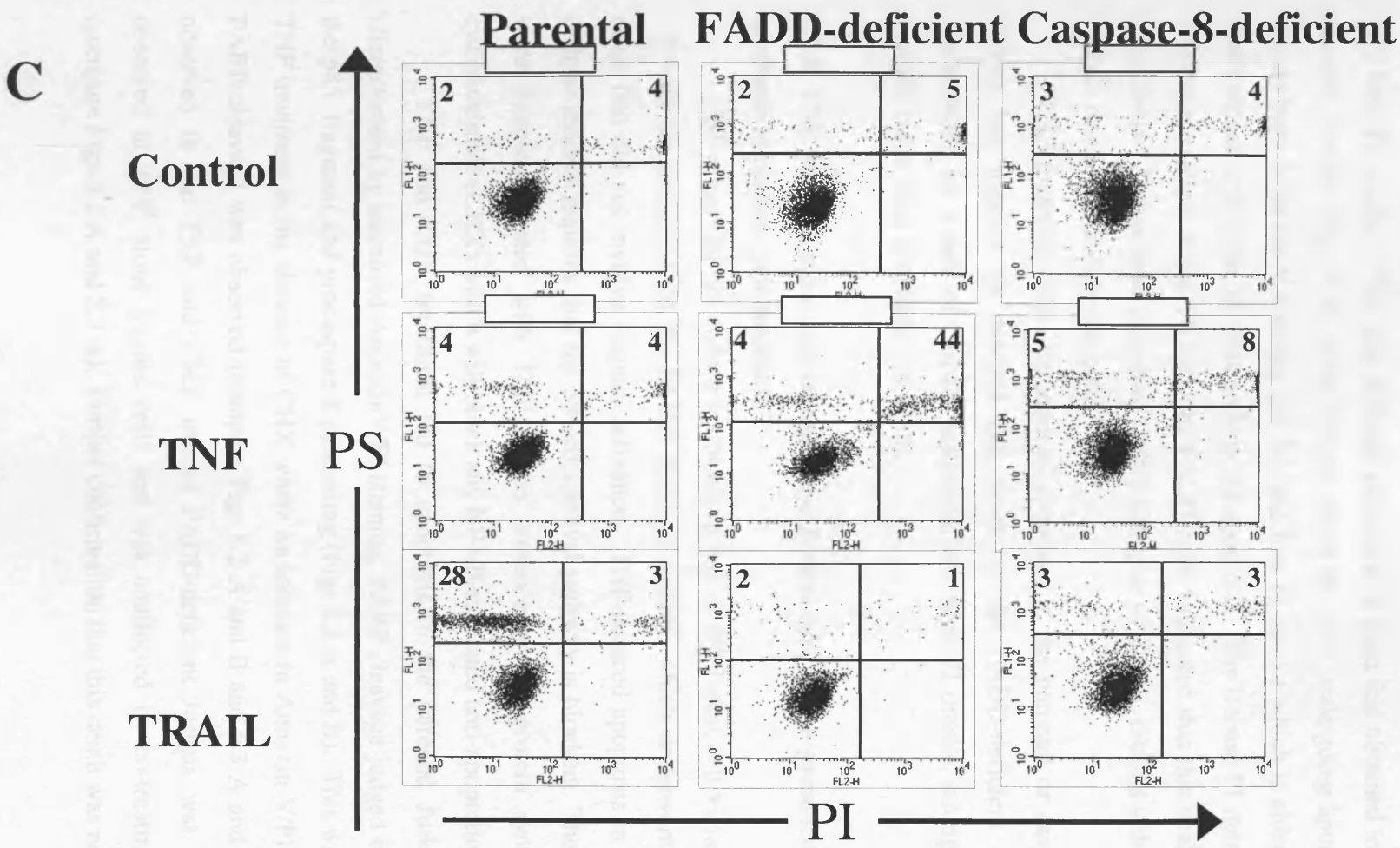


Figure 5.2 Effect of TNF on cell death in wild-type, FADD-deficient and Caspase-8-deficient Jurkats. Parental, FADD- and caspase-8-deficient Jurkat cells were treated for indicated time periods with TNF (200 ng/ml). Cell death was assessed by Annexin V/PI staining as described in *Materials and Methods (2.2.2)*. (A) Graphs of Annexin V/PI results. (B) Cells were also subjected to Western blotting for the apoptotic substrate, PARP. (C) Annexin V/PI plots from TNF and TRAIL (1 μg/ml) treated cells. Numbers in the upper left (Annexin V high) and right quadrants (Annexin V and PI high) of these plots represent the % of cells within these populations. Data shown are representative of 3 independent experiments. Data shown are representative of 3 independent experiments.

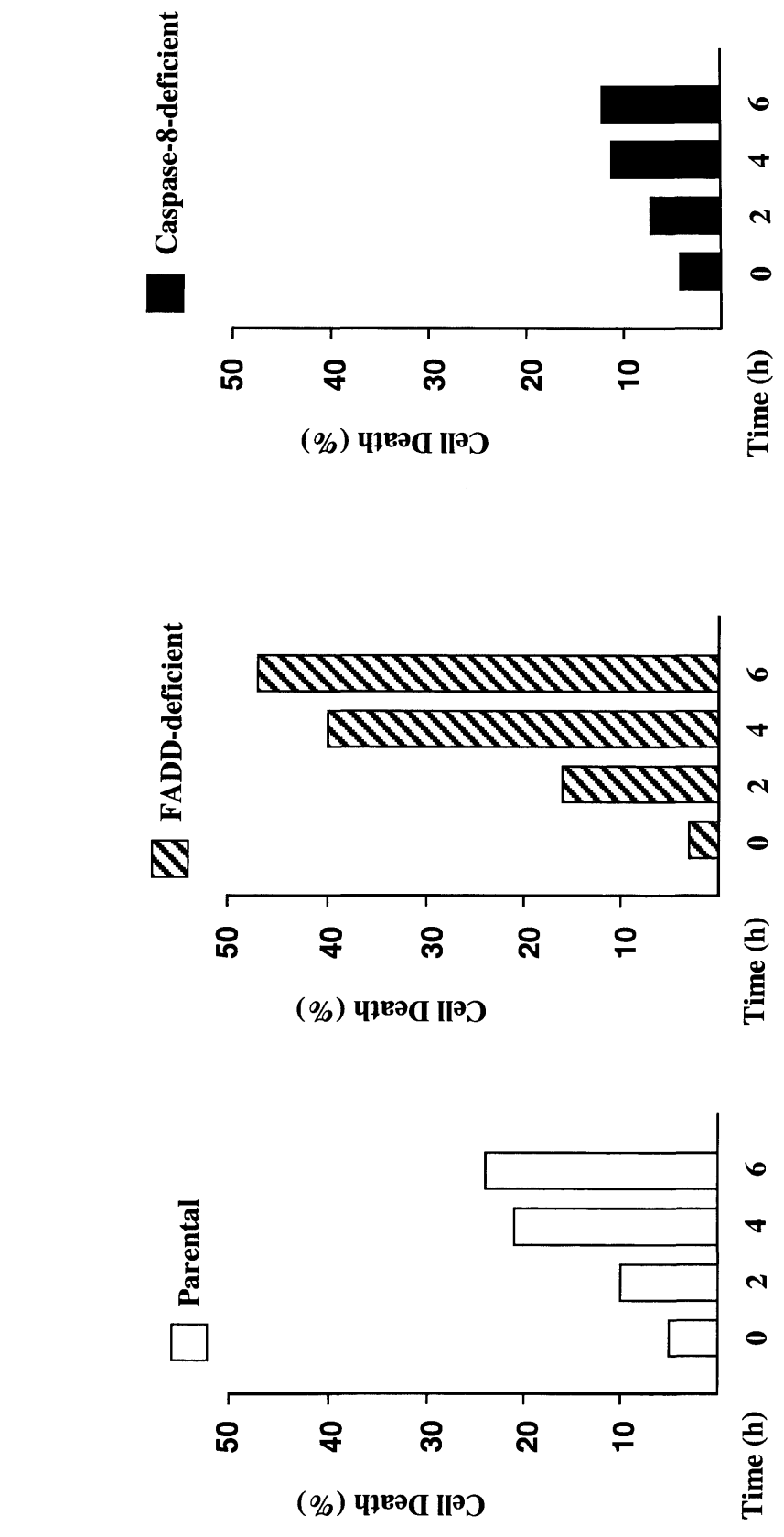
with TNF did not markedly effect the distribution of cells within the plots (Fig. 5.2C middle, far left and far right). When the FADD-deficient cells were treated however, a large proportion (44%) appeared in the far right of the upper right quadrant (Fig. 5.2C middle plot) displaying high Annexin V staining and, due to their position in the quadrant, very high PI uptake. This plot differed considerably from that obtained in TRAIL-treated parental Jurkats (Fig. 5.2C lower left) in which the cells undergoing apoptosis exhibited mainly high Annexin V staining (28 %), and low PI uptake which is characteristic, along with caspase activation, of cells undergoing apoptosis. The intense PI staining and lack of caspase activation in the TNF-treated FADD nulls suggested that this death was probably by necrosis. As has been described before (Chapter 4) TRAIL did not induce cell death in FADD- or caspase-8-deficient cells.

TNF therefore does not induced apoptosis in the parental or caspase-8-deficient Jurkats but appears to mediate cell death in the FADD-deficient cells which is characterised by a lack of caspase activation, and high PI uptake, strongly suggesting a necrotic rather than apoptotic cell death.

5.2.3 TNF induces apoptosis in wild-type Jurkat cells in the presence of the protein synthesis inhibitor cycloheximide

TNF alone did not induce apoptosis in any of the Jurkat cell variants used in Fig. 5.2 with the exception of the FADD-deficient Jurkats, which underwent a necrotic cell death that did not involve caspase activation. TNF-induced apoptosis in the majority of cellular models requires that the NF-κB survival pathway is blocked. The Jurkat variants were therefore treated with TNF in the presence of the protein synthesis inhibitor cycloheximide (CHX) which will block any NF-κB-regulated anti-apoptotic genes.

TNF and CHX treatment led to apoptosis in the parental Jurkats which was characterised by increased AnnexinV/PI staining, PARP cleavage judged by generation of the p85 fragment and procaspase-8 processing (Figs 5.3 A and B). This was in contrast to TNF treatment in the absence of CHX where no increase in Annexin V/PI staining or any PARP cleavage was observed (compare Figs 5.2 A and B and 5.3 A and B). Cell death observed in the TNF and CHX treated FADD-deficient Jurkats was similar to that observed in TNF alone treated cells and was unaffected by co-treatment with CHX (compare Figs 5.2 A and 5.3 A). Further confirmation that this death was non-apoptotic


A
Parental
FADD-deficient
Caspase-8-deficient
Cell Death (%)
10
20
30
40
50
Time (h)
0
2
4
6

B

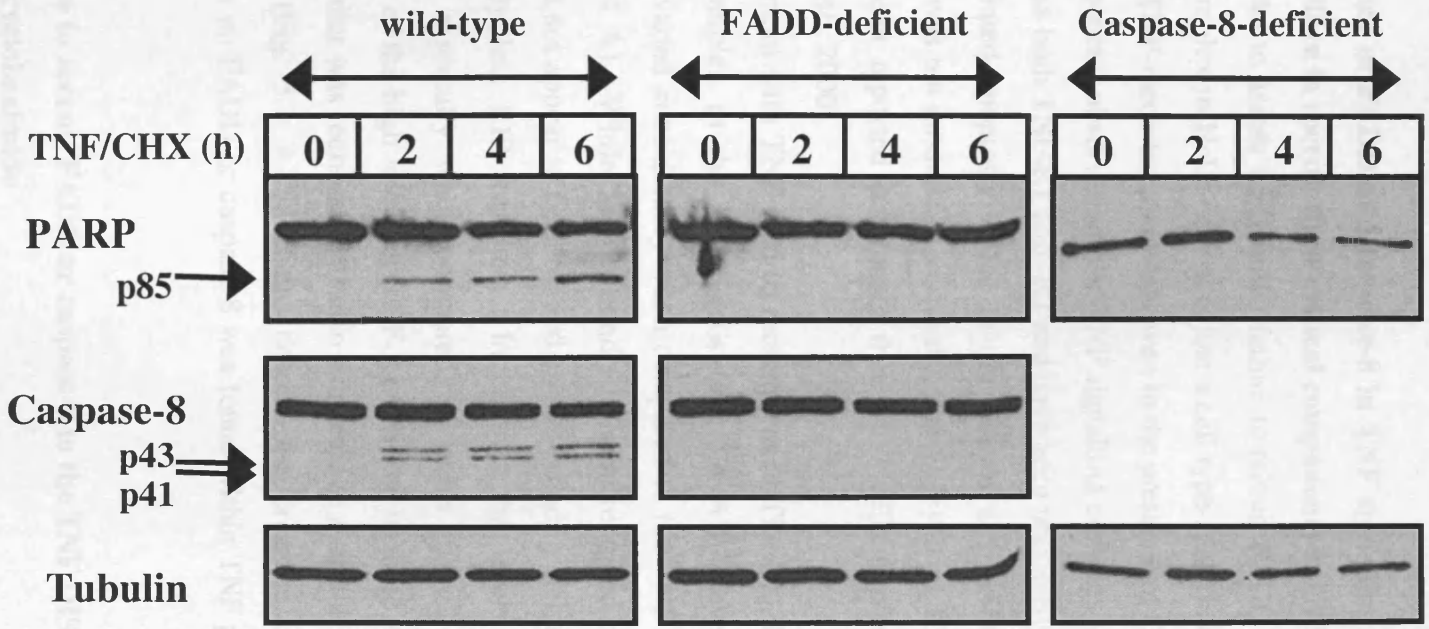


Figure 5.3 Effect of TNF and cycloheximide (CHX) on cell death in wild-type, FADD-deficient and Caspase-8-deficient Jurkats. Parental, FADD- and caspase-8-deficient Jurkat cells were treated for indicated time periods with TNF (200 ng/ml) and cycloheximide (1 μM). Cell death was assessed by Annexin V/PI. (A) Graphs of Annexin V/PI results. (B) Cells were also subjected to Western blotting for the apoptotic substrate, PARP and the initiator caspase, caspase-8. α-Tubulin was used as a protein loading control.

was that caspase-8 was unprocessed within these cells (Fig 5.3 B), which is consistent with the death observed being caspase-independent. No cell death was induced in the FADD- or caspase-8-deficient Jurkats in the presence of TNF and CHX which is consistent with the fact that caspase-8 is the apical caspase in TNF-mediated apoptosis.

5.2.4 Absence of FADD and caspase-8 in TNF signalling complexes is not cell-type specific: Failure to recruit these critical components in U937 cells

In order to assess whether the failure to recruit FADD and caspase-8 into the TNF signalling complex in HeLa cells was just a cell type-specific effect in a cell line that does not undergo TNF-mediated apoptosis even in the presence of CHX (Fig 5.1C) or whether it was a more general observation, the TNF signalling complex was examined in U937 cells. U937s express both TNF-R1 and -R2 and have been previously been shown to be sensitive to TNF-mediated apoptosis. Due to the lack of availability of suitable Western blot antibodies it was not possible to assess the relative levels of TNF-R2 compared to TNF-R1 but it has been reported previously that U937 cells express more TNF-R2 than -R1 (Pryhuber *et al.*, 2000).

Treatment with TNF lead to recruitment of TRADD, RIP and TRAF2 to the TNF signalling complex in the U937 cells (Fig 5.4 A). Levels of recruitment of these components varied considerably when compared to the TNF complexes formed in HeLa cells (Fig. 5.1 A). While TRADD and RIP were recruited to TNF precipitates in these cells they did not appear to be modified as was found in HeLa cells (5.1 A) and also there was markedly less RIP recruited. In contrast the levels of TRAF2 recruited were considerable especially when compared its levels in cell lysates and may be as a consequence of the high levels of TNF-R2 reported in these cells (Pryhuber *et al.*, 2000). The TRAF2 that was recruited was also subjected to a similar modification as was seen in HeLa cells (Fig. 5.1 A). Again, despite the presence of other TNF-R signalling intermediates no FADD or caspase-8 was found within TNF precipitates from U937 cells (Fig.5.4B).

5.2.5 Failure to recruit FADD or caspase-8 to the TNF DISC in U937 cells despite the presence of cycloheximide

When treated with TNF alone U937 cells failed to undergo apoptosis as judged by PS externalisation and PI uptake, caspase-8 and caspase-3 cleavage (Fig. 5.5 A). However, as demonstrated in 293 cells with TRAIL (Chapter 4), the failure of a cell to undergo

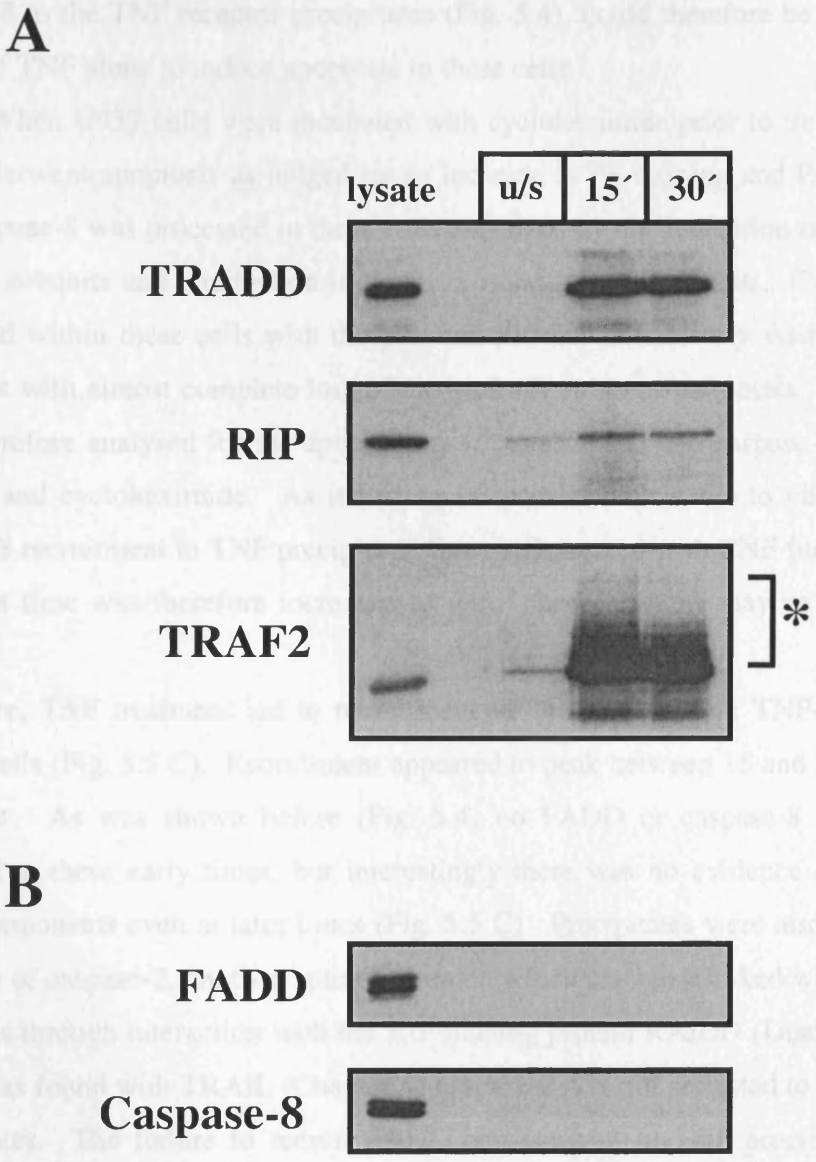


Figure 5.4 Recruitment of TRADD, RIP and TRAF2 but not FADD or caspase-8 to TNF signalling complexes in U937 cells. U937 cells (5×10^7/treatment) were treated for indicated time periods and TNF precipitates were isolated essentially as described for HeLas (Fig. 5.1). Precipitates were analysed for the presence of known TNF signalling components, TRADD, RIP and TRAF2 (A) and for the apoptotic mediators FADD and caspase-8 (B) * indicate modified species of TRAF2 which only appear in precipitates after TNF stimulation.

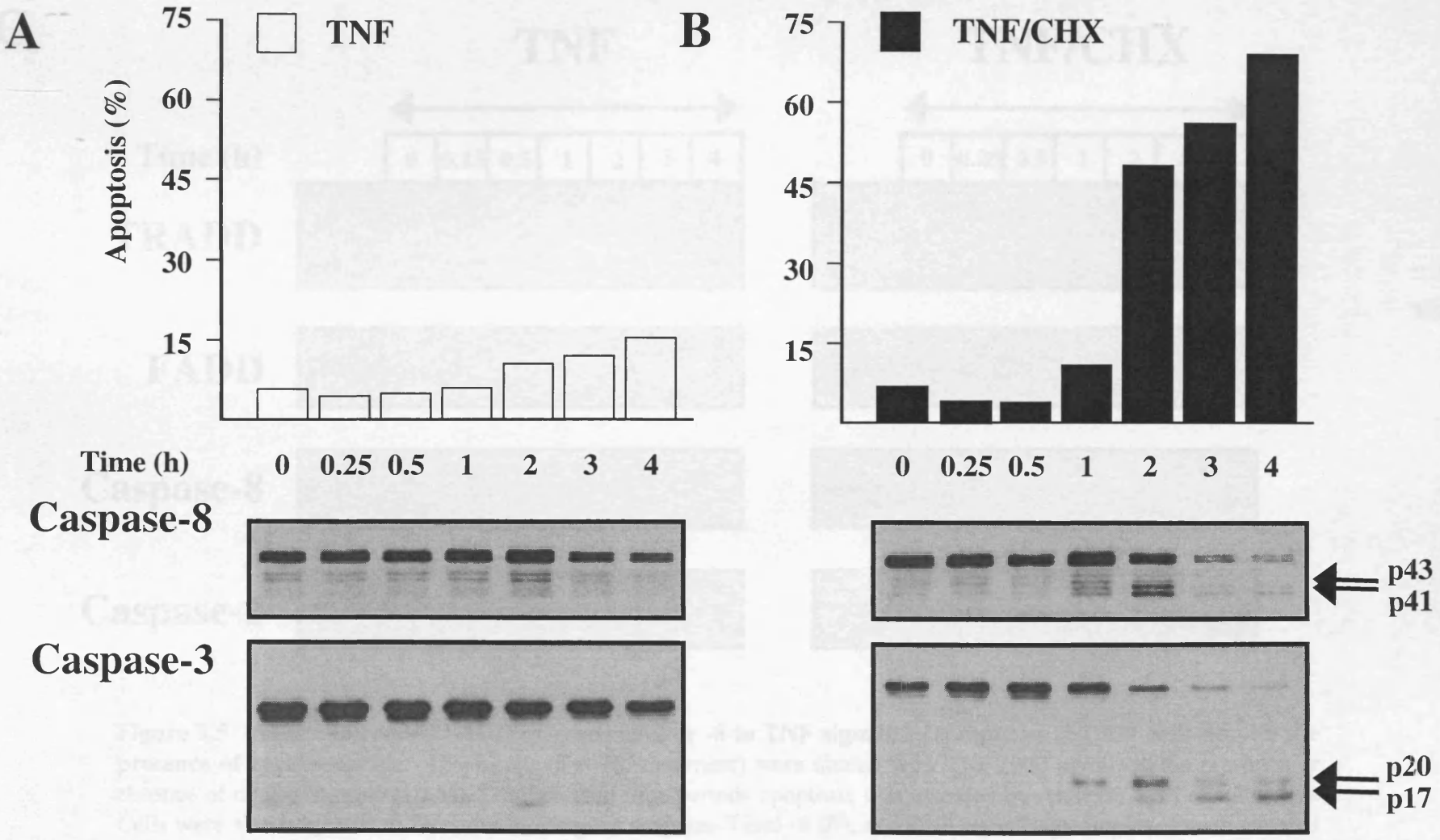
apoptosis does not necessarily imply that apoptotic mediators such as FADD and caspase-8are not recruited upon receptor ligation (Fig. 4.1). The failure to recruit FADD and caspase-8 to the TNF receptor precipitates (Fig. 5.4) could therefore be as a result of the failure of TNF alone to induce apoptosis in these cells.

When U937 cells were incubated with cycloheximide prior to treatment with TNF they underwent apoptosis as judged by an increase in PS staining and PI uptake (Fig. 5.5 B). Caspase-8 was processed in these cells as judged by the generation of the intermediate p43/p41 subunits and a reduction in the proform at later time-points. Caspase-3 was also processed within these cells with the p20 and p17 subunits clearly visible 1 h after TNF treatment with almost complete loss of the proform at later time-points. TNF precipitates were therefore analysed for the apoptotic mediators FADD and caspase-8 in the presence of TNF and cycloheximide. As it had so far proved impossible to observe FADD and caspase-8 recruitment in TNF precipitates from cells treated with TNF for up to 30 min the treatment time was therefore increased to see if these proteins may be recruited at later times.

As before, TNF treatment led to recruitment of TRADD in both TNF- and TNF/CHX-treated cells (Fig. 5.5 C). Recruitment appeared to peak between 15 and 30 min after TNF treatment. As was shown before (Fig. 5.4) no FADD or caspase-8 was found to be recruited at these early times, but interestingly there was no evidence of recruitment of these components even at later times (Fig. 5.5 C). Precipitates were also analysed for the presence of caspase-2, another initiator caspase which has been linked with death receptor apoptosis through interaction with the RIP binding protein RAIDD (Duan & Dixit. 1997), but as was found with TRAIL (Chapter 4) caspase-2 was not recruited to the TNF receptor precipitates. The failure to recruit FADD or caspase-8 in TNF precipitates from TNF sensitive cells even at later time points suggested that these components were in fact not recruited to such receptor complexes.

5.2.6 Proteasome inhibition leads to the retention of RIP in TNF signalling complexes

The modified forms of RIP, TRADD and TRAF2 observed in HeLa cells were unusual as they appeared to be occurring upon recruitment to the receptor and were not present in similarly treated cell lysates (Fig. 5.1 A). Similarly the modification of TRAF2 in U937 cells was also only observed in TNF precipitates (Fig. 5.4 A). It was therefore unlikely that these modifications were an artefact of the precipitation process as they differed considerably, the most striking being those on TRADD and RIP. TRADD


A
TNF
B
TNF/CHX
Apoptosis (%)
75
60
45
30
15
Time (h)
0 0.25 0.5 1 2 3 4
Caspase-8
Caspase-3
p43
p41
p20
p17

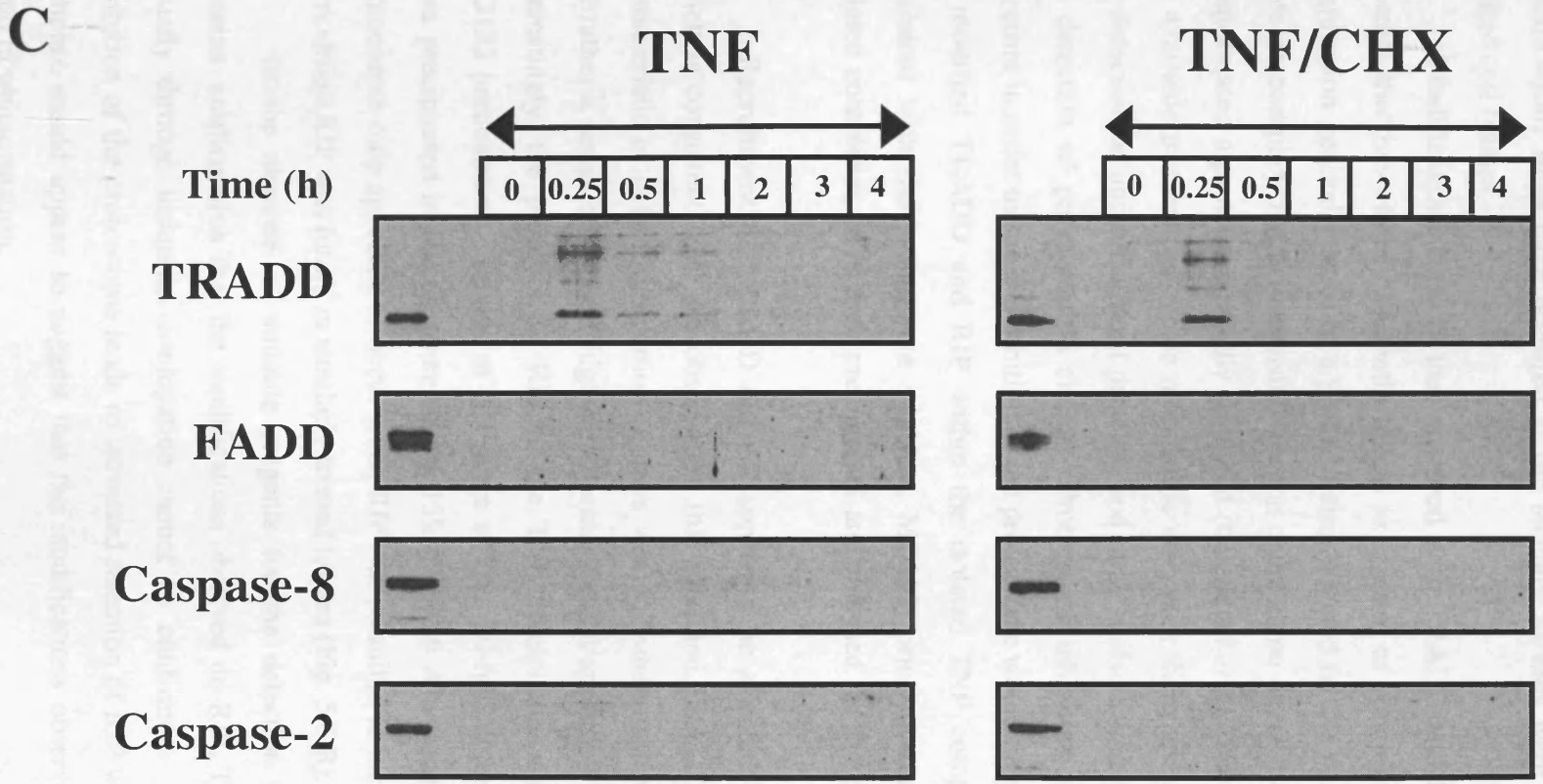


Figure 5.5 Failure to recruit FADD or caspases-2 or -8 to TNF signalling complexes in U937 cells despite the presence of cycloheximide. U937 cells (5×10^7/treatment) were treated with TNF (200 ng/ml) in the presence or absence of cycloheximide (1 μM). At indicated time periods apoptosis was assessed by Annexin V/PI staining (A). Cells were also subjected to Western blotting for caspases-3 and -8 (B), and TNF signalling complexes were isolated and analysed by Western blotting for TRADD, FADD and caspases-2 and -8 (C). Data shown are representative of 3 independent experiments.

appeared to exhibit a "ladder-like" appearance whereas RIP appeared more as a "smear". Although there have been no reports demonstrating modification of TRADD, RIP modification has been demonstrated before. Zhang and co-workers noted that RIP was modified upon recruitment to TNF-R1 (Zhang *et al.*, 2000a). They also concluded that the modifications must have occurred at the receptor as they were not present in similarly treated cell lysates.

Modifications such as that observed with TRADD and RIP are characteristic of proteins that have been covalently linked to a series of ubiquitin molecules. Ubiquitin conjugation generally leads to a protein being targeted by the proteasome, a multisubunit protease complex that is responsible for the degradation of most cytosolic proteins. Once ubiquitinated a protein is rapidly degraded (Glickman *et al.*, 2002). Although antibodies are available that can recognise monomeric ubiquitin, these are generally not suitable for the detection of ubiquitinylated proteins, and there is also a lack of antibodies suitable for the detection of polyubiquitin chains. Proteasome inhibitors are readily available and therefore in order to see if inhibition of the proteasome would have any effect on the levels of modified TRADD and RIP within the isolated TNF complexes HeLa cells were incubated with the proteasome inhibitor, MG132, prior to treatment with TNF. The isolated complexes were then precipitated and analysed for the presence of TRADD and RIP.

Recruitment of TRADD did not appear to be affected by MG132 pretreatment which is consistent with the observation that modified TRADD in HeLa cells was not characteristic of polyubiquitination as there was no "smearing" of the protein on the gels but rather a series of distinct higher molecular weight species (Figs. 5.1 A and Fig. 5.6). Interestingly, the presence of RIP in the TNF precipitates was markedly affected by MG132 pretreatment. Levels of RIP were at least 10-fold higher when TNF complexes were precipitated in the presence of MG132 (Fig. 5.6 A). Again, modification of these components only appeared to occur when RIP was recruited to the TNF-R complex as only unmodified RIP was found in similarly treated lysates (Fig. 5.6 B).

In the absence of suitable reagents for the detection of ubiquitin conjugated proteins confirmation that the modifications observed on RIP, TRADD and TRAF2 was actually through ubiquitin conjugation cannot be confirmed. However, the fact that inhibition of the proteasome leads to increased retention of RIP within the TNF signalling complex would appear to suggest that the modifications observed on RIP may be as a result of ubiquitination.

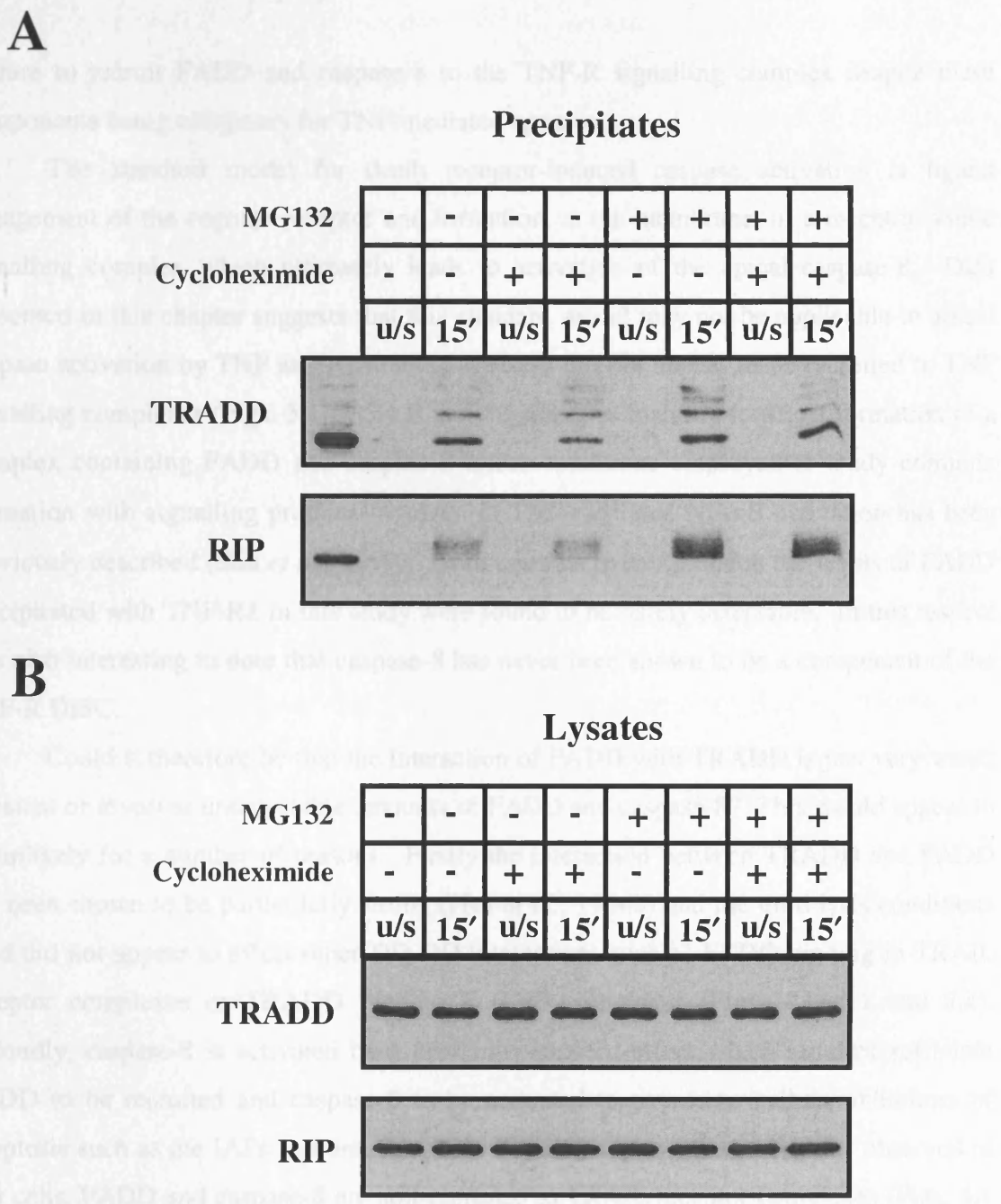


Figure 5.6 Effect of proteasome inhibition on the levels of RIP and TRADD in TNF-signalling complexes. HeLa cells (3×10^7/treatment) were treated with TNF (200 ng/ml) for 15 min as indicated, both in the presence and absence of cycloheximide (1 μM) as a co-treatment and the proteasome inhibitor, MG132 (1 μM) as a 30 min pretreatment. TNF signalling complexes were isolated and Western blotted for TRADD and RIP (A). Lysates from treated cells were also analysed for TRADD and RIP (B).

5.3 DISCUSSION

Failure to recruit FADD and caspase-8 to the TNF-R signalling complex despite these components being obligatory for TNF-mediated apoptosis

The standard model for death receptor-induced caspase activation is ligand engagement of the cognate receptor and formation, at the membrane, of a receptor-bound signalling complex which ultimately leads to activation of the apical caspase-8. Data presented in this chapter suggests that this standard model may not be applicable to apical caspase activation by TNF as FADD and caspase-8 did not appear to be recruited to TNF signalling complexes (Figs. 5.1 B, 5.4 B and 5.5 C). The inability to detect formation of a complex containing FADD and caspase-8 under conditions employed to study complex formation with signalling proteins involved in TNF-mediated NF-κB activation has been previously described (Shu *et al.*, 1997). Even upon its overexpression the levels of FADD precipitated with TNF-R1 in this study were found to be barely detectable. In this respect it is also interesting to note that caspase-8 has never been shown to be a component of the TNF-R DISC.

Could it therefore be that the interaction of FADD with TRADD is just very weak, transient or involves undetectable amounts of FADD and caspase-8? This would appear to be unlikely for a number of reasons. Firstly the interaction between TRADD and FADD has been shown to be particularly strong (Hsu *et al.*, 1996b) and the mild lysis conditions used did not appear to effect other DD-DD interactions such as FADD binding in TRAIL receptor complexes or TRADD binding in TNF complexes (Figs. 4.1, 5.1 and 5.4). Secondly, caspase-8 is activated by a proximity-induced effect which requires sufficient FADD to be recruited and caspase-8 to be activated to overcome cellular inhibitors of apoptosis such as the IAPs and anti-apoptotic Bcl-2 family members. As was observed in 293 cells, FADD and caspase-8 are still recruited to TRAIL receptor complexes (Figs. 4.1 and 4.9) despite these cells being resistant to TRAIL-induced apoptosis. The difference in FADD and caspase-8 recruitment in these cells compared with the TRAIL-sensitive HeLas was clearly not enough to engage apoptosis within these cells (Figs. 4.1 and 4.3). An argument therefore that TNF treatment may lead to the recruitment of undetectable amounts of caspase-8 is untenable as it would be subjected to the same cellular constraints.

How is caspase-8 activated during TNF-mediated apoptosis?

FADD and caspase-8 are clearly required for TNF-induced apoptosis even if they do not appear to be recruited to a membrane-bound DISC (Figs. 5.1 and 5.3). How then is caspase-8 activated during TNF-mediated apoptosis and how does it differ from CD95 and TRAIL?

In unstimulated cells, TNF-R1 is primarily found in the *trans*-golgi network (TGN) and TRADD is loosely associated with the golgi. Following TNF stimulation TRADD relocalises and rapidly associates with TNF-R1 at the plasma membrane (Jones *et al.*, 1999). It has been reported that FADD and caspase-8 form aggregates upon TNF treatment. Aggregation was found to be dependent on myosin II motor activity as it was inhibited in cells expressing a kinase dead myosin light chain kinase (MLCK) (Jin *et al.*, 2001). It was however unclear whether these aggregates were actually associated with TNF-R1. A kinase dead mutant of MLCK was found to delay TNF-mediated apoptosis in these cells therefore providing a role for myosin II motor activity in regulating TNF-mediated apoptosis..

There is also evidence that TNF-mediated signalling may involve calveolae-like domains (CLD) within the plasma membrane. TNF-R1 was found to co-localise with CLD marker proteins such as CD36. Cell surface expression of TNF-R1 and TNF- but not CD95-mediated apoptosis is reduced in CLD-disrupted cells (Ko *et al.*, 1999a). Consistent with the above study TNF-mediated apoptosis has been demonstrated to require receptor internalisation. Preincubation of cells with monodansylcadaverine, a transglutaminase inhibitor, was observed to inhibit TNF-mediated apoptosis but not CD95-mediated apoptosis (Schutze *et al.*, 1999). Interestingly this treatment did not appear to inhibit other TNF-induced signalling pathways such as JNK activation (Bradley *et al.*, 1993; Schutze *et al.*, 1999).

Internalisation results in dissociation of TRADD from the receptor possibly as a consequence of ligand dissociation when the internalised ligand-receptor complex is relocated to the acidic environment of the endosomes (Jones *et al.*, 1999). Internalisation of receptor complexes therefore may act to attenuate TNF-signalling but there is also evidence that TNF signalling may bifurcate pre- and post- internalisation. It is possible that early TNF-mediated signalling events such as NF-κB and JNK activation occur pre-internalisation. Evidence for this is that components required for these processes such as RIP and TRAF2 are precipitated with TNF receptor complexes (Figs. 5.1 and 5.4 and

(Devin *et al.*, 2000; Zhang *et al.*, 2000a)). The internalised receptor or dissociated TRADD may then interact with other TNF signalling components such as FADD and caspase-8 to mediate apoptosis.

The lysosomal compartment has also been implicated in TNF-mediated cell death. Lysosomal enzymes such as the cathepsins have not only been implicated in TNF-induced necrosis but they have also been shown to be capable of inducing an apoptotic pathway either by cleavage of caspases (Leist *et al.*, 2001) or proapoptotic molecules such as Bid (Stoka *et al.*, 2001). TNF treatment has been demonstrated to lead to cathepsin B release from the lysosomes (Leist & Jaatela. 2001) and, in this respect, it is interesting to note that hepatocytes derived from cathepsin B null mice are resistant to TNF-mediated apoptosis (Guicciardi *et al.*, 2001). The cathepsin B inhibitor cystatin A has been demonstrated to inhibit TNF-induced apoptosis in WEHI-S cells (Foghsgaard *et al.*, 2001). Whether these other proteases act upstream or downstream of caspase-8 activation is currently unknown.

TNF-mediated necrosis

As well as being required for TNF-mediated apoptosis (Fig. 5.3), FADD also appears to be required for the prevention of a necrotic TNF-mediated cell death (Figs. 5.2 and 5.3). This necrotic cell death is observed in only a few other cellular systems. The murine fibroblast cell lines, L929 and NIH3T3s all undergo a similar death in response to TNF which, in some cases can be enhanced by z-VAD.fmk pre-treatment (Khwaja *et al.*, 1999; Luschen *et al.*, 2000b). The mechanism of TNF-induced necrosis is unknown but probably involves reactive oxygen species (ROS) (Vercammen *et al.*, 1998a; Vercammen *et al.*, 1998b). The kinase function of RIP has also been demonstrated to be required although how this may relate to ROS production is unknown (Holler *et al.*, 2000). In this respect it is also interesting that TRAF2 has been demonstrated to induce ROS production although again the mechanism behind this is unclear (Chandel *et al.*, 2001a).

Therefore two molecules which are required for the prevention of TNF-mediated apoptosis through activation of NF-κB may also be required for a TNF-mediated caspase-independent cell death. The fact that RIP is a caspase substrate (Fig. 3.11 and (Lin *et al.*, 1999)) suggests that caspase activation may act to block TNF-induced necrosis through cleavage of RIP.

Modifications of components within the TNF-R signalling complex

Although not fully confirmed as ubiquitin modifications (due to a lack of suitable reagents) the observed effect of a proteasome inhibitor on RIP within the TNF-R complex would at least suggest that this modification may be through ubiquitin conjugation (Fig. 5.6). If this is indeed the case then parallels exist with another NF-κB activation pathway, that induced by interleukin-1 (IL-1). IL-1-mediated NF-κB activation requires the presence of another death domain-containing Ser/Thr kinase called IRAK1 (IL-1 receptor Associated Kinase 1) (Cao *et al.*, 1996). Like RIP, in the TNF pathway, its kinase function is not required for its function (Maschera *et al.*, 1999). Interestingly upon IL-1 stimulation IRAK1 has been demonstrated to be hyperphosphorylated, ubiquitinated and subsequently degraded by the proteasome (Yamin *et al.*, 1997). The kinase responsible for this is another IRAK family member, IRAK4, and the introduction of an IRAK4 kinase mutant leads to abrogation of IL-1-induced NF-κB activation (Li *et al.*, 2002a). Clearly then IRAK-1 modification is important for NF-κB activation in response to IL-1 but the role of modified IRAK1 is unknown. It is speculated that the modification allows the IRAK1 and TRAF6 complex to dissociate from the IL-1 receptor complex and may act to limit or attenuate signalling (reviewed in (Janssens *et al.*, 2003)). In this respect it is interesting to note that another member of the RIP family, RIP3, has been demonstrated to be recruited to TNF-R1 and function as a RIP kinase. Overexpression of RIP3 attenuates TNF-mediated NF-κB activation and it was suggested that it acts as an inhibitor of NF-κB activation (Sun *et al.*, 2002a). However, this finding may be an artefact of overexpression and RIP3 may actually play a similar role to IRAK4.

Ubiquitination has also been demonstrated to play an important role in activation of the IKK signalosome in response to IL-1. It has been demonstrated that an atypical ubiquitination site, lysine 63, results in chains which do not target a particular protein for destruction (Glickman & Ciechanover. 2002). This type of modification has been demonstrated to be responsible for activation of the apical kinases in the IL-1 signalling pathway. TRAF6 has been demonstrated to activate a TAK1 (Transforming Growth Factor-beta (TGF-β) Activating Kinase 1)/TAB1/TAB2 complex through a lysine 63 polyubiquitin chain, a step that is required for IL-1 mediated NF-κB activation. It is interesting that in the same studies TRAF2 was found to be capable of generating such chains and activating this complex in a similar manner to TRAF6 and so may play such a role in the TNF signalling pathway (Deng *et al.*, 2000; Wang *et al.*, 2001a).

