Characterisation of CD23 Cleavage by Endogenous and Exogenous Proteases using Neo-Epitope Antibodies

Thesis submitted for the degree of Doctor of Philosophy At the University of Leicester

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by

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For my parents with love

Abbreviations

Α	adenosine
ACE	angiotension converting enzyme
ADAM	a disintegrin and metalloproteinase
APC	antigen presenting cell
APS	ammonium persulphate
APP	β-amyloid precursor protein
BCR	B cell receptor
bp	baes pair
BSA	bovine serum albumin
С	cytosine
°C	degrees Celsius
CD	cluster of differentiation
CD40L	CD40 ligand
CIAP	calf intestinal alkaline phoshatase
CO ₂	carbon dioxide
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribose nucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
EBV-B	Epstein Barr virus-transformed B cell
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunoabsorbent assay
FCS	foetal calf serum
FDC	follicular dendritic cell
G	guanidine
GC	germinal centre
G+C	guanidine + cytosine content

GM-CFS	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IDC	interdigitating cell
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
ITAM	immunoreceptor tyrosine-based activation motif
kDa	kilodalton
KL	kit ligand
LA	Luria-Bertani agar
LB	Luria-Bertani broth
MAb	monoclonal anitbody
MADM	bovine mammalian disintegrin-metalloproteinase
MBS	m-maleimeidobenzoyl-N-hydroxy succinimide ester
mCD23	membrane CD23
MCS	multiple cloning site
MHC	major histocompatibility complex
mIg	membrane immunoglobulin
MOPS	3-[N-Morpholino]propanesulphonic acid
MW	molecular weight
NF-κB	nuclear factor-kapper B
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
РКС	protein kinase C
РМА	phorbal 12-myristate 13-acetate
r	recombinant

RNAase A	ribonuclease A
RT-PCR	reverse transcription-polymerase chain reaction
sCD23	soluble CD23
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSC	saline sodium citrate
ssDNA	single stranded DNA
Т	thymidine
TACE	TNF-α converting enzyme
TAE	tris acetate buffer
tCD23	truncated CD23
TCR	T cell receptor
TE	tris EDTA buffer
TEMED	N,N,N'N'-tetramethylethylenediamine
TGF	transforming growth factor
Th	T helper cell
TIMPs	tissue inhibitors of metalloproteinases
TNF	tumour necrosis factor
TNFR	TNF receptor
Tween-20	polyoxyethylenesorbitan monolaurate
v/v	volume in volume
w/v	weight in volume

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Abstract

Characterisation of CD23 Cleavage by Endogenous and Exogenous Proteases using Neo-Epitope Antibodies

Tracey Jane Wright

CD23 is the low affinity IgE receptor. It is a type II integral transmembrane glycoprotein that can be shed from the cell surface forming soluble products of approximately 37, 33, 29, 25 and 16kDa. Both membrane and soluble CD23 molecules have been attributed with a variety of biological functions including: B cell growth and differentiation, non-specific antigen uptake and presentation, germinal centre B cell survival, and the regulation of IgE synthesis.

It appears that membrane and soluble CD23 have opposing regulatory functions and that inhibition of CD23 shedding may have potential to alleviate both allergic and inflammatory diseases. This has focused attention on the endogenous protease(s) responsible for CD23 shedding, leading to the demonstration that both the 37 and 33kDa sCD23 fragments are cleaved from the cell surface by a metalloprotease.

In order to characterise the cleavage events within CD23, anti-neoepitope antibodies specific for the newly created amino and carboxy termini of the two predominant cleavage sites within CD23 (producing the 37 and 25kDa soluble CD23 products) were raised. Characterisation of these antibodies demonstrated that the proteolytic cleavage events responsible for creating the 37 and 25kDa sCD23 fragments are independent of each other. Furthermore, two different proteases were shown to be responsible for cleaving these two fragments. The work described in this thesis confirms previous reports that 37kDa sCD23 is cleaved by a metalloprotease, however cleavage of the 25kDa fragment was not inhibited by metalloprotease inhibitors.

The production of the two different sized sCD23 molecules by different proteases has important implications for targeting the proteolytic cleavage events to alleviate symptoms of allergic and inflammatory diseases. This emphasises the importance of defining the biological functions of mCD23 and each of the sCD23 molecules.

Chapter 1: CD23

1.1 Introduction

In 1987 it was discovered that the membrane protein described as the low affinity immunoglobulin E (IgE) receptor, FccRII and the B cell differentiation antigen CD23 were in fact the same protein (Kikutani *et al.*, 1986, Ludin *et al.*, 1987). This led to a variety of functions including: B cell growth and differentiation, non-specific antigen uptake and presentation, germinal centre B cell survival, and the regulation of IgE synthesis being attributed to CD23. Interest in this protein first arose due to the proposed antagonistic regulatory functions of membrane and soluble forms of CD23 on IgE synthesis. However, more recently it has been shown that CD23 is not only an important regulator of IgE synthesis, but it is also involved in the progression of an inflammatory response.

CD23 is cleaved from the cell surface forming soluble products of approximately 37, 33, 29, 25 and 16kDa (Letellier et al., 1988). The release of functional soluble products enables CD23 to interact with receptors expressed on the same cell or neighbouring cells without direct cell-cell contact. Thus, sCD23 has been described as possessing cytokine like properties, activating cells through ligand-receptor interactions. CD23 has five known ligands; IgE, CD21 (Aubry et al., 1992), CD11b, CD11c (Lecoanet-Henchoz et al., 1995) and the integrin $\alpha_{\nu}\beta_{5}$ (Matherson *et al.*, 1999); it is the interaction between these ligands and either the membrane or soluble forms of CD23, that determine the functional activity Soluble CD23 (sCD23) appears to activate both allergic and of this molecule. inflammatory responses. Binding of CD21 with sCD23 on B cells stimulates IgE synthesis (Aubry et al., 1992), while the interaction of sCD23 with CD11b/CD11c expressed by monocytes, promotes the release of pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-a (Lecoanet-Henchoz et al., 1995). On the other hand, membrane CD23 appears to function in the negative feedback regulation of IgE synthesis, inhibiting further IgE production through signal transduction pathways mediated by the binding of soluble IgE to membrane CD23.

The identification of the endogenous protease(s) responsible for the shedding of CD23 from the cell surface may highlight points of therapeutic intervention to relieve the symptoms of allergy and inflammation. The role of CD23 shedding in promoting an

allergic reaction is further supported by the finding that the house dust mite allergen Der p1 also cleaves CD23 (Hewitt *et al.*, 1995, Schulz *et al.*, 1995). It has been suggested that this proteolytic event may contribute to the increased synthesis of IgE that is associated with atopic allergy.

In summary although many biological functions have been attributed to CD23, the specific functions of membrane CD23 and each of the soluble products has not yet been clearly defined. Furthermore the identification of a soluble form of CD21, a receptor for CD23, implies that both membrane and soluble forms of CD23 may have overlapping functions. Therefore, further research is needed to ascertain which form of CD23 (membrane or soluble) is responsible for the initiation of different biological effects.

1.2 B Cell Development, Maturation and Differentiation

Throughout life B-lymphocytes are generated and develop in the bone marrow. Here they undergo a series of irreversible changes in their immunoglobulin (Ig) gene arrangements. After successful rearrangement of the Ig heavy chain genes, light chain genes rearrange by VDJ recombination; these immature B lymphocytes express complete IgM molecules on their cell surface (Alt et al., 1984, Reth et al., 1987, and Alt et al., 1987). Membrane bound Ig, expressed on the cell surface forms a complex composed of two heavy chains and two light chains to which antigen can bind. This complex is noncovalently associated with a disulphide-linked heterodimer of Ig α and Ig β subunits, to form the B cell antigen receptor (BCR) (Reth 1992). Newly generated B cells that express self-reactive immunoglobulin are deleted by the induction of programmed cell death (apoptosis), while the remaining B cells then migrate towards the sinusoids to leave the bone marrow (Hermans et al., 1989). Mature naïve B cells which co-express IgM and IgD, leave the bone marrow and circulate through the lymphoid system where they may encounter antigen. Some microbial antigens can directly stimulate B cell activation independent of T cells these are termed thymus independent antigens. However, most require an additional stimulus from an antigen specific T-helper (Th) cell, these are termed thymus dependent antigens.

Foreign antigens are captured by Langerhan's cells or dendritic cells found in the mucosae or skin. These cells process antigen and then migrate into the secondary lymphoid tissues through the draining afferent lymph where they encounter T cells. Langerhan's cells are phagocytic, dendritic like cells. Dentritic cells display a branched or dendritic morphology and are potent stimulators of T cell responses. It should be noted that dendritic cells are different from the follicular dendritic cells which present antigen to B cells. The antigen loaded Langerhan's/dendritic cells now called interdigitating cells (IDC), induce antigen specific naïve T cells to proliferate, secrete cytokines and express CD40 ligand (CD40L/CD154). These activated T cells subsequently co-stimulate antigen specific naïve B cells to proliferate, through an interaction between CD40L (expressed on activated T cells) and CD40 (expressed on B cells) (reviewed in Banchereau *et al.*, 1994). Co-stimulated antigen specific naïve B cells, may either differentiate directly into short lived plasma cells becoming antibody secreting cells, or they may give rise to germinal centres (GC), specialised structures found within lymphoid organs (Liu *et al.*, 1996).