Clearly TRAF2, which contains a RING finger motif, is a candidate ubiquitin ligase for RIP as they are both recruited to the same receptor complex. cIAP-1 and TRAF5 are also implicated by such an association. Modifications were also observed on TRAF2 in both HeLa and U937 cells (Figs. 5.1 A and 5.4) and on TRADD in HeLas (Fig. 5.1 A) only. The nature and significance of these awaits further study and although modification of TRAF2 has been previously reported (Chen *et al.*, 2002) there has been no suggestion that TRADD is similarly modified. It has recently been demonstrated that, at least during TNF-R2 signalling, cIAP-1 can act as an E3 ligase for TRAF2 (Li *et al.*, 2002b).

In summary the data presented in this chapter suggests that although they are obligatory for TNF-mediated apoptosis FADD and caspase-8 do not appear to be recruited to a membrane-bound TNF receptor complex. This would suggest that cell death induction by TNF does not proceed via the ligand-induced formation of a membrane bound-FADD and caspase-8-containing DISC which is the standard model for death receptor-induced apoptosis but rather that caspase-8 must be activated elsewhere. The data also demonstrates that components of the TNF-induced NF-κB activation pathway are modified when recruited to the receptor complex. The modification on RIP is suggested to be as a result of ubiquitin conjugation based on the observed effects of proteasome inhibition on stabilisation of RIP within the receptor complex. The role that these modifications may play in TNF signalling is unknown but parallels can clearly be drawn from the IL-1 signalling pathway.

CHAPTER 6: EFFECT OF PROTEIN KINASE C ACTIVATION ON TRAIL-INDUCED APOPTOSIS

6.1 INTRODUCTION

The importance of TRAIL as a potential therapeutic agent became obvious when it was demonstrated to be selectively toxic to transformed and tumour cells but not to the majority of normal cells (Ashkenazi & Dixit. 1998; Pitti *et al.*, 1996; Wiley *et al.*, 1995). The mechanism of this differential sensitivity was initially attributed to the presence of TRAIL decoy receptors on normal cells, however, recent studies have failed to correlate decoy receptor expression with TRAIL sensitivity (Griffith *et al.*, 1999a; Zhang *et al.*, 1999) and there has been growing evidence to suggest that TRAIL-resistance may be regulated intracellularly.

Several intracellular proteins have been implicated in providing resistance to TRAIL-induced apoptosis. The inactive caspase-8 homolog, c-FLIP, interferes with the activation of procaspase-8 at the DISC (Thome *et al.*, 1997), high basal FLIP expression has been correlated with TRAIL resistance in a number of model systems and modulation of FLIP levels using metabolic inhibitors has been demonstrated to sensitise resistant cells (Griffith *et al.*, 1998a). Other inhibitors include the Inhibitor of Apoptosis Proteins (IAPs), in particular X-linked IAP (XIAP), which blocks apoptosis by directly inhibiting effector caspases (Deveraux *et al.*, 1998) and the Bcl-2 family members, Bcl-2 and Bcl-$_{XL}$, which protect by inhibiting the mitochondrial changes associated with activation of the Apaf-1/caspase-9 caspase-activating pathway (Li *et al.*, 1997).

Pro-survival pathways that have been implicated in TRAIL-resistance include activation of the transcription factor NF-κB (Hu *et al.*, 1999; Jeremias *et al.*, 1998) which in the case of TNF has been shown to act by upregulating several anti-apoptotic genes (Wang *et al.*, 1998). Activation of protein kinase C (PKC) has been demonstrated to protect cells from apoptosis in a number of systems, including those caused by death receptor ligation (Gomez-Angelats *et al.*, 2000; Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001). Inhibition of CD95-mediated apoptosis by PKC is characterised by inhibition of DNA fragmentation and reduced CD95-induced cytochrome *c* release. Together with reduced cleavage of Bid and activation of caspases-3 and -8 this suggests that PKC-mediated inhibition of CD95-mediated apoptosis occurs at an apical point during CD95 signalling (Holmstrom *et al.*, 1999; Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001). CD95-mediated cell shrinkage and K^+ efflux is also inhibited (Gomez-Angelats *et al.*, 2000). Consistent with this there is some suggestion that aggregation of CD95 is also inhibited by PKC however CD95-mediated DISC formation has been demonstrated to be unaffected by PKC (Holmstrom *et al.*, 2000; Ruiz-Ruiz *et al.*, 1999). Activation of PKC has also been

reported to inhibit TRAIL-induced apoptosis (Sarker *et al.*, 2001), but it is unclear where the inhibition was occurring. It has been suggested that the block occurs either at the level of procaspase-8 activation (Meng *et al.*, 2002) or downstream of caspase-8-mediated cleavage of the proapoptotic Bcl-2 homolog, Bid (Sarker *et al.*, 2001) which suggests that PKC could effect mutiple events downstream of TRAIL signalling a combination of which leads to cellular insensitivity to TRAIL.

This chapter studies the effects of PMA on TRAIL-induced apoptosis in HeLa cells in order to try to further examine the mechanism by which phorbol ester-mediated PKC activation can protect against TRAIL-induced cytotoxicity. PMA pretreatment led to inhibition of TRAIL-mediated apoptosis in HeLa cells which was characterised by a reduction in Annexin V/Propidium Iodide staining, PARP and caspase-8 cleavage and incomplete caspase-3 maturation. The effects of PMA were completely abrogated by the PKC inhibitor, bisindoylmaleimide 1 (Bis1) implicating a role for PKC in the inhibitory action of PMA. TRAIL-induced mitochondrial release of the apoptosis mediators cytochrome *c* and Smac was also blocked and together with a reduction in procaspase-8 processing and Bid cleavage suggested that PKC activation was affecting apical events in TRAIL signalling. This led us to examine TRAIL DISC formation which was subsequently found to be disrupted in PMA treated cells as judged by a reduction in FADD recruitment. Disruption of DISC formation therefore provides a probable mechanism by which PKC can protect against TRAIL-induced apoptosis.

6.2 RESULTS

6.2.1 PMA-protects HeLa cells from TRAIL-induced apoptosis.

It has previously been reported that activators of protein kinase C are capable of inhibiting death receptor-induced apoptosis although the mechanism of this protection remains poorly understood. Therefore in order to further elucidate the role that PKC plays in protection from death receptor-induced apoptosis HeLa cells were treated with PMA and the effect of TRAIL-induced apoptosis was assessed.

Pretreatment of HeLa cells with 20 ng/ml PMA completely abrogated TRAIL-induced apoptosis as measured by phosphatidylserine (PS) exposure and propidium iodide staining (Fig. 6.1 A). Consistent with a block in apoptosis, PMA pre-treatment also inhibited TRAIL-induced cleavage of the caspase-3/-7 substrate PARP (Fig. 6.1 B). Treatment with PMA alone was not cytotoxic to HeLa cells at the concentration used (data not shown). In order to further understand the mechanism by which PMA protects cells from TRAIL, cell pellets were subjected to Western blotting for a number of components of the TRAIL receptor signalling pathway.

Caspase-8 is the apical caspase activated during TRAIL signalling and is recruited and processed at the DISC in a FADD- and TRAIL-dependent process (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). The proenzyme exists as two splice-forms, p55 and p53, which are activated in a two-step process involving the removal of the small subunit, p12 to generate p43 and p41 intermediates. A second cleavage then results in the removal of the large p18 subunit (Boldin *et al.*, 1996a; Medema *et al.*, 1997; Srinivasula *et al.*, 1996). When HeLa cells were treated with PMA 30 min prior to the addition of TRAIL, there was a markedly reduced activation of caspase-8 as judged by the reduction in generation of the large (p18) subunit (Fig 6.2).

Caspase-3 was processed in TRAIL-treated cells to its fully mature p17 subunit. Active caspase-8 processes the proform (p32) between its large and small subunits to generate a p20/p12 intermediate. The large subunit (p20) then undergoes an autocatalytic activation step resulting in removal of the prodomain and generation of the fully mature p17 form. Interestingly, although caspase-3 activity was inhibited in PMA-pretreated cells, as judged by inhibition of PARP processing (Fig. 6.1 B), caspase-3 was processed but only to its p20 intermediate (Fig. 6.2). The presence of the p20 but not the p17 subunit suggested that maturation of the p20 subunit was inhibited in PMA-pretreated cells.

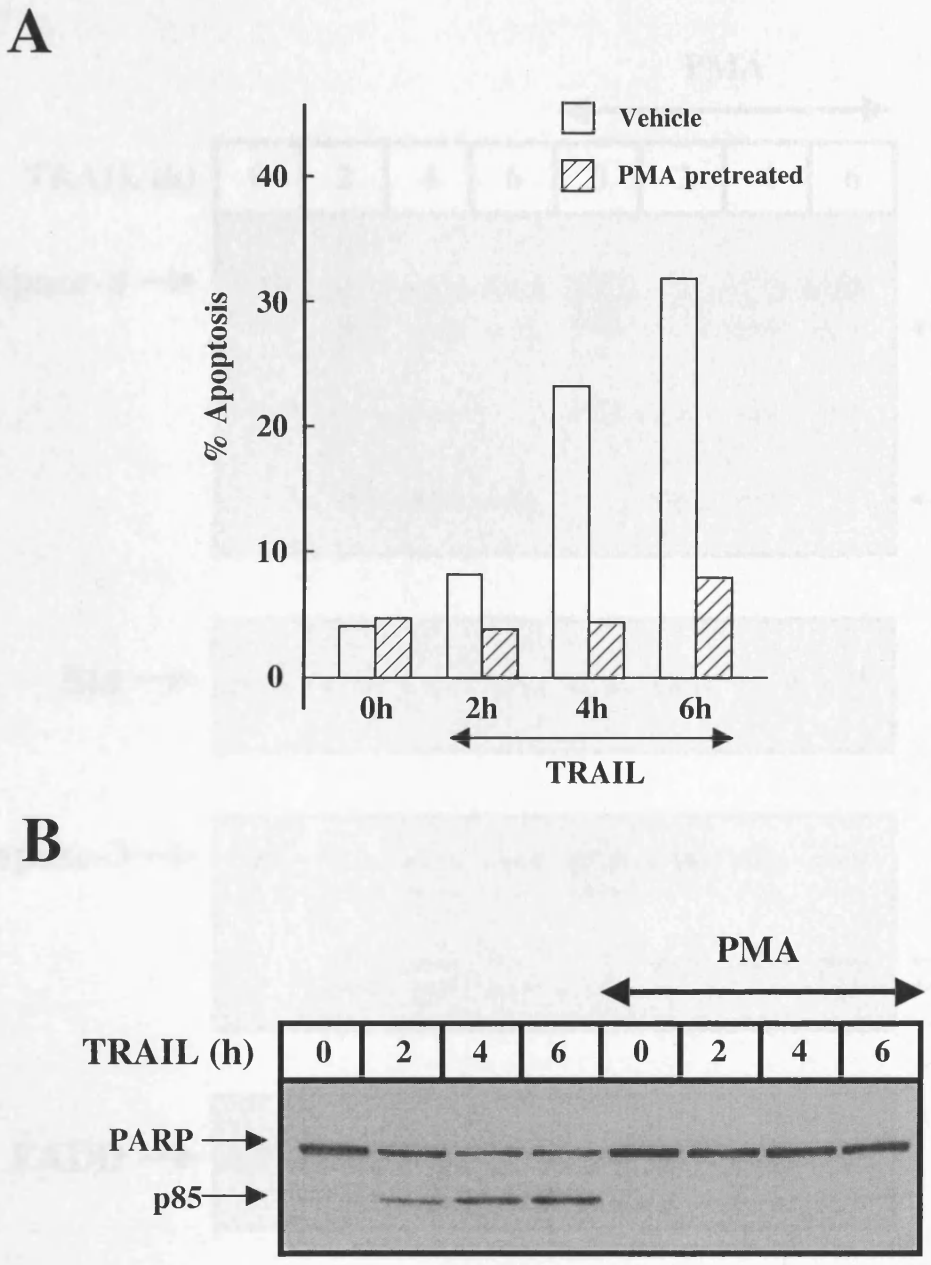


Figure 6.1 Effect of PMA on TRAIL-Induced Apoptosis in HeLas. (A) HeLa cells were pretreated with PMA (20 ng/ml) for 30 min prior to treatment with TRAIL (1 μg/ml) for indicated time periods. Apoptosis was assessed by Annexin V staining and flow cytometry as described in *Materials and Methods*. (B) Treated cells were also subjected to Western blotting using antibodies to the caspase-3/-7 substrate PARP. Data shown are representative of 3 independent experiments.

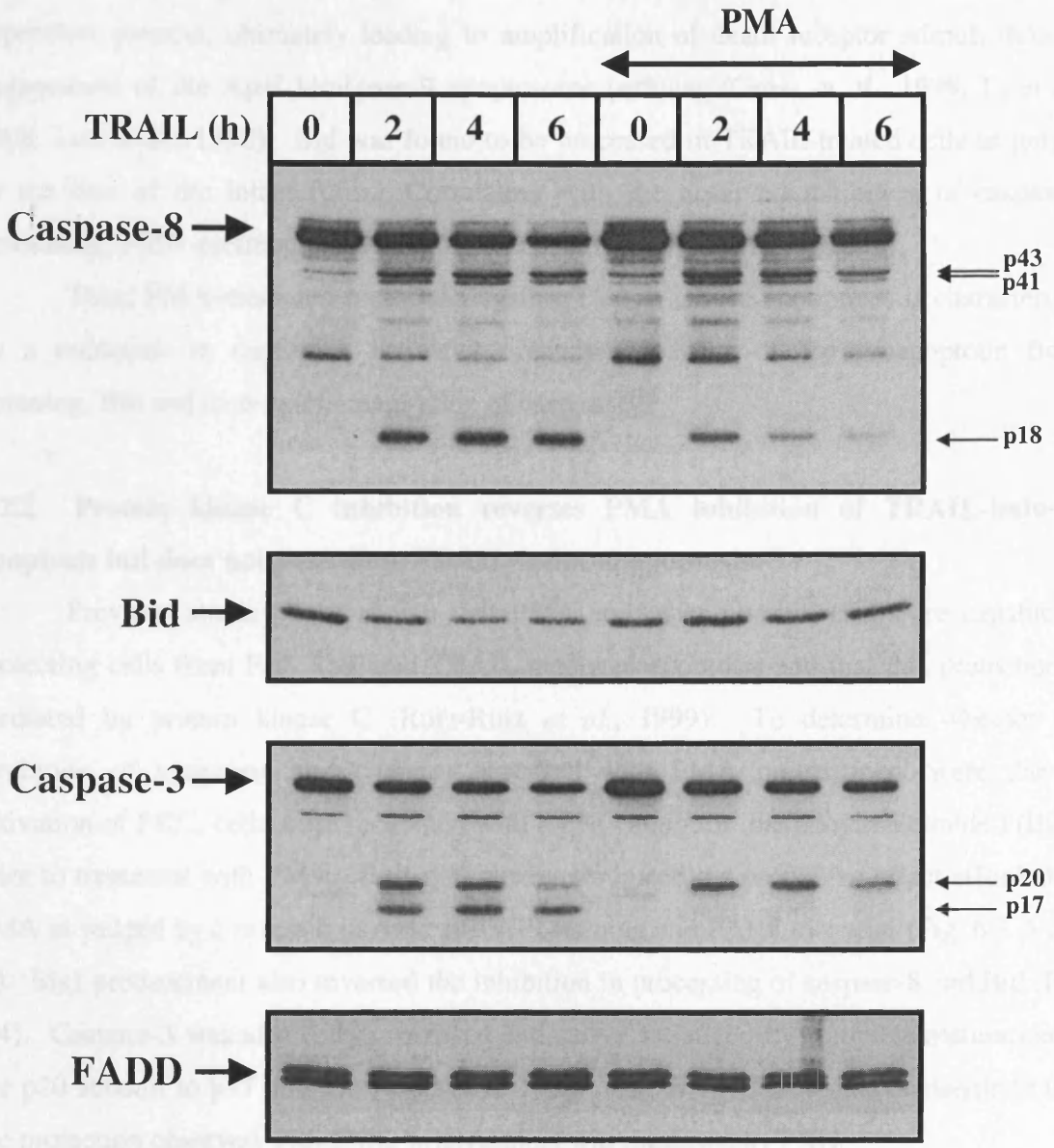


Figure 6.2 Effect of PMA on TRAIL-mediated caspase-8, -3 and Bid cleavage. Cells were treated as in Fig. 6.1. and subjected to Western blotting for Caspases-8 and -3 and the caspase-8-cleaved proapoptotic Bcl-2 family member, Bid. FADD was used as a protein loading control.

Bid, a pro-apoptotic Bcl-2 family member, is another known caspase-8 substrate. Cleavage of full length Bid (p22) leads to the generation of a truncated form (p15, tBid) which is then is capable of triggering mitochondrial disruption via a Bax- and/or Bak-dependent process, ultimately leading to amplification of death receptor stimuli through engagement of the Apaf-1/caspase-9 apoptosome pathway (Gross *et al.*, 1999; Li *et al.*, 1998; Luo *et al.*, 1998). Bid was found to be processed in TRAIL-treated cells as judged by the loss of the intact form. Correlating with the observed inhibition of caspase-8 processing, PMA-pretreatment markedly reduced this loss od Bid (Fig. 6.2).

Thus, PMA-mediated protection against TRAIL-induced apoptosis is characterised by a reduction in caspase-8 activation, reduced cleavage of the proapoptotic Bcl-2 homolog, Bid and incomplete maturation of caspase-3.

6.2.2 Protein kinase C inhibition reverses PMA inhibition of TRAIL-induced apoptosis but does not potentiate TRAIL-induced apoptosis.

Previous studies have shown that PMA and other phorbol esters are capable of protecting cells from Fas, TNF and TRAIL-mediated apoptosis and that this protection is mediated by protein kinase C (Ruiz-Ruiz *et al.*, 1999). To determine whether the inhibition of apoptosis and changes observed with PMA pretreatment were due to activation of PKC, cells were incubated with a PKC inhibitor bisindoylmaleimide I (Bis1) prior to treatment with PMA. Bis1 completely abrogated the protective effect afforded by PMA as judged by a reversal in Annexin V/PI staining and PARP cleavage (Fig. 6.3 A and B). Bis1 pretreatment also reversed the inhibition in processing of caspase-8 and Bid (Fig. 6.4). Caspase-3 was also fully processed and active as judged by complete maturation of the p20 subunit to p17 and the cleavage of PARP (Fig. 6.4). These data demonstrate that the protection observed with PMA-pretreatment was mediated by PKC.

A number of other studies have demonstrated that protein kinase C inhibitors, including bisindoylmaleimides, can potentiate FasL/CD95L-induced apoptosis {Zhou,

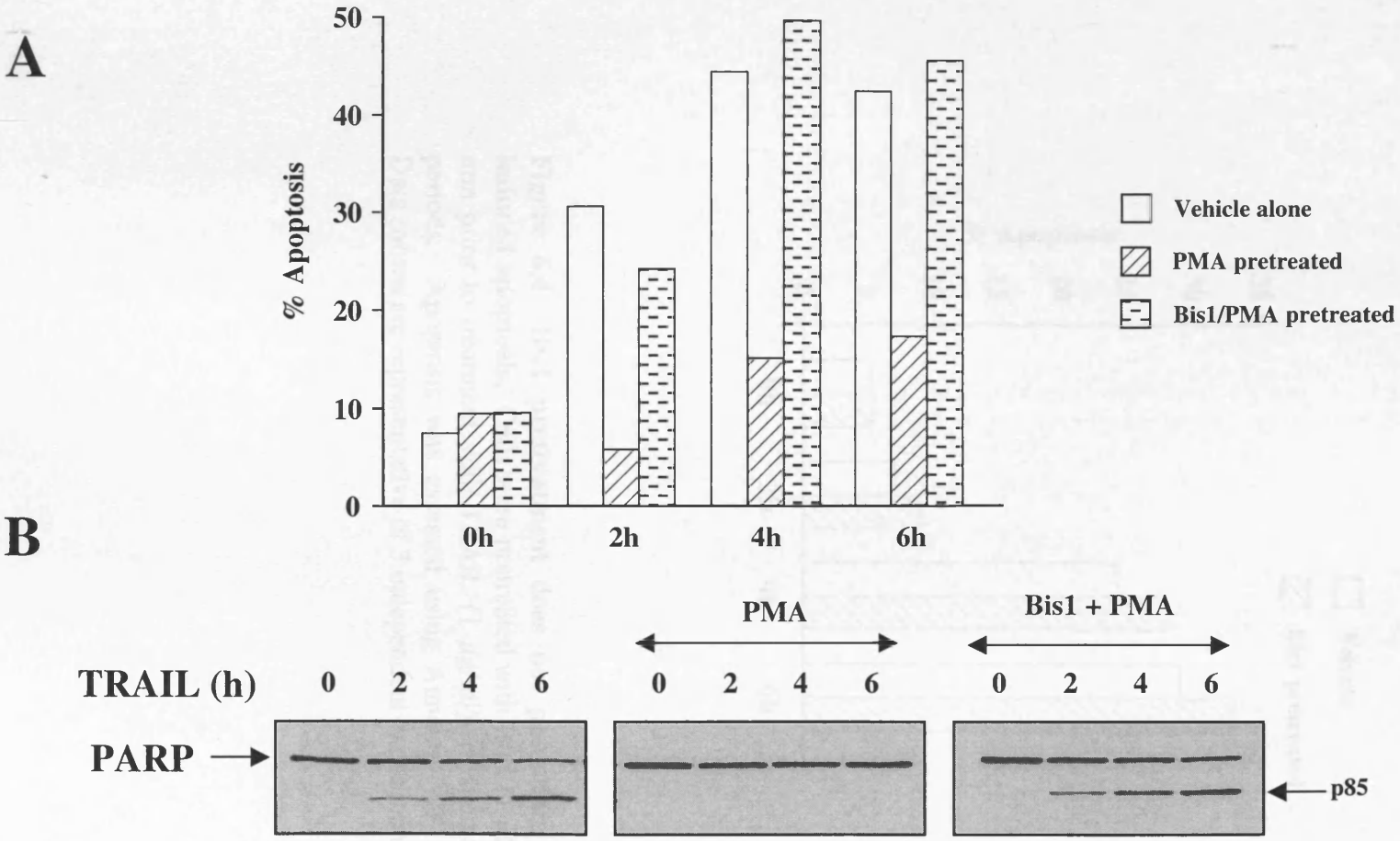


Figure 6.3 PMA-Mediated Protection against TRAIL-Induced Apoptosis can be reversed by a PKC Inhibitor. (A) HeLa cells were pretreated with the PKC inhibitor Bisindolylmaleimide I (GF 109203X) (1 μM) for 30 min prior to a 30 min pre-treatment with PMA and TRAIL treatment. Apoptosis was assessed using Annexin V staining. (B) Cells were also Western blotted for the apoptotic substrate PARP. Data shown are representative of 3 independent experiments.

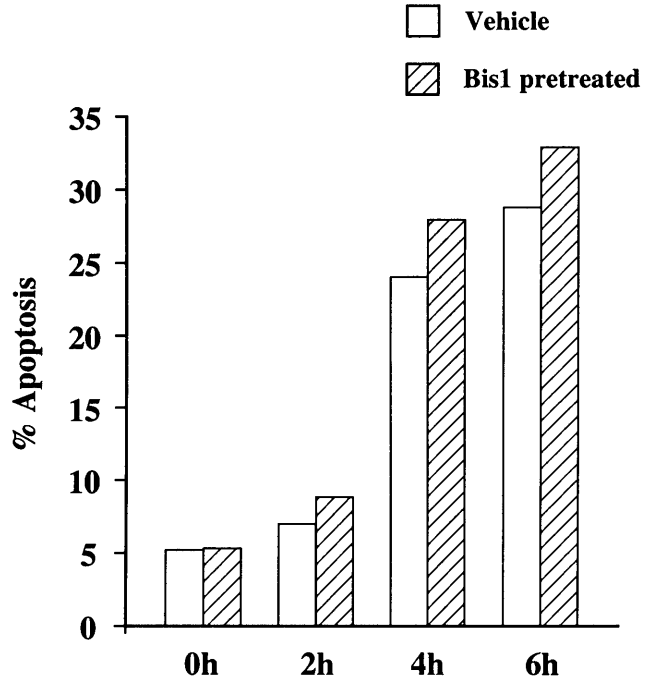


Figure 6.4 Bis1 pretreatment does not potentiate TRAIL-induced apoptosis. Cells were pretreated with Bis1 (1 μM) for 30 min prior to treatment with TRAIL (1 μg/ml) for indicated time periods. Apoptosis was assessed using Annexin V/PI staining. Data shown are representative of 3 independent experiments.

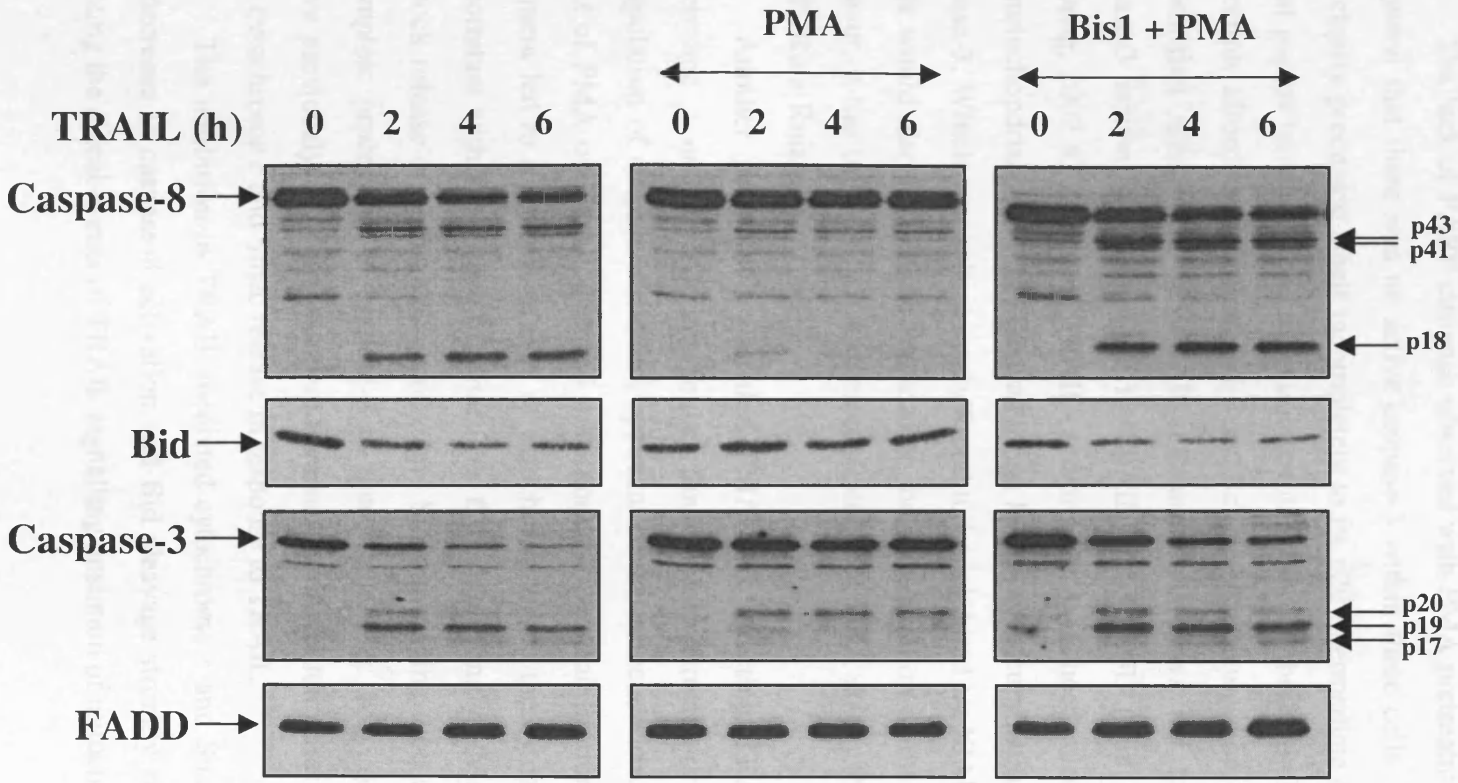


Figure 6.5 Bis1 pretreatment reverses cleavages of critical substrates prevented by PMA. Cells were treated with PMA in the presence or absence of Bis1 pretreatment prior to TRAIL treatment. Activation of procaspase-8 and -3 and cleavage of the Bcl-2 homolog Bid was assessed by Western blotting. FADD was used as a protein loading control.

1999 #116;Willems, 2000 #117}. Although Bis1 was capable of reversing the effects of PMA (Figs. 6.3 and 6.4) it was unable to potentiate TRAIL-induced apoptosis (Fig. 6.5)

6.2.3 Effect of PMA and Bis1 on cytochrome *c* and Smac release

The lack of PARP cleavage observed with PMA pretreatment (Fig. 6.1 B) strongly suggested that there was no active caspase-3 within these cells. Despite this, caspase-3 was clearly processed albeit incompletely to its p20 intermediate (Fig. 6.2). A number of recent papers have provided a possible explanation for these results. In these studies, the protection afforded by either Bcl-2 or Bcl-$_{XL}$ overexpression, lack of Bax or by an unidentified component in TRAIL-resistant melanomas led to a similar incomplete caspase-3 activation in response to TRAIL and CD95L {Sun, 2002 #66;Zhang, 2001 #4;Deng, 2002 #5}. Lack of TRAIL- and/or CD95L-induced release of cytochrome *c* and the mitochondrial XIAP antagonist, Smac led to an accumulation of the p20 subunit of caspase-3. Which was subsequently found to be inhibited by XIAP. An increase in XIAP levels would also be expected to lead to the accumulation of the p20 form of caspase-3, however, it has previously been demonstrated that PMA does not directly affect levels of XIAP (Ruiz-Ruiz *et al.*, 1999).

Another possibility was that PMA was affecting TRAIL-induced release of cytochrome *c* or the IAP antagonist, Smac either directly or indirectly through the manipulation of caspase activation upstream of the mitochondria. To investigate this, the effect of PMA on TRAIL-induced mitochondrial permeabilisation was assessed. TRAIL treatment led to a gradual release of cytochrome *c* into the cytosolic fraction which was concomitant with the release of Smac (Fig. 6.6). Interestingly, PMA-pretreatment appeared to block release of both cytochrome *c* and Smac providing a possible explanation for the incomplete processing of caspase-3 in these cells (Fig. 6.2). Analogous to the results shown previously, Bis1-pretreatment reversed the inhibition observed with PMA restoring both cytochrome *c* and Smac release in response to TRAIL.

The inhibition in TRAIL-mediated cytochrome *c* and Smac release together with the decrease in caspase-8 activation and Bid cleavage strongly suggested that PMA was effecting the apical events of TRAIL signalling upstream of mitochondria.

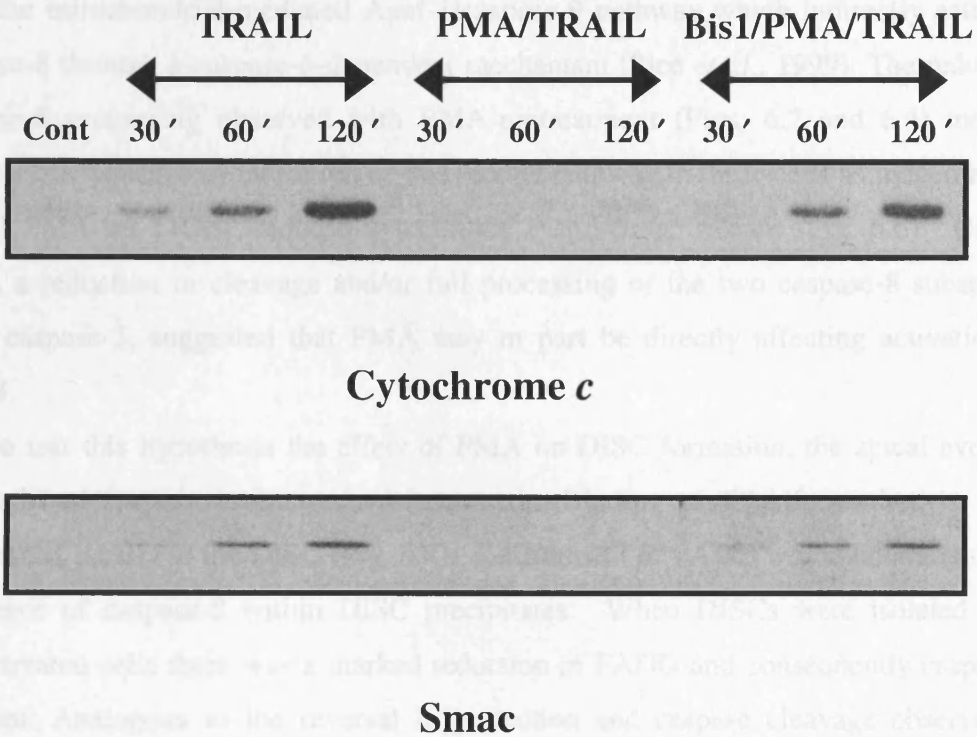


Figure 6.6 TRAIL-induced mitochondrial release of the proapoptotic mediators cytochrome *c* and Smac is blocked by PMA-pretreatment. Cells were treated with PMA (20 ng/ml, 30 min) in the presence or absence of Bis1 pretreatment (1 μM, 30 min) prior to treatment with TRAIL (1000 ng/ml) for the indicated time periods. Cytosolic fractions were then isolated as described in *Materials and Methods* and analysed for the presence of the proapoptotic mediators cytochrome *c* and the IAP anatagonist Smac using Western blotting.

6.2.4 Activation of protein kinase C results in a reduction of FADD recruitment to the DISC

During death receptor-mediated apoptosis caspase-8 can be processed by two mechanisms. The first is the activation of procaspase-8 as the apical caspase in a FADD and ligand-dependent manner at the DISC. The second, which is redundant if the first is inhibited, is an amplification loop in which caspase-3 is activated either directly by caspase-8 or through the mitochondrial-mediated Apaf-1/caspase-9 pathway which indirectly activates procaspase-8 through a caspase-6-dependent mechanism (Slee *et al.*, 1999). The reduction in caspase-8 processing observed with PMA-pretreatment (Figs. 6.2 and 6.4) may be therefore a consequence of inhibition of this second pathway in these cells as judged by the effects of PMA on TRAIL-induced cytochrome *c* and Smac release (Fig. 6.6). Clearly however, a reduction in cleavage and/or full processing of the two caspase-8 substrates, Bid and caspase-3, suggested that PMA may in part be directly affecting activation of caspase-8.

To test this hypothesis the effect of PMA on DISC formation, the apical event in TRAIL-induced caspase activation was assessed. Binding of TRAIL resulted in rapid recruitment of FADD to the DISC (Fig. 6.7). Recruitment of FADD was concomitant with the presence of caspase-8 within DISC precipitates. When DISCs were isolated from PMA-pretreated cells there was a marked reduction in FADD and consequently caspase-8 recruitment. Analogous to the reversal in protection and caspase cleavage observed in whole cell lysates, Bis1 pretreatment resulted in restoration of FADD and caspase-8 recruitment to the DISC (Fig. 6.7).

Clearly the reduction in FADD and caspase-8 recruitment to the DISC provides one mechanism for the protection observed in PMA-pretreated cells and together with the ability of Bis1 to reverse the effects of PMA clearly suggests a major role for PKC in modifying apical events in TRAIL signalling.

6.2.5 PMA pretreatment does not markedly alter cell surface TRAIL receptor expression.

Protein kinase C activators such as PMA have previously been shown to protect cells from TNF-induced cytotoxicity by reducing the number of cell surface receptors available for TNF binding through a process known as receptor shedding (Pinckard JK. 1997). Although as yet no studies have reported evidence of TRAIL receptor shedding, a decrease in cell surface TRAIL receptors would be expected to result in a

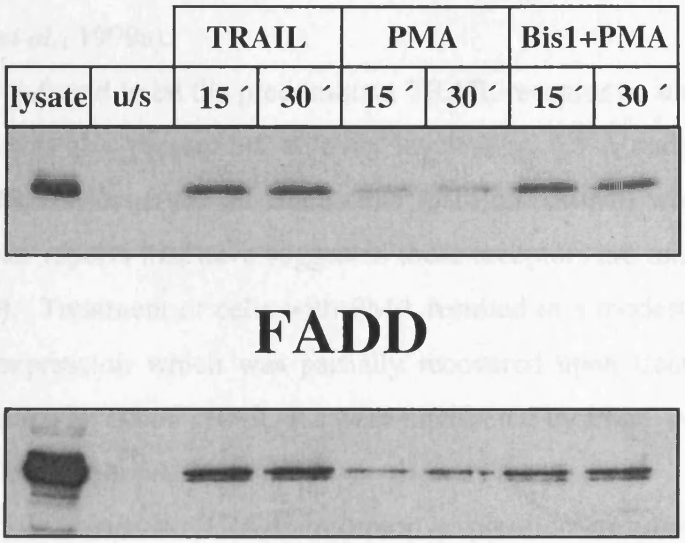


Figure 6.7 PMA pretreatment reduces FADD recruitment to the DISC. HeLa cells (3×10^7/treatment) were either pretreated with Bis1 (1 μM, 30 min) followed by PMA (20 ng/ml, 30 min), PMA alone or vehicle alone followed by biotinylated TRAIL (bTRAIL) 1 μg/ml. After indicated times cells were washed, lysed and TRAIL receptor complexes precipitated using streptavidin-coated agarose beads. Precipitates were then analysed for the presence of the known TRAIL DISC components FADD and Caspase-8. Unstimulated receptors (u/s) were represented by precipitates resulting from the addition of bTRAIL to lysates from untreated cells.

reduction in FADD recruitment and DISC formation such as that observed in PMA treated cells (Fig. 6.7). Therefore, in order to assess whether the decrease in FADD recruitment observed in DISCs isolated from PMA-treated cells was due to a decrease in TRAIL receptors, cell surface expression was assessed by flow cytometry. Cells were treated for indicated times with PMA or pretreated with Bis1 prior to PMA treatment and cell surface TRAIL receptor expression measured by flow cytometry using receptor specific antibodies (2.2.4) and (Griffith *et al.*, 1999a).

TRAIL-R1 was found to be the predominant TRAIL receptor on unstimulated cells, although TRAIL-R2 was also present but at lower levels (Fig. 6.8 A and B). No staining for TRAIL-R3 or -R4 was observed on HeLa cells (data not shown) which is consistent with a number of other reports that have suggested these receptors are mainly intracellular (Zhang *et al.*, 2000b). Treatment of cells with PMA resulted in a modest decrease in cell surface TRAIL-R1 expression which was partially recovered upon treatment with Bis1 (Fig. 6.8 A). In contrast, levels of TRAIL-R2 were unaffected by PMA treatment or PMA in combination with Bis1 (Fig. 6.8 B).

The modest decrease in TRAIL receptor expression resulting from PMA-pretreatment led us to investigate other potential mechanisms by which PMA may affect DISC formation/FADD recruitment.

6.2.6 Effect of PKC activation on cell surface TRAIL receptors and TRAIL receptor aggregation

There appears to be a critical requirement for the aggregation of death receptors in order to initiate apoptotic signalling (Boldin *et al.*, 1995). It has been demonstrated that upon triggering Fas oligomerises and forms sodium dodecyl sulphate stable aggregates which can be resolved by SDS-polyacrylamide gel electrophoresis. These aggregates were found to form almost immediately after CD95 stimulation and corresponded to dimeric and trimeric forms of the receptor(Kischkel *et al.*, 1995). In order to assess whether the ligand-induced formation of aggregates was affected by PMA pre-treatment, DISCs were isolated from unstimulated and ligand-stimulated cells treated with/without PMA-pretreatment. Precipitates were then resolved on SDS-PAGE in the absence of reducing agents followed by immunoblotting with TRAIL-R1 and -R2-specific antibodies. Treatment with TRAIL resulted in the formation of higher molecular weight species which appeared to correspond to dimeric and trimeric forms of the respective TRAIL receptors (Fig. 6.9 A and B). TRAIL treatment also resulted in the loss of monomeric receptors. PMA pretreatment did

not appear to affect the formation of the aggregates. Formation of these higher order aggregates of both TRAIL-R1 and -R2 required TRAIL binding as they were largely absent in the unstimulated receptor controls.

6.2.7 PMA Pretreatment modulates the affinity of FADD for TRAIL-R1 and -R2.

The relatively minor effects of PMA on TRAIL-R1 cell surface expression and the lack of any effect on receptor aggregate formation (Figs. 6.8 and 6.9) could not fully explain the dramatic effects of PMA on TRAIL-induced apoptosis. Another possible explanation for the observed reduction in FADD recruitment in the PMA-pretreated cells (Fig. 6.7) was that the affinity of FADD for the intracellular domain of TRAIL-R1 or -R2 was being affected. In order to test this hypothesis, we fused the intracellular domain (ICD) of TRAIL-R1 and -R2 to a glutathione-*S*-transferase (GST) tag and expressed this chimeric protein in *E-coli*. Coating these fusion proteins to GSH beads and incubating them in lysates from untreated and PMA-pretreated cells allows an assessment of whether PMA affects the affinity of FADD for the respective receptor ICDs. As would be expected from the data obtained in Chapter 4, the GST-TRAIL-R1 ICD and -TRAIL-R2 ICD were both able to precipitate FADD from control lysates (Figs. 6.10 A and B). Interestingly when the respective ICDs were incubated with lysates from PMA-pretreated cells, the level of FADD bound to the beads was greatly reduced consistent with the data previously obtained from native DISC analysis (Fig. 6.7). Data from this *in vitro* biding assay suggested that the reduced FADD recruitment observed in TRAIL DISCs isolated from PMA-preatreated cells may be as a result of a reduced affinity of FADD for the respective TRAIL receptor ICDs.

6.2.8 PMA treatment does not affect the phosphorylation state of FADD.

As PMA-mediated PKC activation has previously been shown to be able to protect against cytotoxicity from other TNF family members there was a possibility that it may be affecting a common component within these three signalling pathways. One such candidate molecule is FADD as it appears to be the universal adaptor for Fas, TNF and TRAIL-mediated death signalling (Figs. 4.2 and 5.3 and (Juo *et al.*, 1999; Yeh *et al.*, 1998)), and its recruitment is clearly reduced by PMA-pretreatment (Fig. 6.7). FADD has been shown to exist in two forms, non- and a serine-phosphorylated form (Medema *et al.*, 1997). In order to assess whether PMA was affecting the phosphorylation

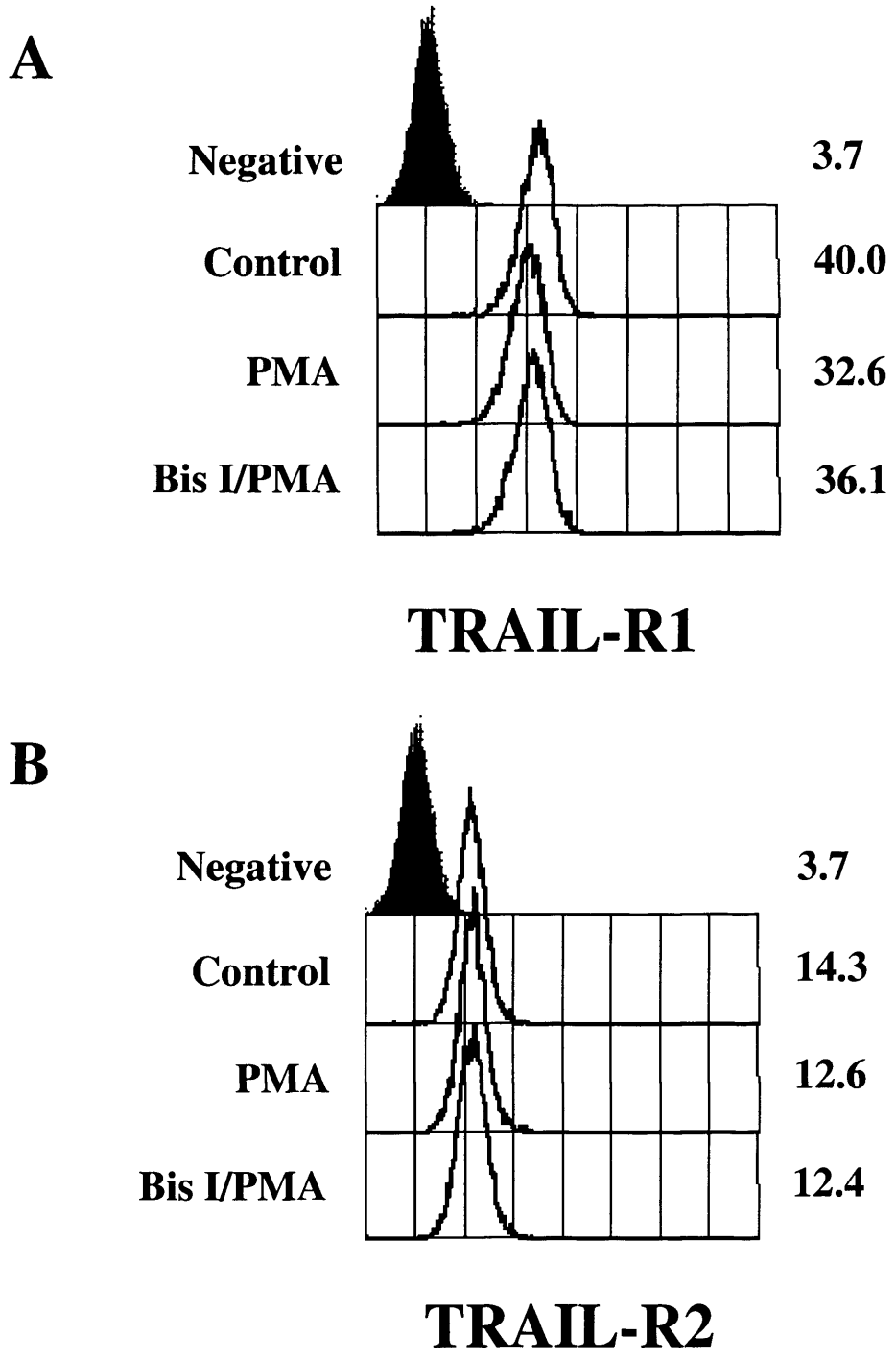


Figure 6.8 The effects of PMA on cell surface TRAIL receptor expression.

After treatment with PMA (20 ng/ml) for 30 min in the presence and absence of a Bis1 (1 μM, 30 min pretreatment) HeLa cells were harvested and cell surface receptor expression analysed by flow cytometry using monoclonal antibodies to either (A) TRAIL-R1 or (B) TRAIL-R2 as described in *Materials and Methods*. Unlabeled cells were used to control for background fluorescence (Negative). Values shown represent mean fluorescence intensity.

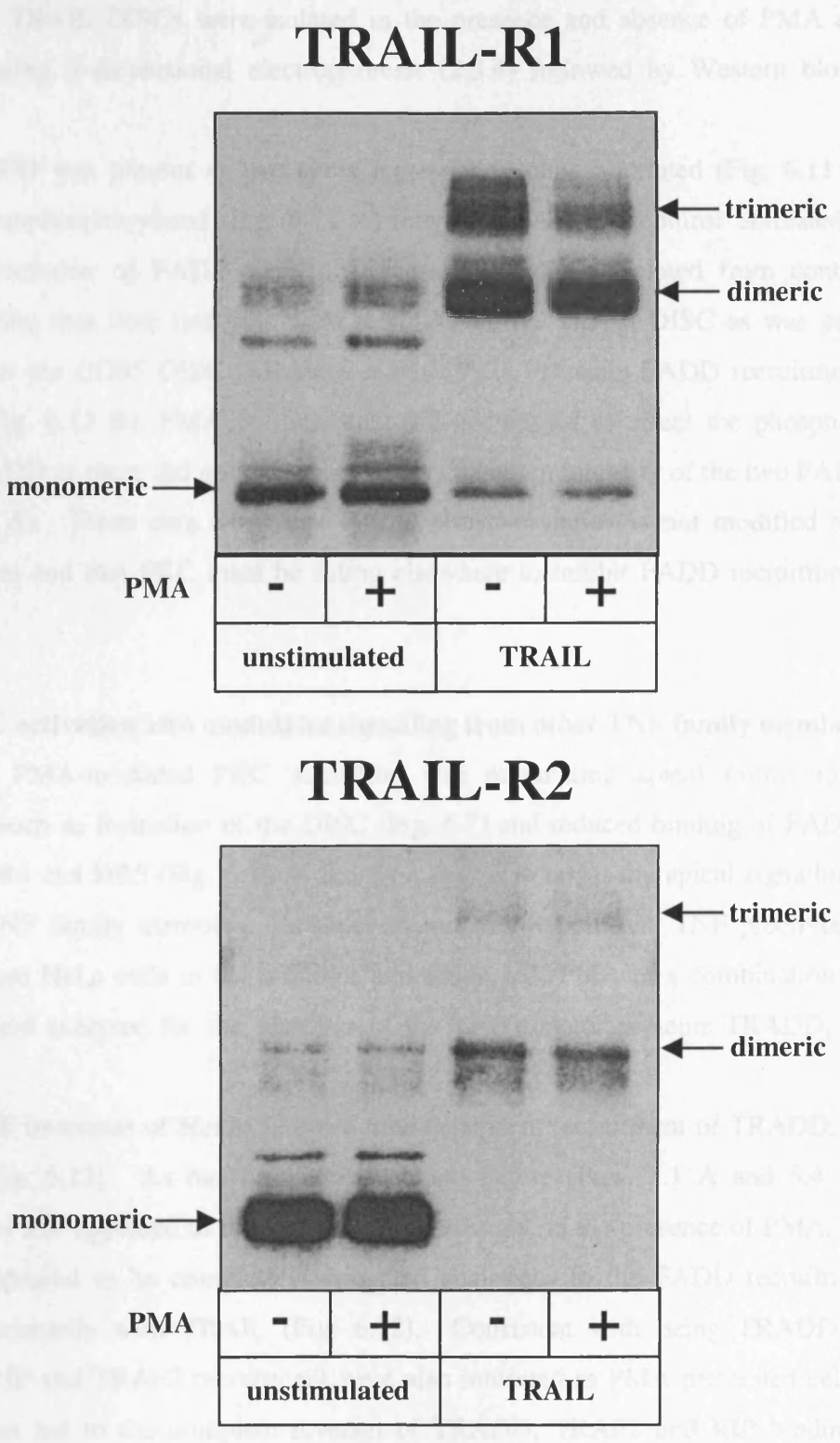


Figure 6.9 PMA pretreatment does not markedly reduce TRAIL receptor aggregation. TRAIL DISCs were isolated in the presence and absence of PMA (20 ng/ml, 30 min) and aggregated TRAIL-R1 (A) and -R2 (B) analysed using Western blotting under non-reducing conditions. Aggregated receptors were only apparent in TRAIL treated cells.

of FADD, TRAIL DISCs were isolated in the presence and absence of PMA and then analysed using 2-dimensional electrophoresis (2.3.4) followed by Western blotting for FADD.

FADD was present as two spots representing phosphorylated (Fig. 6.11 A, right spot) and unphosphorylated (Fig. 6.11 A, left spot) FADD in control untreated lysates. The two isoforms of FADD were also present in DISCs isolated from control cells demonstrating that both isoforms were recruited to the TRAIL DISC as was previously reported for the CD95 DISC (Medema *et al.*, 1997). Despite FADD recruitment being reduced (Fig. 6.11 B), PMA pre-treatment did not appear to affect the phosphorylation state of FADD as there did not appear to be any change in intensity of the two FADD spots (Fig. 6.11 A). These data show that FADD phosphorylation is not modified by PMA-pretreatment and that PKC must be acting elsewhere to inhibit FADD recruitment to the DISC.

6.2.9 PKC activation also modulates signalling from other TNF family members.

Clearly if PMA-mediated PKC activation was modulating apical events in TRAIL signalling such as formation of the DISC (Fig. 6.7) and reduced binding of FADD to the ICDs of DR4 and DR5 (Fig. 6.10 A and B) it may also target the apical signalling events of other TNF family members. In order to test this hypothesis, TNF precipitates were isolated from HeLa cells in the presence and absence of PMA or a combination of PMA and Bis1 and analysed for the presence of the TNF adaptor proteins TRADD, RIP and TRAF2.

TNF treatment of HeLas led to a time-dependent recruitment of TRADD, RIP and TRAF2 (Fig. 6.12). As has been demonstrated before (Figs. 5.1 A and 5.4 A), both TRAF2 and RIP appeared to be modified. Interestingly, in the presence of PMA, TRADD binding appeared to be completely abrogated analogous to the FADD recruitment data obtained primarily with TRAIL (Fig. 6.12). Consistent with being TRADD binding proteins, RIP and TRAF2 recruitment were also inhibited in PMA-pretreated cells. Bis1 pretreatment led to the complete reversal of TRADD, TRAF2 and RIP binding, again suggesting that the mechanism of this inhibition was mediated via PKC activation.

HeLa cells are not sensitive to TNF-mediated cytotoxicity either in the presence or absence of cycloheximide (Fig. 5.1 C) so it was not possible to assess the effects of PMA

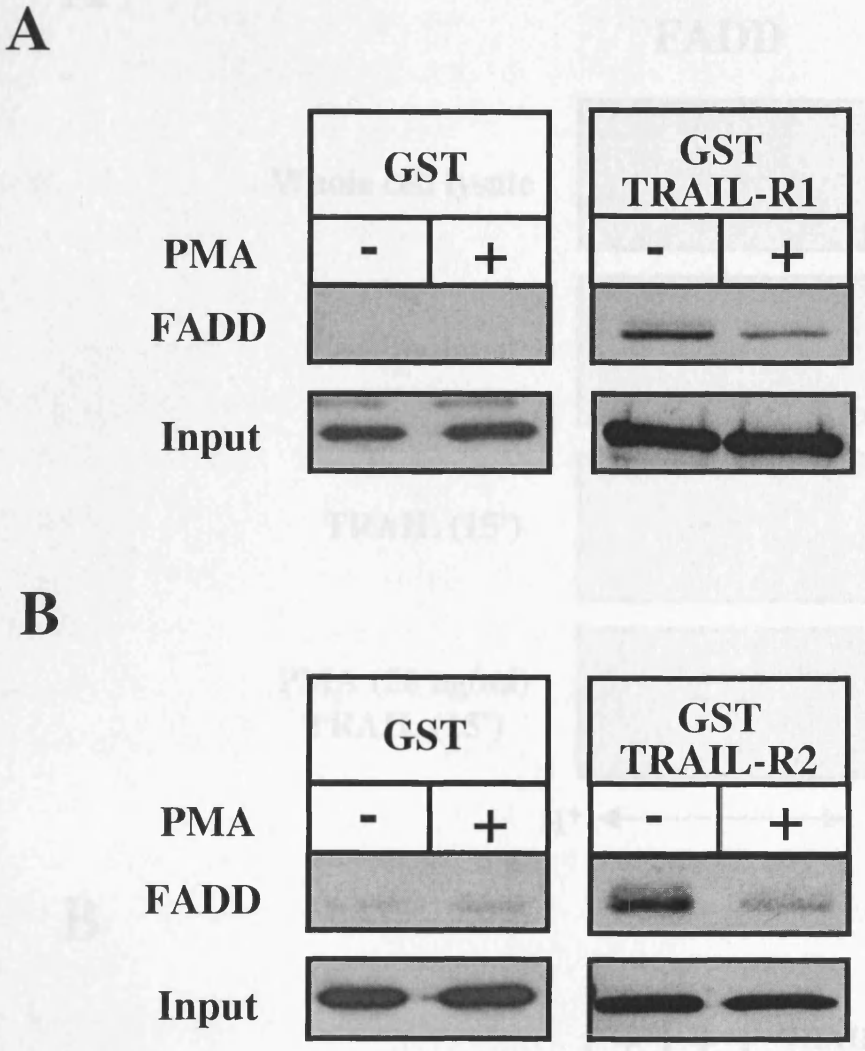


Figure 6.10 PMA inhibits the interaction of FADD with TRAIL-R1 and -R2 -GST fusion proteins in cell lysates. Cells were treated with/without PMA (20 ng/ml) for 30 min. Lysates were prepared from treated cells and used to study the interaction of FADD with the intracellular domains of (A) TRAIL-R1 and (B) TRAIL-R2 fused to GST essentially as described in *Materials and Methods*. GST alone was used as a control for non-specific interactions.

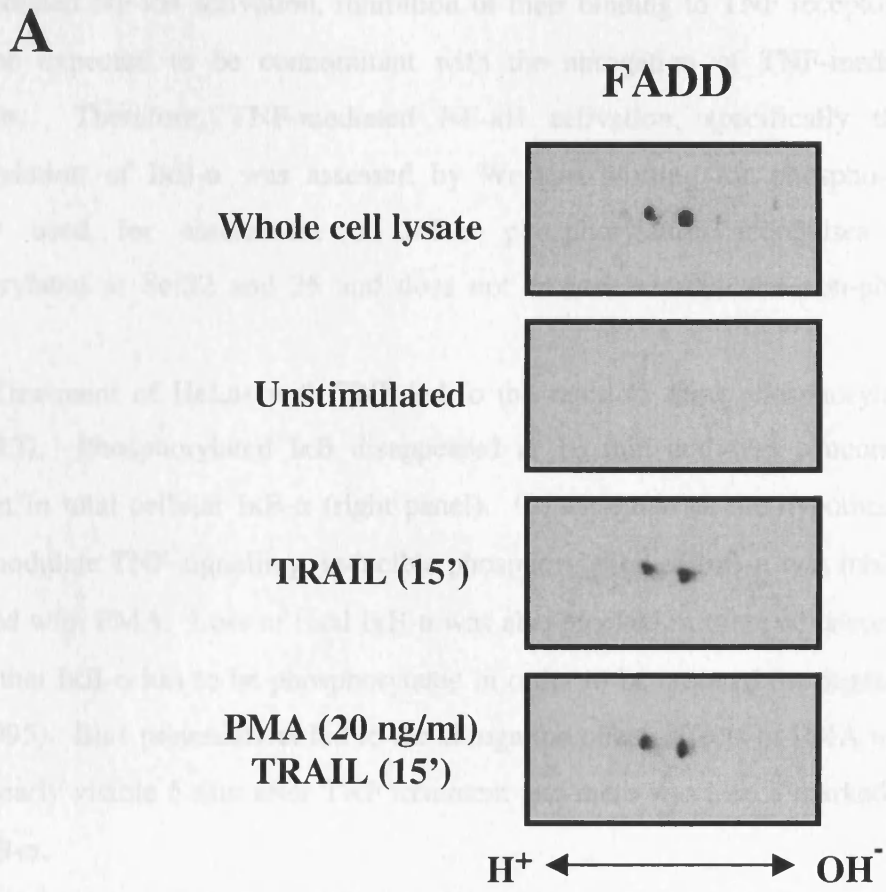


Figure 6.11 FADD phosphorylation is not modified by PMA-pretreatment. (A) Cells were pretreated with PMA (20 ng/ml, 30 min) prior to bTRAIL (1 μg/ml) for 15 min. TRAIL receptor complexes were then precipitated as described in *Materials and Methods*. FADD phosphorylation state was analysed by assessing the mobility of FADD by 2-dimensional electrophoresis and Western blotting. (B) Represents FADD present in the precipitates when analysed by conventional 1-dimensional electrophoresis.

on TNF-mediated apoptosis in the HeLa cells. As both RIP and TRAF2 are required for TNF-mediated NF-κB activation, inhibition of their binding to TNF receptor precipitates would be expected to be concomitant with the abrogation of TNF-mediated NF-κB activation. Therefore, TNF-mediated NF-κB activation, specifically the inducible phophorylation of IκB-α was assessed by Western blotting for phospho-IκB-α. The antibody used for assessment of IκB-α phosphorylation recognises only IκB-α phosphorylated at Ser32 and 36 and does not cross-react with the non-phosphorylated form.

Treatment of HeLas with TNF led to the rapid (5 min) phosphorylation of I-κB (Fig. 6.13). Phosphorylated IκB disappeared at 10 min and was concomitant with a reduction in total cellular IκB-α (right panel). Consistent with the hypothesis that PMA would modulate TNF signalling, inducible phosphorylation of IκB-α was inhibited in cells pretreated with PMA. Loss of total IκB-α was also blocked in these cells compatible with the fact that IκB-α has to be phosphorylated in order to be targeted for degradation (Chen *et al.*, 1995). Bis1 pretreatment led to the abrogation of the effects of PMA with phospho-IκB-α clearly visible 5 min after TNF treatment and there was also a marked reduction in total Iκ-B-α.

In summary it appears that PMA-mediated PKC activation is capable of modulating signalling by other members of the TNF receptor superfamily. In both the TRAIL and TNF systems PMA exerts its effect(s) on apical events of death receptor signalling by inhibiting recruitment of core DISC/signalling components.

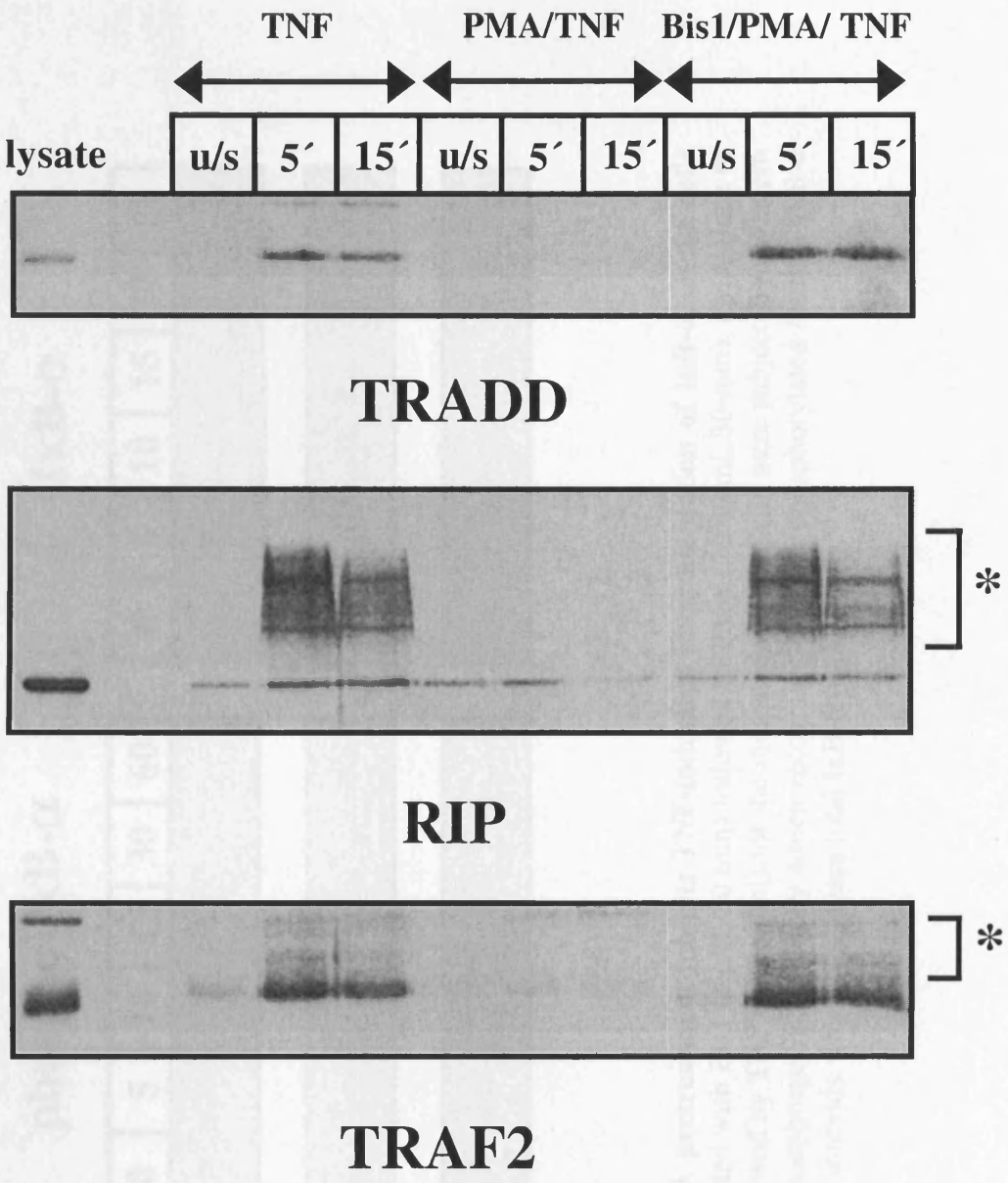


Figure 6.12 PMA pretreatment inhibits TRADD recruitment to the TNF-R1 complex. HeLa cells (3×10^7/treatment) were either pretreated with Bis1 (1 μM, 30 min) followed by PMA (20 ng/ml, 30 min), PMA alone or vehicle alone followed by biotinylated TNF (bTNF) 200 ng/ml. After indicated times cells were washed, lysed and TNF receptor complexes precipitated using streptavidin-coated agarose beads. Precipitates were then analysed for the presence of the TNF adaptor protein TRADD, RIP and TRAF2 Unstimulated receptors (u/s) were represented by precipitates resulting from the addition of bTNF to lysates from untreated cells.

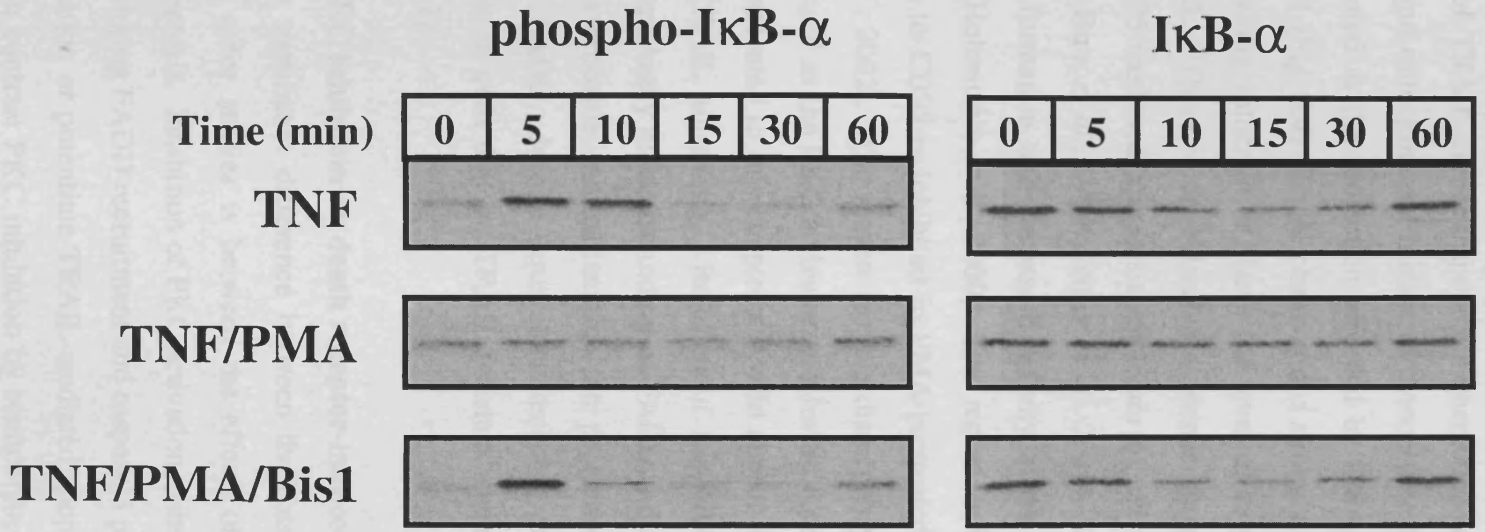


Figure 6.13 PMA pretreatment inhibits TNF-inducible phosphorylation of IκB-α. HeLa cells were either pretreated with Bis1 (1 μM, 30 min) followed by PMA (20 ng/ml, 30 min), PMA alone or vehicle alone followed by TNF (200 ng/ml) for the indicated times. Cells were subjected to Western blotting using a phosphospecific antibody which recognises only the phosphorylated form of IκB-α (left panel) and an antibody which recognises total IκB-α (right panel).

6.3 DISCUSSION

Effect of PKC activation on DISC Formation.

Data in this chapter describe a novel mechanism for inhibition of death receptor signalling pathways by activation of PKC. PMA pre-treatment was found to result in the inhibition of TRAIL-induced apoptosis characterised by inhibition of caspase-8 and -3 activation and mitochondrial release of cytochrome *c* (Figs. 6.2 and 6.6). PKC activation was implicated as the protection afforded by PMA was reversed with a PKC inhibitor, namely Bis1 (Fig. 6.4). These observations strongly suggested that PKC must be affecting apical signalling, either at or above the level of caspase-8 activation, to protect the cells from TRAIL. There is considerable evidence suggesting that PKC activation similarly affects CD95-mediated apoptosis upstream of caspase-8 activation (Holmstrom *et al.*, 1999; Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001). Despite this, it has also been reported that DISC formation and caspase-8 activity at the CD95 DISC is unaffected by PKC activation (Holmstrom *et al.*, 2000). Two recent studies, however, have shown that FADD recruitment to CD95 is inhibited in PMA-pretreated cells (Gomez-Angelats *et al.*, 2001; Meng *et al.*, 2002). The reason for this discrepancy is unclear as in all of these studies PMA was used as the PKC activator and Jurkat T cells were the model system. Together with data presented in this chapter it would appear that PKC activation can also similarly modulate TRAIL signalling. Isolation of the TRAIL DISC in the presence of PMA resulted in markedly reduced binding of FADD and as a consequence caspase-8 (Fig. 6.7) providing a probable mechanism for the protection afforded by PMA. The reduced binding of FADD, despite equivalent receptor expression, was also found to be a characteristic of cells that are TRAIL resistant when compared to a TRAIL sensitive cell line (Fig. 4.1).

Effect of PKC inhibition on death receptor-induced apoptosis.

One significant difference between the data presented in this chapter and that reported in other studies is between the effects of PKC inhibitors on death receptor-induced apoptosis. Inhibition of PKC activation, using Bis1, reversed the inhibitory effects of PMA restoring FADD recruitment and caspase-3 processing but did not did not enhance DISC formation or potentiate TRAIL-mediated apoptosis when used alone (Figs. 6.3, 6.4 and 6.5). In contrast PKC inhibition by bisindolylmaleimides can lead to potentiation of CD95-mediated apoptosis. In one study potentiation was associated with an increase in

FADD recruitment to the Fas receptor (Gomez-Angelats & Cidlowski. 2001), while in the other, no increase in FADD was observed despite an increase in apoptosis (Meng *et al.*, 2002). However, in the latter study another PKC inhibitor, H7 was unable to potentiate CD95-mediated apoptosis. Clearly the results obtained in these studies are as a result of the differing specificites of the PKC inhibitors used. Different PKC isoforms may therefore protect, or lead to potentiation of death receptor-induced apoptosis at many different levels. Consistent with this hypothesis some PKC inhibitors have been demonstrated to modulate CD95-mediated apoptosis by downregulation of c-FLIP (Willems *et al.*, 2000).

Effect of PKC activation on death receptor aggregation.

One of the mechanisms by which PKC has been proposed to protect from CD95-induced apoptosis is through inhibition of receptor aggregation (Ruiz-Ruiz *et al.*, 1999). Inhibition of DISC formation through disruption of the formation of higher order receptor aggregates would in turn lead to reduced binding of FADD and procaspase-8. There is already some evidence that death receptor-mediated apoptosis can be effected by changes in the actin cytoskeleton. PKC activation can lead to drastic changes in cell morphology and membrane dynamics possibly through cytoskeletal changes. MARCKS is an actin cross-linking protein that appears to be regulated by PKC (Hartwig *et al.*, 1991). Inhibitors of actin polymerisation such as latrunculin A have also been demonstrated to protect cells from Fas-mediated apoptosis (Algeciras-Schimnich *et al.*, 2002). In this respect it is interesting to note that the CD95 receptor has been demonstrated to interact with an actin binding protein, ezrin (Parlato *et al.*, 2000). These observations may provide a mechanism for the disruption of CD95-mediated apoptosis by PKC activation through modulation of CD95 membrane mobility and aggregation. This has been demonstrated to be the mechanism by which phosphatidylinositol 3′-kinase (PI3K) activation, after ligation of CD3, can protect Th2 cells from CD95-mediated apoptosis (Varadhachary *et al.*, 2001).

Despite these reports, data presented in this chapter would suggest that it is unlikely that PKC activation is affecting TRAIL receptor aggregation. Firstly, PMA did not markedly affect aggregation of TRAIL-R1 or -R2 when analysed using SDS-PAGE in the absence of reducing agents (Fig. 6.9), a method that has been previously used to assess CD95 aggregation (Kischkel *et al.*, 1995). Secondly, binding of FADD to the intracellular domains of TRAIL-R1 and -R2 was also reduced by PMA in an *in vitro* binding assay

(Fig. 6.10). The mechanism(s) whereby PKC activation inhibits CD95 and TRAIL-induced apoptosis may therefore differ.

Modification of DISC components: role of phosphorylation in death receptor signalling.

One possibility was that PKC was affecting DISC formation by modification of specific DISC components. FADD is a phosphoprotein and migrates as two distinct species when analysed by 2-dimensional gel electrophoresis (Fig. 6.11 A and (Medema *et al.*, 1997)). The function of these distinct forms is unknown. Phosphorylation of FADD has been suggested to be cell cycle-dependent. When cells were arrested in G_1/S only the non-phosphorylated form of FADD was present whereas only phosphorylated FADD was found in G_2/M-arrested cells (Scaffidi *et al.*, 2000). FADD phosphorylation has also been demonstrated to be affected by the PKC inhibitor, BisXIII. As well as observing decreased FADD-CD95 interaction in PMA-treated cells, the authors also noted that when reversing this effect with BisXIII, the FADD in the lysates appeared to be almost completely present in the faster migrating (unphosphorylated form) (Meng *et al.*, 2002). Whether there was as a consequence of the effect of BisXIII on the cell cycle is not known. These authors also noted that despite potentiating CD95-induced apoptosis, the effect of BisXIII was not due to increased FADD binding at the DISC.

Thus the phosphorylation state of FADD does not appear play a role in CD95-mediated apoptosis or PKC-mediated protection from CD95-mediated apoptosis. The demonstration that both forms of FADD are recruited to the TRAIL DISC and again are not affected by PKC activation strongly suggest that the same is true for TRAIL signalling.

There are, however, a number of studies which suggest that phosphorylation may still play a role in death receptor-induced apoptosis and in this respect CD95 function has been demonstrated to be affected by a number of kinases or phosphatases. FAP-1 (Fas-associated phosphatase-1) is a phosphatase which has been demonstrated to interact with the ICD, specifically the last 3 amino acids, of CD95 (Sato *et al.*, 1995). FAP-1 has been demonstrated to be a negative regulator of CD95-mediated apoptosis and caspase-8 activation during CD95-mediated apoptosis is inhibited in FAP-1 transfected cells (Li *et al.*, 2000). Another modulator of CD95 is BTK (Bruton's tyrosine kinase). Targeted disruption of the BTK gene in DT40 cells resulted in cells that were resistant to CD95-mediated apoptosis (Uckun. 1998). Binding of BTK to the ICD of CD95 was subsequently found to lead to reduced binding of FADD and caspase-8 and hence CD95 resistance

(Vassilev *et al.*, 1999). CD95 has also been demonstrated to bind the lipid raft-associated scr kinase fyn and has been demonstrated to become tyrosine phosphorylated upon stimulation (Atkinson *et al.*, 1996; Daigle *et al.*, 2002). However, in other studies the scr kinases and lipid rafts have not been demonstrated to be involved in CD95 signalling (Ko *et al.*, 1999b; Schraven *et al.*, 1995) and the significance of this is therefore unclear. There is a conserved YXXL motif within the death domains of CD95, TNF-R1 and TRAIL-R1 and -R2, which has been demonstrated to bind SHP-1 (Src homology domain 2 (SH2)-containing tyrosine phosphatase-1), SHP-2 and SH2-containing inositol phosphatase (SHIP). Binding of these components has been reported to lead to a caspase-independent disruption of growth factor survival signals and modulation of this motif does not appear to modulate death receptor-induced apoptosis (Daigle *et al.*, 2002). It is of interest to note that SHP-1 translocates from the cytosol to the cell membrane upon PMA stimulation, but whether this is PKC-mediated is unknown (Zhao *et al.*, 1994). Whether PKC activation affects any of the molecules described above or whether any of them are capable of modulating TRAIL receptors and or TRAIL-induced apoptosis is not known.

Summary

In conclusion we have demonstrated that PMA-mediated PKC activation is able to block TRAIL-induced apoptosis. Protection was characterised by inhibition of PARP cleavage as well as a reduction in procaspase-8 activation and Bid cleavage. Interestingly procaspase-3 was processed, but only to its p20 form, which was compatible with the finding that TRAIL-induced mitochondrial release of cytochrome *c* and the IAP-antagonist, Smac was also blocked by PMA. TRAIL DISC analysis showed that FADD recruitment was markedly reduced in cells pretreated with PMA, an effect which could not be explained by a marked decrease in cell surface receptor levels or a reduction in receptor aggregation. Using an in vitro binding assay we show that in PMA-treated cells the affinity of FADD for the intracellular domain of the receptor was reduced by an as yet unknown mechanism. As there are no currently identified cellular inhibitors of caspase-8, disruption of DISC formation and/or reduction in recruitment of key components may be an important mechanism for controlling cellular sensitivity to TRAIL. The precise mechanism of this disruption is unclear but may provide a model whereby phosphorylation of TRAIL signalling pathway components provides protection from death receptor ligation. This would clearly be an important mechanism as it could be utilised by cells to escape the cytotoxic effects of TRAIL and may therefore have the potential to be capable of being manipulated and utilised to sensitise resistant cells to TRAIL.

CHAPTER 7: DISCUSSION

7.1 Overview

Data presented in this thesis is aimed at trying to further understand the molecular mechanisms by which TRAIL interacts with its receptors to induce caspase activation and apoptosis. A summary of TRAIL-induced signaling pathways and the molecules involved is shown in Figure 7.1. The activation of other signaling pathways by other TNF family members, has also been addressed with respect to TRAIL and the molecular mediators of these pathways have been characterised by DISC analysis. The receptor signalling complex of TNF itself has also been isolated and compared to that induced by TRAIL and this has revealed an important difference relating to their primary signalling pathways; for TRAIL in tumour and transformed cells at least this is caspase activation whereas, for TNF NF-κB activation predominates. Finally members of the PKC family are implicated as mediators of a novel protective effect that confers cellular resistance to TRAIL by interfering with proximal signalling events during TRAIL-mediated activation of procaspase-8.

7.2 Role of NF-κB activation in TRAIL signalling

The dominant signalling pathway activated by TNF is the NF-κB pathway, and only when this pathway is inhibited does the apoptotic signalling arm of TNF become apparent (Van Antwerp *et al.*, 1996). TRAIL signalling appears to mirror that of TNF. The dominant arm, at least in TRAIL-sensitive cells is caspase activation and apoptosis and only when this arm is blocked does the NF-κB activation arm become apparent. This clearly has implications for its potential role *in vivo* as TRAIL only appears to induce apoptosis in tumor and transformed cells thus the dominant signalling arm *in vivo* would be predicted to be activation of NF-κB. As well as activating an NF-κB reporter gene, TRAIL also induced expression of an NF-κB-regulated cytokine, IL-8 (section 3.2.1) suggesting that it, like TNF, may act as a pro-inflammatory cytokine albeit with much less potency than TNF. IL-8 secretion was only observed in the TRAIL resistant 293 cell line and required the presence of a caspase inhibitor in the TRAIL sensitive HeLa cells. As mentioned above TRAIL appears to have little effect on normal cells and therefore its effect *in vivo* may be activation of NF-κB-regulated genes such as IL-8. The fact that it appears to be well tolerated *in vivo* when administered to mice suggests that NF-κB activation by TRAIL *in vivo* would be considerably lower than that

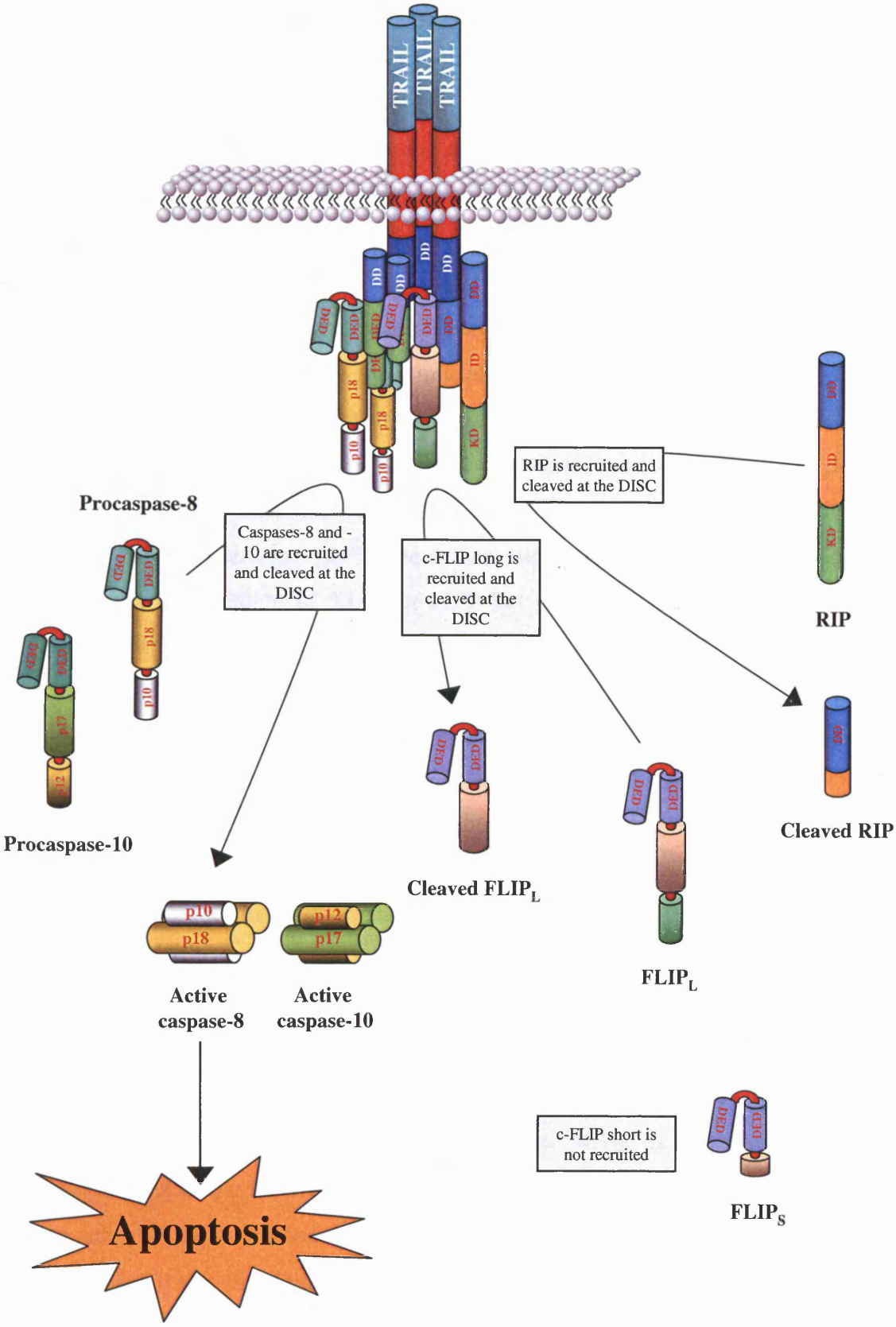


Figure 7.1 Mechanism of TRAIL-induced apoptosis
(see text for details)

induced by TNF which *in vivo* causes massive cell death, not through its apoptotic arm, but as a consequence of NF-κB-mediated inflammatory shock (Ashkenazi *et al.*, 1999).

The exact NF-κB-regulated genes that are responsible for providing protection against TNF-mediated apoptosis are still unknown but obvious candidate proteins include members of the IAP family and anti-apoptotic Bcl-2 family members (Wang *et al.*, 1998). It is however entirely conceivable that TRAIL may activate a totally different subset of genes to TNF.

Does the NF-κB activation pathway play a role in the apparent resistance observed in "normal" cells? Clearly complete inhibition of NF-κB can sensitise resistant cells to TRAIL as observed in 293 cells transfected with an IκB-α "super-repressor" (section 3.2.2). Kothney-Wilkes and colleagues observed that transformed keratinocytes could be protected from TRAIL by treatment with IL-1. This effect was NF-κB-mediated as it was blocked by the IκB-α "super-repressor". The authors suggested that protection afforded by IL-1 was through upregulation of XIAP or FLIP but this was not confirmed (Kothny-Wilkes *et al.*, 1998). It is therefore conceivable that low levels of NF-κB activation by IL-1 and other NF-κB inducers may afford protection to "normal" cells in vivo. Interestingly, the primary keratinocytes used in the same study were resistant to TRAIL. Thus it appears that changes that occur during transformation of a cell can result in sensitisation to TRAIL. It will be important to elucidate the mechanism(s) underlying this sensitisation.

Does TRAIL modulate its own cytotoxicity by activation of NF-κB? This would be difficult to prove experimentally as the specifics of the machinery required by TRAIL receptors to activate NF-κB is unclear. Although introduction of a dominant-negative IκB-α mutant sensitised 293 cells to TRAIL it could be argued that these cells had high constitutive NF-κB activation that was directly suppressed by the IκB-α mutant thereby leading to sensitisation rather than the mutant acting to block TRAIL-induced NF-κB activation. Elucidation of the machinery that is used by TRAIL to activate NF-κB will enable a more targeted approach to disable TRAIL-induced NF-κB activation by TRAIL rather than total NF-κB activation within the cell. The presence of RIP in the TRAIL DISC (section 4.2.5) and the finding by Lin and colleagues that TRAIL-mediated NF-κB activation appears to be abrogated in RIP-deficient cells argues for at least one molecule that is required for NF-κB activation by TRAIL (Lin *et al.*, 2000). Introduction of DN-RIP

into a TRAIL resistant cell would help to answer the question as to whether TRAIL does in fact modulate its own cytotoxicity like TNF, via activation of NF-κB.

In early studies, one proposed role of the decoy receptor TRAIL-R4 was activation of NF-κB. This together with its ability to sequester TRAIL away from the death receptors TRAIL-R1 and -R2 was presented as the mechanism by which TRAIL-R3 and -R4 protected cells from TRAIL-induced apoptosis. In my reporter system I was unable to demonstrate that TRAIL-R4 was capable of activating NF-κB even when greatly overexpressed (section 3.2.6). Further examination of the sequence of TRAIL-R4 reveals that it lacks a number of key residues, the *lpr* mutation in the mouse for example, that have been demonstrated to be required not only for death receptor-induced apoptosis but also for NF-κB activation by death receptors (Chaudhary *et al.*, 1997). This was confirmed by truncating TRAIL-R2 to mimic the cytoplasmic domain of TRAIL-R4 which resulted in a mutant that no longer activated NF-κB. This data therefore confirms the findings of Pan and colleagues (Pan *et al.*, 1998) but disagrees with the findings of Degli-Esposti and Hu and colleagues (Degli-Esposti *et al.*, 1997; Hu *et al.*, 1999) the reason for these discrepancies is not clear. The true role of the two TRAIL "decoy" receptors is becoming increasingly unclear. It was originally suggested that "normal" cells would express higher levels of these receptors on the cell surface to antagonise the cytotoxic effect of TRAIL on TRAIL-R1 and -R2 and in theory this sounded like an entirely plausible argument to explain the differential sensitivity of TRAIL (Sheridan *et al.*, 1997). However, when specific antibodies were generated to each of the four TRAIL receptors it became increasingly clear that in the majority of cells TRAIL-R3 and -R4 are not expressed at the cell surface but are instead intracellular, exhibiting a peri-nuclear distribution (Zhang *et al.*, 2000b). Further, when Legembre and colleagues introduced a GPI-linked CD95 mutant, similar to TRAIL-R3, into cells they observed increased sensitivity to CD95L in cells expressing higher levels of the "so-called" decoy mutant (Legembre *et al.*, 2002). Thus the exact role TRAIL-R3 and -R4 play in TRAIL signaling is currently unclear. But data from Legembre suggests that TRAIL-R3 may also play a pro-apoptotic role.

7.3 The TRAIL DISC

7.3.1 Components of the TRAIL DISC

There was initially much confusion surrounding the adaptor protein used by the two TRAIL death receptors to activate the cell death machinery. Much of this confusion was due to the fact that MEFs are not sensitive to TRAIL and therefore the FADD$^{-/-}$ or caspase-8$^{-/-}$ MEFs could not be used to provide evidence for their respective requirement for TRAIL cytotoxicity (Varfolomeev *et al.*, 1998). Further, many studies disagreed with one another as to whether a FADD-DN mutant was able to protect cells from overexpression of the respective TRAIL death receptors. Progress was also hampered by the lack of specific reagents for immunoprecipitation of native DISC complexes. Data presented in Chapter 4 and from a number of other studies now demonstrate that FADD is recruited to a native TRAIL DISC (i.e. involving proteins expressed at physiological levels) (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Further support is obtained from the observation that FADD-deficient Jurkat cells are refractory to TRAIL-induced apoptosis (section 4.2.2).

Do both TRAIL death receptors use FADD as an adaptor? Only TRAIL-R1 was overexpressed in FADD$^{-/-}$ MEFs so it was speculated at the time that TRAIL-R2 may use an alternative adaptor protein (Yeh *et al.*, 1998). Sprick and colleagues addressed this using antagonising antibodies to TRAIL-R1 and -R2 to independently isolate TRAIL-R1 and -R2 complexes. Both complexes were demonstrated to contain FADD (Sprick *et al.*, 2000). In a separate study Kuang and colleagues returned to the FADD$^{-/-}$ MEFs and stably expressed each respective TRAIL death receptor. Only the "wild-type" cells were sensitive to TRAIL thus proving that FADD was absolutely required for transducing the TRAIL death signal (Kuang *et al.*, 2000).

Caspase-8 also appears to be a common component of death receptor signalling pathways as caspase-8 deficient Jurkat cells are refractory to CD95, TNF and TRAIL-induced apoptosis ((Juo *et al.*, 1998) Figs. 4.2 and 5.3). The role of caspase-10 however is somewhat confusing. It is clearly recruited and processed at the DISC (Fig. 4.3 B) but it is not required for TRAIL- or CD95-mediated apoptosis, both of which are abrogated in caspase-8-deficient Jurkats despite the fact they still express caspase-10 (Sprick *et al.*, 2002). The HeLa cells used in this study were very sensitive to TRAIL-induced apoptosis but western analysis confirmed that they did not contain caspase-10 (data not shown).

Additionally, as mice completely lack the caspase-10 gene its real function is even more unclear.

Although a recent report has suggested that c-FLIP$_L$ can at low concentrations function to promote procaspase-8 activation it is generally believed that the two c-FLIP splice variants c-FLIP$_L$ and c-FLIP$_S$ function to inhibit procaspase-8 processing at the DISC. As a result MEFs generated from FLIP$^{-/-}$ mice (both variants) are more sensitive to death receptors induced apoptosis than "wild-type" cells (Yeh *et al.*, 2000). c-FLIP$_L$ was recruited and processed in TRAIL DISCs isolated from both sensitive and insensitive cells (Figs. 4.4 and 4.9) however, despite both Jurkat and B-CLL expressing c-FLIP$_S$ it did not appear to be recruited to the DISC. The reason behind this is not clear as Jurkat cells expressing high levels of c-FLIP$_S$ are refractory to CD95, TNF and TRAIL-mediated apoptosis (Thome *et al.*, 1997). In addition the structure of c-FLIP$_S$ is almost identical to c-FLIP$_L$ and therefore would have been predicted to be recruited to the DISC to the same extent as c-FLIP$_L$.