Germinal centres have four distinct anatomical compartments as described by Hardie *et al.*, (1993): a dark zone, a basal light zone, an apical light zone and an outer zone. The dark zone is populated by rapidly proliferating centroblasts derived from only one of a few founder B cells, thereby increasing the number of cells specific for the recognised antigen. The Ig genes in these cells undergo somatic hypermutation, in which successive rounds of mutation of the Ig variable (V) region genes, termed somatic hypermutation, are followed by expression of the gene products on the cell surface (MacLennan and Gray 1986).

The basal light zone contains the progeny of centroblast, centrocytes and a rich network of follicular dendritic cells (FDC), stromal cells unique to the primary and secondary lymphoid follicles (MacLennan *et al.*, 1990). Here B cells are selected based upon the affinity of their rearranged IgV gene region for specific antigen, non-selected cells (i.e. those expressing low affinity Ig) die. Lui *et al.*, (1989) demonstrated that these cells die by apoptosis when placed into tissue culture. However, this process can be arrested by positive selection of cells by antibody binding to the rearranged surface antigen receptors (MacLennan and Gray 1986). Antigen binding rescues germinal centre B cells expressing high affinity Ig from apoptosis. These cells then migrate into the apical light zone where they may present specific antigen derived peptides to GC T cells, activation of the GC T cells leads to the expression of a second B cell survival signal CD40L (Lui *et al.*, 1989, Banchereau *et al.*, 1991).

FDC in the apical light zone have been shown to express high levels of CD23, which is cleaved from the cell surface to form soluble CD23 fragments. These soluble products may also rescue GC B cells from apoptosis through an interaction with CD21 in synergy with IL-1 (Lui et al., 1991, Aubry et al., 1992). Apoptosis of germinal centre B cells provides a mechanism for the positive selection of high affinity cells into the B cell memory pool, based on their ability to recognise immune complexes held on FDCs. Only those cells whose antigen receptors have somatically mutated to produce high affinity Ig molecules, receive signals for survival and rescue from apoptosis (MacLennan and Gray 1986). The interaction between these molecules (CD40-CD40L/CD21-CD23) induces expression of the anti-apoptotic proto-oncogene bcl-2, that prolongs cell survival and differentiation into plasma cells or memory cells. The mechanism of plasma cell or memory cell differentiation is not yet fully understood, however it is believed that signals gained from FDCs and T cells determine this as the B cell leaves the germinal centre. CD40 stimulation is believed to promote differentiation into memory cells while CD21/CD23 interactions lead to the generation of plasma cells (Arpin et al., 1995). In both pathways of antigen-induced B cell differentiation, Ig isotype switching occurs.

1.3 Immunoglobulin Isotype Switching

Isotype switching of the constant (C) region of the Ig heavy chain, changes the naïve B cell phenotype from one in which IgM and IgD are co-expressed to the expression of just one of the downstream isotypes; IgG, A or E, without affecting antigenic specificity. Isotype switching permits a more diverse immunological response by altering the effector function of the antibody molecule (Stavnezer 1996). Plasma cells have a life-span of approximately 4 weeks; expression of surface Ig and/or MHC class II molecules are either low or absent on these cells, so they can no longer interact with antigen or helper T cells, which helps to limit the duration of an antibody response. However, memory B cells can be rapidly activated on subsequent challenge producing a rapid effector response to the same antigen.

Germline transcription of various IgH constant region gene segments is regulated by multiple factors including surface Ig crosslinking, CD40 receptor engagement and soluble cytokines such as IL-4 (which enhances IgG_1 and IgE levels in mice and IgG_4 and IgE

levels in humans), IL-5 (which augments the production of IgA in mice), IFN- γ (which enhances IgG₃ and IgG_{2a} levels in mice) and TGF- β (which enhances IgG_{2b} and IgA levels in both humans and mice). B cell isotype switching to IgE and IgG₄ production is activated by IL-4 in conjunction with an interaction between B cell CD40 and CD40L expressed on activated T cells (Jabara *et al.*, 1990, Zhang *et al.*, 1991). The ligation of CD40/CD40L also delivers signals that act synergistically with IL-4 to up-regulate levels of CD23 mRNA and protein on the surface of B cells (Bouget *et al.*, 1993, Paterson *et al.*, 1996).

1.4 FccRI the High Affinity IgE Receptor

FceRI, the high affinity IgE receptor is expressed by a variety of cells including mast cells and basophils (Geha 1992), Langerhan's cells (Bieber *et al.*, 1992), dendritic cells (Maurer *et al.*, 1996), monocytes (Maurer *et al.*, 1994), and eosinophils (Gounni *et al.*, 1994). IgE antibodies bind to FceRI by the Ce3 domain of the IgE molecule to the α chain of high affinity receptor (Keown *et al.*, 1995). Most IgE molecules are bound to FceRI expressed by cells of the mucosal linings, accounting for the low serum concentration of this isotype of antibody. However, it is not until the binding of specific antigen to IgE/FceRI complexes occurs, that these cells are activated. Activation is brought about by the binding of specific antigen to the IgE/FceRI complex facilitating aggregation of adjacent receptors.

In the case of mast cells and basophils, the activation process results in the release of histamine, prostaglandins, leukotrienes, and a range of cytokines (Burd *et al.*, 1989, Plaut *et al.*, 1989, Schroeder *et al.*, 1994), all of which contribute to the characteristic symptoms of type I allergic reactions such as prutitis, oedema, increased mucous production and bronchoconstriction. These mediators not only cause the acute phase of an inflammatory response they also enhance activation of inflammatory cell populations, by maintaining allergen specific IgE production, up-regulating MHC class II and CD23, and inducing class switching of B cells to synthesise IgE (Bieber *et al.*, 1989, Gauchat *et al.*, 1993).

Human FceRI exist in two forms called α and β . FceRI was originally believed to be composed of a tetrameric structure consisting of one α subunit, one β subunit and two disulphide linked γ subunits ($\alpha\beta\gamma_2$) now called FceRI β (Blank *et al.*, 1989). However, the $\alpha\gamma_2$ subunits were later found to exist as a complex on human monocytes, lacking the β chain (Maurer *et al.*, 1994). This form is now referred to as FccRI α . Recently several studies have reported a correlation between atopic disease and serum IgE levels with polymorphism variations with the FccRI β chain gene (Stanford *et al.*, 1993, Shirakawa *et al.*, 1994). Since the relative proportions of FccRI α and β may vary between individuals, how an individual responds to an immune reaction mediated by FccRI may depend on the relative signalling capabilities of the two receptors and possibly why one individual's response is more intense to an allergen than another's. An understanding of the function of the FccRI β is therefore required in order to understand how FccRI β polymorphisms might contribute to the development of atopic disease.

FccRIß spans the plasma membrane four times, therefore both and amino terminus and the carboxy terminus protrude into the cytoplasm (Kuster et al., 1992). The amino terminus contains a number of proline residues but has been ascribed no function. The carboxy terminal tail, however, contains a sequence similar to an immunoreceptor tyrosine-based activation motif (ITAM) implicated in the transduction of signals in antigen receptormediated cell activation (Reth 1989). However, the FceRIB ITAM has a slightly shorter spacing between the two critical tyrosine residues, (6 residues as opposed to 7 residues in the motif consensus). FceRly has a single membrane-spanning domain and exists as a disulphide linked dimer. Its carboxy terminus contains an ITAM motif, which conforms to the motif described by Reth (1989). Current data on ITAM function suggests that multiple motifs function additively to increase the strength of a particular signal or separately to generate parallel signals (Weiss 1993, Weiss and Littman 1994). Comparisons between the signalling functions of $\alpha \gamma_2$ and the $\alpha \beta \gamma_2$ complexes demonstrated that β functions as an amplifier of the Syk tyrosine kinase, significantly increasing cell activation signals above those mediated by $\alpha \gamma_2$ (Lin et al., 1996). These results have important implications for understanding how β gene polymorphisms might participate in the pathogenesis of atopic disease.

1.5 Structure of CD23 the Low Affinity Receptor for IgE

Unlike other Ig receptors CD23 is not a member of the Ig gene superfamily but instead shows homology to a group of calcium dependent C-type lectins of which the asialoglycoprotein is a member (Kikutani *et al.*, 1986, Ludin *et al.*, 1987, and Suter *et al.*,

1987). Homology between these two proteins first lead to the suggestion that CD23 may participate in the endocytosis of IgE immune complexes (Kikutani *et al.*, 1986).

The gene coding for CD23 is located on human chromosome 19 (Wendel-Hansen *et al.*, 1990), it encodes two transcripts named CD23a and CD23b, generated by different transcriptional initiation sites and alternative RNA splicing (Yokota *et al.*, 1988). The two forms, a and b, differ only at the N terminus by their first six or seven amino acids respectively. This variation in amino acid sequence between the two molecules has been suggested to lead to different intracellular signalling pathways (Kolb *et al.*, 1994). CD23a contains a tetrapeptide motif, YSEI; that may act as a signal for endocytosis. Therefore CD23a but not CD23b, which lacks this motif may have a function in antigen presentation (Hopkins 1992). Conversely CD23b contains the dipeptide motif, NP, present in its cytoplasmic tail. This motif may be required to mediate phagocytosis in monocytes (Yokota *et al.*, 1992).

Monomeric human CD23 is a 45kDa molecule. The structure of which differs from many other surface receptors, as it is a type II integral transmembrane glycoprotein with its carboxy terminus located extracellularly (figure 1.1). The amino acid sequence encodes four highly conserved and two partially conserved cysteine residues, with respect to other C-type lectins. The formation of disulphide bonds between these residues gives rise to a compact lectin head (Delespesse et al., 1992). The lectin domain comprises the IgE binding site between cysteine residues 163-282 (Bettler et al., 1992), and is flanked on the C-terminal side by an inverse RGD (Arg-Gly-Asp) motif, which may have a function in cell adhesion (Grangette et al., 1989). Between the lectin head and transmembrane domain is a stalk region, which in humans contains three imperfect heptad hydrophobic repeats. Each repeat is encoded by a separate exon suggesting this may have arisen from gene duplication (Suter et al., 1987). The heptad repeats consist of four leucines separated by seven residues forming a 'leucine zipper' motif (Landshultz et al., 1988) that gives rise to an α helical coiled-coil structure (Beavil *et al.*, 1992). This coiled-coil structure is proposed to encourage oligomerisation at the cell surface leading to the formation of dimers or even trimers (Dierks et al., 1993, Beavil et al., 1995, and figure 1.2).



Figure 1.1 Structural Representation of CD23 Amino Acid Sequence

CD23 is a type II integral transmembrane protein that contains five distinct domains, a Cterminal tail, a lectin domain, an α helical coiled-coil stalk, a transmembrane domain and a N-terminal intracellular domain.

(Figure adapted from an original kindly supplied by Dr. R. Beavil, Kings College, London.)



amino terminal

Figure 1.2 Schematic Representation of Trimeric Membrane CD23

The coiled-coil stalk of CD23 is proposed to encourage oligomeriastion at the cell surface leading to the formation of dimers or even trimers as depicted above. (Figure adapted from an original published by Sutton and Gould 1993)

Expression of CD23 is tightly regulated in a tissue specific manner. CD23a is constitutively expressed at a low level on mature IgM^+/IgD^+ B cells, (Kikutani *et al.*, 1986), monocytes (Melewicz *et al.*, 1982) and eosinophils (Gounni *et al.*, 1998). Whereas, CD23b is expressed on a variety of cell types including B cells, T-cells, eosinophils, platelets, Langerhan's cells, follicular dendritic cells, natural killer (NK) cells, monocytes, and epithelial cells of bone marrow and thymus, all induced by IL-4 (Yokota *et al.*, 1988).

IL-4 is the most potent activator of CD23 expression on all cell types. Both IL-4 and IL-13 up-regulate the expression of CD23a and CD23b on B cells (Punnonen *et al.*, 1993, McKenzie *et al.*, 1993), in conjunction with a CD40/CD40L interaction (Fanslow *et al.*, 1992, Burlinson *et al.*, 1996). In contrast interferon (IFN)- γ , IFN- α , transforming growth factor (TGF)- β and glucocorticoids all inhibit the enhancement of CD23 expression on B cells. While, IL-2 and IFN- α induces CD23b expression on all other cell types (Delespesse *et al.*, 1991). Regulation of CD23b expression differs on monocytes from that of B cells. In monocytes IL-3, IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF) all induce CD23 expression (Alderson *et al.*, 1992), while IL-10 has been shown to specifically inhibit the effects of IL-4 induced CD23 expression (Dugas *et al.*, 1996, Morinobu *et al.*, 1996).

1.6 Soluble CD23 Fragments are Cleaved Products from the Membrane Protein

Shedding of CD23 from the cell surface results in the release of soluble CD23 (sCD23) fragments of the following sizes: 37kDa, 33kDa, 29kDa, 25kDa and 16kDa (Letellier *et al.*, 1988). The cleavage sites of these products are shown in figure 1.2. The smaller sCD23 fragments (29-25kDa) are thought to be derived from proteolytic cleavage of the larger 37kDa molecules, as the molecular weight of sCD23 fragments released from RPMI 8866 cells shifts from 25-27kDa to 33-37kDa in the presence of the cysteine protease inhibitor iodoacetamide (Letellier *et al.*, 1988). Furthermore, three of these sites, that release soluble fragments of 33kDa, 29kDa and 25kDa, cleavage lies within the coiled-coil stalk a region that is predicted to be stable and insensitive to proteolysis. However, at the fourth site where the 37kDa fragment is generated, the heptad repeat pattern breaks down. This may locally disrupt the coiled-coil structure and account for susceptibility to proteolysis at this point (Gould *et al.*, 1991).

Binding of both IgE and anti-CD23 antibodies (to the lectin domain) inhibit the release of sCD23 (Lee et al., 1987). This may be due to an allosteric mechanism that stabilises the coiled-coil stalk and protects against proteolysis (Gould et al., 1991). Binding of CD23 to IgE is thought to be dependent upon oligomerisation of CD23. Oligomerisation is encouraged by the coiled-coil stalk, a region that is lost in some of the smaller soluble fragments leading to initial suggestions that these fragments were unable to bind IgE (Dierks et al., 1993). However, all soluble CD23 fragments (including the 16kDa fragment) retain the lectin domain to which IgE binds, and are thus capable of binding IgE (Sarfati et al., 1992), but with lower affinity/avidity than mCD23 (Kelly et al., 1998). Protein-protein cross-linking analysis indicates that sCD23, including the 16kDa and 25kDa fragments, can form hexamers in solution, and that in addition to the coiled-coil structure this may be brought about by interactions between the lectin head domains (Beavil et al., 1995). However, it has recently been shown that the α helical coiled-coil stalk is important for orientating the lectin head domains of CD23, enabling their interaction with IgE (Kelly et al., 1998). This offers an explanation for the higher affinity/avidity of mCD23 for IgE over its soluble counterparts.

Various biological activities have been attributed to sCD23 fragments. The 37kDa, 33kDa and 16kDa sCD23 forms have all been implicated in the regulation of IgE synthesis by B cells. It is thought that the 37kDa and 33kDa sCD23 fragments promote IgE synthesis, while the 16kDa fragment limits IgE synthesis (Sarfati *et al.*, 1984, Sarfati *et al.*, 1992). The most stable soluble fragment is a 25kDa product, this fragment is reported to possess pleiotropic cytokine-like activities, and in synergy with IL-1 stimulates prothymocyte maturation (Mossalayi *et al.*, 1990), the rescue of germinal centre centrocytes from apoptosis and their differentiation into plasmocytoid cells (Liu *et al.*, 1991). These cytokine-like activities are mediated by an epitope distinct from the IgE binding site (Mossalayi *et al.*, 1992), and indicate that the cytokine like properties attributed to CD23 are mediated through a second receptor other than IgE. Other reported properties of sCD23 molecules include functions that aid B cell growth (Stevens *et al.*, 1991), cell adhesion, antigen presentation (Gordon *et al.*, 1989) and the production of inflammatory cytokines, such as tumour necrosis factor (TNF)- α , IL-1 β and IL-6 by human monocytes *in vitro* (Armant *et al.*, 1994, Herbelin *et al.*, 1994, Plater-Zyberk *et al.*, 1995).

1.7 CD23-CD23 Receptor Interactions

CD23 has five known ligands: IgE, CD21 (Aubry *et al.*, 1992), CD11b and CD11c (Lacoanet-Henchoz *et al.*, 1995), and integrin $\alpha_V\beta_5$ (Matherson *et al.*, 1999). Molecular binding studies have shown that CD23 binds IgE within the lectin head region, close to the calcium binding site (Vercelli *et al.*, 1989). This may explain why the binding of IgE to CD23 is calcium dependent (Richard and Katz 1990). IgE is thought to have two different binding sites for CD23 (Bettler *et al.*, 1992), these have been mapped to the Fcc3-4 domains (Vercelli *et al.*, 1989, Sutton and Gould 1993, Shi *et al.*, 1997). Sedimentation equilibrium studies analysing soluble CD23 bound to Fcc3-4 domains of IgE, indicate that the two sites for CD23 in IgE are different and that binding is co-operative. It has therefore been speculated that IgE binds dimers or trimers of CD23 molecules (Shi *et al.*, 1997). This finding is consistent with previous work on murine CD23 (Dierks *et al.*, 1993), which indicated that only the oligomeric forms of CD23 have sufficient affinity/avidity to bind IgE.

The interaction between CD23 and CD21 is also calcium dependent (Pochon et al., 1992). CD21 is a highly glycosylated membrane protein found on B cells, follicular dendritic cells basophils and a sub-population of peripheral blood T lymphocytes and thymocytes. The CD21 molecule consists of a large extracellular domain organised into 15-16 short consensus repeats (SCR), a transmembrane portion and a short cytoplasmic domain (Ahearn and Fearon 1989, Weis et al., 1988). Since the discovery that CD21 was a ligand for CD23, it has been shown that CD21 mediates many of the cytokine-like activities attributed to CD23 (figure 1.3). These include the rescue of germinal centre B cells from apoptosis (Bonnefoy et al., 1993), the ability of CD23 to promote thymocyte maturation and myeloid cell differentiation, B cell homotypic adhesion (Bjorck et al., 1993), histamine production by basophils (Bacon et al., 1993) and IgE synthesis (Aubry et al., 1992). Mossalayi et al., (1992) demonstrated that CD21 binds to an epitope on CD23 distinct from, but overlapping with that of the IgE binding site. This suggests that IgE and CD21 may compete for binding to CD23. Both membrane and soluble CD23 fragments (37kDa and 25kDa) have been shown to bind CD21 (Aubry et al., 1992, Graber et al., 1992, Bonnefoy et al., 1993). Furthermore, the soluble form of CD21, corresponding to the extracellular domain of this receptor, may also act as a functional ligand for mCD23 (Fremeaux-Bacchi et al., 1998).