RIP is required for TNF-mediated NF-κB activation but has not previously been implicated in TRAIL signalling. It was also observed to be processed at the DISC and may represent the caspase-sensitive component which is cleaved in TRAIL sensitive HeLa cells thus preventing NF-κB activation. One problem with this hypothesis is that RIP is also cleaved in the TRAIL sensitive 293 cells. Two other mediators of death receptor signal transduction are TRADD and TRAF2 which are required for TNF-mediated NF-κB activation and JNK signalling (Hsu *et al.*, 1995; Tada *et al.*, 2001). Although TRAIL is capable of activating both of these pathways none of these components was recruited to the DISC. Although TNF induces JNK activation directly through the TRAFs, TRAIL-induced JNK activation has been demonstrated to be caspase-dependent as it can be blocked by z-VAD.fmk (MacFarlane *et al.*, 2000). TRAIL-induced JNK activation is therefore probably due to active caspases within the cell rather than due to a direct receptor-activated event. How TRAIL activates NF-κB without TRAF2 is unclear but as there appears to be some redundancy between other TRAF family members another TRAF such as TRAF5 could be involved instead.

7.3.2 TRAIL DISC formation in TRAIL-sensitive and -resistant cells

One surprising finding was that DISCs were still formed in cells which appeared to be refractory to TRAIL-induced apoptosis. DISCs isolated from 293 and B-CLL cells contained both FADD and caspase-8 which appeared to be processed in both cell types. The absence of processed caspase-8 when whole cells pellets were analysed suggested that further procaspase-8 activation was attenuated in these cells and that only the small amount of caspase-8 found in the receptor precipitates was in fact processed. During DISC formation in general it is unclear whether a finite amount of procaspase-8 is recruited and processed or whether procaspase-8 is continually recruited, processed and released, and thus effectively "cycled" through the DISC. This would be an interesting concept but would be hard to study experimentally. TRAIL DISC isolates can be made to process exogenously added procaspase-8 but the kinetics of this cleavage is unrealistic requiring overnight incubations (data not shown). Inhibition of further procaspase-8 recruitment, possibly due to the two FLIP variants or due to failure of the DISC to release the processed prodomain could result in an inability to propagate the apoptotic signal. As a result sufficient active caspase-8 would not be generated to overcome the threshold set by the anti-apoptotic proteins and the cell would survive.

The levels of TRAIL-R2 precipitated in the 293 cells may also provide another clue. Despite precipitating comparable levels of TRAIL-R2 to that seen in HeLa cells, there appeared to be considerably less FADD and hence caspase-8 precipitated in 293 cells. A similar effect was observed in the Jurkat model compared to B-CLL cells (Figs. 4.1 and 4.9). Why significantly less FADD was recruited is unclear but it could be due to levels of the other TRAIL receptors in particular TRAIL-R1 (as the other receptors are thought to reside intracellularly). Aggregation of these receptors is another possibility. Receptor aggregation has been demonstrated to be absolutely required for CD95L-mediated cytotoxicity and has also been demonstrated to be inhibited by PI3K activation in primary T cells (Varadhachary *et al.*, 2001). This in turn affected the lateral diffusion of CD95 in the cell membrane leading to inhibition of CD95-mediated apoptosis.

Another possibility has presented itself from data obtained in Chapter 6. PKC activation appears to protect cells from TRAIL-induced apoptosis by disrupting DISC formation. This was shown not to be through inhibition of receptor aggregation or through decreased cell surface receptors levels, but instead by modulating the affinity of FADD for the receptor ICDs. It is tempting to speculate that PKC activation has led to a modification or relocation of a critical known or unknown DISC component which in turn inhibits

FADD binding to the DD of the receptor and subsequently leads to inhibition of TRAIL-induced apoptosis. If levels of this component are upregulated or it is more active in cells resistant to TRAIL this could potentially lead to the reduced FADD binding observed in the 293 cells. As there are no cellular inhibitors of caspase-8 this may provide an additional level of control above that where FLIP functions to inhibit caspase-8 activation and apoptosis.

7.4 TNF signalling

7.4.1 Failure to recruit FADD or caspase-8 to the TNF signalling complex

The standard model for death receptor-induced apoptosis is recruitment and activation of the initiator caspase-8 in a membrane-bound death-inducing signalling complex (DISC). Data presented in Chapter 5, however, suggests that this model may not be applicable to TNF signalling. Isolation of TNF receptor complexes resulted in the precipitation of TRADD, RIP and TRAF2 but not the apoptotic mediators FADD or caspase-8 (sections 5.2.1 and 5.2.4). Despite this observation it has been demonstrated that these components are clearly required for TNF-mediated apoptosis (section 5.2.2 and (Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998).

Death receptors have often been split into two groups based on their primary signalling pathways. The first including CD95, TRAIL-R1 and -R2 consist of those whose primary function appears to be the induction of apoptosis. Following receptor ligation there is rapid formation of a membrane-bound DISC containing both FADD and caspase-8 (section 4.2.1 and (Kischkel *et al.*, 1995)). The second group, including TNF and wsl-1, primarily activate NF-κB which functions to antagonise the activation of their apoptotic arm. In general these receptors will only induce apoptosis in a given cell type if the NF-κB activation pathway is blocked e.g. using the protein synthesis inhibitor, cycloheximide. In this respect, delineation of NF-κB and caspase activation pathways during TNF signalling would appear to better fit observations made about TNF signalling. NF-κB and JNK activation are rapid and occur within seconds of receptor ligation the and as a consequence mediators of these pathways, RIP and TRAF2, are directly recruited to the receptor. Apoptosis only occurs later when *de novo* synthesis of NF-κB regulated genes is inhibited. The apoptotic mediators of TNF-induced apoptosis, FADD and caspase-8, are therefore

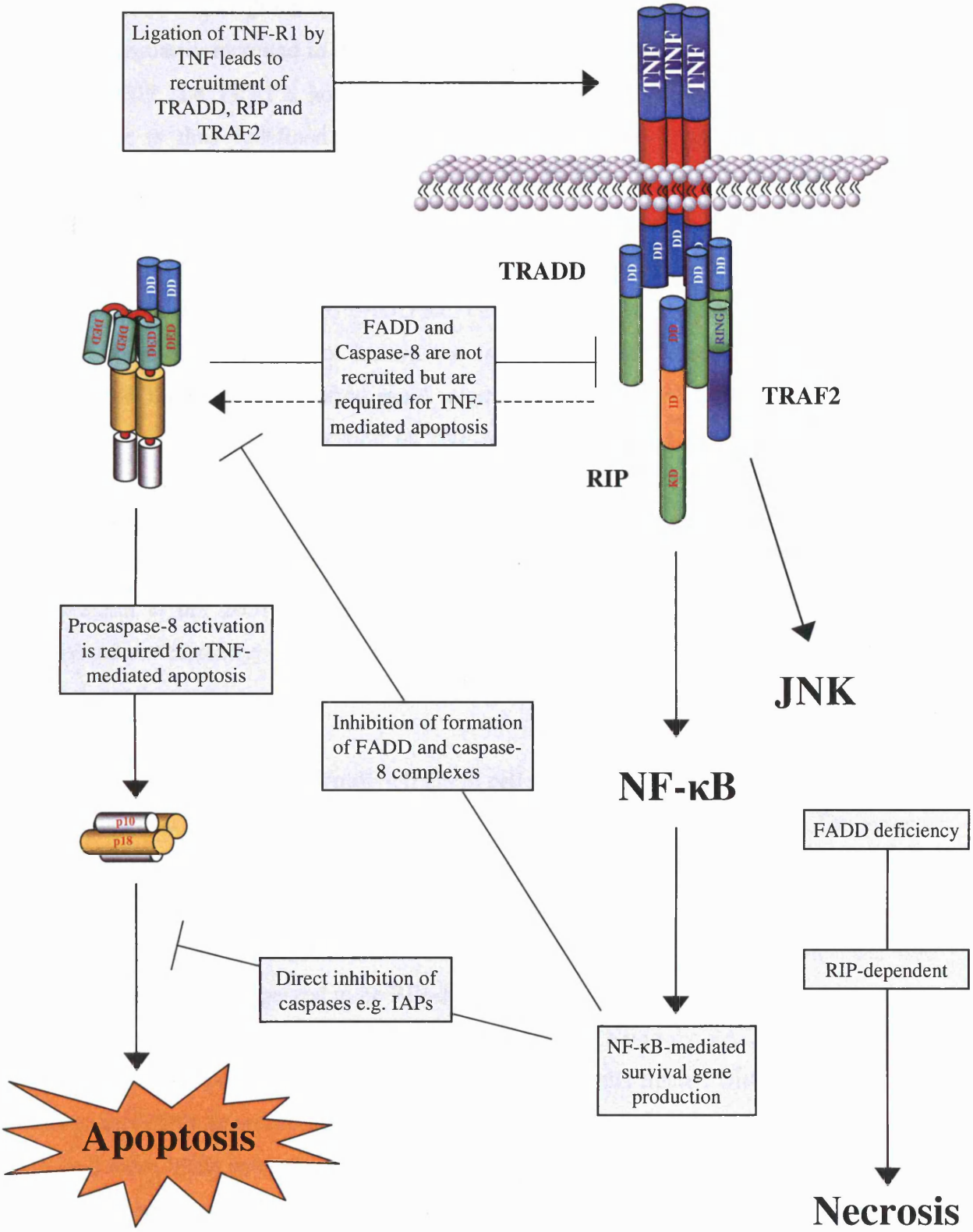


Figure 7.2 TNF-R1 signalling pathways
(see text for details)

not recruited to the receptor and must function elsewhere. Data presented in this thesis further supports this grouping and hypothesis by demonstrating that FADD and caspase-8 are not primarily recruited to the TNF receptor complex. Mediators of NF-κB activation, such as RIP and TRAF2, however, are rapidly recruited. The model for TNF receptor signalling is thus redefined in Figure. 7.2. Ligation of TNF-R1 by TNF leads to recruitment of TRADD. TRADD in turn acts as a platform for the recruitment of the TNF signalling intermediates RIP and TRAF2 which act to mediate the primary TNF signalling pathways i.e. NF-κB and JNK activation. Only when the NF-κB activation pathway is blocked does the cell undergo FADD and caspase-8-dependent apoptosis. Where FADD and caspase-8 interact is not known but is clearly not in a conventional membrane-associated DISC. The reported rapid internalisation of TNF/TNF-R complexes which when inhibited lead to abrogation of TNF-mediated apoptosis suggests that FADD and caspase-8 may interact in the cytosol rather than at the membrane (Schutze *et al.*, 1999). There are also several studies which suggest the involvement of lysosomes and lysosomal proteases such as cathepsins in TNF-mediated cell death (Foghsgaard *et al.*, 2001; Guicciardi *et al.*, 2001). Whether these pathways are apical to FADD and caspase-8, activated concurrently, or downstream of apical caspase activation during TNF signalling remains to be established.

7.4.2 TNF mediates an alternative from of cell death in the absence of FADD

TNF may also be able to mediate a caspase-independent form of cell death. The absence of FADD revealed an alternative form of TNF-mediated cell death which did not appear to involve caspases or phosphatidylserine exposure (sections 5.2.2 and 5.2.3). This form of cell death has been described and characterised recently by Holler and colleagues who demonstrated that it appeared to be RIP-dependent . Although Holler and colleagues noted that TRAIL and CD95 mediated a similar cell death to TNF in the FADD-deficient Jurkat cell model, data presented in Chapter 5 suggests that cells treated with TNF were much more susceptible as no cell death was observed with TRAIL (Fig. 5.2). This would appear to be a more likely scenario, especially if RIP is the effector molecule for this type of cell death, as considerably more RIP is recruited to TNF complexes than the TRAIL DISC (compare Figs 4.5 and 5.1). The significance of the results obtained in FADD-deficient Jurkat cells and any physiological function may be somewhat questionable. If however, the results of Holler and colleagues are confirmed and this form of cell death requires the

apparently redundant kinase function of RIP, it may yet serve some physiological role (Holler *et al.*, 2000). Identification of the substrate for RIP as a kinase will help to further characterise this novel death receptor-induced cell death pathway.

While RIP appears to be the effector molecule in the necrosis-like cell death observed in the FADD-deficient Jurkats and the presence of FADD in some way prevents this, Kawahara and colleagues have also demonstrated that FADD itself can mediate a similar necrosis-like cell death (Kawahara *et al.*, 1998). Dimerisation of FADD in cells lacking caspase-8 led to a cell death characterised by the absence of caspase activation or DNA fragmentation and the authors thus concluded that FADD could mediate two forms of cell death, caspase -dependent and -independent. TNF has been demonstrated to induce a caspase-independent cell death in only a relatively small number of cell lines (NIH3T3, L929 and WEHI-S). In some of these studies the addition of z-VAD.fmk actually enhanced the cell death providing evidence that it may be negatively regulated by caspases (Khwaja & Tatton. 1999; Vercammen *et al.*, 1998a). Reactive oxygen species (ROS), cathepsins (particularly cathepsin B) and the lysosomal compartment have all been implicated as mediators of this alternate cell death (Brunk *et al.*, 2001; Foghsgaard *et al.*, 2001). It is interesting to note that caspase inhibition enhances ROS production after TNF treatment possibly due to caspase-mediated cleavage of phospholipase A_2 (Adam-Klages *et al.*, 1998; Luschen *et al.*, 2000a). It would therfore be of interest to examine the effect ROS scavengers, cathepsin inhibitors or lysosomal stabilisers have on TNF cytotoxicity in the FADD-deficient Jurkats.

7.4.3 Modification of components recruited to the TNF signalling complex

Recruitment of TRADD, RIP and TRAF2 to TNF-R complexes led to them being subjected to some form of modification (Sections 5.2.1 and 5.2.4). TNF-induced modification of RIP and TRAF2 has now also been noted in two other separate studies. Zhang and co-workers noted that RIP assumed a "ladder-like" appearance when immunoprecipitated with TNF-R1 although no further information was provided (Zhang *et al.*, 2000a). Modified RIP and TRAF2 were also observed by Chen and colleagues but again no explanation was provided (Chen *et al.*, 2002). The retention of RIP within these complexes by inhibition of the proteasome at least suggests that this modification is a consequence of ubiquitin ligation. The presence of several potential ubiquitin ligases within the TNF-R1 signalling complex such as TRAF2 (Figs. 5.1 and 5.4) and (based on other studies), cIAPs -1 and –2, would further support this hypothesis (Shu *et al.*, 1996).

Parallels can be therefore be drawn with the role and modification of IRAK, which is phosphorylated and ubiquitinated following IL-1 stimulation (Janssens & Baeyaert. 2003). IRAK is phosphorylated by a related kinase IRAK4 (Li *et al.*, 2002a). The recent discovery of another RIP molecule, RIP3 which functions as a RIP kinase and is recruited to TNF-R1 may provide further evidence that RIP has a parallel function to IRAK in TNF signalling (Sun *et al.*, 2002a). Whether this modification functions to lead to release of RIP and bound components from the TNF signalling complex for termination or potentiation/amplification of signalling, or whether it is required to orientate receptor components to facilitate upstream kinase activation during NF-κB activation requires further study.

7.5 Inhibition of death receptor signalling by protein kinase C activation

It has previously been reported by a number of groups that PMA-mediated PKC activation can provide protection against CD95L and TRAIL-mediated apoptosis (Holmstrom *et al.*, 2000; Holmstrom *et al.*, 1999; Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001). The exact nature of this inhibition however was uncertain and different groups proposed that PKC can inhibit death receptor-mediated apoptosis at different levels. Data presented in Chapter 6 represents a novel mechanism for PMA-mediated inhibition of TRAIL-mediated apoptosis (Fig. 7.3). A series of observations provided evidence that PKC activation appeared to be modulating TRAIL-induced apoptosis at a pre-mitochondrial step. PKC activation blocked TRAIL-induced phosphatidylserine exposure and PARP cleavage, suggesting that caspase-3 activity was blocked (section 6.2.1). This however was an oversimplification and in fact initial cleavage of caspase-3, (i.e. removal of the small subunit) was not fully inhibited by PMA (Fig. 6.2). Rather the second autocatalytic step of caspase-3 processing was inhibited, most likely due to XIAP binding (Sun *et al.*, 2002b). In support of this hypothesis, mitochondrial release of the IAP antagonist Smac and cytochrome *c* was blocked by PMA treatment (section 6.2.3). These data therefore suggested that PKC may be either affecting activation of caspase-8 or cleavage one of its cellular substrates, the proapoptotic Bcl-2 family member, Bid. Other reports on PMA inhibition of TRAIL and CD95-induced apoptosis have failed to report this observation, as effector caspase cleavage was not assessed (Ruiz-Ruiz *et al.*, 1999; Sarker *et al.*, 2001).

Bid has recently been shown to be phosphorylated specifically by casein kinases I and II but also following PMA treatment. The resulting phosphorylated Bid was suggested to be more resistant to caspase-8 cleavage than the non-phosphorylated form (Desagher *et al.*, 2001). PKCs have also been implicated in this process as PKC inhibitors were demonstrated to lead to dephosphorylation of Bid which was subsequently shown to be more sensitive to cleavage by caspase-8. This could therefore provide an alternative explanation for the observed inhibition of TRAIL cytotoxicity by PMA. Bid cleavage was inhibited by PMA but the possibility still existed that the reduced caspase-8 activation observed was instead due to PMA directly affecting activation of procaspase-8. This led to the observation that FADD recruitment to the TRAIL DISC appeared to be inhibited by PMA treatment. The reduction in FADD did not appear to be due to a reduction in TRAIL receptor surface expression or aggregation.

It could be argued that PMA was leading to upregulation of the TRAIL decoy receptors from intracellular stores. This would be expected to result in disruption of the DISC and a reduction in FADD binding. It was not possible to examine this due to reagent availability but this hypothesis is unlikely to be valid when considering the dramatic effect which PMA appears to have in the receptor ICD binding assay (Fig. 6.10). The *in vitro* binding data also supports the previous observations that TRAIL-R1 and -R2 aggregation and cell surface expression are not markedly affected by PMA and are therefore unlikely to be responsible for the reduced recruitment of FADD.

How then does PMA affect the binding of FADD to the respective receptor ICD? Two possibilities exist. Firstly there may be an as yet unidentified DISC component which either facilitates or is required for the interaction of FADD with the DD of the receptor. One candidate is death associated protein 3 (DAP3) which has been implicated in receptor-mediated apoptosis signalling (Kissil *et al.*, 1999). In another study Miyazaki and colleagues suggested that DAP3 was a direct mediator of death receptor signaling and functioned to regulate FADD binding to TRAIL-R4 and CD95 (Miyazaki *et al.*, 2001). This hypothesis was based on a yeast-2-hybrid screen using the ICDs of the respective receptors which resulted in DAP3 being a candidate interactor. DAP3 would therefore be a candidate molecule for the effect of PMA on DISC formation. However, in general, a yeast-2-hybrid screen does not take into account the cellular localisation of a particular protein. As a consequence Berger and colleagues used immunohistochemistry and cell fractionation to show that in fact DAP3 was a mitochondrial protein and was not released

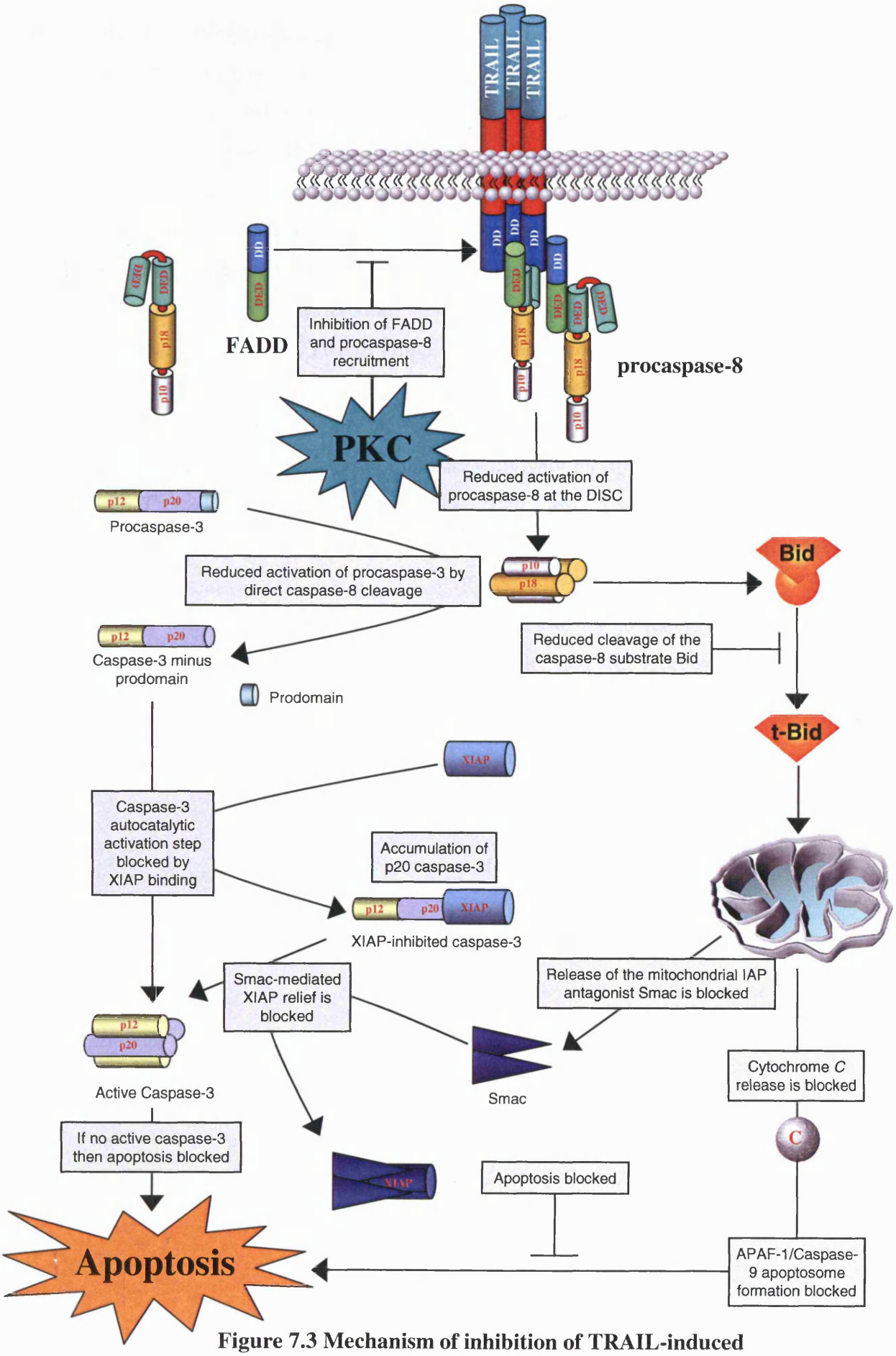


Figure 7.3 Mechanism of inhibition of TRAIL-induced apoptosis by PMA-mediated PKC activation.
(see text for details)

during apoptosis (Berger *et al.*, 2002). It is therefore unlikely to ever have the opportunity to interact with a death receptor.

A second possibility is that PMA/PKC may be causing the modification of either FADD or the receptor ICD which leads to a reduced binding affinity. FADD phosphorylation state was assessed by 2D electrophoresis and appeared unchanged by PMA treatment. This data is backed up by a study demonstrating that the phosphorylation state of FADD does not modulate CD95-induced apoptosis (Scaffidi *et al.*, 2000). Additionally if there was a differential recruitment of phosphorylated compared to non-phosphorylated FADD in the TRAIL DISC, then FADD would not appear as a doublet in TRAIL precipitates. It was not possible to assess TRAIL receptor phosphorylation state in this study due to limited specific antibodies. However, Frankel and colleagues have demonstrated that TRAIL-R1 appears to be constitutively phosphorylated in unstimulated cells while TRAIL-R2 exists in an unphosphorylated form. They also demonstrated that PMA treatment did not affect the basal phosphorylation state of either receptor (Frankel *et al.*, 2001).

It will therefore be important in future studies to try to identify the PMA/PKC sensitive component of CD95, TNF-R1 and TRAIL-R1 and -R2 receptor signalling as it could clearly be utilised by cells as another mechanism to inactivate death receptor signalling.

7.6 Future Directions

Data presented in this thesis leave a number of important unanswered questions.

TRAIL signalling:

- What determines cellular sensitivity or resistance to TRAIL
- Why are there two TRAIL death receptors
- What role do TRAIL-R3 and -R4 play in TRAIL signalling

TNF signalling:

- FADD and caspase-8 are clearly not recruited to a membrane-bound DISC despite being critical components for TNF-induced apoptosis. How/where is caspase-8 activated in a FADD-dependent manner during TNF-mediated apoptosis.
- Does the necrosis seen in the FADD-deficient cells have a physiological/pathological role. How is RIP involved in mediating this pathway? What is the substrate for the kinase activity of RIP.
- What is the nature and function of the modifications observed on TRADD, RIP and TRAF2?

PKC activation:

- How does PKC activation lead to a reduction in binding of FADD in TRAIL signalling? Is the mechanism similar for inhibition of TRADD binding to TNF-R1 and FADD binding to CD95?
- Does PKC activation lead to a modification within the intracellular domain of the respective death receptor?
- Is a specific PKC isoform involved in providing protection?

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CHAPTER 9: APPENDIX

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Modulation of Tumor Necrosis Factor Apoptosis-inducing Ligand-induced NF-κB Activation by Inhibition of Apical Caspases*

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Tumor necrosis factor (TNF) apoptosis-inducing ligand (TRAIL), a member of the TNF family, induces apoptosis in many transformed cells. We report TRAIL-induced NF-κB activation, concomitant with production of the pro-inflammatory cytokine Interleukin-8 in the relatively TRAIL-insensitive cell line, HEK293. In contrast, TRAIL-induced NF-κB activation occurred in HeLa cells only upon pretreatment with the caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.fmk), indicating that this was due to a caspase-sensitive component of TRAIL-induced NF-κB activation. NF-κB activation was mediated by the death receptors, TRAIL-R1 and -R2, but not by TRAIL-R3 or -R4 and was only observed in HeLa cells in the presence of z-VAD.fmk. Receptor-interacting protein, an obligatory component of TNF-α-induced NF-κB activation, was cleaved during TRAIL-induced apoptosis. We show that receptor-interacting protein is recruited to the native TRAIL death-inducing signaling complex (DISC) and that recruitment is enhanced in the presence of z-VAD.fmk, thus providing an explanation for the potentiation of TRAIL-induced NF-κB activation by z-VAD.fmk in TRAIL-sensitive cell lines. Examination of the TRAIL DISC in sensitive and resistant cells suggests that a high ratio of c-FLIP to caspase-8 may partially explain cellular resistance to TRAIL-induced apoptosis. Sensitivity to TRAIL-induced apoptosis was also modulated by inhibition or activation of NF-κB. Thus, in some contexts, modulation of NF-κB activation possibly at the level of apical caspase activation at the DISC may be a key determinant of sensitivity to TRAIL-induced apoptosis.

Tumor necrosis factor apoptosis-inducing ligand (TRAIL)[1] is a recently cloned member of the TNF ligand family. Unlike CD95L and TNF, which have a restricted tissue distribution, TRAIL is constitutively expressed, at least at the mRNA level, in a wide variety of tissues and cell types (1, 2). Due to this ubiquitous distribution, it was postulated that regulation of TRAIL-induced cell death may be mediated by restricted receptor expression. The TRAIL receptor family is unusually complex, comprising at least four membrane-bound members. TRAIL induces apoptosis through TRAIL-R1 (DR4) (3) and TRAIL-R2 (DR5/TRICK2/KILLER) (4–7), both of which contain a cytoplasmic death domain motif that displays homology to the death domains found in CD95 and TNF receptor 1 (TNF-R1). Two additional receptors, TRAIL-R3 (TR3/DcR1/TRID/LIT) (8–11) and TRAIL-R4 (TR4/DcR2/TRUNDD) (12–14), are unable to signal for cell death and have been termed "decoy" receptors (9, 10). TRAIL-R3 lacks an intracellular domain and is a glycosylphosphatidylinositol-linked cell surface protein, whereas TRAIL-R4 contains a truncated intracellular domain and, thus, an incomplete death domain lacking residues critical for engaging apoptosis. Ectopic expression of TRAIL-R3 and -R4 protects cells from TRAIL-induced apoptosis, and it was hypothesized that they antagonize TRAIL-R1 and -R2 death signaling by either competing for limited TRAIL ligand (9, 10, 13) or, in the case of TRAIL-R4, by transduction of an anti-apoptotic signal (12). There is particular interest in the potential use of TRAIL as a novel anticancer agent as it appears to be selectively toxic to transformed and tumor cells but not to the majority of normal cells (1, 2, 15).

Although caspase-8 was identified as the apical caspase in TRAIL-induced apoptosis (16, 17), the mechanism of its recruitment and the adaptor molecule(s) involved have been subject to controversy. Early studies using overexpression of dominant-negative FADD produced conflicting results as to whether FADD and/or another adaptor was involved (9, 18–21). Recently, several studies report the presence of FADD in the native TRAIL death-inducing signaling complex (DISC) (16, 22, 23), suggesting that TRAIL utilizes a similar death-signaling pathway to those activated by CD95L and TNF. However, this does not explain the selective toxicity observed with TRAIL. Although TRAIL resistance sometimes correlates with the relative expression levels of death to decoy receptors, much evidence now points toward alternative models for TRAIL resistance (24), including the presence of intracellular anti-apoptotic molecules such as c-FLIP, which modulates TRAIL signaling (25, 26). Another mechanism of cellular resistance to members of the TNF family is through activation of the transcription factor, NF-κB (27). In many cell types, TNF negatively regulates its own cytotoxicity by up-regulation of NF-κB-regulated anti-apoptotic genes, such as c-IAP1 and c-IAP2 (28). Several studies show that TRAIL activates NF-κB (29, 30) and that this activation is mediated not only by the death receptors, TRAIL-R1 and -R2, but also

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[1] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; NIK, NF-κB-inducing kinase; PARP, poly(ADP-ribose) polymerase; RIP, receptor-interacting protein; TNF, tumor necrosis factor; TNF-R1, TNF receptor 1; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Ab, antibody.

by the truncated decoy receptor, TRAIL-R4 (12, 30).

To further examine the role NF-κB plays in TRAIL signaling, we employed a reporter gene system to study TRAIL-induced NF-κB activation and its relationship to TRAIL-induced apoptosis. We demonstrate that TRAIL-induced NF-κB activation is mediated by TRAIL-R1 and TRAIL-R2 and that this activation is a caspase-sensitive event as it occurs in TRAIL-sensitive cells only in the presence of the cell-permeable broad spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD.fmk). In parallel, we show that the receptor-interacting protein (RIP), which is responsible for TNF-α-induced NF-κB activation, is cleaved during TRAIL-induced apoptosis and that this is inhibited by z-VAD.fmk. We demonstrate that RIP is recruited to the native TRAIL DISC and this recruitment is enhanced in the presence of z-VAD.fmk, thus providing evidence for a direct link between TRAIL receptor engagement and an obligatory component of the NF-κB signaling pathway. Because the C-terminal product of RIP cleavage inhibits NF-κB activation (31, 32), we propose that the ability of z-VAD.fmk to reveal an NF-κB component of TRAIL signaling is mediated in part by its ability to inhibit RIP cleavage and, thus, maintain NF-κB activation in TRAIL-sensitive cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human TRAIL (residues 95–281) was produced as previously described (5). Recombinant human TNF-α was obtained from Sigma (Poole, UK). The caspase inhibitor z-VAD.fmk was from Enzyme Systems Inc. (Dublin, CA). Anti-FADD and anti-RIP monclonal Abs were obtained from BD Transduction Laboratories and BD Pharmingen (Heidelberg, Germany), respectively. Anti-TRAIL-R2 and anti-TRAIL-R4 monoclonal Abs were gifts from Immunex Corp. (Seattle, WA), anti-poly(ADP-ribose) polymerase (PARP) monoclonal Ab C2–10 was a gift from Dr. G. Poirier (Laval University, Quebec, Canada), anti-Bid Ab was a gift from Dr. X. Wang (University of Texas Southwestern Medical Center, Dallas, Texas), and anti-caspase-3 Ab was a gift from Dr D. Nicholson (Merck Frosst, Quebec, Canada). A rabbit polyclonal anti-caspase-8 Ab has been described previously (33), and a mouse monoclonal antibody to caspase-8 (C15) (34), used for DISC analysis, was a gift from Dr. P. H. Krammer (German Cancer Research Center, Heidelberg, Germany). Horseradish peroxidase-conjugated secondary antibodies, goat-anti-mouse and goat-anti-rabbit, were obtained from Sigma and Dako (Cambridge, UK), respectively.

Cell Culture—All cell culture materials were from Life Technologies, Inc. (Paisley, UK), and plastic-ware was from Becton Dickinson Labware (Bedford, MA). HeLa and HEK293 (293) cells were obtained from European Collection of Animal Cell Cultures (Wiltshire, UK). Both cell lines were cultured in Dulbecco's modified Eagle's medium/high glucose containing 10% fetal bovine serum and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. Mock-transfected MCF-7 (MCF-7 (Vector)) and caspase-3-transfected MCF-7 (MCF-7 (caspase-3)) cells were a gift from Dr. Alan Porter (National University of Singapore, Singapore) and have been described elsewhere (35).

Transfections and Reporter Gene Assays—Transfections were performed in 6-well plates using Effectene (Qiagen, Sussex, UK) according to the manufacturer's protocols. Cells were transfected with an NF-κB alkaline phosphatase reporter construct (p(NF-κB)4-tk-sPAP), which produced a secretable placental alkaline phosphatase product (Glaxo Wellcome) together with a β-lactamase expression construct (pRSV lactamase) (36) to assess transfection efficiency. Expression vectors for TRAIL receptors R1, R2, and R3 and WSL-1 have been described elsewhere (5, 37). pcDNA3-TRAIL-R4 was a gift from Dr. E. S. Alnemri (Thomas Jefferson University, Philadelphia, PA). The IκB "super-repressor" pCMV2-IκBM (S32A/S36A), which acts as a dominant-negative inhibitor of NF-κB (38), was obtained from CLONTECH (Hants, UK). mutTRAIL-R2 (ΔSer-324–Ser-369) was constructed using standard molecular biology protocols, and the deletion was confirmed by sequencing. Protein expression was confirmed using Western blotting with a TRAIL-R2-specific antibody.

Alkaline Phosphatase Assay—Heat-inactivated (65 °C, 30 min) conditioned medium (30 μl) from each well was assayed for alkaline phosphatase activity in duplicate in 96-well microtiter plates using (150 μl) (200 μg/ml) *p*-nitrophenyl phosphate in 1 M diethanolamine containing 0.5 mM $MgCl_2$. Plates were then incubated for up to 2 h at room temperature, and the absorbance at 405 nm was measured every 30 min in a Labsystems iEMS plate reader (Labsystems Affinity Sensors, Cambridge, UK).

β-Lactamase Assay—Conditioned medium (30 μl) was assayed for β-lactamase activity in duplicate in 96-well plates. Nitrocefin solution (150 μl) (200 ng/ml) (Glaxo Wellcome) in 50 mM NaK_2PO_4, pH 7.0, containing 0.1% Triton X-100) was added to each well. Plates were then incubated at room temperature, and the absorbance at 492 nm was measured every 30 min for 2 h.

Determination of Apoptosis in Transfected Cells—For apoptosis measurements, cells were cotransfected with a LacZ-containing vector, pRSC (39). Transfected cells expressing LacZ then appeared blue when stained with the β-galactosidase substrate, X-gal. The percentage of apoptotic cells was then expressed as the percentage of blue cells exhibiting apoptotic morphology.

IL-6 and IL-8 Enzyme-linked Immunosorbent Assay—Conditioned medium from transfected cells was analyzed for IL-6 and -8 content using sets of matched antibodies together with a recombinant human IL-6 or -8 standard (R&D Systems, Oxford, UK) essentially according to the manufacturer's instructions. The alkaline phosphatase *p*-nitrophenyl phosphate substrate system (Sigma) was used for detection, and plates were read at 405 nm.

Western Blotting—Discontinuous SDS-polyacrylamide gel electrophoresis was carried out using the Mini-Protean II Cell (Bio-Rad) using a Tris/Glycine buffer system based on the method of Laemmli (40). After electrophoresis, proteins were transferred to Hybond N nitrocellulose membrane (Amersham Pharmacia Biotech) using the Mini-Trans Blot system (Bio-Rad). Protein loading was assessed by Ponceau S staining of membranes. Blots were then stained with primary antibodies using standard protocols followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Immunostained proteins were visualized on x-ray film using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

Isolation of the TRAIL DISC—DISC precipitation was performed using biotin-tagged recombinant TRAIL (Bio-TRAIL) essentially as described by Sprick *et al.* (22). Recombinant TRAIL was biotinylated using a D-Biotin-*N*-hydroxysuccinimide ester (Roche Molecular Biochemicals) according to the manufacturer's instructions. Biotin incorporation was checked by Western blotting and was found to have no significant effect on biological activity. Cells (3×10^7 cells per treatment) were treated with Bio-TRAIL for up to 60 min. DISC formation was then stopped, and unbound Bio-TRAIL was removed by washing the cells three times with ice-cold phosphate buffered saline. Cells were then re-suspended in 5 ml of lysis buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100 (v/v), containing Complete™ protease inhibitors (Roche Molecular Biochemicals)) and lysed for 60 min on ice followed by centrifugation at 15,000 × *g* for 10 min at 4 °C. To provide an unstimulated receptor control, Bio-TRAIL was added to lysates from untreated cells. The TRAIL DISC was then precipitated using 30 μl of streptavidin-agarose beads (Sigma) at 4 °C overnight. Precipitates were washed five times with lysis buffer, and receptor complexes were eluted with 60 μl of sample buffer. Western blotting was performed using 30 μl of eluted complexes representing DISC precipitated from 1.5×10^7 cells.

RESULTS

Activation of NF-κB in HEK293 Cells but Not HeLa Cells—To determine the role of NF-κB in TRAIL signaling, 293 and HeLa cells were transfected with an NF-κB reporter construct, which is linked to a secretable alkaline phosphatase product. Cells were either treated with TNF-α or TRAIL for 24 h, and the medium was analyzed for alkaline phosphatase activity. TRAIL produced a small induction of NF-κB in 293 but not HeLa cells (Fig. 1*A*). TNF-α, the positive control for NF-κB activation, produced a marked increase in reporter activity in both cell lines (Fig. 1*A*). Because the reporter assay showed only a modest increase in NF-κB activation, we attempted to determine whether two NF-κB-inducible genes, IL-8 and IL-6, were also up-regulated. Both TRAIL and TNF-α caused a marked increase in IL-8 production in 293 cells (Fig. 1*B*). Induction of IL-8 by TRAIL was ~7-fold less than that by TNF-α and was similar to the differences observed in NF-κB activation with the reporter assay (Fig. 1*A*). In HeLa cells, basal IL-8 production was increased by a small extent by TRAIL and extensively by TNF-α (Fig. 1*B*). HeLa cells also

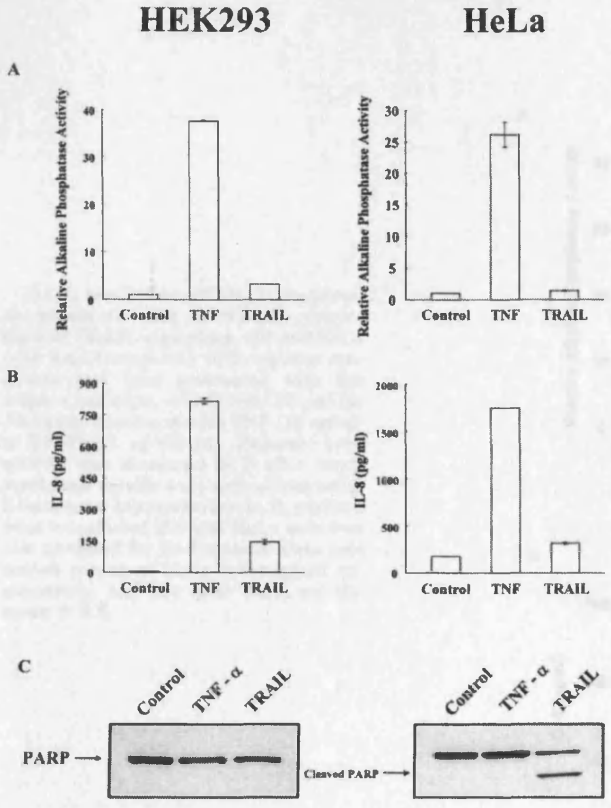

FIG. 1. **Activation of NF-κB in HEK293 but not HeLa cells correlates with their sensitivity to TRAIL-induced apoptosis.** *A*, HeLa and 293 cells were transfected with 0.1 μg of NF-κB-alkaline phosphatase reporter construct and 0.1 μg of β-lactamase reporter construct. Fresh medium was added 16 h after transfection, and the cells were treated with recombinant TNF-α (10 ng/ml) or recombinant TRAIL (1 μg/ml). Reporter gene activity was measured 24 h later, and results were normalized using β-lactamase expression levels. *B*, medium was also assayed for production of the NF-κB-regulated gene product, IL-8. Data are presented as fold-increase above control from three independent experiments, and *error bars* represent the mean ± S.E. *C*, treated cells were subjected to SDS- polyacrylamide gel electrophoresis followed by Western blotting as described under "Experimental Procedures." Membranes were probed with a mouse monoclonal antibody against PARP. The *arrows* represent intact or cleaved PARP.

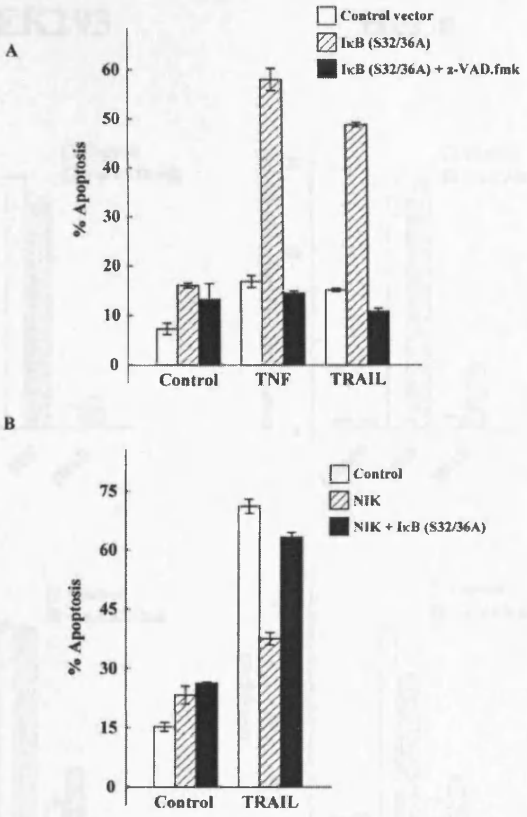

FIG. 2. **Inhibition of NF-κB sensitizes 293 cells to TRAIL-induced apoptosis, whereas up-regulation of NF-κB attenuates TRAIL-induced apoptosis in HeLa cells.** *A*, 293 cells were transfected with 0.1 μg of NF-κB reporter, 50 ng of a β-galactosidase-containing construct, pRSC, together with 0.1 μg of pCMV-IκBM (S32A/S36A). Medium was removed 16 h after transfection, fresh medium was added, and the cells were then treated with TRAIL (1 μg/ml) or TNF (10 ng/ml). Where indicated z-VAD.fmk (20 μM) was used as a 1-h pretreatment. After 24 h, medium was removed, and cells were stained with X-gal. The percentage apoptosis was assessed by comparing total blue cells in each well with the total number of blue cells displaying apoptotic morphology. *B*, HeLa cells were transfected with 0.1 μg of NF-κB reporter and 50 ng of a β-galactosidase-containing construct pRSC together with 5 ng of pcDNA3-NIK. 0.1 μg of pCMV-IκBM (S32A/S36A) was included where indicated to block NIK-induced NF-κB activation. Medium was removed 16 h after transfection and assayed for reporter activity. Fresh medium was added, and cells were further incubated for 2 h either alone or in the presence of TRAIL (1 μg/ml). Cells were then stained with X-gal, and apoptosis was assessed by comparing the total number of normal blue cells in each well to the number of blue cells displaying morphological features of apoptosis such as membrane blebbing and nuclear condensation. All transfections were carried out in duplicate, and the data presented represent three independent experiments. *Error bars* are the mean ±S.E.

produced high basal levels of IL-6, which were not significantly affected by treatment with TRAIL but were markedly increased by TNF-α (data not shown). To further implicate the role of NF-κB activation in TRAIL signaling, cells were transfected with an IκB-α (S32A/S36A) mutant, which completely blocks NF-κB activation in response to a number of stimuli (38). In both cell lines, this mutant completely abolished both TNF-α- and TRAIL-induced reporter gene activity together with IL-6 and IL-8 production (data not shown). This suggested that IL-6 and IL-8 production by both TNF-α and TRAIL was regulated by NF-κB. To investigate whether there was any correlation between NF-κB activation in 293 and HeLa cells and the sensitivity of these cells to TRAIL-induced apoptosis, the cleavage of PARP, a substrate for caspase-3 and -7, was assessed. In apoptotic cells, PARP is cleaved at a DEVD ↓ G motif to yield a characteristic 85-kDa fragment (41). After exposure to TRAIL, PARP was cleaved to an 85-kDa fragment in HeLa but not in 293 cells, indicating that HeLa but not 293 cells were sensitive to TRAIL-induced apoptosis (Fig. 1*C*). Taken together these data suggested that the resistance of 293 cells to TRAIL-induced apoptosis may, in part, be linked to their ability to activate NF-κB.