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B cell activation

Figure 1.3 Soluble CD23 Activates B Cells by Binding to CD21

Binding of soluble CD23 to CD21 has been shown to mediate many of the cytokine-like activities attributed to CD23. These include the rescue of germinal centre B cells from apoptosis (Bonnefoy *et al.*, 1993), the ability of CD23 to promote thymocyte maturation and myeloid cell differentiation, B cell homotypic adhesion (Bjorck *et al.*, 1993), histamine production by basophils (Bacon *et al.*, 1993) and IgE synthesis (Aubry *et al.*, 1992). The above figure depicts sCD23 binding to membrane CD21 on B cells. Here CD21 is present within a molecular complex with surface membrane immunoglobulins, CD19, and TAPA-1.

On the membrane of B cells, CD21 is expressed in a molecular complex associated with CD19 and a cell signalling complex molecule TAPA-1 (Matsumoto *et al.*, 1991, Bradbury *et al.*, 1992) (figure 1.3). CD19 is associated with Ig α and Ig β , the signal transducing components of the antigen receptor on B cells. The co-activation of CD19/TAPA-1/CD21 and that of the BCR complex greatly reduces the amount of antigen needed to reach the threshold for B cell activation (Fearon and Carter 1995). It is thought that CD23 may activate B cells through binding of CD21 in this manner, rescuing germinal centre B cells from apoptosis. Immune complexes held by FDC in germinal centres interact with somatically mutated high affinity membrane IgM expressed by B cells. However, this interaction is insufficient to stimulate the B cells alone, because the antigen concentration and affinity for the membrane Ig is too low. Binding of sCD23 may activate CD19 via CD21, reducing the amount of antigen needed to reach the threshold for B cell activation to within the physiological range of that produced by Ig/antigen interactions (Carter *et al.*, 1991, Carter and Fearon 1992).

High levels of sCD23 have been reported in various chronic inflammatory diseases. The increase in CD23 expression in diseases in which IgE is not implicated, or where the activities of CD23 could not be attributed to an interaction with CD21, led to a hunt for further ligands for CD23. Lecoanet-Henchoz and colleagues (1995) reported that full-length recombinant (r)CD23 incorporated into fluorescent liposomes, bound to a subset of cells that were shown to comprise mainly CD14⁺ cells (i.e. monocytes). Further analysis showed that CD23 specifically interacted with CD11b and CD11c, the α chains of the β 2 integrin adhesion molecule complexes CD11b-CD18 and CD11c-CD18 that were expressed on monocytes. The significance of this pairing was demonstrated by triggering CD11b and CD11c with either recombinant sCD23 or anti-CD11b and anti-CD11c monoclonal antibodies. The results of this experiment showed a marked increase in the release of nitrite-oxidative products and proinflammatory cytokines (TNF- α , IL-1 β and IL-6).

CD11b and CD11c are adhesion molecules that participate in many cell-cell and cellmatrix interactions (Springer 1990). Within the C-terminal tail of CD23 is an inverse RGD (Arg-Gly-Asp) motif, which is a common recognition motif for integrin receptors, and has been suggested to function in cell adhesion. It was therefore an obvious suggestion that CD23 may interact with CD11b/CD11c via this motif. However, CD23 interactions with CD11b/CD11c were not inhibited by a polyclonal antibody directed against this tripeptide, indicating binding was not via the inverse RGD motif. Instead the interaction between CD23 and CD11b/CD11c appears to involve carbohydrate, since the addition of tunicamycin was shown to decrease CD23 binding to monocytes. In addition IgE, which is known to bind to the lectin domain, also partially inhibits CD23 binding to monocytes. This suggests that the interaction of CD23 with CD11b/CD11c is through its C-type lectin domain, similar to the interaction between CD23 and CD21 (Lecoanet-Henchoz *et al.*, 1995).

A fifth ligand for CD23, the integrin $\alpha_v\beta_5$ has recently been detected on human B cell precursor cell line, SMS-B cells (Matheson *et al.*, 1999). This integrin has been shown to bind to sCD23, however the significance of this interaction has not yet been determined.

1.8 The Role of CD23 in the Regulation of IgE Synthesis

CD23 appears to serve two primary roles in an allergic reaction. The first is in CD23/IgE mediated antigen presentation (Heyman et al., 1993) and the second in the role of regulating IgE synthesis. Interest in this protein has increased due to the proposed antagonistic role of CD23 on IgE synthesis. It is thought that IgE and IgE-antigen complexes bind to and cross-link mCD23 expressed on the B cell surface and that this interaction down-regulates IgE synthesis through a negative feedback mechanism (figure 1.4). Sherr et al., (1989) first reported that IgE immune complexes are capable of inhibiting ongoing IgE synthesis in the human plasma cell line U266/AF-10 and human B cells (isolated from highly atopic individuals), as measured by isotype specific ELISA. Evidence to support a role for mCD23 in the inhibition of IgE production was identified by Bonnefoy et al., (1990). Using a panel of monoclonal antibodies known to be specific for the lectin head of CD23, it was shown that blocking of CD23 inhibited IL-4 induced IgE production in cultured human lymphocytes. Further research by this group (Flores-Romo et al., 1993), has shown that a rat anti-CD23 antibody Rb55, not only inhibited IL-4 induced IgE production in human mononuclear cells, but also inhibited IgE production in vivo. These results suggest that the binding of IgE and IgE immune complexes to CD23
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y down regulater lgE synthesis, and that lgE is theil proposible for regulation that is control over LgE synthesis invogin in CD20.

Feedback inhibition of IgE synthesis via CD23



IgE-allergen immune complex

of CD23 expression invels on hill

-ve signal to IgE-secreting B cell

Figure 1.4 Binding of IgE to the Low Affinity IgE Receptor, CD23, Inhibits Further IgE Production Through a Negative Feedback Inhibition Mechanism Soluble IgE binds to membrane CD23 sending a negative feedback signal to the B cell,

which down regulates further IgE synthesis.

directly down-regulates IgE synthesis, and that IgE is itself responsible for regulating homeostatic control over IgE synthesis through mCD23.

This result is supported by both CD23 deficient and transgenic mice studies. Although CD23 deficient and transgenic mice do not display many of the predicted defects based upon *in vitro* observations, they do show alterations in IgE production. CD23 transgenic mice are shown to produce similar basal levels of secreted IgE when compared to their control littermates. However, when mCD23 transgenic mice are infected with the parasite *Nippostrongylus brasiliensis* (Nb) to induce an immune response their production of IgE is impaired. This impaired IgE production was shown to be greater than 2 fold when compared to the increased levels of serum IgE produced from infected sCD23 transgenic or control mice (Texido *et al.*, 1994). Additionally, CD23 deficient mice have been found to have double the concentration of serum IgE when compared to control animals (Yu *et al.*, 1994). Implicating CD23 as a negative feedback regulator of IgE synthesis.

The cellular mechanism by which CD23 limits IgE production appears to be linked with apoptosis. Cho et al., (1997) studied the effects of CD23 expression levels on IgE production in vitro. Purified resting B cells were co-cultured with CHO cells transfected with either CD23, ICAM-1, or CD23 plus ICAM-1, and then activated with IL-4, IL-5 and recombinant CD40L expressed on CHO cells. The results clearly showed a dosedependent decrease of IgE production and B cell proliferation with increasing numbers of CHO cells transfected with CD23. This was investigated further by co-cross linking CD23 and surface (s)Ig (Campbell et al., 1997). The stimulation of B cells by cross-linking their sIg receptors has been previously used to study B cell activation events (Brunswick et al., 1988). In this study (Campbell et al., 1997) monoclonal anti-IgD was coupled to DNPdextran, and co-cross linking of sIg and CD23 was achieved by the addition of anti-DNP IgE to B cells activated by the anti-IgD-DNP-dextran (figure 1.5). The results of this investigation revealed that B cell mRNA levels of the proto-oncogene c-myc were significantly elevated one hour after inducing B cell activation. The overexpression of cmyc has frequently been implicated in the mechanism of apoptosis. In addition apoptotic B cells were observed three days after stimulation, this was completely blocked in cells from transgenic mice expressing the oncogene *bcl*-2, a known cell survival signal. In conclusion



Figure 1.5 Co-Cross Linking Membrane CD23 and Surface (s)Ig

Co-cross linking of sIg and CD23 was achieved by the addition of anti-DNP IgE to B cells activated by the anti-IgD-DNP-dextran. The results of this investigation revealed that B cell mRNA levels of the proto-oncogene c-*myc* were significantly elevated one hour after inducing B cell activation.

(Figure adapted from an original published by Campbell et al., 1997)

this study demonstrated evidence to suggest that co-cross linking of CD23 with B cell sIg mediates accelerated programmed cell death - apoptosis (Campbell *et al.*, 1997).