Inhibition of NF-κB Sensitizes HEK293 Cells to TRAIL-induced Apoptosis—To assess whether there is a relationship between the activation of NF-κB by TRAIL in 293 cells (Fig. 1*A*) and their relative insensitivity to TRAIL-induced apoptosis (Fig. 1*C*), we overexpressed the IκB-α (S32A/S36A) mutant, which blocks NF-κB signaling, thereby sensitizing cells to TNF-induced apoptosis (27). Transfected cells were treated with TNF-α, TRAIL, or vehicle alone for 24 h before apoptosis measurements. In control vector-transfected cells, TNF-α or TRAIL induced a small amount of apoptosis, only ~5% above that observed in untreated control-transfected cells (Fig. 2*A*). However, in IκB-α (S32A/S36A)-transfected cells, both TNF-α and TRAIL induced marked apoptosis in 50–60% of transfected cells, which was completely abrogated by z-VAD.fmk (Fig. 2*A*). These data demonstrate that blocking the NF-κB

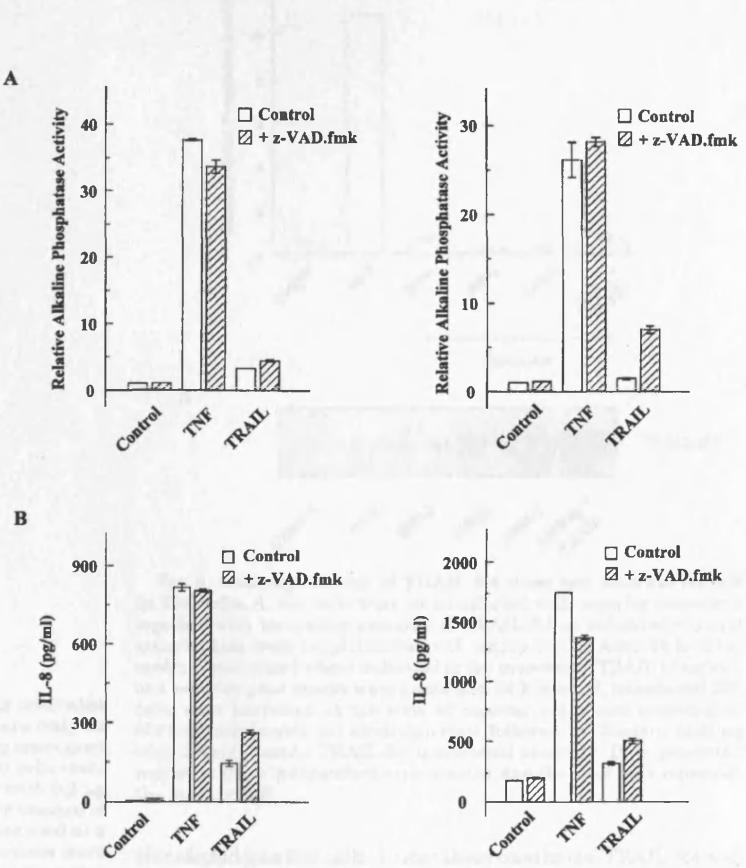

FIG. 3. **Inhibition of TRAIL-induced apoptosis reveals an NF-κB component of TRAIL signaling.** 293 and HeLa cells were transfected with reporter constructs and then pretreated with the caspase inhibitor, z-VAD.fmk (20 μM) for 1 h before treatment with TNF (10 ng/ml) or TRAIL (1 μg/ml) (*A*). Reporter gene activity was measured 24 h after treatment, and results were normalized using β-lactamase expression levels. *B*, medium from transfected 293 and HeLa cells was also analyzed for IL-8 content. Data presented represent three independent experiments, and the *error bars* are the mean ± S.E.

pathway in 293 cells sensitizes them to both TNF- and TRAIL-induced apoptosis.

Up-regulation of NF-κB Protects against TRAIL-induced Apoptosis—To determine the potential role of NF-κB in ameliorating TRAIL-induced apoptosis, HeLa cells were transfected with NF-κB-inducing kinase (NIK). Overexpression of NIK potently activates NF-κB (42), and catalytically inactive forms of NIK block NF-κB activation in response to a number of stimuli including TNF-α (43). NF-κB was activated in cells co-transfected with the reporter system and NIK (data not shown). When these cells were then exposed to TRAIL for 2 h, ~70% apoptosis was evident in control-transfected cells over this period (Fig. 2*B*). Although transfection of NIK alone induced some background apoptosis, the NIK-transfected cells were much less sensitive to TRAIL-induced apoptosis than control-transfected cells (Fig. 2*B*). Co-expression of NIK with the IκB-α (S32A/S36A) mutant completely blocked NIK-induced NF-κB activation (data not shown) and restored the TRAIL sensitivity of the cells (Fig. 2*B*). These results demonstrate that an NIK-mediated NF-κB pathway can protect HeLa cells against TRAIL-induced apoptosis.

Inhibition of TRAIL-induced Apoptosis Reveals an NF-κB Component of TRAIL Signaling—The lack of activation of NF-κB by TRAIL in HeLa cells may have been related to the sensitivity of these cells to TRAIL-induced apoptosis. To test this hypothesis, cells were incubated with the pan-caspase inhibitor, z-VAD.fmk, which inhibits TRAIL-induced apoptosis at an early stage by blocking the activation of the apical caspase, caspase-8 (17). Pre-incubation of HeLa cells for 1 h with z-VAD.fmk before treatment with TRAIL resulted in a marked increase in both NF-κB activation (Fig. 3*A*) and IL-8 production (Fig. 3*B*). In contrast, similar treatment of 293 cells with z-VAD.fmk before TRAIL treatment resulted in only a small increase in NF-κB activation over untreated cells (Fig. 3*A*). TNF-α-induced NF-κB activation and IL-8 production were not increased by z-VAD.fmk pretreatment in either cell type (Figs. 3, *A* and *B*), which is consistent with the inability of TNF to induce apoptosis at this time point. Taken together these data clearly demonstrated that TRAIL also activated NF-κB in HeLa cells but that activation was only apparent when caspase activity was blocked.

TRAIL-R1 and -R2 Activate NF-κB in HeLa Cells Only in the Presence of z-VAD.fmk—To examine the contribution of individual TRAIL receptors to the TRAIL-induced NF-κB activation observed, we overexpressed each of the four TRAIL receptors in the presence of the NF-κB reporter system. Expression of the two "death-inducing" TRAIL receptors, TRAIL-R1 and -R2, caused NF-κB activation in 293 (Fig. 4*A*) but not HeLa cells (Fig. 4*B*). No NF-κB activation was observed in either cell type after overexpression of TRAIL-R3 or -R4 (Figs. 4, *A* and *B*). Activation of NF-κB by a related death receptor, WSL-1/Apo-3/TRAMP/LARD (37, 44–46), occurred in both cell lines and was ~3-fold higher than that induced by TRAIL-R1 or -R2 in 293 cells (Fig. 4*A*). The lack of NF-κB activation in TRAIL-R3-transfected cells was unsurprising as this TRAIL receptor lacks a cytoplasmic domain and is therefore presumed to be incapable of engaging any intracellular signal transduction pathways. In contrast to some other reports (12, 30), overexpression of TRAIL-R4 did not cause NF-κB activation in 293 cells (Fig. 4*A*).

In the presence of z-VAD.fmk, both TRAIL-R1 and -R2, which alone had no effect on NF-κB reporter activity, caused a

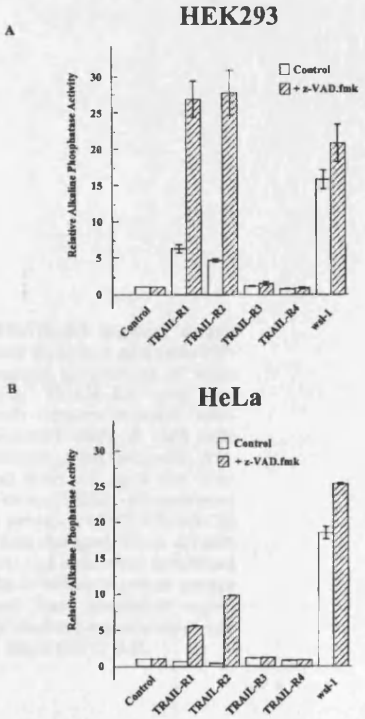

FIG. 4. **Overexpression of TRAIL-R1 and TRAIL-R2 activates NF-κB in 293 cells, but in HeLa cells, activation occurs only in the presence of z-VAD.fmk.** To study the NF-κB signaling component of the individual TRAIL receptors, 293 (*A*) and HeLa (*B*) cells were transfected with 0.1 μg of each reporter construct together with 0.2 μg of the indicated TRAIL receptor construct in the presence or absence of z-VAD.fmk (20 μM). A wsl-1-containing construct (0.2 μg) was used as a positive control for NF-κB activation, and control transfections were supplemented with 0.2 μg of empty control vector. The medium was changed 16 h after transfection and, where indicated, fresh z-VAD.fmk was added, and then reporter gene assays were performed after a further 24 h. Results were normalized using β-lactamase expression levels. Data presented represent three independent experiments, and the *error bars* represent the mean ± S.E.

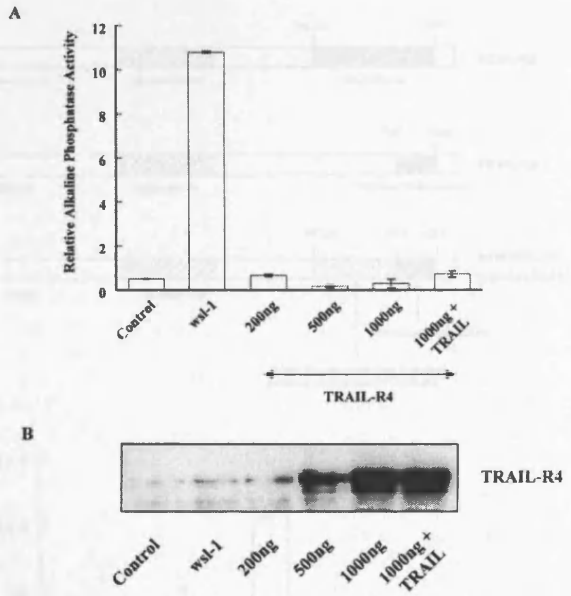

FIG. 5. **Overexpression of TRAIL-R4 does not activate NF-kB in 293 cells.** *A*, 293 cells were co-transfected with reporter constructs together with increasing amounts of TRAIL-R4 as indicated. Control transfections were supplemented with empty vector. After 16 h, fresh medium was added where indicated in the presence of TRAIL (1 μg/ml), and reporter gene assays were performed 24 h later. *B*, transfected 293 cells were harvested at the time of reporter assay and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with an anti-human TRAIL-R4 monoclonal antibody. Data presented represent three independent experiments, and the *error bars* represent the mean ± S.E.

significant increase in reporter gene activity in HeLa cells (Fig. 4*B*). NF-κB activation by WSL-1 was also potentiated by z-VAD.fmk. Transfection of TRAIL-R1 and -R2 in 293 cells in the presence of z-VAD.fmk caused a large potentiation in reporter gene activity (Fig. 4*A*) together with IL-8 production (data not shown) when compared with untreated cells. NF-κB activation induced by WSL-1 was also potentiated but to a lesser extent. Although 293 cells are relatively resistant to TRAIL-induced apoptosis (Fig. 1*C*), overexpression of death receptors such as TNF-R1 and TRAIL-R1 and -R2 resulted in ligand-independent receptor trimerization and, thus, extensive apoptosis (5, 47), irrespective of the inherent TRAIL sensitivity of these cells. No NF-κB activation was evident in either cell line in response to TRAIL-R3 or -R4 overexpression in the presence or absence of z-VAD.fmk (Fig. 4, *A* and *B*). This inability of z-VAD.fmk to reveal an NF-κB component of TRAIL-R3 and -R4 signaling is presumably because these receptors do not induce caspase recruitment or activation. Taken together these data provide indirect evidence that TRAIL-R1- and -R2-induced NF-κB activation is, in part, a caspase-sensitive process.

A TRAIL-R2 Partial Death Domain Mutant Does Not Activate NF-κB—The lack of NF-κB activation in either 293 or HeLa cells by TRAIL-R4 (Figs. 4, *A* and *B*) is in contrast to a number of other reports. To ensure that sufficient TRAIL-R4 had been expressed, increasing concentrations of TRAIL-R4 (200–1000 ng) were transfected into 293 cells. Under these conditions, TRAIL-R4 was unable to induce NF-κB activation even in the presence of exogenous TRAIL (Fig. 5*A*) and despite there being a concomitant increase in protein levels in these cells (Fig. 5*B*). One mechanism by which TRAIL-R4 is purported to act as a decoy receptor is through activation of an NF-κB-mediated survival pathway (12, 30). However, TRAIL-R4 contains only a partial death domain lacking 56 amino acids at the N terminus, which are present in the death domain of both TRAIL-R1 and -R2 and are believed to be responsible for engaging downstream signaling pathways. To test the hypothesis that this N-terminal region of the death domain may be responsible for NF-κB activation, we created a TRAIL-R2 mutant lacking this region (Fig. 6*A*). Western blotting with a TRAIL-R2-specific monoclonal Ab clearly showed that the mutant was expressed (data not shown). When overexpressed in 293 cells in the presence of z-VAD.fmk, mutTRAIL-R2 (ΔSer-324–Ser-369) failed to induce NF-κB activation, whereas both wild-type TRAIL-R2 and WSL-1 produced marked activation (Fig. 6*B*). These results suggested that motifs required for adaptor binding and/or NF-κB activation by wild-type TRAIL-R2 were no longer present in mutTRAIL-R2 (ΔSer-324–Ser-369).

RIP Is Cleaved in HeLa Cells during TRAIL-induced Apoptosis—Our results with z-VAD.fmk in HeLa cells implicated the presence of a protein, whose cleavage by caspases prevented TRAIL-induced NF-κB activation (Fig. 3*A*). In TNF-R1 signaling, RIP, a death domain-containing kinase, has been implicated in NF-κB activation (48). In addition, cells derived from $RIP^{-/-}$ mice were unable to activate NF-κB in response to TNF-α, and these mice were hypersensitive to the cytotoxic effects of TNF (49). RIP is also cleaved during TNF-induced apoptosis by caspase-8 to produce a dominant-negative fragment, which inhibits TNF-induced NF-κB activation (31, 32).

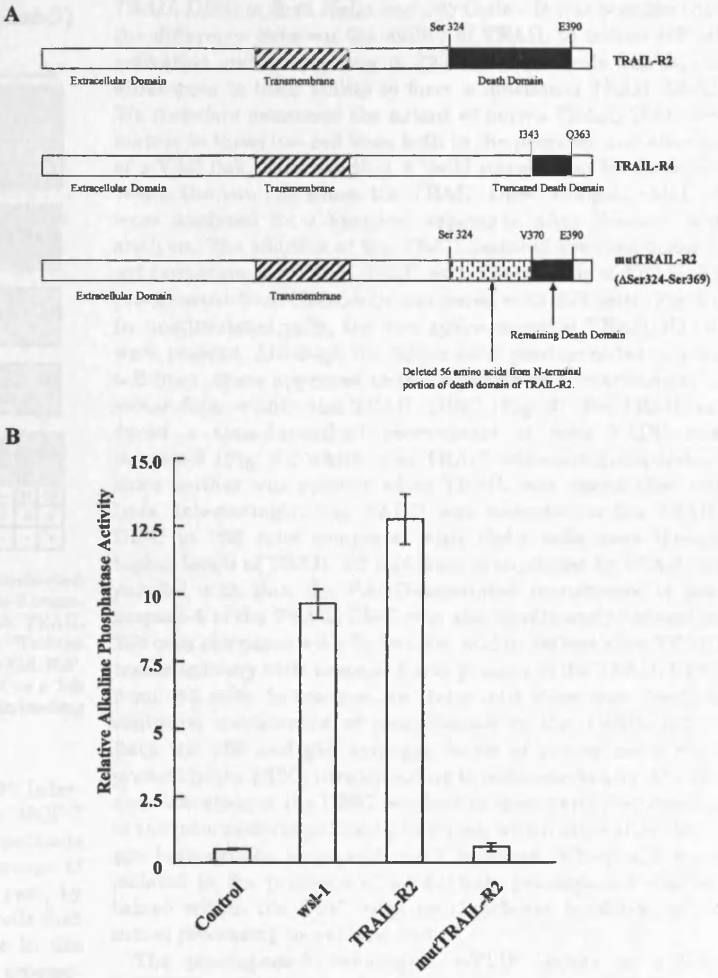

FIG. 6. **A TRAIL-R2 partial death domain mutant does not activate NF-κB.** *A*, a schematic illustration of wild-type TRAIL-R2, TRAIL-R4, and the TRAIL-R2 death domain mutant, mutTRAIL-R2 (ΔSer-324–369). *B*, 293 cells were co-transfected with reporter constructs together with 0.2 μg of the indicated receptor or mutTRAIL-R2 construct for 16 h in the presence of z-VAD.fmk (20 μM). Medium was changed, fresh z-VAD.fmk was added, and cells were incubated for a further 24 h before reporter assays were performed. Data presented represent three independent experiments, and *error bars* are the mean ± S.E.

By analogy, cleavage of RIP during TRAIL-induced apoptosis could explain our observation that activation of NF-κB in TRAIL-sensitive HeLa cells only occurred when TRAIL-induced apoptosis was blocked (Fig. 3*A*). We therefore studied the cleavage of RIP as well as the activation of caspase-8 in HeLa cells. Treatment with TRAIL resulted in the loss of the proform of caspase-8 accompanied by processing to its p43/41 and p18 forms (Fig. 7). TRAIL treatment also resulted in the cleavage of RIP to a 42-kDa immunoreactive fragment, corresponding to the reported RIP cleavage product (31) and cleavage of PARP to its well characterized 85-kDa product. Processing of caspase-8 and cleavage of RIP and PARP were completely inhibited by z-VAD.fmk (Fig. 7). No cleavage of caspase-8, PARP, or RIP was observed in 293 cells under the same conditions, in agreement with the relative resistance of these cells to TRAIL-induced apoptosis (Fig. 7). These data demonstrated that a caspase-dependent cleavage of RIP was associated with TRAIL-induced apoptosis in HeLa cells and supported the hypothesis that extensive RIP cleavage prevented concomitant TRAIL-induced NF-κB activation within the same cell.

To determine whether the cleavage of RIP was mediated entirely by caspase-8 as proposed (31, 32) or whether effector caspases such as caspase-3 also played a role, we utilized MCF-7 cells, which do not express functional caspase-3 (35). In MCF-7 cells, TRAIL induced a time-dependent processing of caspase-8 to its p41/p43 and p18 fragments as well as cleavage of Bid (Fig. 8 *lanes 1–7*), a preferred caspase-8 substrate (50),

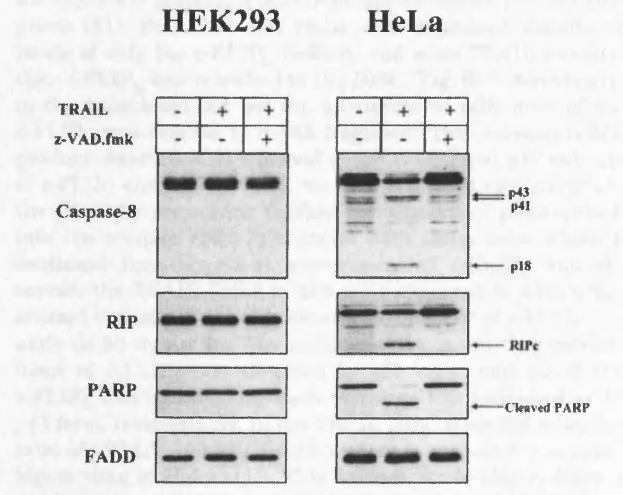

FIG. 7. **Caspase-8, RIP, and PARP are cleaved after TRAIL treatment in HeLa cells.** *A*, 293 or HeLa cells were treated with TRAIL (1 μg/ml) for 6 h either in the presence or absence of z-VAD-fmk (20 μM). Cells were then harvested and analyzed by Western blotting using antibodies to caspase-8, RIP, and PARP. The adaptor protein FADD was used as a protein loading control. RIPc, cleaved RIP.

both of which were completely inhibited by z-VAD.fmk (Fig. 8, *lane 8*). This was consistent with the ability of z-VAD.fmk to inhibit death receptor-induced apoptosis by inhibiting the proc-

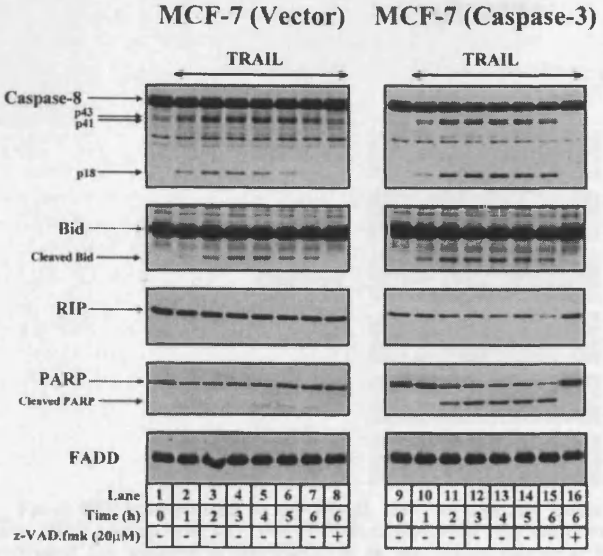

FIG. 8. **Enhanced processing of RIP in caspase-3 transfected MCF-7 cells.** Mock-transfected (*MCF-7 (Vector)*) and caspase-3-transfected (*MCF-7 (Caspase-3)*) MCF-7 cells were treated with TRAIL (1 μg/ml) for the indicated time periods and subjected to Western blotting using antibodies to caspase-8, the caspase-8 substrate Bid, RIP, and PARP. z-VAD.fmk (20 μM) was included where indicated as a 1-h pretreatment. The adaptor protein FADD was used as a protein loading control.

essing of the apical caspase-8 to its active tetramer (33). Interestingly, no cleavage of RIP or PARP was observed in MCF-7 cells (Fig. 8, *lanes 2–7*). These results support the hypothesis that caspase-8 was not solely responsible for the cleavage of RIP but rather that this cleavage was mediated, in part, by caspase-3. To test this hypothesis, we utilized MCF7 cells that had been stably transfected with caspase-3 (35). As in the caspase-3 null cells, TRAIL induced a time-dependent processing of caspase-8 and its substrate Bid; however, generation of the active caspase-8 p18 subunit and truncated Bid was enhanced in the cells transfected with caspase-3 (Fig. 8, *lanes 9–15*). Enhanced cleavage of these proteins could be due either to a direct effect of caspase-3 or to the engagement of a positive feedback loop whereby active caspase-3 directly or indirectly activates caspase-8, hence leading to the generation of increasing amounts of active caspase-8 and cleaved Bid. In the caspase-3-transfected MCF-7 cells, PARP also displayed a time-dependent processing to its 85-kDa product as a consequence of the presence of caspase-3 activity within these cells (Fig. 8, *lanes 10–15*). Interestingly, RIP was also cleaved in these cells, and although no cleavage fragment was evident, there was a time-dependent loss of the full-length form, which was almost complete at 6 h (Fig. 8, *lane 15*). The lack of an observable RIP fragment in MCF-7 cells may have been due to its rapid degradation within these cells. In agreement with RIP cleavage being a caspase-mediated event, no loss of intact RIP was observed in the presence of z-VAD.fmk (Fig. 8, *lane 16*).

No loss of intact RIP was observed in caspase-3 null cells, although caspase-8 and Bid were cleaved. When caspase-3 was introduced, cleavage of these components was enhanced, and there was also loss of intact RIP. This suggested that RIP cleavage in these cells was mediated directly by caspase-3 or indirectly through enhanced cleavage of caspase-8. These data support the hypothesis that caspase-8 is not solely responsible for cleavage of RIP, but that RIP cleavage is also mediated by caspase-3.

z-VAD.fmk Enhances the Recruitment of RIP to the Native TRAIL DISC in Both HeLa and 293 Cells—It was possible that the difference between the ability of TRAIL to induce NF-κB activation and/or apoptosis in 293 and HeLa cells was due to differences in their ability to form a functional TRAIL DISC. We therefore examined the extent of native TRAIL DISC formation in these two cell lines both in the presence and absence of z-VAD.fmk. In order that a valid comparison be made between the two cell lines, the TRAIL DISC samples obtained were analyzed from identical exposures after Western blot analysis. The addition of Bio-TRAIL induced the time-dependent formation of a TRAIL DISC with lower levels of TRAIL-R2 precipitated from HeLa cells compared with 293 cells (Fig. 9). In unstimulated cells, the two splice forms of TRAIL-R2 (6) were present. Although the larger form predominated in both cell lines, there appeared to be no preferential recruitment of either form within the TRAIL DISC (Fig. 9). Bio-TRAIL induced a time-dependent recruitment of both FADD and caspase-8 (Fig. 9), which was TRAIL stimulation-dependent since neither was present when TRAIL was added after cell lysis. Interestingly, less FADD was recruited to the TRAIL DISC in 293 cells compared with HeLa cells even though higher levels of TRAIL-R2 had been precipitated by TRAIL. In parallel with this, the FADD-dependent recruitment of procaspase-8 to the TRAIL DISC was also significantly reduced in 293 cells compared with HeLa cells, and by 60 min after TRAIL treatment very little caspase-8 was present in the TRAIL DISC from 293 cells. In contrast, in HeLa cells there was clearly a continual recruitment of procaspase-8 to the TRAIL DISC. Both the p55 and p53 zymogen forms of procaspase-8 were present in the DISC, corresponding to caspases-8a and -8b (34), and activation at the DISC resulted in their partial processing to the intermediate p43 and p41 forms, which arise after cleavage between the large and small subunits. When cells were isolated in the presence of z-VAD.fmk, procaspase-8 was retained within the DISC with no significant inhibition of its initial processing to p41 and p43.

The procaspase-8 homologue, c-FLIP, exists as a long (c-FLIP$_L$) and a short (c-FLIP$_S$) splice variant, both of which are capable of protecting cells from death receptor-induced apoptosis (51). Both 293 and HeLa cells expressed significant levels of only the c-FLIP$_L$ isoform, and after TRAIL stimulation, c-FLIP$_L$ was recruited to the DISC (Fig. 9). Interestingly, in the stimulated but not the unstimulated cells most of the c-FLIP$_L$ was cleaved to a p43 fragment. This represents the product obtained after removal of the C-terminal p12 subunit of c-FLIP, and like c-FLIP$_L$, the p43 fragment can inactivate the DISC by preventing further recruitment of procaspase-8 into the complex (52). In contrast with HeLa cells, where a continual time-dependent recruitment of c-FLIP$_L$ was observed, the TRAIL DISC in 293 cells appeared to have a decreased capacity for the continual recruitment of c-FLIP$_L$. As early as 30 min after TRAIL stimulation, no further recruitment of c-FLIP$_L$ was detected in 293 cells, and all of the c-FLIP$_L$ that had initially been recruited was processed to its p43 form. Interestingly, in the TRAIL DISC from 293 cells, the ratio of c-FLIP$_L$ and its cleaved product to caspase-8 was much higher than in HeLa cells. This balance would clearly favor a much greater inhibition of TRAIL-induced apoptosis in 293 cells than in HeLa cells.

Surprisingly, the adaptor protein RIP was associated with unstimulated TRAIL receptors isolated from both 293 and HeLa cells. After TRAIL stimulation, there was a time-dependent increase in the recruitment of RIP to the TRAIL DISC (Fig. 9). As early as 60 min after the addition of TRAIL, some cleavage of RIP was apparent in the DISC formed in both cell lines. In the presence of z-VAD.fmk, this cleavage was com-

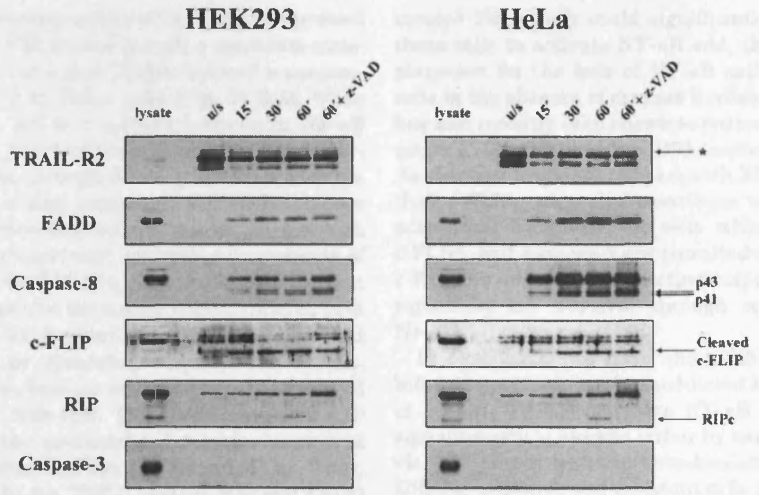

FIG. 9. **RIP is recruited to the TRAIL DISC in 293 and HeLa cells.** 293 and HeLa cells (3×10^7) were treated with biotinylated TRAIL (Bio-TRAIL) for up to 60 min, and where indicated, cells were pretreated for 60 min with z-VAD.fmk (20 μM). Unstimulated receptor controls (*u/s*) represent the addition of Bio-TRAIL to an equivalent volume of lysate isolated from unstimulated cells. TRAIL receptor complexes were precipitated with streptavidin-conjugated agarose beads and analyzed by Western blotting for the known TRAIL DISC components, TRAIL-R2, FADD, and caspase-8. Precipitates were also analyzed for the presence of c-FLIP, RIP, and as a negative control, caspase-3. Lysates isolated from unstimulated control cells were included as a positive control for the expression of all these proteins in both 293 and HeLa cells. To enable comparison of the relative amounts of each component recruited to the DISC, equivalent exposures are shown. The *asterisk* indicates a minor nonspecific band detected by the TRAIL-R2 antibody.

pletely inhibited, resulting in a significant increase in the amount of RIP retained within the TRAIL DISC isolated from either cell line. As a negative control for these experiments, we used caspase-3, which has never been shown to be a constituent of any DISC. Consistent with this, no caspase-3 was present in any of the DISC samples analyzed despite there being high levels of this protein expressed in the cell lysates (Fig. 9, *bottom panels*).

DISCUSSION

TRAIL-induced Apoptosis Can Be Modulated by NF-κB—In most cell types the predominant downstream signaling event of TNF is not apoptosis but NF-κB activation. TNF can negatively regulate its own cytotoxic ability through the up-regulation of NF-κB-regulated anti-apoptotic genes (28), and inhibition of NF-κB activation restores its cytotoxicity (27). In this study we show that NF-κB can similarly modulate TRAIL-induced apoptosis. Increased NF-κB activation, by overexpression of NIK, markedly decreased TRAIL-induced apoptosis in HeLa cells (Fig. 2), similar to the protection reported previously in transformed keratinocytes by IL-1-induced NF-κB activation (53). Conversely, inhibition of NF-κB activation by overexpression of an IκB-α (S32A/S36A) mutant sensitized 293 cells to TRAIL-induced apoptosis (Fig. 2). This mutant also sensitizes TRAIL-resistant primary leukemic and melanoma cells (29, 54) but did not sensitize HeLa-TL-R cells that had become resistant after long term culture in TRAIL (30). This suggests that the ability of NF-κB to modulate TRAIL sensitivity by may be model-dependent. It is unclear whether TRAIL modulates its own cytotoxicity by activation of NF-κB in a manner similar to that reported for TNF or whether resistant cells may have a high constitutive NF-κB activity that offers protection. Taken together these data demonstrate that modulation of NF-κB activation is a key determinant of the sensitivity of some cells to TRAIL-induced apoptosis.

Activation of NF-κB by TRAIL Is Mediated by TRAIL-R1 and TRAIL-R2 but Not by TRAIL-R4—Another mechanism by which cells may modulate their sensitivity to TRAIL is through the expression of the putative decoy receptors TRAIL-R3 and -R4. These decoy receptors are purported to act by either trimerizing with TRAIL-R1 or -R2 to form inactive signaling complexes or by sequestering ligand from TRAIL-R1 or -R2. In this study we found that TRAIL-R1 and -R2 mediated TRAIL-induced NF-κB activation in a ligand-independent manner. However, contrary to some other reports (12, 30), we were unable to demonstrate TRAIL-R4-mediated NF-κB activation even upon gross overexpression of TRAIL-R4 and the subsequent addition of TRAIL (Fig. 5). Previous studies have proposed a model for the protection of cells by TRAIL-R4 via the activation of a NF-κB-mediated survival pathway (12). This model is not easily explained because TRAIL-R4 contains a truncated death domain, which lacks a number of key residues conserved throughout the TNF-R family that have been implicated in cytotoxicity signaling (55). In the present study a TRAIL-R2 mutant, containing a truncated death domain resembling that found in TRAIL-R4, was unable to activate NF-κB (Fig. 6). This suggested that residues or motifs required for NF-κB activation were absent in this TRAIL-R2 mutant. When one such residue, Ile-225, is mutated to Asn in CD95, it is responsible for the lymphoproliferative (lpr) phenotype in mice (56). This residue is conserved in both TNF-R1 and TRAIL-R2 (Leu-351 and Leu-334, respectively) and, when similarly mutated, results in loss of receptor cytotoxicity (19, 55). Interestingly, this mutation has also previously been demonstrated to abolish TRAIL-R2-mediated NF-κB activation (19). The lack of any NF-κB activation by TRAIL-R4 observed in this study is in agreement with an earlier study (13) and a very recent study (57), which demonstrated that TRAIL-R4 is capable of protecting colon carcinoma cells from TRAIL-R2- and p53-mediated apoptosis. This protective effect was localized to the first 43 amino acids of the cytoplasmic domain and not within the remaining portion of the death domain (57). Thus, TRAIL-R4 may mediate as yet unknown signaling pathways that protect against TRAIL-induced apoptosis.

Caspase Inhibition Potentiates TRAIL-induced NF-κB Activation and Enhances Recruitment of RIP to the Native TRAIL DISC—TRAIL-induced NF-κB activation appeared to require a molecule(s) that was inactivated after caspase cleavage, since no NF-κB activation was apparent in HeLa cells with TRAIL or

TRAIL-R1 or -R2 overexpression unless z-VAD.fmk was present (see Fig. 3*A* and Fig. 4*B*). RIP is clearly such a candidate molecule, based on the observations that TRAIL induced a caspase-dependent cleavage of RIP in HeLa cells (Fig. 7) that, when prevented by z-VAD.fmk, led to a marked increase in NF-κB activation (Figs. 3 and 4). RIP has been implicated in receptor-mediated NF-κB activation through direct interaction with the IκB kinase signalosome complex component, NEMO/IκB kinase γ (58). Although RIP has previously been shown to associate with TRAIL receptors after overexpression of various components of the TRAIL signaling pathway (19, 20), we now show for the first time that RIP is a component of the native TRAIL DISC in both HeLa and 293 cells (Fig. 9). A recent study has also provided evidence that RIP may be absolutely required for TRAIL-mediated NF-κB activation, because no activation was observed in TRAIL-treated $RIP^{-/-}$ cells (59). The observation that RIP was pre-associated with the unstimulated receptor control in both cell lines was unexpected, and the significance of this, if any, remains to be elucidated. In the TNF-R system, RIP and FADD are both recruited through the intermediate adaptor TNFR-associated death domain in a stimulation-dependent manner (48). In previous studies, TNFR-associated death domain was not found to be a component of the TRAIL DISC (22, 23), and whether RIP requires such an intermediate adaptor or directly associates with TRAIL-R2 remains to be elucidated. Some cleaved as well as full-length RIP was present within the TRAIL DISC isolated from both cell lines, compatible with some RIP cleavage occurring within the DISC. In HeLa cells, where caspase-3 was also activated, some processing of RIP could almost certainly have occurred outside the DISC, thereby diminishing the pool of full-length RIP available for NF-κB activation. The implications of extensive RIP cleavage became more evident from studies where the DISC was isolated in the presence of z-VAD.fmk. Under these conditions caspase-dependent cleavage of RIP was blocked, and as a consequence, more RIP accumulated within the TRAIL DISC (Fig. 9). Taken together, these data provided a potential mechanism for the potentiation of TRAIL-induced NF-κB signaling observed in the presence of z-VAD.fmk.

The Ratio of c-FLIP to Caspase-8 in the DISC May Determine Sensitivity to TRAIL-induced Apoptosis—Analysis of the TRAIL DISC also provided a potential explanation for the differential sensitivity of 293 and HeLa cells to TRAIL-induced apoptosis. Although 293 cells were not sensitive to TRAIL-induced apoptosis (Fig. 7), a TRAIL DISC was formed that contained small amounts of FADD and some processed caspase-8 (Fig. 9). However, when compared with HeLa cells, the recruitment of FADD to the TRAIL DISC in 293 cells was clearly less efficient even though higher levels of TRAIL-R2 were precipitated from these cells. Levels of c-FLIP may in some cells determine resistance to CD95-induced apoptosis (52), since in the presence of c-FLIP, procaspase-8 is no longer able to replace the cleavage products at the DISC and become activated. We now show that in 293 cells, the ratio of $c\text{-}FLIP_L$ to caspase-8 within the TRAIL DISC is much greater than in HeLa cells and may contribute to inactivation of the TRAIL DISC, as evidenced by the lack of caspase-8 or RIP processing detected in 293 whole cell extracts (Figs. 7 and 9). Taken together our data suggest that the differential sensitivity of 293 and HeLa cells to TRAIL-induced apoptosis may be in part explained by the efficiency of recruitment and activation of integral DISC components such as FADD and procaspase-8. The differential sensitivity of these cells to TRAIL-induced NF-κB activation may also be, in part, explained by the differential activation of apoptotic signaling molecules within their TRAIL DISCs. For example, the extensive caspase-dependent processing of the NF-κB-activating kinase RIP in TRAIL-treated HeLa cells could significantly inhibit the capacity of these cells to activate NF-κB and, thus, could provide an explanation for the lack of NF-κB activation detected in these cells in the absence of caspase inhibitors. Interestingly, c-FLIP has also recently been shown to possess some NF-κB-activating activity (60–62), and in CD95-treated cells both $c\text{-}FLIP_L$ and its cleavage fragment interact with RIP. It is therefore possible that $c\text{-}FLIP_L$ may also contribute to TRAIL-induced NF-κB activation. Therefore, in cells where comparable levels of $c\text{-}FLIP_L$ and caspase-8 are recruited to the TRAIL DISC, then c-FLIP, in addition to inhibiting caspase-8 activation, may also signal for cell survival through concomitant activation of NF-κB.

In conclusion, we have shown that sensitivity to TRAIL-induced apoptosis can be modulated by activation or inhibition of NF-κB. TRAIL activates NF-κB only when its apoptotic signaling arm is blocked either by use of a caspase inhibitor or via endogenous resistance mechanisms. Analysis of the TRAIL DISC in sensitive and resistant cells revealed that a high ratio of c-FLIP to caspase-8 may explain the resistance of some cells to TRAIL-induced apoptosis. We demonstrate for the first time the recruitment of the NF-κB-activating kinase RIP to the native TRAIL DISC and propose that caspase-mediated cleavage of RIP can inhibit the capacity of TRAIL-sensitive cells to activate NF-κB. By contrast, in cells that are relatively resistant to TRAIL-induced apoptosis, the predominant TRAIL-signaling event is NF-κB activation. Whether recruitment of other as yet unidentified components and/or additional adaptors are required for TRAIL-induced NF-κB activation is under investigation.

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Mechanisms of resistance to TRAIL-induced apoptosis in primary B cell chronic lymphocytic leukaemia

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Primary B cells from B cell chronic lymphocytic leukaemia (B-CLL) were resistant to the novel selective cytotoxic agent, TNF-related apoptosis-inducing ligand (TRAIL). Low levels of the death-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2 but not the putative 'decoy' receptors, TRAIL-R3 and TRAIL-R4, were expressed on the surface of B-CLL cells. Resistance to TRAIL was upstream of caspase-8 activation, as little or no caspase-8 was processed in TRAIL-treated B-CLL cells. Low levels of a TRAIL death-inducing signalling complex (DISC) were formed in these cells, accompanied by the recruitment of endogenous FADD, caspase-8 and c-FLIP$_L$ but not c-FLIP$_S$. Both caspase-8 and c-FLIP$_L$ were cleaved to form two stable intermediates of ~43 kDa, which remained associated with the DISC. Caspase-8 was not further processed to its active heterotetramer. Thus the resistance of B-CLL cells to TRAIL may be due partly to low surface expression of the death receptors resulting in low levels of DISC formation and also to the high ratio of c-FLIP$_L$ to caspase-8 within the DISC, which would prevent further activation of caspase-8. Our results highlight the possibility of sensitising B-CLL cells to TRAIL by modulation of c-FLIP levels or by upregulation of surface expression of death receptors.
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Keywords: Caspases; CD95; c-FLIP; IAPs; DISC

Introduction

B-cell chronic lymphocytic leukaemia (B-CLL), one of the most common haematological malignancies in the western world, is characterized by the accumulation of mature non-proliferating B cells that co-express CD5 and CD23 (Rozman and Montserrat, 1995). This accumulation results from a failure of cells to undergo apoptosis rather than by excessive cellular proliferation.

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Several members of the tumour necrosis factor (TNF) family induce apoptosis. Induction of apoptosis is restricted to receptors that contain a protein–protein interaction motif, termed the 'death domain', and include those activated by CD95/FasL and TNF-related apoptosis-inducing ligand (TRAIL). Intracellular molecules that couple CD95 to the apoptotic pathway include the adaptor molecule FADD/MORT1 (Chinnaiyan *et al.*, 1995; Kischkel *et al.*, 1995), which recruits caspase-8 into the death-inducing signalling complex (DISC) (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Similarly, TRAIL engages apoptosis via recruitment and rapid activation of caspase-8 (MacFarlane *et al.*, 2000), which may in some cases be independent of FADD (Yeh *et al.*, 1998). However, recent studies demonstrate both FADD and caspase-8 are integral components of the TRAIL DISC (Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Importantly, TRAIL induces apoptosis in a wide range of tumour cell lines but not in most normal cells (Wiley *et al.*, 1995; Marsters *et al.*, 1996). TRAIL interacts with four distinct membrane-bound receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5/TRICK2), TRAIL-R3 (TRID/DcR1/LIT) and TRAIL-R4 (DcR2/TRUNDD) (reviewed in Ashkenazi and Dixit, 1998). TRAIL-R1 and TRAIL-R2 are death receptors and contain the cytoplasmic 'death domain' necessary to mediate apoptosis. TRAIL-R3 and TRAIL-R4 lack or contain a truncated death domain and consequently do not mediate apoptosis and have been proposed to inhibit TRAIL-induced apoptosis either by acting as decoy receptors or via transduction of an anti-apoptotic signal. TRAIL- and CD95-induced apoptosis is also regulated by inhibitory molecules, which interfere with signalling from the DISC. Such molecules include cellular Flice inhibitory protein (c-FLIP), which competes with caspase-8 for binding to FADD (Scaffidi *et al.*, 1999), and cIAP-1 and -2 that can be induced by NF-κB and may act to inhibit caspase-8 (Wang *et al.*, 1998). c-FLIP is structurally similar to caspase-8, containing two death effector domains and a caspase-like domain, which lacks the active-site cysteine of caspase-8 (Irmler *et al.*, 1997; Scaffidi *et al.*, 1999). Two endogenous forms are present, c-FLIP$_L$ and c-FLIP$_S$; both are recruited to the CD95 DISC and inhibit

CD95-induced apoptosis (Irmler *et al.*, 1997; Scaffidi *et al.*, 1999; Krueger *et al.*, 2001). Activation of normal human B cells up-regulates c-FLIP expression and delays apoptosis in CD95-sensitive B cells, suggesting that c-FLIP may prevent inappropriate cell death (Hennino *et al.*, 2000).

Exploitation of receptor-mediated apoptosis may represent a novel therapeutic strategy for many forms of cancer, although the use of TNF or CD95 has been prohibited by their toxicity toward normal tissues. In contrast, TRAIL may represent a more suitable ligand for exploitation, as it does not generally induce cell death in normal tissues. B-CLL and other B cell malignancies, such as B-cell non-Hodgkin's lymphoma (B-NHL), are commonly resistant to CD95-mediated apoptosis (Wang *et al.*, 1997; Xerri *et al.*, 1997; Plumas *et al.*, 1998). The mechanism(s) of CD95 resistance in these cells is unclear, but may include lack of expression of functional receptors or overexpression of inhibitory molecules.

Results

Resistance of B-CLL cells to receptor-mediated apoptosis

Primary B-CLL cells from patients were generally not sensitive to either TRAIL or anti-CD95, whereas three tumour cell lines were sensitive (Figure 1 and Table 1). Even at higher concentrations of TRAIL (2000 ng/ml), B-CLL cells were resistant to apoptosis. Control and TRAIL-induced apoptosis in B-CLL cells were 21 ± 12 and $22 \pm 11\%$, respectively (mean $\pm$ s.e. from three different patients). However, extensive apoptosis was observed in Jurkat cells exposed to TRAIL (100 ng/ml), a 20-fold lower concentration (Figure 1). As there has been some controversy over the form of TRAIL used to induce apoptosis (Lawrence *et al.*, 2001), we also used a commercially available form, TRAIL Biomol. B-CLL cells were also resistant to this form of TRAIL (2000 ng/ml). In contrast, B-CLL cells were sensitive to apoptosis induced by the proteasome inhibitor MG132 as assessed by increased externalization of phosphatidylserine (PS) (Figure 1a) and caspase activation (Almond *et al.*, 2001), which confirmed that the effector arm of apoptosis was present and operational in these cells. These results demonstrated that B-CLL cells were resistant to TRAIL and raised the possibility that this may be due to a defect in cell surface expression of TRAIL receptors.

Cell surface expression of TRAIL receptors

In the tumour cell lines, two patterns of cell surface death receptor expression were observed: in Jurkat cells TRAIL-R2 > TRAIL-R1 > > TRAIL-R4 whereas in both SKW and MC116 cells TRAIL-R1 $\geqslant$ TRAIL-R2 > TRAIL-R4 (Figure 2). No cell surface expression of TRAIL-R3 was detected in any of the cell lines (Figure 2). In B-CLL cells, lower levels of TRAIL

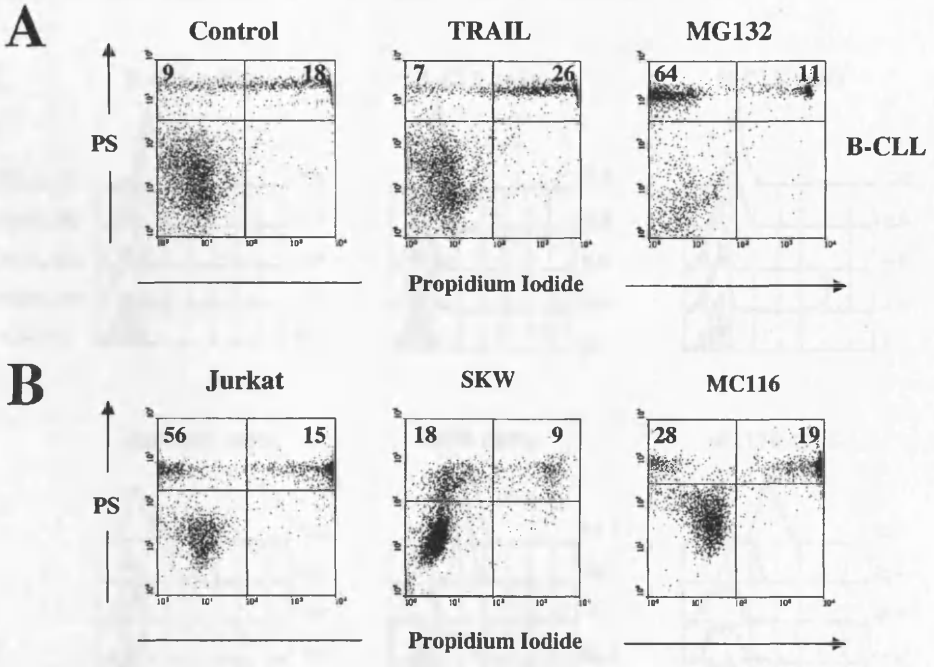

Figure 1 Primary B-CLL cells are resistant to TRAIL-induced apoptosis. (**a**) Freshly isolated B-CLL cells were incubated in the presence of LZ-TRAIL (500 ng/ml; case 5 is shown) or the proteasome inhibitor MG132 (1 μM) for 24 h. Cell death was quantified by flow cytometric analysis after staining with Annexin V-FITC/propidium iodide (PI), and the percentage of cells with increased PS exposure is indicated. (**b**) Cell lines were incubated with LZ-TRAIL (100 ng/ml) for 6 h, and apoptosis assessed by PS externalization. The percentage of apoptotic cells in untreated cells was typically less than 10%. PS; externalized phosphatidylserine. The numbers in the left and right hand quadrants indicate the percentage of PS^+ PI^- and PS^+ PI^+ cells, respectively

death receptor expression (TRAIL-R1≈TRAIL-R2) were generally observed compared to the cell lines, with no detectable expression of TRAIL-R3 or TRAIL-R4 (Figure 2). Thus the resistance of the B-CLL cells to TRAIL-induced apoptosis was not probably due to increased cell surface expression of 'decoy' receptors in agreement with other studies (Griffith *et al.*, 1998; Leverkus *et al.*, 2000).

Both FADD and caspase-8 are expressed in B-CLL cells

FADD was detected as two immunoreactive products of ~28 kDa, most probably corresponding to different phosphorylated forms of FADD observed in both human and mouse cells (Kischkel *et al.*, 1995; Zhang and Winoto, 1996). Lower levels of FADD were detected in B-CLL cells compared to the cell lines (Figure 3). Significant levels of caspase-8 were observed in all B-CLL samples and in the cell lines. In all B-CLL samples, caspase-8 was detected as a doublet of ~55 kDa (Figure 3), possibly corresponding to the two isoforms, caspases-8a and -8b (Scaffidi *et al.*, 1997). When equivalent numbers of cells from the patients or cell lines were examined, either by Ponceau S staining (data not shown) or immunoblotting for the housekeeping protein GAPDH (Figure 3), a greater cellular protein content was observed in the cell lines, which correlated with their larger cell diameter (12.5 μm) compared to B-CLL cells (7.5 μm). Thus, based on loading of equivalent cell numbers, more FADD and caspase-8 were clearly evident in the transformed cell lines

Table 1 Clinical information and the effects of TRAIL and anti-CD95 on the viability of purified B-CLL cells

					% Apoptosis (24 h)		
Case	*Sex*	*Age*	*WBC × 10⁶*	*Binet stage*	*Control*	*Anti-CD95*	*TRAIL*
1	F	83	66	C	24	ND	23
2	M	78	36	A	14	ND	14
3	F	77	35	A	25	ND	23
4	F	81	30	A	22	ND	24
5	M	79	20	A	9	10	11
6	F	59	20	A	14	10	18
7	F	78	68	C	38	35	51
8	M	80	18	A	26	17	32
9	M	63	83	A	41	40	44
10	M	79	116	A	12	7	14
11	M	72	42	A	21	16	23
12	M	64	10	A	19	21	21
13	M	65	105	A	25	16	26
14	M	83	22	A	87	88	88
15	F	76	68	A	14	13	12
16	F	83	60	C	24	ND	27
17	F	64	30	A	27	29	28
18	F	69	101	A	29	27	32
19	F	84	59	C	24	ND	26
20	M	73	110	C	48	41	48
21	F	78	35	A	6	ND	5
22	M	64	40	C	53	ND	53
23	M	69	85	C	23	ND	24
24	M	79	92	A	19	ND	18
25	F	91	56	A	15	ND	17
26	M	69	90	C	23	ND	23
27	M	80	111	C	43	ND	39

Freshly isolated B-CLL cells were incubated *in vitro* in the presence of LZ-TRAIL (500 ng/ml) or anti-CD95 (500 ng/ml) for 24 h. Apoptosis in control and treated samples was determined by Annexin V binding. The white blood count (WBC) and Binet stage of each patient is indicated, together with the sex and age of each patient. ND; not determined

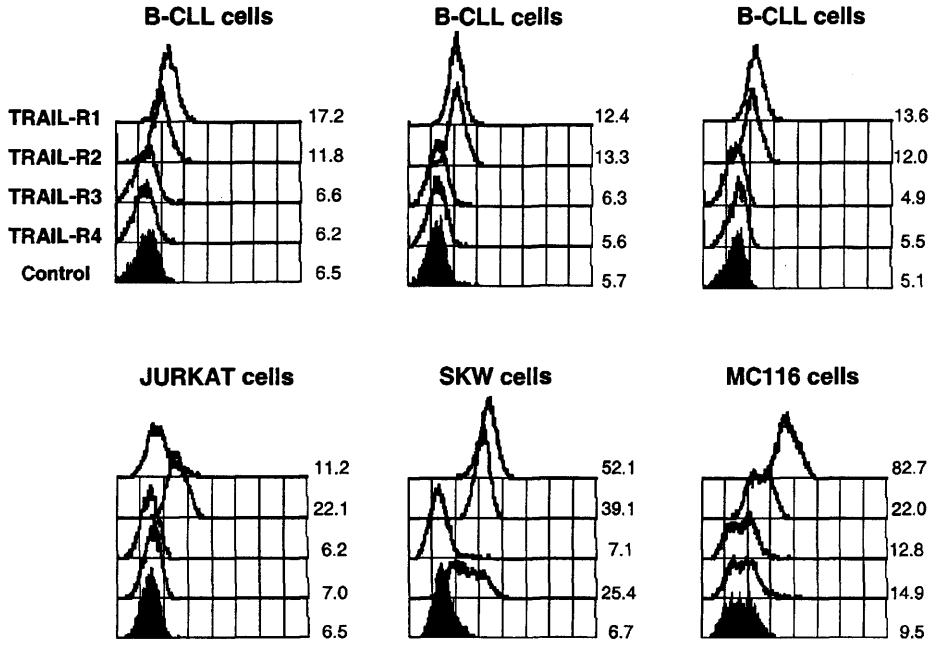

Figure 2 Cell surface expression of TRAIL receptors in B-CLL cells. Receptor expression was detected by single-colour flow cytometry performed on freshly isolated B-CLL cells or the cell lines (representative cases 12, 14 and 20 are shown). Cells were stained with monoclonal antibodies to TRAIL-R1-R4, or with an isotype-matched antibody (negative control) followed by a FITC-conjugated secondary antibody. The values shown indicate the mean fluorescence intensity

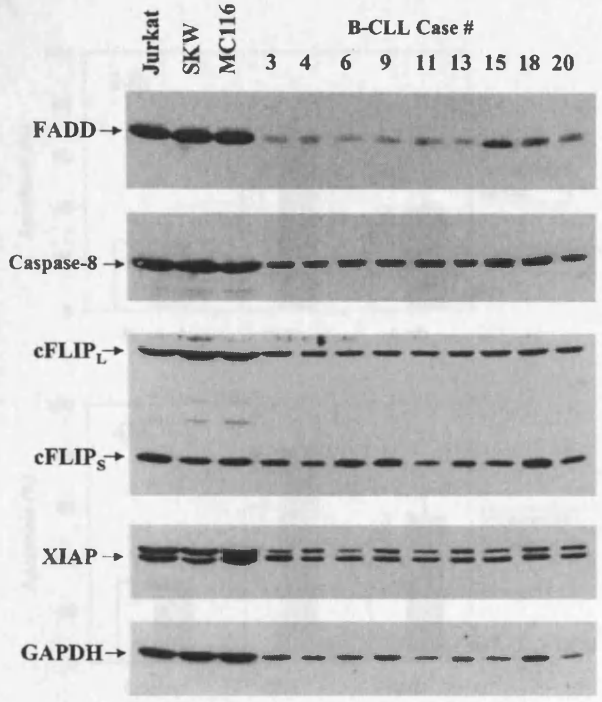

Figure 3 The signalling molecules FADD and caspase-8 together with the inhibitory molecules c-FLIP and XIAP are expressed in B-CLL cells. Freshly isolated B-CLL cells and the tumour cell lines were analysed by Western blot analysis as described in Materials and methods. Immunoblotting for the housekeeping protein GAPDH was used as an indication of protein loading. A greater cellular protein content was observed in the cell lines compared to the clinical samples. Lower exposure of the FADD blots revealed that FADD in the cell lines was detected as a doublet (data not shown)

compared to the B-CLL cells but this merely reflected the difference in cell size. Thus in B-CLL cells both FADD and caspase-8 were present in sufficient amounts to engage apoptosis by the death receptor machinery.

The inhibitory molecules c-FLIP and XIAP are expressed in B-CLL cells

In order to explain the resistance of B-CLL cells to TRAIL, the expression of c-FLIP and XIAP was examined. c-FLIP was detected in all clinical samples, migrating as two isoforms of ~55 and ~28 kDa (Figure 3), corresponding to c-FLIP$_L$ and c-FLIP$_S$ (Irmler *et al.*, 1997; Scaffidi *et al.*, 1999). Expression of the two isoforms was approximately equal. The anti-apoptotic mechanisms of some IAP proteins are unknown although XIAP, cIAP-1 and cIAP-2 appear to inhibit both stress- and death receptor-induced apoptosis through direct inhibition of distinct caspases (reviewed in Deveraux and Reed, 1999). In B-CLL cells and the cell lines, XIAP was detected as a band of ~57 kDa together with a previously reported non-specific immunoreactive product of ~58 kDa (Deveraux *et al.*, 1999) (Figure 3). In order to determine whether FLIP and XIAP were involved in TRAIL resistance, B-CLL cells were treated with inhibitors of transcription and translation, which increase the sensitivity of certain target cells that are otherwise resistant to CD95L, TNF or TRAIL-induced apoptosis (Griffith *et al.*, 1998; Mori *et al.*, 1999; Leverkus *et al.*, 2000).

Cycloheximide sensitizes B-CLL cells to TRAIL-induced apoptosis but not by decreasing c-FLIP levels

B-CLL cells, pre-treated for 18 h with either actinomycin D (2–8 nM) or cycloheximide (1–10 μM), were sensitized to TRAIL-induced apoptosis in a time- and concentration-dependent manner (Figure 4a and data not shown). Treatment of B-CLL cells with cycloheximide (5 and 10 μM) inhibited protein synthesis by 53 ± 5 and $75 \pm 10\%$, whereas treatment with actinomycin D (4 and 8 nM) inhibited RNA synthesis by 57 ± 19 and $71 \pm 6\%$, respectively (mean $\pm$ s.e. of cells from three different patients). Higher concentrations of cycloheximide and actinomycin D could not be used as they induced extensive apoptosis. Although both cycloheximide and actinomycin D alone induced some apoptosis, the synergistic effect of their pre-treatment on TRAIL-induced apoptosis in B-CLL cells was evident. Cycloheximide was more potent than actinomycin D and on average potentiated apoptosis by ~1.5-fold (Figure 4a). However, levels of c-FLIP, XIAP, cIAP-1, cIAP-2 or cell surface TRAIL receptors were unaltered following exposure to actinomycin D or cycloheximide for 18 h (Figure 4b and data not shown). Thus sensitization of B-CLL cells to TRAIL by cycloheximide or actinomycin D was not due to changes in levels of IAPs or c-FLIP$_L$.

Bisindolylmaleimides do not alter c-FLIP levels or sensitize B-CLL cells to TRAIL-induced apoptosis

In order to better understand the regulation of c-FLIP$_L$ in B-CLL cells and to exclude its role in resistance, cells were pre-treated with bisindolylmaleimide derivatives, which can sensitize tumour cells, T lymphocytes and dendritic cells to CD95 by decreasing c-FLIP expression (Zhou *et al.*, 1999; Willems *et al.*, 2000). B-CLL cells were pre-treated with two concentrations of each bisindolylmaleimide, one sub-toxic and the other displaying low toxicity, and then exposed to TRAIL (Figure 5a). Higher concentrations of all three bisindolylmaleimides induced apoptosis (data not shown). No increase in TRAIL sensitivity was observed following pre-treatment with the bisindolylmaleimides (Figure 5a). In addition no significant decrease in c-FLIP$_L$ or c-FLIP$_S$ or XIAP levels was observed except with bisindolylmaleimide III (10 μM), where a small decrease in c-FLIP$_L$ (Figure 5b) reflected a small induction of apoptosis. Thus, bisindolylmaleimide derivatives neither decreased c-FLIP levels nor sensitized B-CLL cells to TRAIL. Taken together with the cycloheximide data, these results suggest that the regulation of c-FLIP in B-CLL cells is different from many other cell types studied.

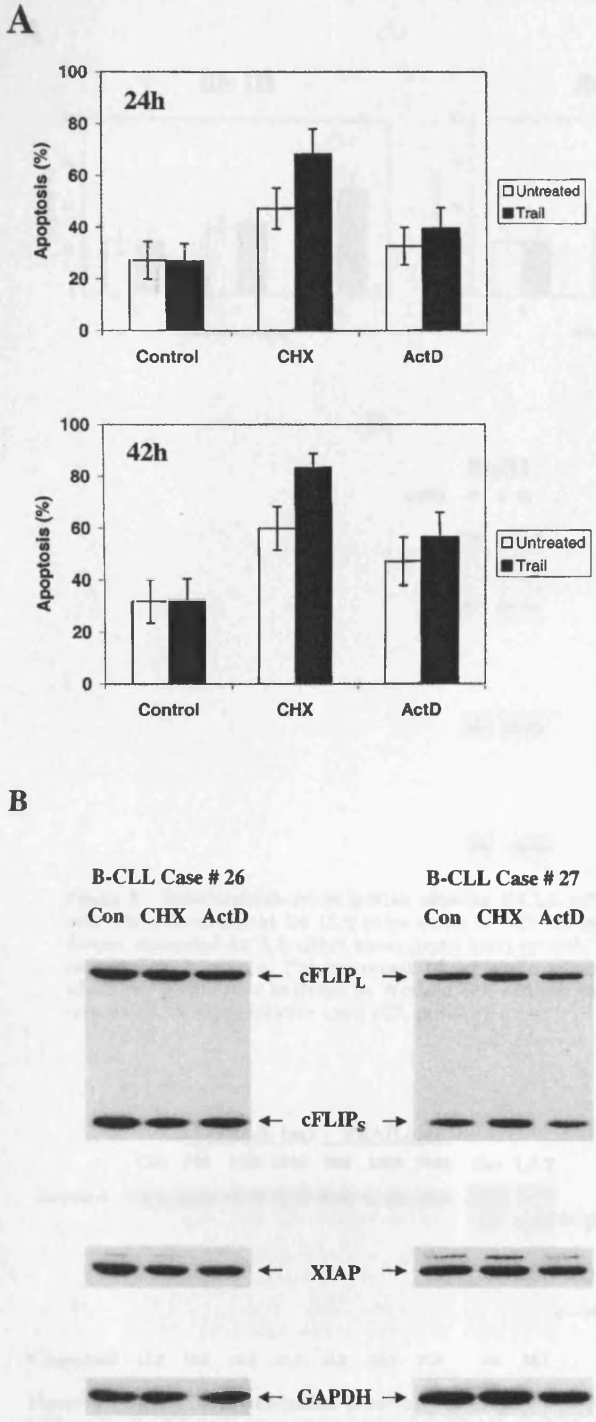

Figure 4 Preincubation with cycloheximide sensitizes primary B-CLL cells to TRAIL-induced apoptosis but does not alter c-FLIP or XIAP protein levels. Primary B-CLL cells were pre-incubated for 18 h either alone or with cycloheximide (CHX) (5 μM) or actinomycin D (ActD) (4 nM). These cells were then further incubated for 6 or 24 h, either alone (open bars), or in the presence of TRAIL (500 ng/ml) (filled bars). (**a**) The percentage of apoptotic cells at both 24 and 42 h was determined by Annexin V binding. Results are the mean of six independent cases $\pm$ s.e. (**b**) After 18 h pre-incubation, whole cell pellets were analysed by Western blot analysis. Equivalent protein loading was confirmed by analysis of GAPDH expression levels. Two representative cases are shown

TRAIL does not induce processing of caspase-8 in B-CLL cells

In order to test whether the resistance of B-CLL cells to TRAIL-induced apoptosis was due to their inability to process caspase-8, we examined B-CLL cells exposed to TRAIL. No processing of caspase-8 was observed using two different forms of TRAIL (500–2000 ng/ml), whereas Jurkat cells exposed to TRAIL (100 ng/ml) showed extensive processing (Figure 6). In the Jurkat cells, caspase-8 was processed both to its p43 and p41 forms, which arise following cleavage of caspases-8a and -8b between their large and small subunits, and also to its p18 catalytically active large subunit in agreement with our earlier studies (Sun *et al*., 1999). These results demonstrated that the resistance of B-CLL cells to TRAIL-induced apoptosis was upstream of their ability to process caspase-8.

TRAIL induces a DISC in B-CLL cells

As the resistance to TRAIL occurred prior to caspase-8 activation, it was possibly due to the inability of the B-CLL cells to form a functional DISC. As a positive control we used Jurkat T cells, which are sensitive to TRAIL (Figure 1b) and form a DISC with recruitment of caspase-8 and FADD (Bodmer *et al*., 2000; Sprick *et al*., 2000). TRAIL induced the formation of a DISC in Jurkat cells together with the recruitment of FADD and caspase-8 (Figure 7, lane 3). Within the DISC, caspase-8 was also processed both to its p43 and p41 forms and also to its p18 catalytically active large subunit (Figure 7, lane 3). Biotinylated-TRAIL also induced formation of a DISC in B-CLL cells together with recruitment of FADD and caspase-8. However, $\sim$10 times more B-CLL than Jurkat cells were required to form a DISC, which comprised approximately similar levels of FADD and c-FLIP$_L$ (Figure 7, compare lane 3 with lanes 6 and 7). Thus the formation of low levels of a TRAIL DISC may in part be the basis for the resistance of B-CLL cells to TRAIL. Less TRAIL-R2 was associated with the DISC in B-CLL cells compared to Jurkat cells (Figure 7, compare lanes 3 and 6), in agreement with the finding of lower surface expression of TRAIL-R2 in B-CLL cells (Figure 2). The association of FADD and caspase-8 were both TRAIL stimulation-dependent since they were not recruited when biotinylated-TRAIL was added after cell lysis, whereas the non-stimulated TRAIL-R2 was precipitated (Figure 7, lane 5). Interestingly, in B-CLL cells, caspase-8 was recruited to the DISC and was present both as p55 and p53 unprocessed zymogens corresponding to caspases-8a and -8b, as well as their partially processed forms of p43 and p41 but without any detectable p18 large subunit (Figure 7, lanes 6 and 7). Thus, partially processed caspase-8 was detected within the DISC (Figure 7), but not in B-CLL cells treated with TRAIL (Figure 6), most probably due to the increased sensitivity provided by analysing the trimerised death receptors and associated proteins within the DISC.

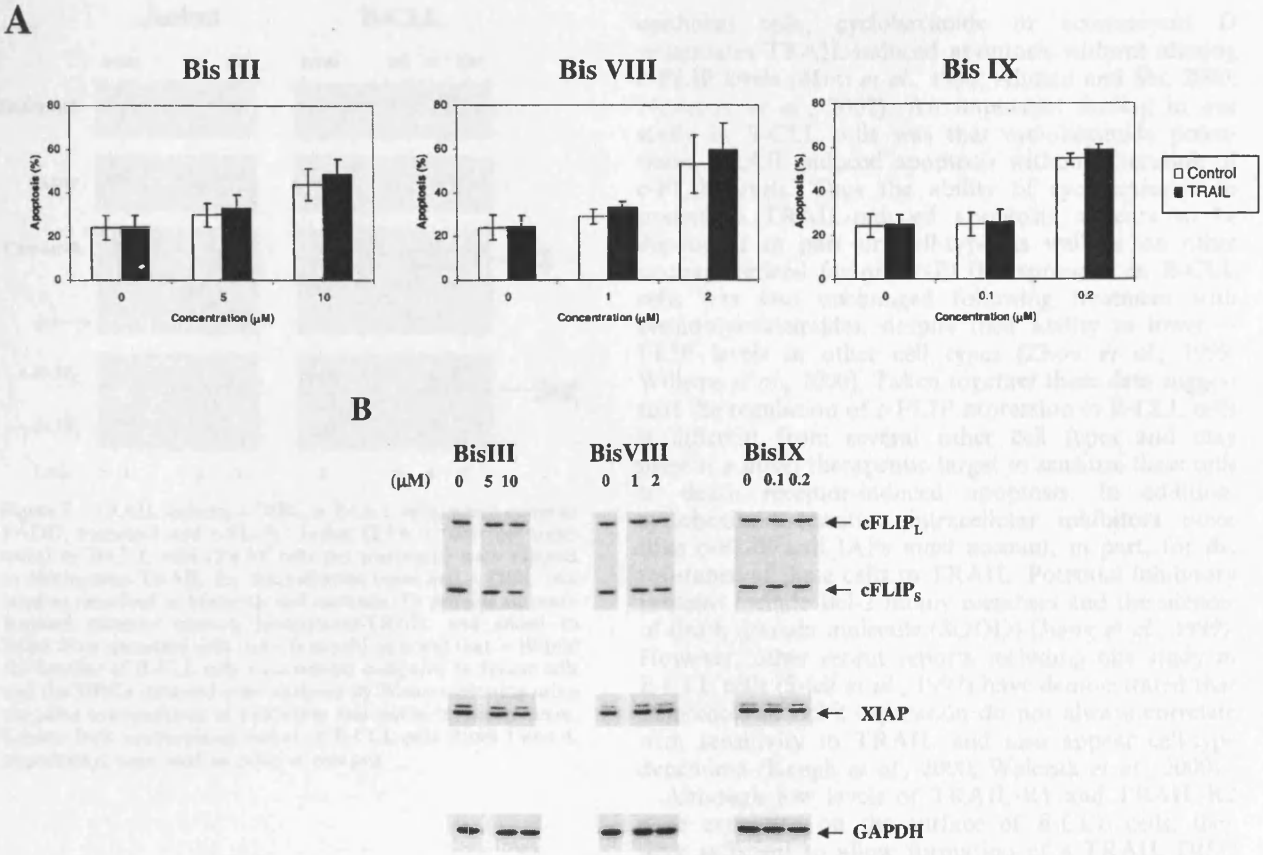

Figure 5 Bisindolylmaleimides neither sensitize B-CLL cells to TRAIL-induced apoptosis nor decrease c-FLIP levels. (**a**) B-CLL cells were pre-incubated for 18 h either alone or with the indicated concentrations of Bisindolylmaleimide III, VIII or IX and then further incubated for 6 h either alone (open bars) or with TRAIL (500 ng/ml) (filled bars). The data represent the mean of three independent cases ± s.e. The percentage of apoptotic cells was determined by Annexin V binding. (**b**) After 18 h pre-incubation, whole cell pellets were analysed by Western blot analysis and equivalent protein loading confirmed by analysis of GAPDH expression levels. A representative case, #27, is shown

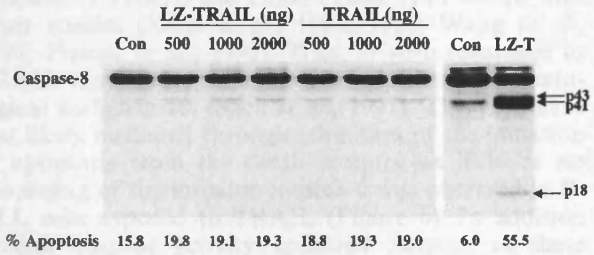

Figure 6 TRAIL does not induce processing of caspase-8 in B-CLL cells. B-CLL cells were treated with LZ-TRAIL (LZ-T) or TRAIL Biomol (TRAIL) at the indicated concentrations (500–2000 ng/ml) for 24 h. Jurkat T cells were treated with LZ-TRAIL (100 ng/ml) for 4 h. Cell lysates were examined by Western blot analysis for processing of caspase-8. Apoptosis was detected by Annexin V staining

It was also possible that some of the resistance to TRAIL was due to c-FLIP$_L$ and c-FLIP$_S$, both of which are expressed in B-CLL cells (Figure 3). These molecules are recruited to the DISC and inhibit CD95-induced apoptosis (Scaffidi *et al*., 1999; Kirchoff *et al*., 2000; Krueger *et al*., 2001). Following TRAIL stimulation of B-CLL cells, c-FLIP$_L$ but not c-FLIP$_S$ was recruited to the DISC (Figure 7). Interestingly, in the stimulated but not the unstimulated cells, most of the c-FLIP$_L$ was cleaved to an ~p43 fragment (Figure 7, lanes 6 and 7), which most likely represents the product obtained following removal of its C-terminal p10 subunit (Scaffidi *et al*., 1999). Recruitment of c-FLIP$_L$ to the DISC might also contribute to the resistance of B-CLL cells to TRAIL.

Discussion

TRAIL has recently emerged as a novel anti-cancer agent, based on its induction of cell death in many tumour cell lines and its lack of toxicity to most normal cells (Ashkenazi *et al*., 1999; Walczak *et al*., 1999). TRAIL may be toxic to normal human

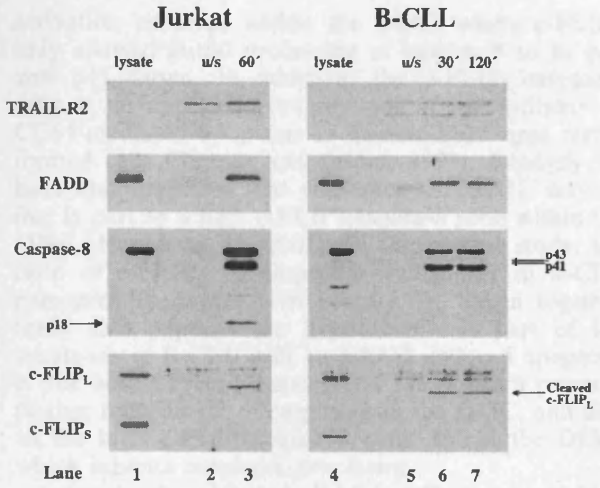

Figure 7 TRAIL-induces a DISC in B-CLL cells, which contains FADD, caspase-8 and c-FLIP$_L$. Jurkat (2.5×10^7 cells per treatment) or B-CLL cells (3×10^8 cells per treatment) were exposed to biotinylated-TRAIL for the indicated times and a DISC isolated as described in Materials and methods. To provide an unstimulated receptor control, biotinylated-TRAIL was added to lysate from untreated cells (u/s). It should be noted that ~10-fold the number of B-CLL cells were treated compared to Jurkat cells and the DISCs obtained were analysed by Western blotting using the same concentration of antibodies and similar exposure times. Lysates from unstimulated Jurkat or B-CLL cells (lanes 1 and 4, respectively) were used as positive controls

hepatocytes *in vitro* (Jo *et al.*, 2000) although its *in vivo* toxicity remains to be established. Importantly, the *in vitro* sensitivity of normal primary astrocytes to LZ-TRAIL was not observed *in vivo* (Walczak *et al.*, 1999). In order to assess the value of TRAIL, we have determined its toxicity to primary B-CLL cells from patients as opposed to the use of cell lines, which have been used in most other studies. B-CLL cells were resistant to TRAIL and CD95 (Table 1) in accord with other studies (Xerri *et al.*, 1997, 1998; Wang *et al.*, 1997; Plumas *et al.*, 1998). Thus, *in vitro* resistance to TRAIL may be common to many primary haematological malignancies (Snell *et al.*, 1997). This resistance was likely mediated through inhibition of the initiation of apoptosis from the death receptor as little or no processing of the initiator caspase-8 was observed in B-CLL cells exposed to TRAIL (Figure 6). In addition effector caspase activity appeared normal, as these same cells were susceptible to chemically induced apoptosis (Figure 1) with concomitant processing of caspases-3 and -7 (Almond *et al.*, 2001).

Although many studies show potentiation of CD95 or TRAIL-induced apoptosis in the presence of cycloheximide or actinomycin D, few of these have investigated the mechanism. In some cell types, there is undoubtedly a correlation of decreased FLIP levels with increased TRAIL sensitivity (Griffith *et al.*, 1998; Leverkus *et al.*, 2000; Fulda *et al.*, 2000; Munshi *et al.*, 2001). However in other cell types, including thyroid cells, Kaposi sarcoma cells and human prostate epithelial cells, cycloheximide or actinomycin D potentiates TRAIL-induced apoptosis without altering c-FLIP levels (Mori *et al.*, 1999; Ahmad and Shi, 2000; Nesterov *et al.*, 2002). An important finding in our study in B-CLL cells was that cycloheximide potentiated TRAIL-induced apoptosis without alteration of c-FLIP levels. Thus the ability of cycloheximide to potentiate TRAIL-induced apoptosis appears to be dependent in part on cell-type as well as on other uncharacterized factors. c-FLIP expression in B-CLL cells was also unchanged following treatment with bisindolylmaleimides, despite their ability to lower c-FLIP levels in other cell types (Zhou *et al.*, 1999; Willems *et al.*, 2000). Taken together these data suggest that the regulation of c-FLIP expression in B-CLL cells is different from several other cell types and may present a novel therapeutic target to sensitize these cells to death receptor-induced apoptosis. In addition, cycloheximide-sensitive intracellular inhibitors other than c-FLIP and IAPs must account, in part, for the resistance of these cells to TRAIL. Potential inhibitory proteins include Bcl-2 family members and the silencer of death domain molecule (SODD) (Jiang *et al.*, 1999). However, other recent reports including one study in B-CLL cells (Snell *et al.*, 1997) have demonstrated that differences in Bcl-2 expression do not always correlate with sensitivity to TRAIL and also appear cell-type dependent (Keogh *et al.*, 2000; Walczak *et al.*, 2000).

Although low levels of TRAIL-R1 and TRAIL-R2 were expressed on the surface of B-CLL cells, they were sufficient to allow formation of a TRAIL DISC (Figure 7). These low levels may partly explain the small amount of DISC formation in B-CLL cells compared to Jurkat cells (~10 times more B-CLL cells were required; Figure 7), thereby contributing to the resistance of B-CLL cells to TRAIL. In cell lines stably overexpressing c-FLIP$_L$ or c-FLIP$_S$, both isoforms are recruited to the DISC but inhibit CD95-induced apoptosis in subtly different ways (Krueger *et al.*, 2001). The presence of c-FLIP$_S$ prevents cleavage of caspase-8, whereas with c-FLIP$_L$ the initial processing of caspase-8 to its p43/41 fragments is observed and the cleavage intermediates of both caspase-8 and c-FLIP$_L$ remain bound to the receptor, so preventing further recruitment of procaspase-8 to replace the processed caspase-8 at the DISC (Krueger *et al.*, 2001). The data in B-CLL cells (Figure 7) is compatible with the latter model of c-FLIP$_L$ preventing further recruitment of caspase-8 to the DISC. In addition, c-FLIP has a higher affinity for the DISC than caspase-8 and is rapidly recruited to the DISC and readily cleaved (Scaffidi *et al.*, 1999), in agreement with our current finding that most of the c-FLIP$_L$ recruited to the DISC was cleaved to its ~p43 fragment (Figure 7). These results also demonstrate that caspase-8 was initially catalytically active. In B-CLL cells exposed to TRAIL, caspase-8 in the DISC was processed only to its p43 and p41 forms, whereas p43, p41 and p18 forms of caspase-8 were detectable both in the DISC and the lysates of Jurkat cells (Figures 6 and 7). These results imply that in B-CLL cells the block in caspase-8

activation occurred within the DISC, where c-FLIP$_L$ only allowed initial processing of caspase-8 to its p43 and p41 forms. In addition, the c-FLIP$_L$/caspase-8 ratio is an important determinant of susceptibility to CD95-mediated apoptosis in Epstein-Barr virus transformed cells (Tepper and Seldin, 1999). Recently we have also suggested that resistance to TRAIL may be due in part to a high c-FLIP/caspase-8 ratio within the DISC (Harper *et al.*, 2001). In the present study, the ratio of c-FLIP$_L$ to caspase-8 was higher in B-CLL compared to Jurkat cells (Figure 7). Taken together these data support the hypothesis that part of the resistance of B-CLL cells to TRAIL-induced apoptosis is due both to recruitment of c-FLIP$_L$, which prevents further recruitment of caspase-8 to the DISC, and also to the high c-FLIP/caspase-8 ratio within the DISC, which inhibits caspase-8 processing.

Interestingly, although B-CLL cells expressed high levels of both c-FLIP$_L$ and c-FLIP$_S$ (Figures 3 and 7), only c-FLIP$_L$ was recruited to the TRAIL DISC. As both c-FLIP$_L$ and c-FLIP$_S$ contain two N-terminal death effector domains, these results suggest that the caspase-like C-terminal domain present in c-FLIP$_L$ but not in c-FLIP$_S$ is involved in part in efficient recruitment to the DISC.

Resistance to TRAIL may also be due to mutations, such as those of TRAIL-R2 in some forms of cancer (Pai *et al.*, 1998; Lee *et al.*, 1999). A polymorphic mutation in the death domain of TRAIL-R1 is also present in about 20% of the normal population (Kim *et al.*, 2000). Resistance to TRAIL-induced apoptosis in B-CLL cells does not exclude its future use, particularly if the sensitization seen *in vitro* can be mimicked *in vivo*. Subtoxic concentrations of chemotherapeutic drugs can sensitize resistant tumour cells by upregulation of death-inducing TRAIL receptors (Pai *et al.*, 1998; Lee *et al.*, 1999; Keane *et al.*, 1999). Such an approach could lead to a sensitization of B-CLL cells, together with the added benefit of reduced toxicity and a lower risk of developing drug resistance. This approach may be particularly valuable with B-CLL cells because of their low surface expression of death receptors (Figures 2 and 7).

In summary, we have demonstrated that primary B-CLL cells are resistant to TRAIL. This resistance was upstream of caspase-8 activation, as little or no caspase-8 was processed in TRAIL-treated B-CLL cells. Low expression of TRAIL death receptors on the cell surface resulted in the formation of low levels of a TRAIL DISC, which contained FADD, caspase-8 and c-FLIP$_L$. Thus the resistance of B-CLL cells appears to be due in part to low levels of DISC formation and also to the high ratio of c-FLIP$_L$ to caspase-8 within the DISC, which would prevent further activation of caspase-8. While our results highlight the possibility of sensitizing B-CLL cells to TRAIL either through upregulation of death receptors or by decreasing c-FLIP levels, the precise mechanism(s) underlying TRAIL resistance in B-CLL cells requires further investigation.

Materials and methods

Patients

Blood samples were obtained from B-CLL patients, staged according to the Binet system, during routine diagnosis at the Leicester Royal Infirmary (UK) with patient consent and local ethical committee approval. Samples were collected into Li-Heparin tubes.

Materials

Media and serum were from Life Technologies, Inc. (Paisley, UK). Anti-CD95 monoclonal antibody (CH-11 clone) was from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Human recombinant TRAIL was from Biomol Research Laboratories (Plymouth Meeting, USA). LZ-TRAIL (a TRAIL-leucine-zipper fusion protein) and the TRAIL-R1-R4 antibodies (clones M271, M413, M430 and M444, respectively) were kind gifts from Immunex Corp., Seattle, WA, USA (Griffith *et al.*, 1999). The proteasome inhibitor MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal) was from Calbiochem (La Jolla, CA, USA). Annexin V-FITC was from Bender MedSystems (Vienna, Austria). Bisindolylmaleimides III, VIII and IX were from Alexis Biochemicals (Nottingham, UK). The sensitivity of B-CLL cells to TRAIL-induced apoptosis was compared to sensitive human cell lines, namely Jurkat T cells (clone E6-1) and the B cell Burkitts lymphoma cell line MC116, both obtained from ECACC and the B lymphoblastoid cell line, SKW6.4 (SKW), kindly provided by Dr M Peter (Heidelberg, Germany). Other reagents were from Sigma (Poole, UK).

Lymphocyte purification and culture

Whole blood was layered over Histopaque and B-CLL cells purified as previously described (King *et al.*, 1998; Almond *et al.*, 2001) except that T cells were removed using anti-CD3 Dynabeads (Dynal, Merseyside, UK) resulting in a mean average purity of 98.2% $CD19^+CD5^+$B-CLL cells. The purified B cells were resuspended in RPMI 1640 medium (2×10^6/ml) in 6-well plates at 37°C in an atmosphere of 5% CO_2 and incubated for up to 24 h with anti-CD95 (500 ng/ml), LZ-TRAIL (500–2000 ng/ml) or MG132 (1 μM). Where indicated, lymphocytes were preincubated for 18 h in the presence or absence of actinomycin D, cycloheximide, or bisindolylmaleimides III, VIII and IX and then incubated with TRAIL (500 ng/ml) for a further 6–24 h as indicated. Samples were either analysed for apoptosis or pelleted and stored at −80°C for subsequent Western blotting.

Quantification of apoptosis and Western blot analysis

Apoptotic cells were quantified by an increase in externalized phosphatidylserine as assessed by Annexin V labelling (King *et al.*, 1998). Samples for Western blot analysis were prepared as described (Sun *et al.*, 1999). Caspase-8, FADD (Transduction Laboratories, Lexington, KY, USA), XIAP (Transduction Laboratories), c-FLIP (Rasper *et al.*, 1998) and GAPDH (Advanced Immunochemical Inc, CA, USA) were detected as described previously (Sun *et al.*, 1999).

Assessment of cell surface receptor expression

Purified B-CLL cells were resuspended in blocking buffer (10% normal goat serum in PBS) and incubated for 30 min on ice to block non-specific binding, then incubated with

anti-TRAIL receptor antibodies (diluted 1 : 50) or an isotype-matched control antibody for 1 h on ice. The samples were washed twice by centrifugation at 400 *g* for 5 min with ice-cold PBS, then incubated with goat anti-mouse FITC-conjugated antibody ($F(ab)_2$ fragment (DAKO) (diluted 1 : 20 in blocking buffer)) for 1 h at 4°C. The samples were then washed twice, resuspended in PBS and analysed immediately by flow cytometry.

Protein and RNA synthesis assays

B-CLL cells were incubated for 18 h either alone or in the presence of cycloheximide (5–10 μM) or actinomycin D (4–8 nM) and samples then assayed for protein and RNA synthesis essentially as described previously (O'Brien *et al.*, 1987) except that B-CLL cells (3×10^6/ml) were pulsed for 30 min with 1 μCi [^{3}H]leucine or [^{3}H]uridine (Amersham Pharmacia, Bucks, UK). Results were expressed as a percentage of the level of protein or RNA synthesis measured in control cultures.

DISC analysis

DISC precipitation was performed using biotin-tagged recombinant TRAIL (biotinylated-TRAIL) essentially as described (Sprick *et al.*, 2000; Harper *et al.*, 2001). B-CLL cells (3×10^8 cells per treatment) were treated with biotinylated-TRAIL for either 30 or 120 min and DISC formation determined (Harper *et al.*, 2001). Western blotting was performed using 30 μl of eluted complexes from streptavidin-agarose beads representing DISC precipitated from 3×10^8 cells. Jurkat cells (2.5×10^7 cells per treatment) were similarly exposed to TRAIL as a positive control for DISC formation.

Abbreviations

B-CLL, B-cell chronic lymphocytic leukaemia; Bis, bisindolylmaleimide; DISC, death-inducing signalling complex; FADD, Fas-associated death domain protein; c-FLIP, cellular FLICE inhibitory protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRAIL, Tumour necrosis factor-related apoptosis-inducing ligand; PS, phosphatidylserine; TRAIL-R, TRAIL receptor; XIAP, X-linked inhibitor of apoptosis protein

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Fas-associated Death Domain Protein and Caspase-8 Are Not Recruited to the Tumor Necrosis Factor Receptor 1 Signaling Complex during Tumor Necrosis Factor-induced Apoptosis*

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Death receptors are a subfamily of the tumor necrosis factor (TNF) receptor subfamily. They are characterized by a death domain (DD) motif within their intracellular domain, which is required for the induction of apoptosis. Fas-associated death domain protein (FADD) is reported to be the universal adaptor used by death receptors to recruit and activate the initiator caspase-8. CD95, TNF-related apoptosis-inducing ligand (TRAIL-R1), and TRAIL-R2 bind FADD directly, whereas recruitment to TNF-R1 is indirect through another adaptor TNF receptor-associated death domain protein (TRADD). TRADD also binds two other adaptors receptor-interacting protein (RIP) and TNF-receptor-associated factor 2 (TRAF2), which are required for TNF-induced NF-κB and c-Jun N-terminal kinase activation, respectively. Analysis of the native TNF signaling complex revealed the recruitment of RIP, TRADD, and TRAF2 but not FADD or caspase-8. TNF failed to induce apoptosis in FADD- and caspase-8-deficient Jurkat cells, indicating that these apoptotic mediators were required for TNF-induced apoptosis. In an *in vitro* binding assay, the intracellular domain of TNF-R1 bound TRADD, RIP, and TRAF2 but did not bind FADD or caspase-8. Under the same conditions, the intracellular domain of both CD95 and TRAIL-R2 bound both FADD and caspase-8. Taken together these results suggest that apoptosis signaling by TNF is distinct from that induced by CD95 and TRAIL. Although caspase-8 and FADD are obligatory for TNF-mediated apoptosis, they are not recruited to a TNF-induced membrane-bound receptor signaling complex as occurs during CD95 or TRAIL signaling, but instead must be activated elsewhere within the cell.

Death receptors such as CD95 (Fas/Apo1), tumor necrosis factor (TNF)[1] receptor-1 (TNF-R1) and the TNF-related apoptosis-inducing ligand (TRAIL) receptors, TRAIL-R1 and TRAIL-R2, belong to a subgroup of the TNF/nerve growth factor family. They are characterized by multiple conserved cysteine-rich domains within their extracellular domain and an intracellular death domain (DD) motif (1, 2). The DD is a highly conserved region of ~80 amino acids, and mutation of a number of key residues within this region abrogates the cytotoxicity induced by receptor ligation (3). The best-characterized death receptor pathway is that induced by CD95. Engagement of CD95 by CD95L results in aggregation of the receptor and recruitment of the DD-containing adaptor protein mediator of receptor-induced toxicity (MORT1)/FADD (Fas-associated death domain) to its intracellular domain through a homophilic DD interaction. FADD is a bipartite molecule, containing an N-terminal death effector domain (DED) and a C-terminal DD (4, 5). FADD recruits the initiator caspase-8 via a homophilic DED interaction. Initiator caspases are activated by proximity-induced activation facilitated by adaptor-mediated clustering of zymogens (6–9). The complex of proteins recruited to the CD95 receptor is termed the death-inducing signaling complex (DISC) (10).

TNF exerts its diverse biological effects through two receptors, TNF-R1 and TNF-R2, and though they exhibit extensive homology in their extracellular domains, their intracellular domains are unrelated with only that of TNF-R1 containing a DD (11). TNF-mediated apoptosis differs from that induced by CD95 in that TNF-R1 initially recruits a different adaptor protein, TNF receptor-associated DD protein (TRADD) (12, 13), which is then believed to recruit FADD, thereby recruiting and activating procaspase-8 in a manner similar to CD95. Both FADD and caspase-8 are absolutely required for TNF and CD95-induced apoptosis as fibroblasts derived from mice where the FADD or caspase-8 gene has been ablated are completely resistant to both CD95 and TNF-induced cytotoxicity, as are cells from transgenic mice expressing a dominant-negative form of FADD (14–17). TRADD acts as a platform for recruitment into the TNF-R1 signaling complex of other signaling intermediates, such as receptor-interacting protein (RIP), a DD-containing kinase, and TNF receptor-associated factor 2 (TRAF2), a member of the TRAF family (18, 19). TNF-induced signaling is believed to diverge at this point; TRAF2/RIP recruitment leads to activation of downstream kinases in the NF-κB and c-Jun N-terminal kinase (JNK) pathways, whereas FADD recruitment leads to apoptosis (20). RIP is critical for TNF-mediated NF-κB activation as cells derived from RIP-deficient mice or RIP-deficient cells obtained through mutation are unable to activate NF-κB in response to TNF and are

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2; DD, death domain; DED, death effector domain; DISC, death inducing signaling complex; FADD, Fas-associated death domain protein; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; TBS, Tris-buffered saline; RIP, receptor-interacting protein; JNK, c-Jun N-terminal kinase; b, biotinylated; IRAK, IL-1 receptor-associated kinase; E3, ubiquitin-protein isopeptide ligase; PARP, poly(ADP-ribose) polymerase; IL-1, interleukin-1; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone.

hypersensitive to TNF-mediated cytotoxicity (21, 22). Deletion of TRAF2 results in only a modest decrease in NF-κB activation but a complete abrogation of TNF-mediated JNK activation (23, 24). However, in response to TNF, TRAF2/5 double-knockout animals are both hypersensitive and unable to activate NF-κB, indicating that some redundancy exists between TRAF2 and TRAF5 (25). TRAF2 also recruits the IκB kinase complex, which is activated by RIP by an as yet unknown mechanism and is independent of its kinase activity (26, 27).

Following exposure to CD95L or TRAIL, there is rapid formation of the corresponding DISC, together with caspase-8 activation resulting in a relatively fast induction of apoptosis (10, 28–30). TNF negatively regulates its own apoptotic activity through activation of NF-κB and cannot mediate apoptosis unless this pathway is blocked (31). This is commonly accomplished by inhibitors of transcription or translation, such as cycloheximide, which block induction of NF-κB-regulated survival genes (32). Thus TNF-induced apoptosis would be expected to occur more slowly than that induced by CD95L or TRAIL, and this could be reflected in TNF-mediated activation of caspase-8. In this respect, the native TNF signaling complex has never been demonstrated to recruit caspase-8, and the majority of work done on characterizing the TNF-R1 signaling complex has been carried out using overexpressed proteins.