The binding of IgE to mCD23 blocks the release of sCD23 and inhibits interactions with CD21, as the two binding sites on CD23, for CD21 and IgE overlap. This has important implications, as soluble CD23 is reported to promote IgE production in the presence of IL-4 (Paterson *et al.*, 1996) through an interaction with CD21 (Aubry *et al.*, 1992 Fremeaux-Bacchi *et al.*, 1996) (figure 1.3). Aubry *et al.*, (1992) showed that the triggering of CD21 by purified full-length recombinant CD23, incorporated into fluorescent liposomes increases IL-4 induced IgE production from peripheral blood mononuclear cells. However, the role of CD23 (membrane or soluble) in promoting IgE production is contentious. Despite repeated *in vitro* examples from studies with human B cells, there is no evidence that CD23 promotes IgE production in murine studies *in vivo*. Although it should be noted that there are many differences between human and murine membrane and soluble CD23, which may account for this (these will be discussed in greater detail in section 1.11).

The anti-CD20 monoclonal antibody B1, is known to inhibit B cell proliferation (Tedder *et al.*, 1985, and 1986), and completely blocks the production of IgE by peripheral blood mononuclear cells (PBMCs) cultured in the presence of IL-4. In addition this antibody reduces the level of mCD23 by stimulating the release of sCD23 (Bourget *et al.*, 1995). Bourget and colleagues compare this to the effects of glucocorticoids, which decreases sCD23 formation and stimulates IL-4 induced IgE synthesis (Fisher and König 1990). They suggest that this data, in relation to their own findings indicates that IgE production correlates with the level of mCD23 molecules at the cell surface and not with the amount of sCD23 that is released. That is IgE production is associated with mCD23 expression, and therefore it is possible that mCD23 binds with CD21 to promote IgE synthesis. These studies propose contrasting roles for mCD23 decreases further IgE production, while mCD23/CD21 interactions promote IgE synthesis.

1.9 The Role of CD23 in Antigen Presentation

Binding of IgE to CD23 may also enhance allergic responses by mediating uptake of IgE immune complexes, thus playing a role in antigen presentation by B cells. The role of CD23 in antigen presentation could provide non-specific B cells with a possible mechanism to specifically bind and uptake allergens at low concentrations and present them to antigen specific T cells. This was first demonstrated in mice by Kehry and Yamashita (1989), and then later in human EBV transformed B cells, which were shown to internalise and present IgE complexes via CD23 (Pirron *et al.*, 1990). The role of naïve B cells in antigen presentation has been heavily debated. Several studies have demonstrated that antigens targeted to CD23 induce T cell stimulation, provided that surface IgD molecules on B cells are adequately cross-linked by anti-IgD antibodies (Eynon and Parker 1992, Eynon and Parker 1993, Morris *et al.*, 1994). From these finding Mudde *et al.*, (1995) postulated that naïve B cells generally induce tolerance in naïve T cells, unless the B cells have been activated by antigen.

In contrast, both activated or naïve B cells will activate memory T cells. This model has important implications when applied to the interactions between B and T cells during the initiation of an allergic response, and may result in the development of multiple allergies as proposed by Mudde and colleagues (1995). A detailed overview of this model has been published (Mudde *et al.*, 1995, Mudde *et al.*, 1996). In summary, in a patient with an allergy to Der p1 a naïve B cell, activated by a specific antigen (called AgX in this example), may also present Der p1/IgE complexes via CD23, to specific T cells. In allergy the responding T cells specific for Der p1 are most likely to be Th2 cells, therefore the AgX B cells will receive signals from the Th2 cells (IL-4 and CD40L interaction) inducing isotype switching to IgE. The AgX B cell will now produce IgE antibodies to AgX. Hence, a patient with an original allergy to Der p1 will now also produce IgE antibodies against the previously innocuous AgX (figure 1.6). In addition, the production of IL-4 by the Der p1 specific Th2 cells may also skew phenotype differentiation of the AgX specific T cells.

A second role for CD23 in antigen presentation was suggested by the finding that CD23 is spatially associated with the major histocompatability complex (MHC) class II human leukocyte antigen (HLA)-DR molecules on human B cells (Bonnefoy *et al.*, 1988). In



Figure 1.6 IgE Mediated Antigen Presentation in Allergy

B cells which bind allergen specific IgE (e.g. for Der p1), via CD23¹ will after recognition of their specific antigen (e.g.AgX) will present the AgX as well as the Der p1 to specific T cells². In the case of Der p1, the responding T cells are usually of Th2 type, which will induce B cells to switch to IgE production. Consequently the B cell will produce new IgE antibodies specific for AgX³. In addition IL-4 produced by the Der p1 specific Th2 cells⁴ may influence the phenotype of AgX naïve T cells, differentiating these into Th2 cells⁵. (Figure adapted from an original published by Mudde *et al.*, 1995)

order to investigate whether CD23 plays a role in antigen presentation, Flores-Romo *et al.*, (1990) used a model system of alloantigen presentation to investigate if CD23 occupancy blocked the capacity of B cells to stimulate T cells. Their finding showed that the addition of anti-CD23 monoclonal antibodies MHM6 and EBVCS 4 (both of which bind to the lectin domain of CD23), but not IgE where able to block alloantigen stimulation. From this, it was speculated that CD23 may not only be important in B-T cell conjugation but may also provide a co-stimulatory signal to the antigen primed T cells. However, as this interaction does not involve IgE a second receptor expressed on T cells may be involved, possibly CD21.

However, the role of CD23 in IgE mediated antigen presentation is contentious. Firstly, many researchers believe that B cell presentation to naïve T cells induces tolerance, regardless of the B cell activation state (Fuchs and Matzinger 1992), this directly contrast the assumption made by Mudde *et al.*, (1995). Secondly, the majority of IgE in humans is bound to the high affinity IgE receptor FceRI. This receptor is thought to be the main receptor involved in IgE mediated antigen presentation (Maurer *et al.*, 1995).

1.10 The Role of CD23 in Inflammation

High levels of CD23 have been reported in various non-allergic chronic inflammatory diseases such as systemic lupus erythematosus (Bansal *et al.*, 1992), inflammatory bowel diseases (Kaiserlian *et al.*, 1993), Sjogren's syndrome (Yano *et al.*, 1992) and rheumatoid arthritis (Hellen *et al.*, 1991). Further evidence to support a role for CD23 in inflammatory disease *in vivo* was demonstrated in a murine model of human rheumatoid arthritis. Treatment of arthritic DBA/1 mice with monoclonal antibody B3B4, specific for CD23 showed a dose-related amelioration of arthritis, possibly as a result of inhibiting the interaction between CD23 and the β_2 integrins CD11b/CD11c have been shown to induce secretion of TNF- α , IL-1 β and IL-6 (Lecoanet-Henchoz *et al.*, 1995). It is therefore possible that the secretion of these pro-inflammatory cytokines was suppressed by the addition of anti-CD23 antibody (Plater-Zyberk and Bonnefoy 1995). However, the exact interaction, either between the anti-CD23 antibody and membrane or soluble CD23, or between CD23 and possible receptors, was not determined.

An alternative pathway by which CD23 has been described to mediate an inflammatory response is through the ligation of mCD23 on monocytes, by either IgE/anti-IgE complexes or anti-CD23 monoclonal antibodies. The addition of anti-CD23 monoclonal antibodies and IgE/anti-IgE immune complexes to human monocytes stimulated with IL-4, triggered a dose dependent increase in intracellular cyclic (c)AMP and provoked the production of inflammatory products such as TNF- α and nitric oxide (Paul-Eugene *et al.*, 1992, Mossalayi *et al.*, 1994, Bayon *et al.*, 1998).

Nitric oxide is the final product of the oxidation of guanidine nitrogen of L-arginine by nitric oxide synthase (NOS) (Paul-Eugene *et al.*, 1994). In promonocytic cells, binding of CD23 elicits a rapid stimulation of L-arginine-dependent accumulation of cGMP and cAMP, followed by activation of NF- κ B, induction of proto-oncogenes c-*fos*, c-*jun*, *jun*B and c-*fms*, production of TNF- α and terminal differentiation into monocytes/macrophages (Ouaaz *et al.*, 1994). The production of pro-inflammatory cytokines and nitric oxide by cross-linking CD23 with IgE-antigen immune complexes has recently been demonstrated in malaria to contribute to the release of TNF- α and the immuno-pathogenesis of this disease (Perlmann *et al.*, 1999).

The exact role of CD23 in inflammatory disease has been confused further by the discovery that sCD21 activates mCD23 on IL-4 pre-treated monocytes, causing the up-regulation of inducible (i)NOS, HLA-DR and CD40 (Fremeaux-Bacchi *et al.*, 1998). Altogether, it appears that binding of CD23 on monocytes up-regulates the production of pro-inflammatory cytokines. Although many pathways have been described that could lead to the production of these cytokines, it is not yet known if all these pathways are relevant in disease situations, or even how these pathways may interact *in vivo*.

IL-10 may have an important role in regulating CD23 dependent immune and inflammatory responses. The expression of CD23b by monocyte/macrophages is tightly regulated by IL-10. Triggering of mCD23 expressed by macrophages induces the expression of IL-10. IL-10 expression, is in turn, down regulated by nitric oxide, which is also produced by monocytes, upon activation of CD23 (Dugas *et al.*, 1996). IL-10 itself has been shown to down regulate CD23b mRNA and protein levels in IL-4 treated monocytes (Marinobu *et al.*, 1996).

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1.11 Murine CD23 Differs in Structure and Function to Human CD23

The functions attributed to membrane and soluble CD23 remain ambiguous, especially as many of the functions ascribed to human CD23 *in vitro* have not been found in CD23 transgenic or knockout murine models. Although, one feature that does appear to be consistent is that mCD23 mediates the inhibition of IgE production (Texido *et al.*, 1994, Lamers and Yu 1995, Hacku *et al.*, 1997).

One possible reason for the ambiguity is that murine CD23 has only limited similarity to human CD23. Murine CD23 has only 57% homology at the amino acid level to its human counterpart, with a further 17% of homology taking into account conservative substitutions. The structure slightly differs from human CD23, in that the stalk region contains four imperfect heptad repeats, has two N-glycosylation sites and one O-linked glycoside, instead of three imperfect repeats and one N-glycosylation site in human CD23 (Conrad *et al.*, 1990). Cleavage of murine CD23 from the cell surface results in the production of 38kDa and 28kDa soluble products (Gould *et al.*, 1991). Although these soluble forms are similar in size to their human counterparts there are no obvious relationships between the cleavage sites (Bartlett *et al.*, 1995). The expression of murine CD23 is found primarily on B lymphocytes, as only murine CD23a is expressed at detectable levels (Conrad *et al.*, 1994). In addition murine CD23 does not contain the intracellular signalling motifs associated with either endocytosis or phagocytosis (Kondo *et al.*, 1994).

1.12 Shedding of the CD23 Ectodomain

Initial studies by Letellier *et al.*, (1990) on the shedding (cleavage) of the CD23 ectodomain, indicated that recombinant full-length CD23 was degraded into 37 and 29kDa proteins when incubated in the presence of CD23 positive cells (RPMI 8866), but not in the presence of CD23 negative cells. This suggested CD23 was autoproteolytic, although there is no precedence for autoproteolytic activity to be associated with C-type lectins, which are generally considered to be non-enzymatically active (Drickamer 1988). Additionally, incubation of mCD23 expressed on RPMI 8866 cells with protease inhibitors, was shown to shift the molecular weight of sCD23 fragments from 25-27kDa to 33-37kDa (Letellier *et al.*, 1988). From this result, it was suggested that the 25-27kDa

responsible for generating the 25-27kDa sCD23 actively cleaved the 37kDa sCD23 fragment. Although later research by Moulder *et al.*, (1993) showed the presence of a 25kDa CD23 molecule, without the presence of the 37kDa fragment in the culture supernatants of RPMI 8866 cells grown in serum free media.

More recently the endogenous protease(s) responsible for CD23 shedding has been postulated to belong to a family of membrane bound metalloproteases. This was first suggested due to the similarity between CD23 shedding and the shedding of a wide range of membrane proteins that are cleaved from the cell surface to form soluble products. These include the β -amyloid precursor protein (APP) (Sisodia and Price 1995), angiotension converting enzyme (ACE) (Ramchandran and Sen 1995), tumour necrosis factor (TNF)- α (Vassalli 1992) and L-selectin (Gearing and Newman 1993). The mechanism of action has been termed ectodomain shedding, and has been recognised as an important aspect of cell regulation and cell-cell interactions (Arribas *et al.*, 1996, Ehlers *et al.*, 1996).

Ectodomain shedding converts membrane receptors into soluble competitors of their own ligands (Fernandez-Botran 1991). In general, this process appears to occur near to the cell surface and is often stimulated by protein kinase C (PKC) activators such as phorbol 12-myristate 13-acetate (PMA) (Hooper *et al.*, 1997). However, the correlation between cleavage sites ends there, as there are no similarities in amino acid sequence surrounding cleavage sites. In addition ectodomain shedding can be found in both type I and type II proteins (Hooper *et al.*, 1997). Despite a lack of sequence similarities in cleavage sites throughout this family of membrane proteins, sequence comparisons indicates that there is a preference for cleavage following a lysine or arginine residue. Although this has not been supported by mutational studies, as amino acid substitutions in APP showed that most amino acids would be tolerated (Zhong *et al.*, 1994). Given the diversity of amino acid sequences cleaved, it was first postulated that many different proteases must be involved. This theory was reinforced by reports that the shedding of different ectodomains appeared to be inhibited by protease inhibitors that targeted different classes of proteases (Arribas *et al.*, 1996).

Due to the wide variety of cleavage site sequences found within this group of proteins, and the tolerance of amino acid base changes at the site of cleavage (illustrated by mutational studies), research has been geared towards identifying the factors that govern ectodomain shedding. One common factor that has been identified in many shedding events is that of conformational or topological requirements, such as cleavage within an open stalk at a minimum length or distance from the transmembrane domain. An example of this is demonstrated in the shedding of the β -amyloid precursor protein. Deletion mutants of this protein showed that cleavage was constrained by a distance of 9-13 residues from the membrane (Zhong *et al.*, 1994). Ectodomain shedding of angiotension converting enzyme is also governed by a similar factor (Ehlers *et al.*, 1996). Other proteins already shown to fit within this group are the p55 TNF-receptor (Brakebrusch 1994) and L-selectin (Chen 1995, Migaki 1995).

Recent research has now shown that ectodomain shedding and PMA induced ectodomain shedding is blocked by hydroxamic-acid based inhibitors of metalloproteases, but not by other class specific protease inhibitors, including tissue inhibitors of metalloproteases (TIMPs) 1 and 2. This led to original suggestions that the group of metalloproteases responsible for ectodomain shedding, are distinct from the matrix metalloproteases (Mohler et al., 1994). However, it has recently been reported that TIMP 3, as well as synthetic hydroxamic acid based inhibitors block shedding of L-selectin (Borland et al., 1999). As yet, many of the sheddases responsible for cleavage of these proteins remain unidentified. One of those already cloned is the TNF- α converting enzyme (TACE), a membrane associated 85kDa enzyme that cleaves membrane-bound TNF- α , to release a 17kDa soluble protein (Black et al., 1997). The primary amino acid sequence of TACE shows 29% homology with bovine mammalian disintegrin-metalloprotease (MADM) and a conserved overall structure with the ADAM (a disintegrin and metalloprotease) family of proteases (Yamamoto et al., 1999). TACE is synthesised in an inactive pro-form and is subsequently cleaved between the cysteine switch and the catalytic domain to generate an active mature enzyme (Moss et al., 1997). The sheddase responsible for cleaving ACE (called ACE secretase), also shares these characteristics. Studies by Ramshandren and Sen (1995) showed, that in a cell free membrane assay, the release of ACE was carried out by an integral transmembrane metalloprotease that is specifically inhibited by a synthetic hydroxamic acid derivative (compound 3).

Several researchers have now shown that broad-spectrum metalloprotease inhibitors also prevent cleavage of the 37 and 33kDa CD23 fragments from mCD23. Research by Christie *et al.*, (1997) showed that Batimastat prevented shedding of sCD23 from RPMI 8866 cells, purified human monocytes stimulated with IL-4, and human tonsillar B cells stimulated with IL-4 and anti-CD40. Inhibition of shedding was accompanied by the loss of IgE production *in vitro* and *in vivo* (in mice). The same conclusions were also drawn by Wheeler *et al.*, (1998) who showed that a standard hydroxamate-type Zn²⁺ metalloproteinase inhibitor prevented sCD23 and IgE release from human tonsillar B cells stimulated with IL-4 and anti-CD40 after up to 3 days of incubation. By day 14 the activity of the compound was reduced allowing release of sCD23 fragments, however IgE production remained inhibited. These results suggest that the addition of inhibitor upon stimulation interferes with the induction of an IgE response.

The identification of the proteolytic mechanism of action leading to the shedding of 37kDa and 33kDa sCD23 products allowed further characterisation of the protease responsible. Marolewski *et al.*, (1998) demonstrated that the CD23 sheddase exits as an integral membrane protein with functional molecular mass of approximately 63kDa. The identification of the protease in the membrane suggests that cleavage could be facilitated, and controlled by, the orientation of the protease with respect to CD23 in the membrane, and may be governed by conformational or topological requirements of the target sequence.

1.13 The Proteolytic Properties of Allergens may Provoke an Allergic Response

Many parasites invade their host by the secretion of proteolytic enzymes that break down connective tissue, allowing the parasite access to host tissues. It has been proposed that these enzymes are particularly active at promoting Th-2 cell responses (Svetic *et al.*, 1993). Th-2 cells promote IgE expression through the secretion of IL-4 and the interaction of CD40 with CD40L, as discussed in detail above. Therefore, it has been suggested that the biochemical functions of proteins, in particular proteolytic activity, may be related to allergenicity and the generation of an IgE response (Stewart and Thompson 1996). Several well characterised house dust mite allergens have been shown to possess enzymatic

characteristics and four of the major house dust mite *Dermatophagoides pteronyssinus* allergens are proteases (Der p1, Der p3, Der p4 and Der p9).

The finding that Der p1 proteolytically cleaves CD23 from the B cell surface (Hewitt *et al.*, 1995, Schulz *et al.*, 1995) provides a mechanism by which the proteolytic activity of Der p1 may potentiate an allergic response. It was speculated that the loss of CD23 from the cell surface by Der p1 could enhance immune responses through IgE production, by ablating the negative feedback inhibitory mechanism, which normally limits IgE synthesis. In addition, the resulting sCD23 fragments cleaved by Der p1, may promote IgE synthesis through an interaction with CD21 (as discussed above). Characterisation of the proteolytic activity of Der p1 has shown it to possess either a cysteine activity or a mixed mechanistic class of cysteine and serine proteases, depending on the source of extract. Subsequent analysis has indicated that the presence of both cysteine and serine activity is probably due to a mixed mite population or possibly a contaminating serine protease, recognised by the anti-Der p1 monoclonal antibody used in its purification, rather than dual activity (Hewitt *et al.*, 1997).