To better understand the apoptotic arm of TNF-R1 signaling, we have examined the TNF signaling complex in Jurkat T cells, which express only TNF-R1 (33), so permitting exclusive study of TNF-R1 complexes. In addition, signaling complexes have been isolated from cells expressing endogenous levels of proteins, so obviating any artifacts introduced by overexpression of key proteins. Such overexpression may be particularly problematic with DD- and DED-containing proteins, which can artificially oligomerise through homophilic interactions. Using this model, engagement of TNF-R1 by TNF resulted in the rapid recruitment of endogenous TRADD, RIP, and TRAF2 but not of the apoptotic mediators FADD and procaspase-8. In marked contrast both FADD and caspase-8 were recruited to the native TRAIL DISC. Thus, activation of the apical caspase-8 in TNF-induced apoptosis occurs by a different mechanism from that utilized by CD95L and TRAIL.

EXPERIMENTAL PROCEDURES

Materials—Antibodies were sourced as follows: anti-FADD, -TRADD, -RIP, and -TRAF2 were from BD Transduction Labs (Heidelberg, Germany); anti-PARP (clone C2–10) was from Alexis Corp. (San Diego, CA); and anti-tubulin was from Amersham Biosciences. Caspase-8 monoclonal antibody C-15 has been described previously (34) and was a kind gift from Dr. P. H. Krammer (German Cancer Research Center, Heidelberg, Germany). Horseradish peroxidase-conjugated secondary antibodies, goat-anti-mouse, and goat-anti-rabbit, were obtained from Sigma and DAKO (Cambridge, UK), respectively. All other chemicals were of analytical grade and purchased from Sigma or Fisher.

Cell Culture—All cell culture materials were from Invitrogen, and plasticware was from BD Biosciences. Jurkat T cells, parental (A3), FADD-deficient, and caspase-8-deficient have been described elsewhere (35, 36) and were kindly provided by Dr. J. Blenis (Harvard Medical School, Boston, MA). HeLa and U937 cells were obtained from European Collection of Animal Cell Cultures (Wiltshire, UK). Jurkat and U937 cells were cultured in RPMI medium containing 10% fetal bovine serum and 1% Glutamax™ and HeLa cells in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere by routine passage every 3 days.

Determination of Apoptosis by Annexin V Staining—Using phosphatidylserine (PS) and propidium iodide (PI), apoptotic (PS^+ PI^-) and necrotic (PS^+ PI^+) cells were assessed by Annexin V labeling (Bender Medsystems, Vienna, Austria) as described previously (37).

Western Blotting—SDS-PAGE was carried out using a Tris/glycine buffer system based on the method of Laemmli (38). After electrophoresis, proteins were transferred to "Hybond N" nitrocellulose membrane (Amersham Biosciences). Membranes were blocked in Tris-buffered saline (TBS) containing 5% Marvel™ and 0.1% Tween 20 (TBSMT) prior to incubation with the primary antibody for 1 h. Membranes were then washed with TBSMT followed by TBST for 5 min, respectively, followed by the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Immunostained proteins were visualized on Kodak x-ray film (Sigma) using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Preparation and Biotinylation of Recombinant TRAIL and TNF—Biotinylated TRAIL (residues 95–281) (bTRAIL) was prepared as previously described (39, 40). To generate TNF-α, an expressed sequence tag containing the full-length TNF-α cDNA was obtained from Human Genome Mapping Project (HGMP) (Hinxton, Cambridge, UK). The extracellular domain of TNF-α (Val^{55}-Leu^{233}) was cloned by PCR into pet28(b), in-frame with N-terminal His and T7 tags, using specific primers. Recombinant TNF-α was then produced and biotin-labeled essentially as described for recombinant TRAIL. The resulting biotinylated TNF (bTNF) retained the properties of unlabelled TNF-α (data not shown).

Isolation of TNF and TRAIL Signaling Complexes—Isolation of TRAIL and TNF signaling complexes was performed essentially as previously described (40). Briefly, cells (5×10^7 cells per treatment) were treated with bTNF (200 ng/ml) or bTRAIL (500 ng/ml) for the indicated times. Cells were then washed three times with ice-cold PBS to remove any unbound ligand and lysed in 3 ml of lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100 (v/v), containing Complete™ protease inhibitors (Roche) for 30 min on ice. Lysates were then cleared by centrifugation (13,000 × *g*, 30 min) and bTNF/bTRAIL complexes precipitated overnight at 4 °C using streptavidin conjugated to Sepharose™ beads (Amersham Biosciences).

Glutathione-S-transferase (GST) Receptor Intracellular Domain Fusion Protein Interactions—GST receptor fusion proteins were created by cloning the N terminus of the intra-cellular domains of TNF-R1 (Gln^{237}-Arg^{455}), CD95 (Lys^{191}-Val^{335}), and TRAIL-R2 (Lys^{191}-Val^{411}) into pGEX 4T.1 in-frame with GST. Receptor fusion proteins were overexpressed in XA-90 cells kindly provided by Prof. D. Riches (National Jewish Medical and Research Center, Denver, CO), and the cells were lysed by sonication in 1.5% (w/v) sarkosyl containing 5 mM dithiothreitol and complete protease inhibitors (Roche). The lysate was bound to 1.5 ml of washed Glutathione-Sepharose beads (50% slurry) at 4°, the beads were washed twice in ice-cold PBS and the amount of purified GST fusion protein quantified by Coomassie Blue staining with comparison against bovine serum albumin standards. Jurkat cells (1.2×10^9) were washed in cold PBS and incubated on ice for 45 min in 5 ml lysis buffer (see previous section). Lysates were cleared by centrifugation and aliquots of the supernatant containing 5 mg protein at 10 mg/ml were incubated at 20° with 10 μg purified GST receptors bound to Sepharose beads. Control pulldowns were carried out with purified GST alone. Bound proteins were pelleted by centrifugation at 1000 rpm for 3 min, washed five times in PBS containing protease inhibitors, and released from the beads by boiling for 5 min in SDS sample buffer. Western blotting was used to assess the binding of the respective adaptor proteins.

RESULTS

TRADD, RIP, and TRAF2, but Not FADD or Caspase-8, Are Recruited to the TNF-induced Signaling Complex—Treatment of Jurkat T cells with bTNF, which retained the properties of unlabelled TNF-α, led to the rapid recruitment of TRADD, RIP, and TRAF2 to the precipitated complexes (Fig. 1*A*). Although both RIP and TRAF2 migrated as single species in the lysates of treated cells, both exhibited a number of higher molecular weight species when TNF complexes were analyzed (Fig. 1*A*, *lane 6*). This "ladder-like" appearance of both RIP and TRAF2 has recently been described (27, 41), although there was no indication of how the proteins were modified. Although this appearance is characteristic of ubiquitinated proteins, we have been unable to confirm this using several ubiquitin antibodies, most probably due to their low sensitivity (data not shown). Although the TNF-R1 signaling complex contained TRADD, RIP, and TRAF2, there was no evidence of recruitment of either FADD or caspase-8 (Fig. 1*A*, *lanes 6–8*). This was surprising, as both FADD and caspase-8 are required for death receptor-induced apoptosis including TNF-mediated apoptosis (15–17). We therefore compared the TNF-R1 signaling complex to the TRAIL DISC, which we had characterized in our previous

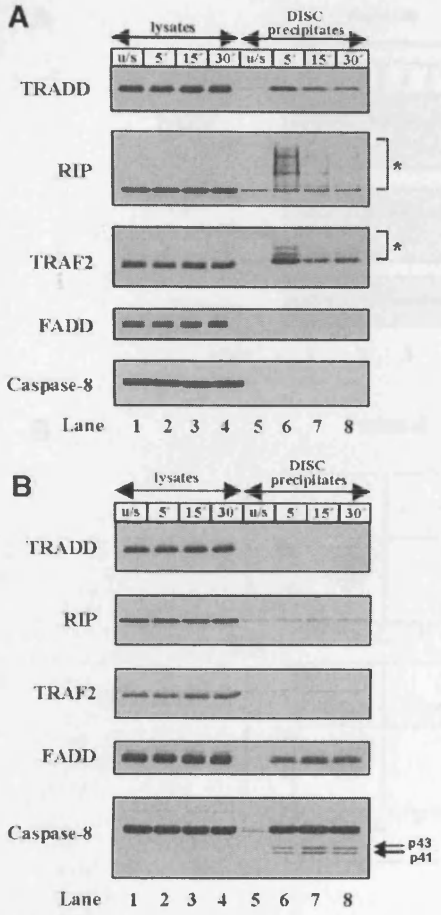

FIG. 1. **Failure to recruit FADD or caspase-8 to the TNF-R1 signaling complex in Jurkat cells.** Jurkat cells (5×10^7) were treated with bTNF (200 ng/ml) (*A*) or bTRAIL (500 ng/ml) (*B*) for the indicated times, and TNF receptor or TRAIL receptor complexes (*DISC*) were isolated as described under "Experimental Procedures." The addition of beads alone to unstimulated cell lysates (*u/s*) was used to control for nonspecific interactions. Cell lysates, prior to receptor complex isolation, and receptor complexes were then analyzed for the presence of TRADD, RIP, TRAF2, FADD, and caspase-8 by Western blotting. The *asterisk* indicates modified species of RIP and TRAF2 seen only in TNF receptor complexes.

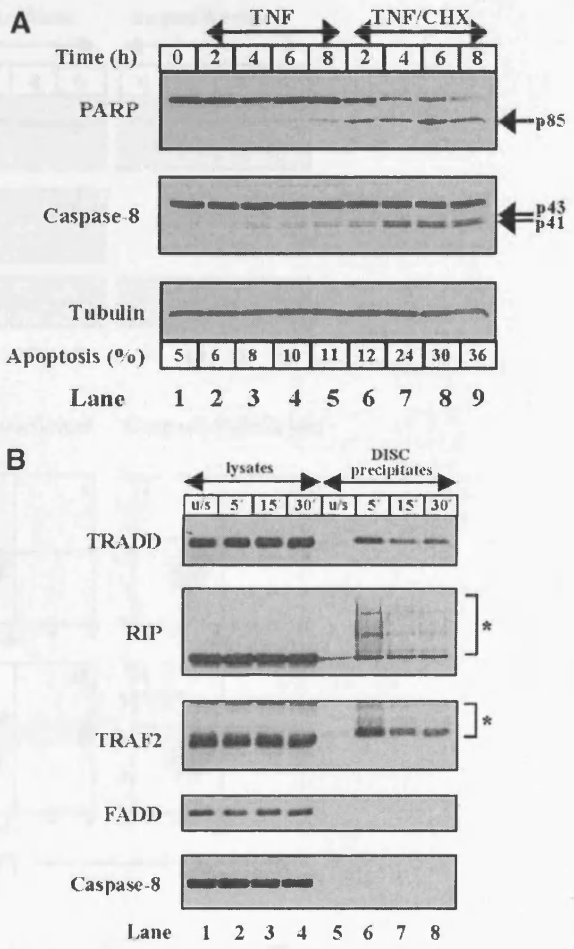

FIG. 2. **TNF-R1 complexes isolated from cycloheximide pretreated cells do not contain FADD or caspase-8.** *A*, Jurkat cells (2×10^6) were treated with TNF (200 ng/ml) for the indicated times in the presence and absence of cycloheximide (*CHX*, 1 μM, 30 min pretreatment). Cells were analyzed by Western blotting for cleavage of PARP or processing of caspase-8. α-Tubulin was used as a protein loading control. Apoptosis was quantified by measuring the percentage of PS^+ PI^- cells as described under "Experimental Procedures." *B*, Jurkat cells (5×10^7) were pre-treated with cycloheximide (1 μM) for 30 min prior to stimulation with bTNF (200 ng/ml) for the indicated times. TNF receptor complexes were then isolated and analyzed as described in the legend to Fig. 1. The *asterisk* indicates modified species of RIP and TRAF2 seen only in TNF receptor complexes.

studies (40). In marked contrast, treatment with TRAIL led to the rapid recruitment of both FADD and caspase-8 to the TRAIL DISC (Fig. 1*B*, *lanes 6–8*). Both the p55 and p53 zymogen forms of caspase-8, corresponding to caspase-8a and -8b (34), and their partially processed p43/p41 intermediates, obtained following removal of the small (p12) subunit, were present within the TRAIL DISC (Fig. 1*B*). Neither TRADD nor TRAF2 were associated with the TRAIL DISC (Fig. 1*B*), in agreement with other reports suggesting that they do not play a role in TRAIL signaling (28, 29, 39). Little if any RIP was present within the TRAIL DISC, consistent with our previous study (40) in HeLa cells, when its accumulation was only observed in the presence of the caspase inhibitor, z-VAD.fmk. In summary, we could find no evidence for the recruitment of FADD or caspase-8 to the TNF-R1 signaling complex although known TNF signaling intermediates, such as TRADD, RIP, and TRAF2, were present.

Failure to Recruit FADD or Caspase-8 in the Presence of Cycloheximide—One possibility for our failure to recruit FADD or caspase-8 to the TNF-R1 signaling complex was the necessity to block the TNF-induced NF-κB survival pathway to induce apoptosis. This was a distinct possibility as TNF treatment alone failed to induce apoptosis in Jurkat cells as assessed by an absence of PS^+ cells, PARP cleavage, or processing of caspase-8 (Fig. 2*A*, *lanes 1–5*). In the presence of cycloheximide, to block the synthesis of NF-κB regulated survival genes, TNF induced apoptosis as assessed by an increase in PS^+ PI^- cells accompanied by processing of caspase-8 and cleavage of PARP (Fig. 2*A*, *lanes 6–9*). Examination of the TNF-R1 signaling complex from these cells revealed recruitment of TRADD, RIP, and TRAF2, but not FADD or caspase-8, similar to that seen in cells treated with TNF alone (compare Figs. 1*A* and 2*B*). Thus neither FADD nor caspase-8 were recruited to the TNF-R1 signaling complex, even when the cells were subjected to TNF in the presence of cycloheximide.

TNF-induced Apoptosis Requires FADD and Caspase-8—The failure to recruit FADD and caspase-8 to the TNF-R1 signaling complex was clearly surprising and at odds with the widely accepted mechanism of TNF-induced apoptosis. To try

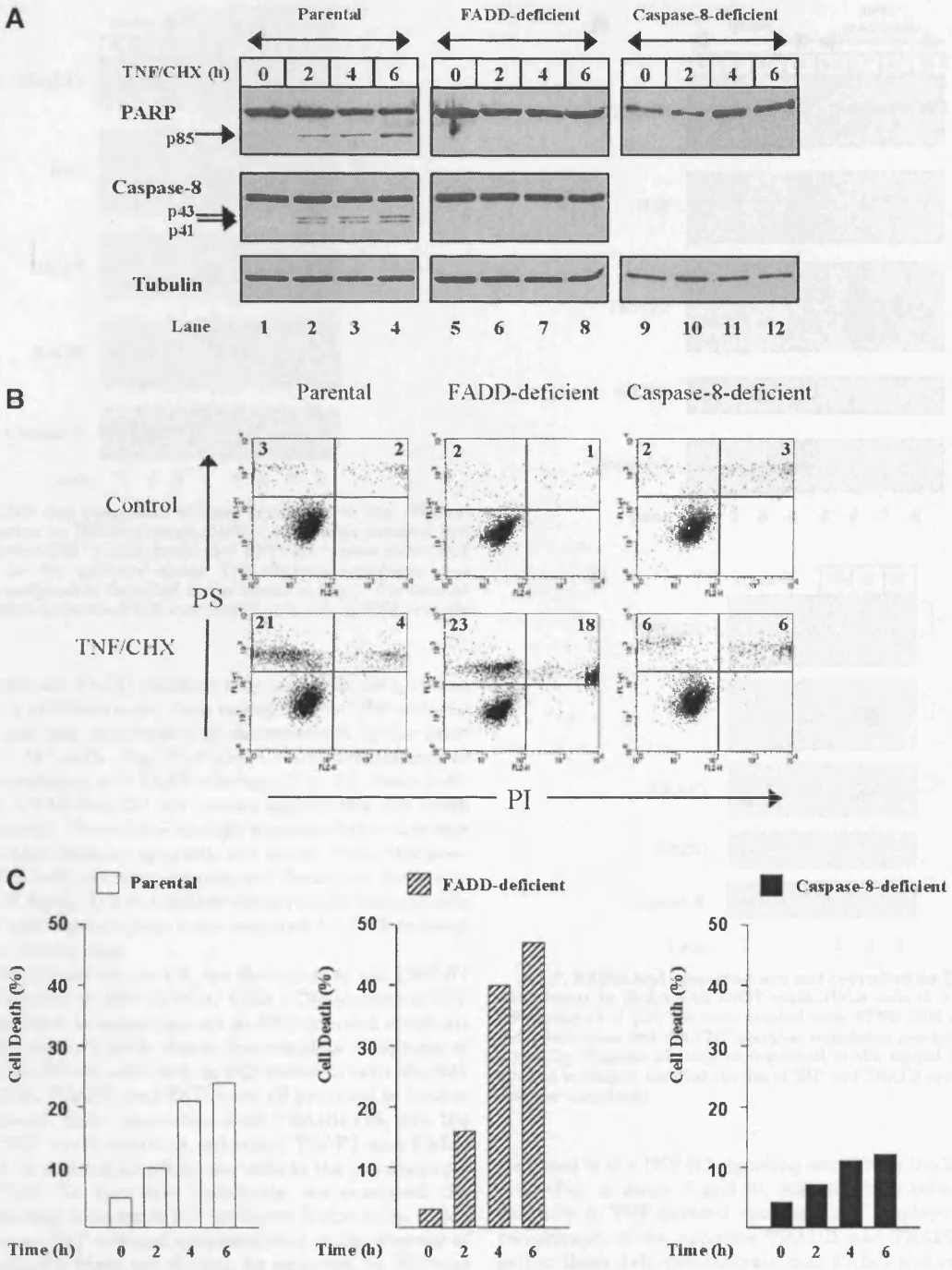

FIG. 3. **FADD and caspase-8 are required for TNF-induced apoptosis.** Parental, FADD-deficient, and caspase-8-deficient Jurkat cells were treated with TNF (200 ng/ml) in the presence of cycloheximide (*CHX*, 1 μM, 30 min pre-treatment) for the indicated times. *A*, cells were analyzed by Western blotting for cleavage of PARP and processing of caspase-8. α-Tubulin was used as a protein loading control. *B*, cells were stained with Annexin V and PI and analyzed by flow cytometry. The *numbers* shown depict the percentage of cells, which were either PS^+ PI^- (*upper left quadrant*) or PS^+ PI^+ (*upper right quadrant*). *C*, time-course of cell death in the different cell lines. TNF mediates time-dependent apoptotic and necrotic cell death in parental and FADD-deficient Jurkat cells, respectively.

and resolve this discrepancy, we utilized FADD- and caspase-8-deficient Jurkat cells to ascertain whether these molecules are essential for TNF-mediated apoptosis in the Jurkat cell model. TRAIL-induced apoptosis was abrogated in both FADD- and caspase-8-deficient Jurkat cells, thus demonstrating the critical requirement for these molecules in TRAIL-induced apoptosis (data not shown), in agreement with previous studies (28–30). TNF in the presence of cycloheximide again induced apoptosis in the parental Jurkat cells as assessed by processing of caspase-8 and cleavage of PARP (Fig. 3*A*, *lanes 1–4*) and an increase in the percentage of PS^+ PI^- cells (Fig. 3, *B* and *C*). All these apoptotic characteristics were inhibited by z-VAD.fmk (data not shown). In the caspase-8-deficient Jurkat cells, no cleavage of PARP (Fig. 3*A*, *lanes 9–12*) or increase in PS^+ cells (Fig. 3, *B* and *C*, and data not shown) was observed, demonstrating that caspase-8 is required for TNF-mediated apo-

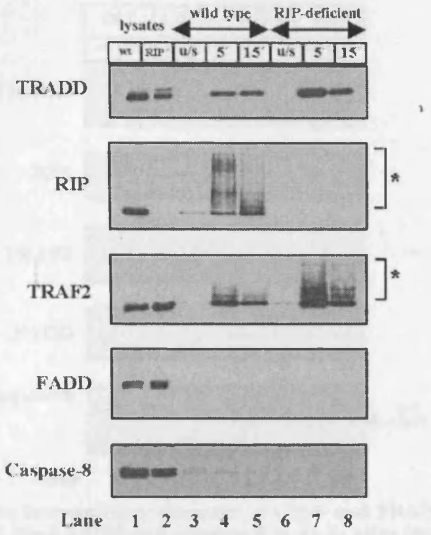

FIG. 4. **FADD and caspase-8 are not recruited to the TNF receptor complex in RIP-deficient Jurkat cells.** Both parental (*wt*) and RIP-deficient (*RIP*$^{-}$) Jurkat cells (5×10^7) were treated with bTNF (200 ng/ml) for the indicated times. TNF receptor complexes were isolated and analyzed as described in the legend to Fig. 1. The *asterisk* indicates modified species of RIP and TRAF2 seen only in TNF receptor complexes.

ptosis. In contrast, FADD-deficient cells, either in the presence or absence of cycloheximide, were susceptible to TNF-induced cell death, and this cell death was characterized by the presence of PS^+ PI^+ cells (Fig. 3, *B* and *C*) and the absence of caspase-8 processing and PARP cleavage (Fig. 3*A*, *lanes 5–8*). In addition, z-VAD.fmk did not protect against this cell death (data not shown). These data strongly suggested that this was a necrotic rather than an apoptotic cell death. Thus, the presence of FADD both prevents necrotic cell death and facilitates apoptotic cell death. Taken together these results demonstrate that both FADD and caspase-8 are required for TNF-induced apoptosis in Jurkat cells.

Neither FADD nor Caspase-8 Are Recruited to the TNF-R1 Signaling Complex in RIP-deficient Cells—The absence of RIP would be expected to sensitize cells to TNF-induced apoptosis by two mechanisms. Firstly, due to the complete abrogation of TNF-mediated NF-κB activation in RIP-deficient cells (21, 22). Second, as RIP, TRAF2, and FADD are all proposed to bind to TNF-R1 through their interaction with TRADD (18, 20), the absence of RIP could result in enhanced TRAF2 and FADD binding and as a result sensitize the cells to the pro-apoptotic effects of TNF. To test this hypothesis, we examined the TNF-R1 signaling complex in RIP-deficient Jurkat cells, which are sensitive to TNF-induced apoptosis even in the absence of cycloheximide (22) (data not shown). As expected, no RIP was present in the RIP-deficient cells (Fig. 4, *lane 2*), but both TRADD and TRAF2 were recruited to the TNF-R1 signaling complex in these cells (Fig. 4, *lanes 7* and *8*). Somewhat more TRADD was recruited in RIP-deficient compared with wild type cells, supporting the suggestion of competition between RIP and TRADD for recruitment to the receptor complex. Higher levels of modified TRAF2 were also found in the TNF-R1 signaling complex in RIP-deficient compared with wild type cells (Fig. 4, compare *lanes 7* and *8* with *lanes 4* and *5*), compatible both with TRAF2 binding to TRADD and also with competition between RIP and TRAF2 for modification within the complex. Similar levels of both FADD and caspase-8 were expressed in wild type and RIP-deficient cells (Fig. 4, compare *lanes 1* and *2*). However, neither FADD nor caspase-8 were

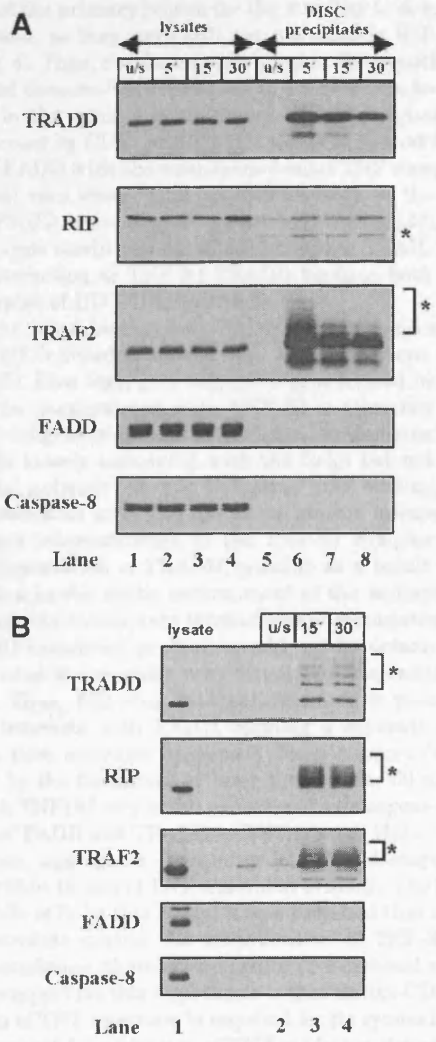

FIG. 5. **FADD and caspase-8 are not recruited to TNF receptor complexes in HeLa and U937 cells.** HeLa cells (3×10^7) (*A*) and U937 cells (5×10^7) (*B*) were treated with bTNF (200 ng/ml) for the indicated times and the TNF receptor complexes precipitated and analyzed by Western blotting as described in the legend to Fig. 1. The *asterisk* indicates modified species of RIP and TRAF2 seen only in TNF receptor complexes.

recruited to the TNF-R1 signaling complex in the RIP-deficient cells (Fig. 4, *lanes 7* and *8*), despite these cells being both sensitive to TNF-induced apoptosis and displaying increased recruitment of the adaptors TRADD and TRAF2. Taken together these data demonstrate that FADD and caspase-8 are not recruited to the same TNF-R1 signaling complex that recruits TRADD, RIP and TRAF2.

The TNF-R Complexes in HeLa and U937 Cells Do Not Recruit FADD or Caspase-8—We wished to determine whether the failure of the TNF-R1 signaling complex to recruit FADD and caspase-8 was restricted to Jurkat cells. Exposure of HeLa cells, which express TNF-R1, and U937 cells, which express both TNF-R1 and TNF-R2, to TNF resulted in formation of TNF-R signaling complexes, which recruited TRADD, RIP, and TRAF2, but not FADD or caspase-8 (Fig. 5). In the TNF-R signaling complex in U937 cells, modification of TRAF2 was evident, but RIP did not appear to be modified (Fig. 5*A*, *lanes 6–8*), whereas in HeLa cells both RIP and TRADD were modified (Fig. 5*B*, *lanes 3–4*). Thus the native TNF-R signaling

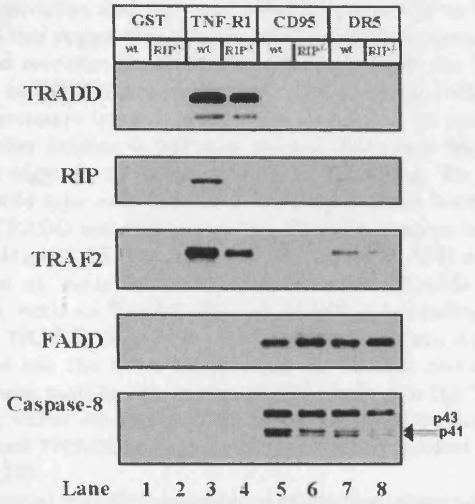

FIG. 6. **The intracellular domains of CD95 and TRAIL-R2 but not TNF-R1 bind FADD and caspase-8 in an *in vitro* interaction assay.** GST-TNF-R1, -CD95, and -TRAIL-R2 (*DR5*) fusion proteins (10 μg) bound to Glutathione-Sepharose beads were incubated in parental (*wt*) and RIP-deficient ($RIP^{-/-}$) Jurkat cell lysates (5 mg at 10 mg/ml) at room temperature for 20 h. After washing with PBS, receptor intracellular domain-interacting proteins were eluted by boiling beads in SDS sample buffer and analyzed by Western blotting. Control pulldowns were carried out with purified GST alone.

complexes, isolated from Jurkat, HeLa, and U937 cells, do not contain FADD or caspase-8.

The Intracellular Domains of CD95 and TRAIL-R2 but Not TNF-R1 Interact with FADD and Caspase-8—To further understand the role of FADD in TNF-R1 signaling, the intracellular domains of TNF-R1, CD95, and TRAIL-R2 were labeled with an N-terminal GST tag. The *in vitro* interactions of these proteins with lysates from wild type and RIP-deficient Jurkat cells were then studied. The intracellular domains from both CD95 and TRAIL-R2 interacted with FADD and caspase-8 but not with TRADD or RIP (Fig. 6, *lanes 5–8*). A small amount of TRAF2 was associated with the intracellular domain of TRAIL-R2 (Fig. 6, *lanes 7* and *8*). In contrast, the intracellular domain of TNF-R1 interacted with TRADD, RIP, and TRAF-2 but not FADD or caspase-8 (Fig. 5, *lanes 3* and *4*), in agreement with the results from TNF-treated cells. Little difference was observed between lysates from wild type or RIP-deficient cells in any of the *in vitro* interactions (Fig. 5). No modification of TRAF2 or RIP was observed in the *in vitro* interactions (Fig. 5), as the cofactors required for such modifications were unlikely to be optimal in cell lysates. The lack of interaction of FADD or caspase-8 with GST-TNF-R1 further supports the hypothesis that the role of these molecules in TNF-mediated cytotoxicity is clearly different from their role in TRAIL- and CD95- induced apoptosis.

DISCUSSION

FADD and Caspase-8 Are Not Components of the TNF-R1 Signaling Complex—Clearly the most significant finding of the present study was that FADD and caspase-8 were not recruited to the TNF-R1 signaling complex, whereas they are recruited to the TRAIL DISC (Figs. 1–5). The inability to detect formation of a stable complex of TNF-R1 with FADD and caspase-8 under the conditions employed to monitor complex formation with signaling proteins involved in NF-κB activation has been reported previously (42). It is possible that the presence of RIP within the TNF-R1 signaling complex, could have either prevented or decreased the recruitment of FADD and caspase-8 by competing for binding to TRADD (18). However, this was clearly not the primary reason for the inability to detect FADD or caspase-8, as they were still not recruited in RIP-deficient cells (Fig. 4). Thus, our data do not support the hypothesis that FADD and caspase-8 are recruited to a membrane-bound TNF complex in the same way as occurs with the signaling complexes formed by CD95 and TRAIL. Could it be that the interaction of FADD with the membrane-bound TNF receptor complex is just very weak? This appears unlikely, as the reported TRADD/FADD interaction is particularly strong (20), and our mild cell lysis conditions did not affect either TRAIL receptor-FADD interaction or TNF-R1-TRADD binding, both of which are examples of DD-DD interactions.

However, we show that both FADD and caspase-8 are essential for TNF-induced apoptosis (Fig. 3) in agreement with others (15–17). How then may caspase-8 be activated in response to TNF? In unstimulated cells, TNF-R1 is primarily found in the *trans*-Golgi network and in caveolae-like domains, whereas TRADD is loosely associated with the Golgi but not with the *trans*-Golgi network (43–45). Following TNF binding, TRADD rapidly associates with TNF-R1 at the plasma membrane (20). Subsequent internalization of the TNF-R1 complex then results in dissociation of TRADD, possibly as a result of ligand dissociation in the acidic environment of the endosomes (43). As a result any subsequent interactions of dissociated TRADD or TRADD-associated proteins would not be detected by the methods used in our study or by direct immunoprecipitation of TNFR-1. Thus, following internalization, it is possible that TRADD interacts with FADD, forming a separate complex, which in turn activates caspase-8. Some support for this is provided by the formation at later times (after 60 min treatment with TNF) of very small amounts of a detergent-resistant complex of FADD and TRADD in TNF-treated HeLa cells (46). In addition, aggregates containing FADD and caspase-8 are formed within 15 min of TNF treatment of Madin-Darby canine kidney cells (47). In this study, it was proposed that myosin II motor activities control the translocation of TNF-R1 to the plasma membrane, thereby regulating TNF-induced apoptosis. Further support for this hypothesis is that unlike CD95, internalization of TNF receptors is required for its cytotoxic activity (48, 49) but not for activation of TNF-mediated signaling pathways, such as JNK activity. Taken together these studies and our present results highlight the possibility that, following TNF treatment, recruitment of FADD and activation of caspase-8 may occur in a separate distinct complex following ligand dissociation rather than occurring directly in a membrane-associated DISC as observed with TRAIL or CD95.

Role of the TNF-R1 Signaling Complex in TNF-induced Necrosis—Under some circumstances ligation of death receptors can result in induction of RIP-dependent necrosis (50, 51). In agreement with these studies, we observed a caspase-independent necrotic cell death in TNF-treated but not in TRAIL-treated FADD-deficient Jurkat cells (Fig. 3). Interestingly, no differences were observed in the TNF-R1 signaling complexes isolated in wild type and FADD-deficient Jurkat cells (data not shown). Thus the commitment of the cell to die by apoptosis or necrosis was not determined by formation of the initial TNF-R1 signaling complex but rather at some later stage. Except for the involvement of RIP (50), little is known about the mechanism by which TNF induces necrotic cell death, but it may involve a role for lysosomes, reactive oxygen species or other proteases, such as cathepsins or granzymes (52, 53).

Binding and Modification of TNF-R1 Adaptor Proteins—RIP and TRADD interact strongly and it has been proposed that RIP is recruited indirectly to TNF-R1 through interaction with TRADD and not through a direct homophilic DD interaction (18). The finding that ectopic expression of TRADD results in

NF-κB activation and increased recruitment of RIP to TNF-R1 supports this suggestion (12, 18). Our results demonstrating an enhanced recruitment of TRADD and TRAF2 to the TNF-R1 complex in RIP-deficient compared with parental Jurkat cells (Fig. 4, compare *lanes 7* and *8* with *4* and *5*) is in agreement with earlier studies in RIP null murine embryonic fibroblasts (26) and suggests an alternative mode of binding. We propose that in wild type cells there may be a competition between the DDs of TRADD and RIP to bind the TNF-R1 complex following TNF treatment. In the absence of RIP, more TRADD is able to bind and so leads to an increase in other TRADD-binding proteins, such as TRAF2 (Fig. 4). If RIP was binding solely through TRADD, then in the RIP-deficient cells one would not expect to see the observed increase in TRADD recruitment. Thus, there may be two modes of RIP binding to the TNF-R1 complex; either directly through binding of its DD to the DDs of aggregated TNF-R1 or as generally believed by indirect binding via TRADD.

Of interest was the extensive modification observed of RIP and TRAF2 following recruitment to the TNF-R1 receptor complex in different cells (Figs. 1, 2, 4, and 5). Such modification has been previously reported, and although its nature and significance were not characterized, it was proposed to be characteristic of ubiquitination (27, 41). We were unable to confirm this using various ubiquitin antibodies, most probably due to their low sensitivity. However, an increase in modified RIP was observed when TNF complexes were isolated in the presence of the proteasome inhibitor MG132 (data not shown), strongly suggesting ubiquitin modification. If confirmed, ubiquitination of RIP would suggest interesting parallels between TNF- and IL-1-induced NF-κB activation. Both RIP and IRAK1 (IL-1 receptor-associated kinase 1) are DD-containing kinases required for TNF- and IL-1-induced NF-κB activation, respectively, although the kinase function of both is dispensable for NF-κB activation (18, 22, 26, 54). RIP3, another member of the RIP family, is recruited to TNF-R1, and phosphorylates RIP (55). Similarly IRAK4, another member of the IRAK family, phosphorylates IRAK1. Phosphorylation of IRAK1 and RIP promotes IL-1-induced or attenuates TNF-mediated NF-κB activation, respectively, and these events play important, if somewhat opposing, roles in the regulation of NF-κB activation. Whether RIP is actually ubiquitinated and degraded like IRAK1, and the relationship of these effects to TNF-mediated NF-κB activation is currently under investigation. Currently, the E3 ligase for IRAK1 is unknown. However, in the TNF-R1 signaling pathway, there are several potential E3 ligases for RIP, such as cellular inhibitor of apoptosis 1 (cIAP-1) and TRAF2, both of which are recruited to the TNF-R1 signaling complex and possess E3 ligase activity (56, 57).

In summary, we have shown that FADD and caspase-8 are not recruited to the TNF-R1 signaling complex, whereas they are recruited to the TRAIL DISC. These findings highlight that the mechanism for caspase-8 activation in TNF-induced apoptosis is clearly different from the commonly accepted mechanism of initial recruitment of TRADD, followed by binding of FADD and subsequent activation of caspase-8 in a membrane-bound DISC. The precise mechanism of caspase-8 activation and the role of FADD in TNF-induced apoptosis are currently under investigation.

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Protein Kinase C Modulates Tumor Necrosis Factor-related apoptosis-inducing ligand-induced Apoptosis by Targeting the Apical Events of Death Receptor Signaling*

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We have further examined the mechanism by which phorbol ester-mediated protein kinase C (PKC) activation protects against tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity. We now report that activation of PKC targets death receptor signaling complex formation. Pre-treatment with 12-*O*-tetradecanoylphorbol-13-acetate (PMA) led to inhibition of TRAIL-induced apoptosis in HeLa cells, which was characterized by a reduction in phosphatidylserine (PS) externalization, decreased caspase-8 processing, and incomplete maturation and activation of caspase-3. These effects of PMA were completely abrogated by the PKC inhibitor, bisindolylmaleimide I (Bis I), clearly implicating PKC in the protective effect of PMA. TRAIL-induced mitochondrial release of the apoptosis mediators cytochrome *c* and Smac was blocked by PMA. This, together with the observed decrease in Bid cleavage, suggested that PKC activation modulates apical events in TRAIL signaling upstream of mitochondria. This was confirmed by analysis of TRAIL death-inducing signaling complex formation, which was disrupted in PMA-treated cells as evidenced by a marked reduction in Fas-associated death domain protein (FADD) recruitment, an effect that could not be explained by any change in FADD phosphorylation state. In an *in vitro* binding assay, the intracellular domains of both TRAIL-R1 and TRAIL-R2 bound FADD: activation of PKC significantly inhibited this interaction suggesting that PKC may be targeting key apical components of death receptor signaling. Significantly, this effect was not confined to TRAIL, because isolation of the native TNF receptor signaling complex revealed that PKC activation also inhibited TNF receptor-associated death domain protein recruitment to TNF-R1- and TNF-induced phosphorylation of IκB-α. Taken together, these results show that PKC activation specifically inhibits the recruitment of key obligatory death domain-containing adaptor proteins to their respective membrane-associated signaling complexes, thereby modulating TRAIL-induced apoptosis and TNF-induced NF-κB activation, respectively.

Death receptors belong to a subgroup of the tumor necrosis factor (TNF)[1] receptor/nerve growth factor superfamily. Members of this subfamily are characterized by a number of conserved cysteine-rich repeats within the extracellular domain and the presence of a "death domain" (DD) motif within the intracellular domain. The DD was first identified in the intracellular domain of TNF receptor-1 (TNF-R1) and was described as a region of ~80 amino acids containing a number of key residues that appeared to be critical for TNF-mediated cytotoxicity. These residues also appear to be highly conserved within other members of the death receptor subfamily (1).

TNF-related apoptosis-inducing ligand (TRAIL) was identified because of its high sequence homology to other TNF family members (2, 3). The TRAIL receptor family is unusually complex in that it consists of at least four membrane-bound members. TRAIL-R1 (DR4) and -R2 (DR5, Killer, and TRICK2) are death receptors and contain a DD motif within the intracellular domain similar to CD95/Fas/APO-1 and TNF-R1 (1). Two other receptors TRAIL-R3 (DcR1, LIT, and TRID) and -R4 (DcR2 and TRUNDD) lack or contain an incomplete DD and are therefore incapable of inducing apoptosis. Ectopic expression of TRAIL-R3 and -R4 has been demonstrated to attenuate TRAIL-induced apoptosis, and they are therefore often described as "decoy receptors" (4).

TRAIL signals apoptosis through formation of a death-inducing signaling complex (DISC). Ligation of TRAIL-R1 and -R2 by TRAIL leads to rapid recruitment of the adaptor protein FADD/MORT1 (5–7). FADD is a bipartite molecule with an N-terminal DD and a C-terminal death effector domain and appears to be a universal adaptor protein, because it is required for cytotoxicity induced by other death receptor subfamily members (8, 9). FADD binding to the receptor occurs through a homophilic DD interaction while the death effector domain mediates the interaction with similar motifs in the prodomain of the initiator caspase, caspase-8. Procaspase-8 is activated at the DISC by an autocatalytic mechanism, which is dependent on the proximity of other procaspase-8 molecules (10). Following activation, caspase-8 mediates apoptosis either by the direct activation of downstream effector caspases or through cleavage of pro-apoptotic molecules such as the Bcl-2 homolog, Bid (11, 12).

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[1] The abbreviations used are: TNF, tumor necrosis factor; mAb, monoclonal antibody; DD, death domain; DISC, death-inducing signaling complex; FADD, Fas-associated death domain protein; GST, glutathione *S*-transferase; PARP, poly(ADP-ribose) polymerase; PS, phosphatidylserine; RIP, receptor-interacting protein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF-R1, TNF receptor 1; TRAIL-R, TRAIL receptor; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2; IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Bis I, bisindolylmaleimide I; b, biotin; PBS, phosphate-buffered saline; ICD, intracellular domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; JNK, c-Jun N-terminal kinase; FAP-1, Fas-associated phosphatase-1; BTK, Bruton's tyrosine kinase.

The importance of TRAIL as a potential therapeutic agent became obvious when it was demonstrated to be selectively toxic to transformed and tumor cells but not to the majority of normal cells (1–3). These observations were confirmed in a number of *in vivo* studies where TRAIL was effective at reducing solid tumor growth and inducing regression of tumor cell xenografts (13–15) in the absence of the systemic toxicity observed with anti-Fas/CD95 or TNF treatment (16, 17). The mechanism of this differential sensitivity was initially attributed to the presence of TRAIL "decoy receptors" on normal cells, however, recent studies have failed to correlate decoy receptor expression with TRAIL sensitivity (18, 19), and there has been growing evidence to suggest that TRAIL resistance may instead be regulated intracellularly.

Several intracellular proteins have been implicated in providing resistance to TRAIL-induced apoptosis. The inactive caspase-8 homolog, c-FLIP, interferes with the activation of procaspase-8 at the DISC (20), with high basal FLIP expression often correlating with resistance to TRAIL. In support of this, modulation of c-FLIP levels by metabolic inhibitors has been demonstrated to sensitize some of these resistant cell types to TRAIL-induced apoptosis (18, 21). Other inhibitors include the inhibitor of apoptosis proteins (IAPs), in particular X-linked IAP (XIAP), which blocks apoptosis by directly inhibiting effector caspases (22), and the Bcl-2 family members, Bcl-2 and Bcl-x_L, which protect cells by inhibiting the mitochondrial changes that lead to activation of the Apaf-1/caspase-9 "apoptosome" complex (23).

Pro-survival signaling pathways that have been implicated in TRAIL resistance include activation of the transcription factor NF-κB (24, 25), which in the case of TNF has been shown to act by up-regulating several anti-apoptotic genes, including c-IAP1 and -2 (26). More recently, we have shown that TRAIL sensitivity can be modulated by activation or inhibition of NF-κB and that, in some contexts, modulation of NF-κB activation at the DISC may be a key determinant of sensitivity to TRAIL-induced apoptosis (27). Recent observations have also implicated the protein kinase C (PKC) pathway in the protection of cells from apoptosis in a number of systems, including those induced by death receptor ligation (28–31). Activation of PKC abrogates CD95-induced apoptosis through inhibition of cell shrinkage and K^+ efflux (28), but additionally modulates CD95 DISC formation by blocking FADD recruitment and thus caspase-8 activation (29, 30). Activation of PKC has also been reported to inhibit TRAIL-induced apoptosis (30, 31), but in this case the block was suggested to occur either at the level of proteolytic cleavage of procaspase-8 (30) or downstream of caspase-8-mediated Bid cleavage (31). These observations prompted us to further examine the mechanism by which 12-*O*-tetradecanoylphorbol-13-acetate (PMA)-mediated PKC activation modulates TRAIL-induced cytotoxicity.

In the present study, we demonstrate that pre-treatment with PMA inhibits TRAIL-induced apoptosis in HeLa cells. TRAIL DISC formation was found to be disrupted in PMA-treated cells as judged by a reduction in recruitment of the obligatory adaptor molecule FADD and the initiator caspase-8. These effects of PMA were completely abrogated by the PKC inhibitor, bisindolylmaleimide I (Bis I), thus implicating a key role for PKC in the modulation of DISC components. Further support for this was provided by the observation that formation of the TNF receptor signaling complex was similarly affected with a decrease in TRADD recruitment being evident in PMA-treated cells. In summary, our results demonstrate that PKC activation modulates the ability of obligatory death domain-containing receptor signaling components to interact with one another, thus providing another potential mechanism whereby cells can modulate TNF-induced NF-κB activation and TRAIL-mediated cytotoxicity, respectively.

EXPERIMENTAL PROCEDURES

Materials—Bisindolylmaleimide I (GF 109203X) and PMA were purchased from Calbiochem (Nottingham, UK) and Sigma (Poole, UK), respectively. Recombinant human TRAIL (residues 95–281) was produced as previously described (32), and recombinant human TNF (residues 55–233) was generated using the same methods (33). Antibodies were sourced as follows: anti-FADD mAb and anti-TRADD mAb were from BD Transduction Laboratories (Heidelberg, Germany); anti-RIP mAb and anti-TRAF2 mAb were from BD Pharmingen; anti-poly(ADP-ribose) polymerase (PARP) mAb and anti-TRAIL-R1 and -R2 were from Alexis Corp. (Nottingham, UK); rabbit anti-Bid, rabbit anti-IκBα, and rabbit anti-phospho-IκBα (Ser 32) were from Cell Signaling (Beverley, MA). Anti-caspase-3 was a kind gift from Dr. D. Nicholson (Merck Frosst, Quebec, Canada), and the caspase-8 mAb (C-15) (34) was a kind gift from Dr. P. H. Krammer (German Cancer Research Center, Heidelberg, Germany). TRAIL receptor antibodies (clones M271, M413, M430, and M444, respectively), used for flow cytometry, were a kind gift from Immunex Corp. (Seattle, WA) (35). Horseradish peroxidase-conjugated secondary antibodies, goat anti-mouse and goat anti-rabbit, were obtained from Sigma and DAKO (Cambridge, UK), respectively. All other chemicals were of analytical grade and were from Sigma or Fisher (Loughborough, UK).

Cell Culture—All cell culture materials were from Invitrogen (Paisley, UK) and plasticware from BD Biosciences Labware (Bedford, MA). HeLa cells (ECCAC, Wiltshire, UK) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and maintained at 37 °C with 5% CO_2 in a humidified atmosphere by routine passage every 3 days.