Der p1 has been shown to cleave CD23 at two locations, Ser155-Ser156 and Glu298-Ser299 producing a 17kDa fragment containing the lectin domain and part of the C-terminal tail (Shulz *et al.*, 1997). The serine residues 155 and 156 lie buried within the coiled-coil stalk, close to the endogenous cleavage sites responsible for the release of 29, 25 and 16kDa sCD23 products. The Glu298-Ser299 site lies within the C-terminal tail, a region of the molecule that remains to be modelled. Schulz *et al.*, (1997) speculated that the C-terminal tail may contribute to the stability of the trimer and suggest that Der p1 cleaves CD23 in a sequential manner, cleaving the C-terminal tail first, thus destabilising the trimer allowing access into the second cleavage site within the coiled-coil stalk.

Alternatively, it has been suggested that the proteolytic activity of Der p1 may facilitate penetration of the epithelial cell barrier. It is thought that the proteolytic activity of Der p1, may disrupts the tight junctions and other intercellular junctions between epithelial cells, increasing epithelial permeability, and allowing the antigen access to the antigen-presenting cells in the submucosal tissue (Herbert *et al.*, 1995). The serine protease inhibitor α 1-antiprotease, protects the lower respiratory tract against damage by proteases released in the lung during inflammation. Studies by Hewitt *et al.*, (1995) showed that α 1-

antiprotease can inhibit the proteolytic activity of Der p1, although, others have shown that Der p1 can inactivate α 1-antiprotease by cleavage within the reactive centre loop (Kalsheker *et al.*, 1996). This suggests that a balance between proteolytic activity of Der p1 and α 1-antiprotease may be important for controlling allergen induced IgE synthesis and that the inactivation of α 1-antiprotease may be important in the pathogenesis of allergic disease. However, Jeannin *et al.*, (1998) have recently reported that α 1antiprotease acts as a co-stimulus for IgE and IgG₄ synthesis through induction of increased expression of CD23 and CD21, an unexpected finding given the experiments detailed above. Thus it appears that both proteases, as well as protease inhibitors may be involved in the regulation of an IgE response. However, whether the allergenicity of these molecules or antigens delivered with them, is influenced by their proteolytic or antiproteolytic activities remains unclear.

House dust mite excretions have also been reported to stimulate IL-4 production from mast cells and basophils (Machado *et al.*, 1996) thus stimulating Th-2 cell differentiation (Paul and Seden 1994) and B cell class switching to both IgG and IgE. Der p1 also cleaves the α subunit of the IL-2 receptor CD25 expressed on T cells and diminishes the proliferation and IFN- γ secretion of these cells following stimulation by anti-CD3 antibody (Schulz *et al.*, 1998). The IL-2 receptor is pivotal for the propagation of Th1 cells (Abbas *et al.*, 1996), hence cleavage of CD25 by Der p1 proposes a second mechanism by which Der p1 may enhance skewing of T cells to Th-2 in an immune response.

1.14 The Opposing Functions of CD23

There is still great debate over the biological functions attributed to CD23. This debate is fuelled by reports showing opposing results from human and murine, and *in vitro* and *in vivo* systems. Our knowledge of the regulatory mechanisms of IgE production is fragmentary, but it does appear that IL-4 and CD40L not only stimulate IgE expression but also promote CD23 production, providing a negative feedback control mechanism for IgE. If as predicted, this is a main function of mCD23 as shown by CD23 knockout and transgenic mice studies, then it is difficult to understand why mCD23 also functions in a mechanism that enhances an IgE response by facilitating antigen presentation? Further conflicting scenarios are predicted by the shedding of CD23 from the cell surface producing sCD23 fragments, these fragments may compete with mCD23 in the interaction

with receptors. Individuals suffering from an allergic disease often display elevated levels of sCD23 in serum (Melewicz *et al.*, 1981, Delespesse *et al.*, 1991). However, whether these fragments are involved directly in promoting IgE synthesis through an interaction with CD21, or whether they interfere with the binding of IgE-containing immune complexes to mCD23, and block effective feedback control, is still not clear.

Other reports demonstrate a role for sCD23 in the production of pro-inflammatory cytokines, through an interaction with CD11b and CD11c on monocytes. However even this interaction has been shown to be complex, as pro-inflammatory cytokines may also be produced by the activation of mCD23 by IgE complexes and soluble CD21. Although to what relevance these interactions have in an *in vivo* situation remains unclear. Despite these problems, these findings suggest that a possible mechanism for the treatment of allergic and inflammatory diseases might be achieved by inhibiting the protease(s) responsible for the shedding of CD23 from the cell surface. This strategy would increase the expression of membrane CD23 controlling IgE production and reduce the release sCD23 thus preventing the stimulation of pro-inflammatory cytokines.

Some of the difficulties in analysing the role of CD23 may stem from the limitations of *in vitro* experimentation. Firstly, the dynamics of CD23 are highly complicated, IL-4 and CD40 not only regulate IgE synthesis but also stimulate CD23 expression and the generation of sCD23 molecules. It is therefore difficult to match *in vitro* conditions to those found *in vivo*, which may vary depending upon the disease. Secondly, functions attributed to CD23 from *in vitro* experiments, are not found in CD23 transgenic or knockout models. This may be due to differences between species or because of redundancy in the genome, with multiple genes performing overlapping functions and the ability of other genes to compensate for the absent function of a targeted gene.

1.15 Aims

Inhibition of the shedding of CD23 may have potential to alleviate both allergic and inflammatory diseases. However, it is clear that the functions of both mCD23 and sCD23 *in vivo* have not yet been clearly defined, with so many opposing functions attributed *in vitro*, it is difficult to predict what effects, if any, the inhibition of CD23 shedding will have. Furthermore, although both membrane and soluble CD23 fragments have been implicated in specific functions, it is not known whether this represents the situation *in vivo* or indeed in humans.

In order to address some of these problems, analysis of the cleavage mechanisms within CD23 is required. Analysis of these cleavage events and their significance in allergic and inflammatory disease are complicated further by the inability to distinguish between the different sized sCD23 products produced, often due to the limited quantities of material available. Therefore the aim of this research is to characterise the cleavage events within CD23, by the development of antibodies specific for the newly created amino and carboxy termini of the two predominant cleavage sites within CD23 (anti-neoepitope antibodies). The development of these reagents will then enable us to determine the mechanisms of cleavage and to assess the effects of the exogenous protease Der p1 on these cleavage events.

Preliminary research within our laboratory and experimental evidence published by others at the start of this research has lead to the following hypotheses:-

- (i) CD23 is cleaved by at least two distinct endogenous proteases
- (ii) The cleavage events by these two proteases are independent
- (iii) A membrane metalloproteinase is responsible for shedding the 37kDa sCD23 fragment and this protease is related to other ectodomain sheddases

The work outlined in this thesis attempts to provide answers to the aims outlined above by testing these hypotheses. More specifically the experimental objectives of this study are to:-

- (1) Clone and express human CD23a
- (2) Generate anti-neoepitope antibodies in rabbits, specific for the amino and carboxy termini of the two predominant cleavage sites, i.e. those producing the 37 and 25kDa cleavage fragments
- (3) Characterise the specificity of the anti-neoepitope antibodies generated
- (4) Use the anti-neoepitope antibodies to determine the mechanism of cleavage at the two predominant cleavage locations, i.e. those responsible for producing the 37 and 25kDa CD23 fragments
- (5) Determine whether CD23 is sequentially cleaved, i.e. cleavage of the smaller 25kDa fragment is dependent upon prior cleavage of the 37kDa fragment
- (6) Determine the proteolytic effect that the house dust mite protease Der p1 has on CD23 in relation to the endogenous cleavage events

Chapter 2: Materials and Methods

2.1 Suppliers and Reagents	
ABI Prism, Applied Biosystems PE, Warrington, Cheshire, UK	
Dye deoxy terminator ready mix	1463-011
Amersham Life Science, Little Chalfont, Bucks, UK	
ECL TM Western blotting detection reagents	RPN 2109
Hybond-N nylon membrane	RPN 303N
Multiprime DNA labelling system	RPN 1601Y
Rainbow [™] coloured protein molecular weight markers	RPN 756
(Range 14.3kDa-220kDa)	
Amicon Ltd, Stonehouse, Gloucestershire, UK	
Centricon-10	4205
Beckman Instruments, High Wycombe Bucks, UK	
Ultracentrifuge tubes	
Beckton Dickinson, Oxford, UK	
Falcon tubes	2070
BDH, Merck Ltd, Lutterworth, Leics, UK	
Ethanol	28304ER
Glacial acetic acid	27013
Hydrochloric acid	10125
Binding Site, Birmingham, UK	
Donkey anti-sheep IgG (H&L) FITC	AF360
Mouse anti-human CD23 (IgG.) BU38	MH112
Mouse anti-human CD23 (IgG.) BU38 FITC	MF112
Sheep anti-human IgE	AU014