Determination of Apoptosis by Annexin V Staining—Apoptotic cells were quantified by measuring externalized phosphatidylserine (PS) assessed by Annexin V labeling and by propidium iodide uptake as described previously (36).

Western Blotting—SDS-PAGE and immunoblotting were carried out as described previously (27).

Analysis of Cytochrome c and Smac Release from Mitochondria—Mitochondrial release of cytochrome *c* and Smac was performed using a digitonin lysis method essentially as described previously (37, 38).

Precipitation of the TRAIL DISC and TNF Signaling Complex—DISC precipitation was performed using biotin-labeled recombinant TRAIL (bTRAIL) or TNF (bTNF) essentially as described previously (7, 27, 33), with the following modifications: cells were lysed with "lysis buffer" containing 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100 (v/v), 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 mM NaF, containing Complete™ protease inhibitors (Roche Applied Science), and streptavidin-Sepharose™ beads were used to precipitate TRAIL·TNF receptor complexes (Amersham Biosciences).

Analysis of TRAIL Receptors by Flow Cytometry—Control and treated HeLa cells were subjected to limited trypsinization, resuspended in blocking buffer (10% normal goat serum in phosphate-buffered saline (PBS)) and incubated for 30 min on ice to block nonspecific antibody binding. Cells were then labeled with anti-TRAIL receptor antibodies and analyzed by flow cytometry as described previously (39).

Analysis of TRAIL Receptor Aggregation—To observe aggregated TRAIL receptors, DISCs were precipitated as described above and subjected to gel electrophoresis in the absence of reducing agents followed by immunoblotting with specific antibodies as has been described previously (40–42). Receptor aggregates appeared as higher molecular weight bands, corresponding to either dimers or trimers of the respective TRAIL receptors.

Expression of GST Fusion Proteins and in Vitro Interactions—GST fusion proteins were created by fusing glutathione *S*-transferase (GST) to the N terminus of the intracellular domains (ICDs) of TRAIL-R1 (residues 269–468) and TRAIL-R2 (residues 209–411). DNA encoding these residues was cloned in-frame into the *Eco*RI and *Xho*I sites of pGEX4T (Amersham Biosciences, Bucks, UK). Receptor fusion proteins were overexpressed in XA-90 cells, kindly provided by Prof. D. Riches (National Jewish Medical and Research Center, Colorado), by inducing with 1 mM isopropyl-D-thiogalactoside for 3 h, and cells were then lysed by sonication in 1.5% (w/v) Sarkosyl, containing 5 mM dithiothreitol and Complete™ protease inhibitors (Roche Applied Science). The dialyzed lysate was bound to 1.5 ml of washed glutathione-Sepharose beads (50% slurry in PBS) for 1 h at 4 °C, the beads were washed twice in ice-cold PBS, and the amount of purified GST fusion protein was quantified by Coomassie Blue staining and comparison with bovine serum albumin

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standards. To assess GST-TRAIL-R-ICD protein binding, HeLa cells were either left untreated or treated with PMA (20 ng/ml) for 30 min, then washed once with PBS and harvested by trypsinization. Cell pellets were resuspended in 3 ml of lysis buffer (see above) and incubated on ice for 45 min. Lysates were cleared by centrifugation, and aliquots of the supernatant containing 5 mg of protein (10 mg/ml) were incubated for 16 h at 4 °C with 10 μg of purified GST-ICD fusion proteins bound to Sepharose beads. Control pull-down assays were carried out using beads coated with purified GST alone. Bound proteins were pelleted by centrifugation at 200 × *g* for 3 min, washed five times in PBS containing protease inhibitors, and released from beads by boiling for 5 min in SDS sample buffer. The interaction of FADD with GST-TRAIL-R-ICDs was assessed by Western blotting.

Analysis of FADD by Two-dimensional Gel Electrophoresis—Two-dimensional electrophoresis was carried out using the IPGphor system (Amersham Biosciences). Briefly, TRAIL DISC samples were resuspended in rehydration buffer (8 M urea, 2% CHAPS, 2% IPG buffer, pH 4–7, trace bromphenol blue) and mixed at room temperature for 4 h. Samples were then applied to IPG (Immobilized pH Gradient) strips with a pH range of 4–7. Strips were then subjected to active rehydration at 50 V for 12 h followed by isoelectric focusing (500 V for 30 min, 1000 V for 30 min, then 8000 V for 1 h). After focusing, strips were equilibrated in SDS equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 30% (w/v) glycerol, 2% SDS, trace bromphenol blue) for 15 min at room temperature. Second dimension electrophoresis and Western blotting were then carried out as described above.

RESULTS

PMA Inhibits TRAIL-induced Apoptosis in HeLa Cells—To further elucidate the role of PKC in death receptor-induced apoptosis, HeLa cells were treated with the PKC activator, PMA, and its effect on TRAIL-induced apoptosis was assessed. Pre-treatment of HeLa cells with 20 ng/ml PMA completely abrogated TRAIL-induced apoptosis as measured by inhibition of both phosphatidylserine (PS) exposure and TRAIL-induced cleavage of the caspase-3/-7 substrate PARP (Fig. 1*A*). Treatment with PMA alone was not cytotoxic to HeLa cells at the concentration used (data not shown).

To investigate the mechanism by which PMA protects cells from TRAIL, cell pellets were subjected to Western blotting for a number of components of the TRAIL receptor signaling pathway. Caspase-8 is known to be the apical caspase activated during TRAIL-induced apoptosis and is recruited and processed at the DISC in a FADD- and TRAIL-dependent manner (5–7). The caspase-8 zymogen exists as two splice forms, p55 and p53, which are activated in a two-step process involving the removal of the small subunit, p12, to generate p43 and p41 intermediates. A second cleavage then results in the removal of the large p18 subunit (43, 44). When HeLa cells were treated with PMA for 30 min prior to the addition of TRAIL, there was a marked reduction in the activation of caspase-8 as judged by a decrease in generation of the large (p18) subunit and a concomitant increase in the amount of procaspase-8 (Fig. 1*B*).

During receptor-mediated apoptosis procaspase-3 is processed by active caspase-8 between its large and small subunits to generate a p20/p12 caspase-3 intermediate. The large subunit (p20) then undergoes an autocatalytic activation step resulting in removal of the prodomain and generation of the fully mature p17 form (45). In TRAIL-treated HeLa cells caspase-3 was processed to its fully mature p17 subunit (Fig. 1*B*). However, although caspase-3 activity was inhibited in PMA-treated cells, as judged by inhibition of PARP processing (Fig. 1*A*), caspase-3 was still processed but only to its p20 intermediate (Fig. 1*B*). The presence of the p20, but not the p17, subunit suggested that maturation of the p20 subunit of caspase-3 was inhibited in cells pre-treated with PMA.

The pro-apoptotic Bcl-2 family member, Bid, is another known caspase-8 substrate. Cleavage of full-length Bid (p22) leads to the generation of a truncated form (p15, tBid), which is then is capable of triggering mitochondrial disruption via a Bax- and/or Bak-dependent process, ultimately leading to amplification of death receptor signaling through engagement of the Apaf-1/caspase-9 apoptosome pathway (11, 12, 46). Bid was found to be processed in TRAIL-treated cells as judged by loss of the intact form. PMA pre-treatment markedly reduced this loss of Bid (Fig. 1*B*), in agreement with the PMA-mediated inhibition of caspase-8 processing. Thus, PMA-mediated protection against TRAIL-induced apoptosis is characterized by inhibition of PS externalization, a reduction in caspase-8 activation, reduced cleavage of the pro-apoptotic Bcl-2 homolog, Bid, and incomplete maturation and activation of caspase-3.

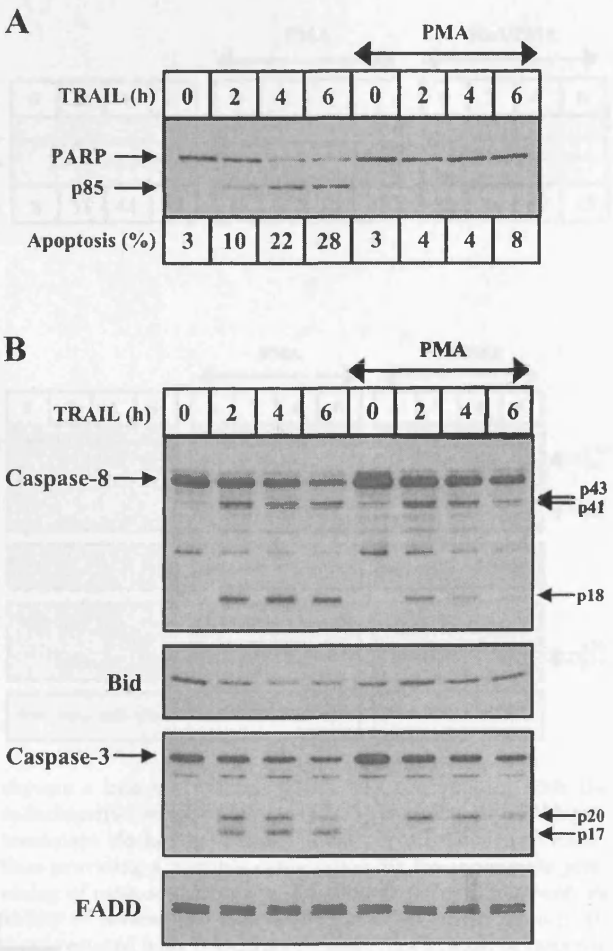

FIG. 1. **PMA inhibits TRAIL-induced apoptosis in HeLa cells.** HeLa cells were pre-treated with PMA (20 ng/ml) for 30 min prior to exposure to TRAIL (1 μg/ml) for the indicated time periods. *A*, apoptosis was assessed by Annexin V staining and flow cytometry as described under "Experimental Procedures" and by Western blotting using an antibody to the caspase-3/-7 substrate PARP. *B*, cells were further analyzed by Western blotting using antibodies to caspases-8 and -3 and the caspase-8 substrate Bid. FADD was used as a protein loading control and migrates as a doublet representing the phosphorylated and non-phosphorylated forms of this protein.

Inhibition of PKC Can Reverse PMA-mediated Resistance to TRAIL-induced Apoptosis—To determine whether the inhibition of apoptosis and the changes observed following pre-treatment with PMA were the result of PKC activation, HeLa cells were preincubated with the PKC inhibitor, bisindolylmaleimide I (Bis I), prior to the addition of PMA. Bis I completely abrogated the protective effect afforded by PMA as judged by a reversal in Annexin V/PI staining (Fig. 2*A*). Preincubation with Bis I also reversed the PMA-mediated inhibition of caspase-8 processing and Bid cleavage. In addition, caspase-3 was fully processed and enzymatically active as judged by complete mat-

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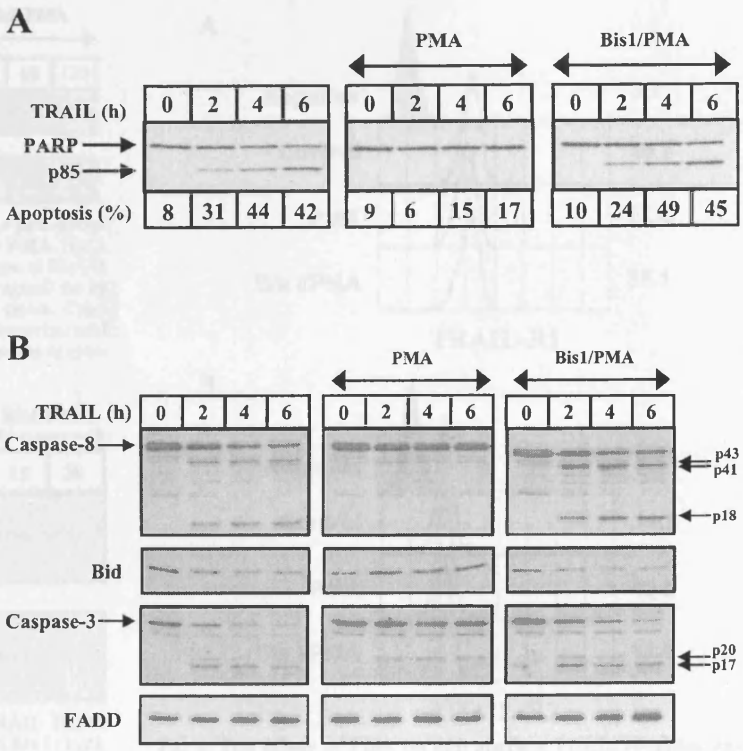

FIG. 2. **PMA-mediated inhibition of TRAIL-induced apoptosis can be reversed by a PKC inhibitor.** HeLa cells were pre-treated with the PKC inhibitor Bis I (1 μM) for 30 min prior to a 30-min incubation with PMA (20 ng/ml) before the addition of TRAIL (1 μg/ml). *A*, apoptosis was assessed by Annexin V staining and by Western blotting using an antibody to PARP. *B*, activation of procaspases-8 and -3 and cleavage of Bid was assessed by Western blotting as described under "Experimental Procedures." FADD was used as a protein loading control.

uration of the p20 subunit to p17 (Fig. 2*B*) and cleavage of PARP (Fig. 2*A*). These data demonstrate that the protection observed with PMA pre-treatment was indeed mediated by PKC.

Several other reports have demonstrated that protein kinase C inhibitors, including the bisindolylmaleimides, can potentiate FasL/CD95L-induced apoptosis (29, 30, 47, 48). However, in our model, though Bis1 was able to specifically reverse the effects of PMA, co-treatment of cells with Bis I and TRAIL did not significantly potentiate TRAIL-induced apoptosis (data not shown). AQ: K

Pre-treatment with PMA Blocks TRAIL-induced Mitochondrial Release of Cytochrome c and Smac/DIABLO—The lack of PARP cleavage observed with PMA pre-treatment (Fig. 1*A*) strongly suggested that there was no active caspase-3 within these cells. Despite this, caspase-3 was clearly processed, albeit incompletely to its p20 intermediate (Fig. 1*B*). Several recent reports have provided a possible explanation for these results. In these studies, the protection afforded by either Bcl-2 or Bcl-x_L overexpression, lack of Bax, or by an unknown component in TRAIL-resistant melanomas led to incomplete caspase-3 activation in response to TRAIL or CD95L (38, 49–51). Lack of TRAIL- and/or CD95L-induced release of cytochrome *c*, and the mitochondrial XIAP antagonist Smac led to accumulation of the p20 subunit of caspase-3, which was subsequently shown to be inhibited by XIAP. By analogy, in our model, any PMA-mediated increase in XIAP levels would be predicted to lead to a similar accumulation of the p20 form of caspase-3. However, this is unlikely to be the case, because others have reported that PMA does not modulate the level of XIAP (41). Another possibility was that PMA was instead inhibiting TRAIL-induced release of cytochrome *c* or the IAP antagonist, Smac, either directly or indirectly through inhibition of caspase activation upstream of the mitochondria.

To investigate this, the effect of PMA pre-treatment on TRAIL-induced mitochondrial perturbation was assessed. TRAIL treatment led to a time-dependent release of cytochrome *c* into the cytosol, which was concomitant with the mitochondrial release of Smac (Fig. 3). Significantly, PMA pre-treatment blocked the release of both cytochrome *c* and Smac, thus providing a possible explanation for the incomplete processing of caspase-3 in our model (Fig. 1*B*). Consistent with its ability to reverse the effects of PMA (Fig. 2), Bis I pre-treatment restored both cytochrome *c* and Smac release in response to TRAIL (Fig. 3). Thus, the ability of PMA to inhibit TRAIL-induced release of cytochrome *c* and Smac, together with the decrease observed in caspase-8 activation and Bid cleavage, strongly suggested that PMA was targeting the apical events of TRAIL signaling, upstream of mitochondria. F3

PMA Pre-treatment Inhibits the Recruitment of FADD and Caspase-8 to the TRAIL DISC—During death receptor-mediated apoptosis, caspase-8 can be processed by two mechanisms. The first is the activation of procaspase-8 as the apical caspase in a FADD and ligand-dependent manner at the DISC (44, 52, 53). The second, which is redundant if the first is inhibited, is mediated via a cytochrome *c*/Smac-mediated amplification loop in which caspase-3 indirectly activates procaspase-8 through a caspase-6-dependent mechanism (54). The reduction in caspase-8 processing observed with PMA pre-treatment (Figs. 1*B* and 2*B*) could, therefore, be a consequence of inhibition of this second pathway as judged by the effects of PMA on TRAIL-induced release of cytochrome *c* and Smac (Fig. 3). However, the PMA-mediated reduction in cleavage and/or full processing of two known caspase-8 substrates, namely Bid and caspase-3 (Figs. 1*B* and 2*B*), suggested to us that PMA was instead modulating the initial activation of caspase-8.

To address this directly, we investigated the effect of PMA on DISC formation, the apical event in TRAIL-induced caspase activation. Binding of TRAIL resulted in rapid recruitment of the adaptor protein FADD to the DISC (Fig. 4). Recruitment of FADD was concomitant with the presence of caspase-8 within the DISC precipitates. When TRAIL DISCs were isolated from cells pre-treated with PMA there was a marked reduction in FADD, and consequently caspase-8, recruitment. Consistent F4

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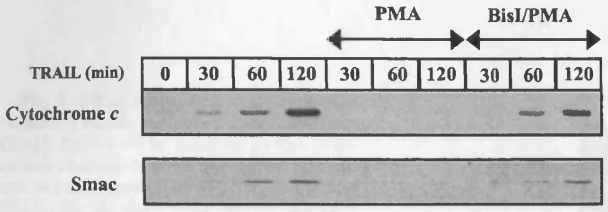

FIG. 3. **TRAIL-induced mitochondrial release of the pro-apoptotic molecules cytochrome *c* and Smac is blocked by PMA.** HeLa cells were preincubated for 30 min in the presence or absence of Bis I (1 μM). Cells were then pre-treated with or without PMA (20 ng/ml) for 30 min before exposure to TRAIL (1 μg/ml) for the indicated times. Cytosolic fractions were then isolated as described under "Experimental Procedures" and analyzed by Western blotting for the presence of cytochrome *c* and Smac.

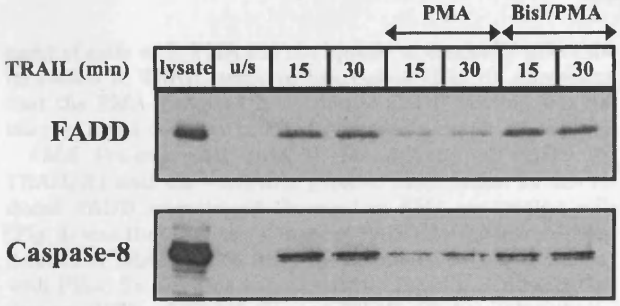

FIG. 4. **PMA inhibits FADD recruitment to the TRAIL DISC.** HeLa cells (3×10^7/treatment) were either pre-treated with Bis I (1 μM, 30 min) followed by PMA (20 ng/ml), PMA alone, or vehicle alone followed by bTRAIL (1 μg/ml). After the indicated times TRAIL receptor complexes (DISCs) were isolated as described under "Experimental Procedures." The addition of beads alone to unstimulated cell lysates (*u/s*) was used to control for nonspecific interactions. Cell lysates, prior to receptor complex isolation, and receptor complexes were then analyzed for the presence of FADD and caspase-8.

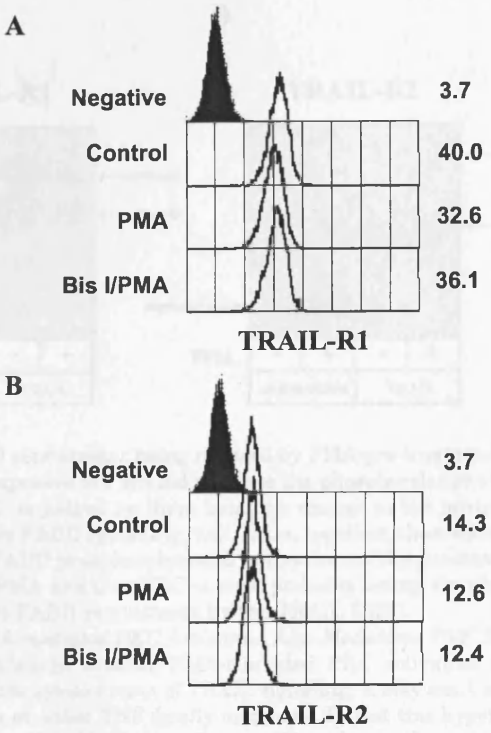

FIG. 5. **The effect of PMA on cell surface TRAIL receptor expression.** HeLa cells were incubated either alone (*Control*) or with PMA (20 ng/ml) for 30 min, in the presence or absence of Bis I (1 μM, 30 min). Cells were then harvested, and cell surface receptor expression was assessed by flow cytometry using monoclonal antibodies to TRAIL-R1 (*A*) or TRAIL-R2 (*B*) as described under "Experimental Procedures." Cells labeled with secondary antibody alone were used to control for background fluorescence (*Negative*). Values shown represent the mean fluorescence intensity.

with that shown previously in whole cell lysates (Fig. 2) preincubation of these cells with the PKC inhibitor, Bis I, reversed the effects of PMA and restored FADD and caspase-8 within the DISC (Fig. 4).

Clearly the observed reduction in FADD and caspase-8 recruitment to the DISC provides a mechanism whereby PMA pre-treatment may protect cells from TRAIL. This, together with the ability of Bis I to inhibit these effects, implicates a major role for PKC in modulating the apical events of TRAIL signaling.

PMA Pre-treatment Does Not Markedly Alter Cell Surface TRAIL Receptor Expression—Protein kinase C activators, such as PMA, can protect cells from TNF-induced cytotoxicity by reducing the number of cell surface receptors available for TNF binding through a process known as "receptor shedding" (55, 56). Although, to date, there have been no reports of TRAIL receptor shedding, any decrease in cell surface TRAIL receptors would be predicted to result in a reduction in FADD recruitment and TRAIL DISC formation similar to that observed in PMA-treated cells (Fig. 4).

To investigate this possibility, HeLa cells were treated either with PMA alone, or with Bis I prior to PMA, and cell surface TRAIL receptor expression was then assessed by flow cytometry using receptor-specific antibodies (35). TRAIL-R1 was the predominant TRAIL receptor on unstimulated cells, although TRAIL-R2 was also present but at lower levels (Fig. 5). No staining for TRAIL-R3 or -R4 was observed on HeLa cells (data not shown), consistent with a number of other reports suggesting that these receptors are mainly expressed intracellularly (57). Treatment of cells with PMA resulted in a very modest decrease in cell surface TRAIL-R1 expression, which was partially recovered upon pre-treatment with Bis I (Fig. 5*A*). In contrast, levels of TRAIL-R2 were largely unaffected by treatment with PMA or by PMA in combination with Bis I (Fig. 5*B*).

The modest decrease in TRAIL receptor expression resulting from PMA pre-treatment suggested that this was not the primary mechanism by which PMA reduced TRAIL DISC formation/FADD recruitment. This led us to investigate other potential mechanisms by which PMA may modulate the TRAIL DISC.

Effect of PMA on TRAIL Receptor Aggregation—There appears to be a critical requirement for the aggregation of death receptors to initiate apoptotic signaling (58, 59). Upon triggering, CD95 oligomerizes and forms SDS-stable aggregates, which can be resolved by SDS-PAGE in the absence of reducing agents. These aggregates form almost immediately after CD95 stimulation and correspond to dimeric and trimeric forms of the receptor (40). To assess whether the ligand-induced formation of death receptor aggregates was affected by PMA pre-treatment, TRAIL DISCs were isolated and resolved by SDS-PAGE in the absence of reducing agents followed by immunoblotting with TRAIL-R1 and -R2-specific antibodies.

Treatment with TRAIL resulted in the formation of higher molecular weight species that appeared to correspond to dimeric and trimeric forms of the respective TRAIL receptors (Fig. 6). TRAIL treatment also resulted in the loss of monomeric receptors, which is consistent with their aggregation into dimeric and trimeric forms. Formation of these higher order aggregates of TRAIL-R1 and -R2 required TRAIL binding at the cell surface, because they were largely absent in the unstimulated receptor controls (Fig. 6). Significantly, pre-treat-

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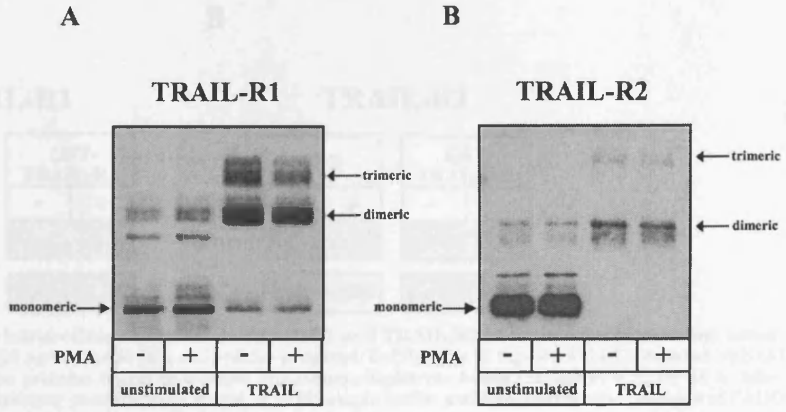

FIG. 6. **PMA does not significantly reduce TRAIL receptor aggregation.** TRAIL DISCs were isolated in the presence or absence of PMA (20 ng/ml, 30 min) and the aggregation of TRAIL-R1 (*A*) and TRAIL-R2 (*B*) then analyzed by Western blot analysis under non-reducing conditions as described under "Experimental Procedures." Aggregated receptors (labeled *dimeric* and *trimeric*) were only evident in TRAIL-treated cells.

ment of cells with PMA did not appear to markedly affect the formation of TRAIL receptor aggregates (Fig. 6), suggesting that the PMA-mediated inhibition of FADD binding was not the result of a decrease in TRAIL-induced receptor aggregation

PMA Pre-treatment Inhibits the Affinity of FADD for TRAIL-R1 and -R2—Another possible explanation for the reduced FADD recruitment observed in PMA-pre-treated cells (Fig. 4) was that that the affinity of FADD for the intracellular domain of TRAIL-R1 or -R2 was modulated by pre-treatment with PMA. To test this hypothesis, we fused the intracellular domain (ICD) of TRAIL-R1 and TRAIL-R2 to a glutathione *S*-transferase (GST) tag and expressed these GST-TRAIL-R1/2-ICD fusion proteins in *Escherichia coli*. By incubating reduced glutathione beads pre-coated with these fusion proteins in lysates from untreated or PMA-treated cells we could assess whether PMA was in any way affecting the affinity of FADD for the respective TRAIL receptor ICDs.

Consistent with our earlier findings in the native TRAIL DISC (Fig. 4), both GST-TRAIL-R1-ICD and GST-TRAIL-R2-ICD were able to precipitate FADD from control cell lysates (Fig. 7). Interestingly, when the respective TRAIL-R ICDs were incubated with lysates from PMA-treated cells, the proportion of FADD precipitated was greatly reduced. Data from this *in vitro* binding assay support the suggestion that the reduced FADD recruitment observed in TRAIL DISCs isolated from cells pre-treated with PMA may be due to a decreased affinity of FADD for the ICDs of TRAIL-R1 and -R2.

PMA Treatment Does Not Alter the Phosphorylation State of FADD—The observation that PMA-mediated PKC activation can protect against TRAIL (Figs. 1 and 2), CD95, and TNF-induced cytotoxicity suggests that PMA may be targeting a component that is common to all three of these death ligand signaling pathways. One such candidate molecule is FADD, because it functions as the universal adaptor protein for CD95-, TNF-, and TRAIL-induced cell death signaling (8, 60). Furthermore, in our model, recruitment of FADD into the TRAIL DISC was markedly reduced by pre-treatment by PMA (Figs. 4 and 7). The FADD protein exists in two forms, non- and a serine-phosphorylated (40). To assess whether PMA was in some way altering the phosphorylation state of FADD, native TRAIL DISCs were isolated and precipitates were analyzed by two-dimensional gel electrophoresis followed by Western blotting for FADD.

FADD was present as two discrete spots in control cell lysates, representing the phosphorylated (Fig. 8*A*, *right spot*) and unphosphorylated (Fig. 8*A*, *left spot*) forms of the protein. These two forms were also present in DISCs isolated from cells exposed to TRAIL, indicating that both forms of FADD are recruited to the TRAIL DISC in a ratio similar to that previously reported for the CD95 DISC (40, 44). Despite overall FADD recruitment being reduced by PMA pre-treatment (Fig. 8*B*), exposure to PMA did not alter the phosphorylation state of FADD as judged by there being no change in the intensity of the two FADD spots (Fig. 8*A*). Taken together, these data show that FADD phosphorylation is not modulated by pre-treatment with PMA and that PKC is most probably acting elsewhere to inhibit FADD recruitment by the TRAIL DISC.

PMA-mediated PKC Activation Also Modulates TNF Signaling—Clearly, because PMA-mediated PKC activation modulates the apical events of TRAIL signaling, it may exert similar effects on other TNF family members. To test this hypothesis, we investigated whether PMA could modulate those signaling events mediated by TNFα. TNF signaling differs from TRAIL and CD95 in that its predominant signaling pathway is activation of NF-κB. TNF negatively regulates its own cytotoxicity through an NF-κB-mediated survival pathway (26). Engagement of TNF-R1 by TNF leads to the recruitment of an intermediate adaptor protein, TRADD (TNF receptor-associated death domain protein), which then acts as a platform for the recruitment of other signaling molecules (61, 62). RIP and TRAF2 are required for activation of the transcription factor NF-κB, whereas FADD is reported to be responsible for apical caspase recruitment (8, 63–65). TRAF2 recruitment is also required for TNF-mediated activation of c-Jun N-terminal kinase (JNK) (65).

TNF receptor complexes were isolated using a similar method to that used previously for TRAIL, and because HeLa cells do not express TNF-R2, TNF-R1 complexes could be studied exclusively (66). Treatment of cells with TNF led to immediate recruitment of TRADD, TRAF2, and RIP to the TNF precipitates (Fig. 9*A*). Surprisingly, although TRADD was present in the precipitates as a single species, RIP and TRAF2 were subjected to some form of modification. Such modifications have been noted elsewhere and may be characteristic of polyubiquitination (33, 67, 68). However, it should be noted that only those RIP and TRAF2 molecules recruited to the receptor precipitates were modified, because both proteins migrated as single species in similarly treated whole cell lysates (Fig. 9*A*) (33). When TNF precipitates were isolated in the presence of PMA, the binding of TRADD was completely abolished (Fig. 9*A*). RIP and TRAF2 binding was also inhibited by PMA pre-treatment, consistent with their recruitment being dependent on the obligatory intermediate adaptor protein, TRADD (69, 70). Significantly, the binding of TRADD, RIP, and TRAF2 to TNF precipitates was restored when receptor complexes were isolated from cells pre-treated with Bis1 prior to the addition of PMA (Fig. 9*A*). These data clearly demonstrate that PMA-mediated PKC activation can modulate the apical events of TNF signaling.

HeLa cells are not susceptible to TNF-mediated cytotoxicity,

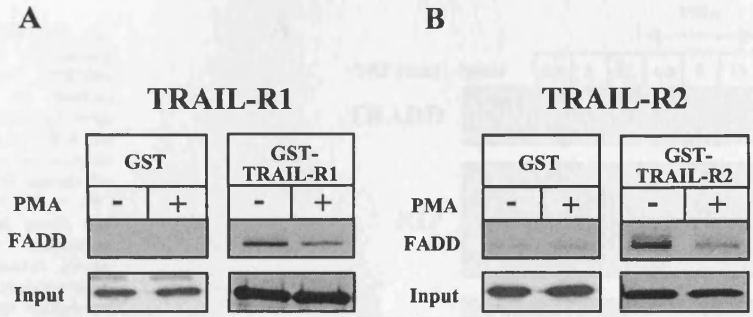

FIG. 7. **PMA inhibits FADD recruitment by the intracellular domains of TRAIL-R1 and TRAIL-R2 in an *in vitro* interaction assay.** HeLa cells were treated either with or without PMA (20 ng/ml) for 30 min and lysates prepared. Cell lysates (5 mg) were then incubated with (*A*) GST-TRAIL-R1-ICD or (*B*) GST-TRAIL-R2-ICD fusion proteins bound to reduced glutathione-Sepharose beads (10 μg) at 4 °C for 16 h. After washing with PBS, receptor intracellular domain-interacting proteins were eluted in SDS sample buffer and analyzed for the presence of FADD by Western blotting. Control pull-down assays were carried out with purified GST alone.

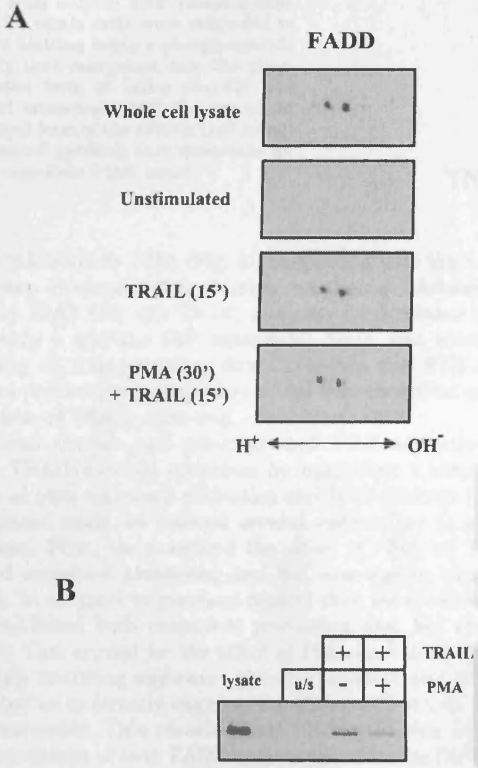

FIG. 8. **FADD phosphorylation status is not modified by pre-treatment with PMA.** HeLa cells were pre-treated with PMA (20 ng/ml, 30 min) prior to treatment with bTRAIL (1 μg/ml) for 15 min. TRAIL receptor complexes were then precipitated as described under "Experimental Procedures." *A*, FADD phosphorylation status was analyzed by assessing the mobility of FADD by two-dimensional gel electrophoresis and Western blotting. *B*, the FADD content of whole cell lysates, lysates containing beads alone (*u/s*), and the TRAIL receptor complexes was also assessed by Western blotting following one-dimensional gel electrophoresis.

even in the presence of protein synthesis inhibitors (data not shown), thus, we could not directly assess the effect of PMA on TNF-induced apoptosis. However, because both RIP and TRAF2 are required for TNF-mediated NF-κB activation, any inhibition of their recruitment to the TNF receptor complex would be predicted to impair TNF-mediated NF-κB activation. The effect of PMA on TNF-mediated NF-κB activation, specifically the inducible phosphorylation of IκB-α at Ser-32, was therefore assessed. Treatment of HeLa cells with TNF led to phosphorylation of IκB-α within 5 min, which then decreased between 10 and 15 min with some recovery of phospho-IκB-α levels evident by 60 min (Fig. 9*B*, *left panel*). The well-documented loss and subsequent recovery of phospho-IκB-α (71) was concomitant with an initial decrease in total cellular IκB-α followed by its re-synthesis at 60 min (Fig. 9*B*, *right panel*). Consistent with the observation that PMA inhibited the binding of RIP and TRAF2 to TNF-R1, PMA pre-treatment abolished TNF-induced phosphorylation of IκB-α and the subsequent reduction in total cellular IκB-α. Significantly, preincubation with the PKC inhibitor, Bis I, completely restored TNF-induced phosphorylation of IκB-α and the TNF-induced decrease and subsequent re-synthesis of IκB-α (Fig. 9*B*).

Taken together, these data suggest that PMA-mediated PKC activation modulates apical events in TNF signaling in a manner similar to that already observed for TRAIL, *i.e.* by inhibiting the recruitment of obligatory core components to the TNF receptor signaling complex, and implicates a more general role for PKC in modulating signaling by other TNF family members.

DISCUSSION

Previous reports have demonstrated that PMA-mediated PKC activation can protect cells from CD95-mediated apoptosis (29, 30, 41, 72, 73). The exact nature of this inhibition was however disputed with different groups proposing that PKC modulated CD95-induced apoptosis at different levels. Some early reports suggested that PKC activation inhibited CD95-induced apoptosis upstream of caspase-8 activation (41, 73); however, Holmstrom and colleagues (72) subsequently reported that CD95 DISC formation and caspase-8 activity were unaffected by PKC activation. More recently it has been shown that FADD recruitment by CD95 is inhibited in PMA pre-treated cells (29, 30), suggesting that PKC in some way modulates CD95 DISC formation. Although several recent reports have indicated that PKC also inhibits apoptosis induced by TRAIL (30, 31, 74, 75) and TNFα (30, 75), the mechanism of this effect is still not well understood. Moreover, it is not known whether a common mechanism could explain the inhibitory effects of PMA on the signaling pathways activated by these closely related death receptor ligands.

We show for the first time that activation of PKC leads to inhibition of TRAIL-induced apoptosis by interfering with a key apical event in TRAIL signaling, namely, recruitment of FADD to the DISC (Fig. 4). A series of observations suggested to us that PKC activation was modulating TRAIL signaling at a step prior to mitochondrial engagement. First, PKC activation blocked TRAIL-induced PS exposure and PARP cleavage suggesting that caspase-3 activity was inhibited (Fig. 1). Second, although caspase-3 activity was blocked, initial cleavage of caspase-3 to yield the immature p20 subunit was not com-

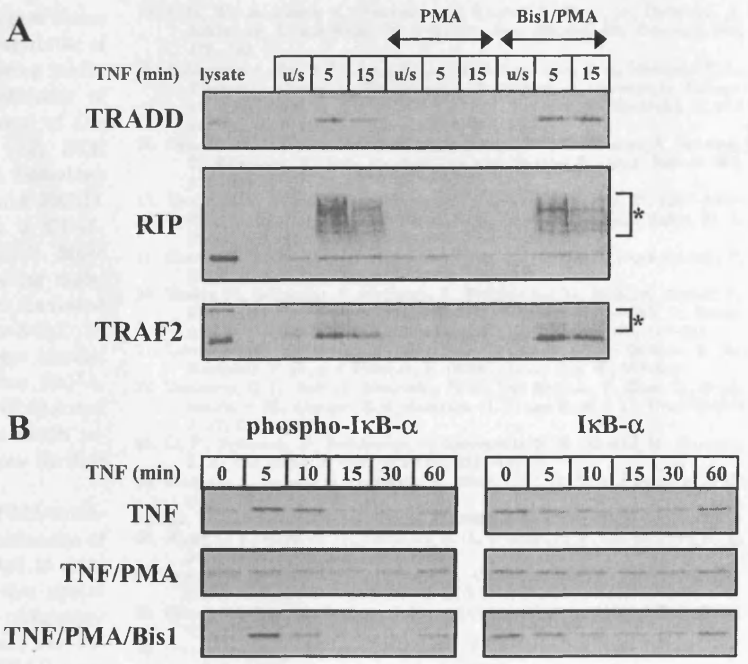

FIG. 9. **PMA inhibits TRADD recruitment by the TNF-R1 signaling complex and subsequent TNF-induced phosphorylation of IκB-α.** HeLa cells were either pre-treated with Bis I (1 μM, 30 min) followed by PMA (20 ng/ml, 30 min), PMA alone, or vehicle alone followed by bTNF (200 ng/ml) for the indicated times. *A*, cells (3×10^7/treatment) were washed and lysed, and TNF receptor complexes were isolated as described under "Experimental Procedures." Cell lysates, prior to receptor complex isolation, and receptor complexes were then analyzed for the presence of TRADD, RIP, and TRAF2. Unstimulated receptors (*u/s*) represent precipitates resulting from the addition of beads alone to lysates from untreated cells. The *asterisk* indicates modified species of RIP and TRAF2 seen only in TNF receptor complexes. *B*, whole cells were subjected to Western blotting using a phosphospecific antibody that recognizes only the phosphorylated form of IκB-α (Ser-32) and does not cross-react with the non-phosphorylated form of the protein (*left panel*) and a second antibody that recognizes total IκB-α protein (*right panel*).

pletely inhibited by PMA (Fig. 2), suggesting that the autocatalytic step of caspase-3 processing was being inhibited most likely by XIAP (38, 49). Third, mitochondrial release of both cytochrome *c* and the IAP antagonist Smac was blocked by PMA (Fig. 3), thus providing direct evidence that PKC activation was preventing engagement of the mitochondrial amplification arm of TRAIL signaling.

Previous studies had reported that PKC activation prevented TRAIL-induced apoptosis by inhibiting a step downstream of both caspase-8 activation and BID cleavage (31). In the present study we utilized several approaches to address this issue. First, we examined the effect of PMA on TRAIL-induced caspase-8 processing and Bid cleavage in whole cell lysates. In contrast to previous reports (31), we observed that PMA inhibited both caspase-8 processing and Bid cleavage (Fig. 2). This argued for the effect of PMA on TRAIL-induced apoptosis occurring upstream of both caspase-8 and Bid, and prompted us to directly examine the effects of PMA on TRAIL DISC assembly. This revealed that PKC activation inhibited the recruitment of both FADD and caspase-8 to the DISC (Fig. 4), thus demonstrating that a primary effect of PMA is mediated via inhibition of the "DD-DD-dependent" interaction between TRAIL-R1/-R2 and FADD at the DISC. Further support for this hypothesis was obtained using our *in vitro* binding assay, which revealed that recruitment of FADD to the intracellular domains of TRAIL-R1/-R2 was similarly significantly impaired in PMA-treated lysates (Fig. 7).

Collectively, these observations inferred that a PMA-sensitive signaling mechanism was involved in modulating TRAIL-induced FADD recruitment to the DISC. One possibility was that activation of PKC was either directly, or indirectly, modifying specific DISC components via a phosphorylation dependent-mechanism. Given that FADD is known to be phosphorylated (40), we hypothesized that PMA may modulate FADD phosphorylation and that this in turn may influence the affinity of FADD for TRAIL-R1/-R2. However, our observation that FADD phosphorylation status was unaltered by PMA (Fig. 8), together with the finding that both forms of FADD were efficiently recruited to the TRAIL DISC (Fig. 8), suggests that the phosphorylation state of FADD does not play a major role in TRAIL-induced apoptosis or in the PKC-mediated protection reported here. This is in agreement with that previously reported for CD95, where modulation of FADD phosphorylation status had no effect on the affinity of FADD for the CD95 DISC (30, 76). Further support for the hypothesis that FADD itself is not the primary target of PKC is provided by our observation that TNF-induced recruitment of TRADD to TNF-R1 was similarly disrupted by pre-treatment with PMA (Fig. 9*A*). This, together with the fact that TRADD is not phosphorylated, argues for PKC acting elsewhere to modulate the recruitment of these obligatory adaptor proteins to their respective membrane-associated signaling complexes.

How then does PMA affect the binding of FADD to the ICDs of TRAIL-R1/-R2? At least two possibilities exist. First, PMA-mediated PKC activation may in some way modify the TRAIL-R1/-R2 receptor ICD, which results in a reduced binding affinity for FADD. However, Frankel and colleagues (77) have already demonstrated that TRAIL-R1 is constitutively phosphorylated in unstimulated peripheral blood lymphocytes, whereas TRAIL-R2 exists in an unphosphorylated form. These authors also demonstrated that PMA treatment did not affect the basal phosphorylation state of either TRAIL-R1 or -R2. Although we cannot exclude the possibility that in our model PMA is in some way modulating the phosphorylation state of TRAIL-R1/-R2, the observation that PMA inhibits FADD recruitment by TRAIL-R1/-R2-ICD in an *in vitro* binding assay (Fig. 7) suggests that modification of the TRAIL receptor ICDs may not be the primary mechanism whereby PMA inhibits TRAIL DISC formation. Second, there may be an as yet unidentified DISC component that either facilitates or is required for the interaction of FADD with the death domain of TRAIL-R1/-R2. In this respect, a number of studies suggest that phosphorylation may still play a role in death receptor-induced apoptosis as a number of novel proteins and kinases have been identified that associate specifically with the membrane proximal cytoplasmic tails of CD95 or TNF-R1 but not with their respective death domains (78, 79). Furthermore, CD95 function has been reported to be affected by a number of kinases or phosphatases. One candidate is the phosphatase, Fas-associated phosphatase-1 (FAP-1), which has been reported to inter-

act with the intracellular domain, specifically, the last three amino acids of CD95 (80). FAP-1 acts as a negative regulator of CD95-induced apoptosis with caspase-8 activation being inhibited in FAP-1-transfected cells (81). Another modulator of CD95 is Bruton's tyrosine kinase (BTK), a member of the Src-related Tec family of protein-tyrosine kinases (82). BTK associates with CD95 via its kinase and pleckstrin homology domains and prevents the interaction of CD95 and FADD. Significantly, targeted disruption of the BTK gene in CD95-resisiatnt B cells sensitizes these cells to CD95 (82). More recently, it was reported that FAF1, a Fas-associating molecule, is also a component of the CD95 DISC, an effect mediated via FAF1 binding directly to both CD95 and caspase-8 (83). In this context it may be significant that FAF1 shows similar protein-interacting characteristics to FADD. Whether FAP-1, BTK, or FAF1 are similarly involved in TRAIL/TNF-induced signaling or whether PKC activation can modulate death receptor-FAP-1/BTK/FAF1 binding or activity requires further investigation.

In this study we report a novel mechanism for PMA-mediated inhibition of TNF-induced signaling. The significance of this observation is 2-fold. First, it is the only report to date demonstrating that PKC activation modulates the apical events of TNF signaling, namely recruitment of the obligatory adaptor molecule TRADD to TNFR-1 (Fig. 9). Second, our observation that PKC activation can modulate both TRAIL and TNF signaling by targeting the recruitment of obligatory adaptor protein(s) to the signaling complex, together with the finding that the CD95 DISC is similarly targeted (29, 30), suggests a common mechanism underlying this phenomenon. Although the protein or factor that mediates this effect is currently unknown, further studies on the regulation of death receptor signaling may unveil a mechanism whereby phosphorylation of common signaling components confers cellular resistance to death ligand-induced apoptosis. Furthermore, our results with TNF highlight a potentially important role for PKC in modulating other signaling pathways activated by death receptor engagement, namely TNF-induced NF-κB activation. Taken together, our results emphasize the importance of determining how protein kinase C activation functions to alter the ability of "death receptor" signaling components to interact with one another.

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