Biogenesis Ltd, Bournemouth, UK	
RNAzol B	CS104
Boehringer Mannheim, Lewes, East Sussex, UK	
Acrylamide/Bisacrylamide	1-685-821
Rapid DNA ligation kit	1-635-379
Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK	
L-trans-epoxysuccinyl-leucylamide- (4-guanidino)-butane (E-64)	324890
Leupeptin, hemisulphate	108975
N^{α} -tosyl-lys chloromethyl ketone, Hydrochloride (TLCK)	616382
Phenyl methyl sulfonyl fluoride (PMSF)	52332
Cambio, Cambridge, UK	
Chicken anti-sheep biotinylated antibody	IMS-06-065
Clontech Laboratories UK Ltd, Basingstoke, Hampshire, UK	
Mouse anti-6xHis monoclonal antibody	8904-1
TALON™ metal affinity resin	8901-2
ECACC, Porton Down, Wiltshire, UK	
RPMI 8866 cells	
RPMI 8226 cells	87012702
Fisher Scientific UK, Loughborough, Leics, UK	
Methanol	M/3900/17
Silver nitrate (AgNO ₃)	S/1240/46
Sulphuric acid	S/9240/17
Tri-sodium citrate	S/3320/53
Vacuum Pouches	220-2938

Gibco BRL, Paisley, UK	
Calf intestinal alkaline phosphatase (CIAP)	18009-019
Deoxyadenosine triphosphate (dATP)	10216-018
Deoxycytosine triphosphate (dCTP)	10217-016
Deoxyguanosine triphosphate (dGTP)	10218-014
Deoxythymidine triphosphate (dTTP)	10219-012
DH5α [™] Competent cells	18263-012
DNAase I, supplied with 10x DNAase I reaction buffer	18068-015
Dpn I supplied with REact 4 buffer	15242-019
Dulbecco's Modified Eagle Medium	41966-029
EcoR I supplied with REact 3 buffer	15202-013
F-10 Hams nutrient media	21765-029
Geneticin® G418 sulphate	11811-015
Glutamax	35050-038
Non-essential amino acids	11140-035
Not I supplied with REact 3 buffer	15441-025
Penicillin and Streptomycin Solution	15140-114
Phosphate buffered saline (PBS)	14200-067
Pvu II supplied with REact 6 buffer	15412-018
Random Hexamers	48190-011
Recombinant Taq DNA polymerase,	10342-020
supplied with: 10x PCR buffer and magnesium chloride	
RPMI 1640 medium	31870-108
Superscript™ RNase H reverse transcriptase	18064-022
supplied with: 5x 1 st Strand buffer and 0.1M DDT	
T4 DNA Ligase supplied with: 5x Ligase buffer	15224-025
Xho I Restriction enzyme supplied with REact 2 buffer	15231-012
Xba I Restriction enzyme supplied with REact 2 buffer	15226-012
1kb ladder	15615-016

ICN Pharmaceuticals Inc, California, USA	
Dulbecco's Modified Eagle Medium (methionine and cysteine free)	1642454
Trans ³⁵ S label™	51006
Millipore Corporation, Bedford, MA, USA	
Immobilon-P membrane	IPVH00010
New England Biolabs Inc. Hitchin, Herts, UK	
Sca I supplied with 10x buffer	122S
Novex, R&D Systems Europe Ltd, Abingdon, Oxon, UK	
SeeBlue™ prestained standards	LC5625
Nunc (Gibco BRL) Paisley, UK	
Cryotubes	375418
Maxisorp 96 well plates	442404A
Nycomed (UK) Ltd, Sheldon, Birmingham, UK	
Lymphoprep	01001967
Oxiod, Unipath Ltd, Basingstoke, Hampshire, UK	
Bacto-tryptone	L42
Yeast extract	L21
Perkin Elmer PCR Products, PE Ltd, Warrington, Cheshire, UK	
ULTma proof-reading thermostable polymerase,	E5073
supplied with 10x PCR buffer and magnesium chloride	E1244/F0468
Pierce & Warringer (UK) Ltd, Chester, UK	
m-maleimeidobenzoyl-N-hydroxy succinimide ester (MBS)	22311

Pharmacia Biotech, St Albans, Herts, UK		
Probe Quant G50 Micro Columns	27-5335-01	
rProtein-A Sepharose® Fast Flow	17-1279-01	
Sephaglas™ Bandprep kit	27-9285-01	
Promega, Southampton, UK		
Wizard™ Plus SV Miniprep kit	A1330	
Qiagen, Crawley, West Sussex, UK		
Qiaex II gel extraction kit	20021	
Serotec Ltd, Oxford, UK		
Rabbit anti-mouse IgG FITC	STAR9B	
Sigma Chemicals, Poole, Dorset, UK		
Agar	A5054	
Agarose	A9539	
Ammonium persulphate (APS)	A9164	
Ampicillin	A9518	
Avidin horseradish peroxidase (HRP)	A3151	
Bovine serum albumin (BSA)	A2153	
Bromophenol blue	B8026	
Calcium chloride (CaCl ₂)	C3306	
Chloroform	C2432	
Dimethyl formamide	D4254	
Dimethyl sulphoxide (DMSO)	D2650	
Dithiothreitol (DTT)	D6052	
Donkey anti-sheep IgG peroxidase	A3415	
Ethidium bromide	E8751	
Ethyl acetate	E7770	
Ethylenediaminetetraacetic acid (EDTA)	E5134	
Ficoll 400	F2637	
Foetal bovine serum (FBS)	F7524	
Formaldehyde	F1268	

Formamide	F7503
Freund's complete adjuvant	F5881
Freund's incomplete adjuvant	F5506
Gluteraldehyde	G7526
Glycerol	G5516
Glycine	G4392
Goat anti-rabbit IgG peroxidase	A4914
Heparin	H4898
Imidazole	I0125
Isoamyl alcohol	10640
Isopropanol	405-7
Kodak BioMax film	Z235394-9
Lithium chloride (LiCl)	L9650
Lysozyme	L7651
Manganese chloride (MnCl ₂)	M3634
Magnesium chloride (MgCl ₂)	M0250
3-[N-Morpholino]propanesulphonic acid (MOPS)	M8899
Mineral oil	M5904
NP-40	N0896
O-Phenylenediamine dihydrochloride (OPD)	P4664
Ovalbumin	A2153
Pepstatin	P4265
Phosphate citrate buffer	P4922
1, 10-Phenanthroline	P9375
Phenol	P4557
Polyethylene glycol (PEG)-8000	P2139
Potassium acetate	P1190
Potassium chloride (KCl)	P9541
Potassium hydroxide (KOH)	P5958
Potassium phosphate	P0662
Proteinase K	P2308
RNAase A	R6513
Saponin	S7900
Sheep anti-mouse IgG peroxidase	A6782

Sodium dodecyl sulphate (SDS)	L4390
Sodium acetate	S2889
Sodium bicarbonate (NaHCO3)	S6014
Sodium carbonate (Na ₂ CO ₃)	S1641
Sodium chloride (NaCl)	S3014
Sodium hydroxide (NaOH)	S0899
Sodium phosphate, monobasic (Na ₂ HPO ₄)	S9638
Sodium phosphate, dibasic (NaH ₂ PO ₄)	S9763
Sodium nitrite (NaNO ₂)	S2252
Sucrose	S0389
N,N,N',N'-Tetramethylethyleediamine (TEMED)	T7024
Thyroglobulin	T1001
Tris acetate EDTA buffer (TAE)	T9650
Trizma base	T8524
Trypan blue	T6416
Polyoxyethylene sorbitan monolaurate (Tween-20)	P1379
Xylene cyanol	X4126
Water (RNAase and DNAase free)	W4502
-used in all molecular biology methods	

Stratagene Ltd, Cambridge, UK

Cloned PfU DNA polymerase supplied with 10x PCR buffer	600153
E.coli strain XL-1 Blue	212207
pBluescript® II KS	200301
Taq 2000 [™] DNA polymerase supplied with 10x PCR buffer	600195

Whatman International Ltd, Maidstone, UK

Whatman 3MM chromatography paper	3030917
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2.2 Amplification of CD23 by Reverse Transcription-Polymerase Chain Reaction

2.2.1 RNA Extraction

CD23 was cloned by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using RNA extracted from peripheral blood mononuclear cells (PBMCs) or EBV-DR B cells (donated by Dr. W. J. Lawley, Leicester University, Leicester, UK). Total RNA was extracted from a pellet of approximately 10x10⁶ viable cells (as determined by trypan blue exclusion). The cells were then washed in 5ml Phosphate Buffered Saline (PBS) and total RNA was extracted using RNAzol[™]B kit following manufacturers instructions.

2.2.2 First Strand cDNA Synthesis using Superscript[™]II Reverse Transcriptase

Isolated RNA was used in reverse transcription cDNA synthesis reactions. First strand cDNA transcriptions were carried out using a variety of primers (500ng oligo dT, 50-250ng Random Hexamers and 2pmole CD23 specific primers), all following the standard methodology outlined below. First strand cDNA was synthesised using Superscript[™]II reverse transcriptase which has an associated RNAase H activity. Reactions were carried out in 20µl volume using 1-5µg of total RNA and 500ng oligo dT primer. The mixture was heated to 70°C for 10 minutes and then chilled on ice for 10 minutes to ensure denaturation of the RNA template, before the addition of 4µl 5X first strand buffer (250mM Tris HCl at pH 8.3, 375mM KCl, 15mM MgCl₂), 2µl 0.1M dithiothreitol (DTT) and 1µl 10mM deoxynuclotide 5'-triphosphate (dNTP) mix (10mM dATP, 10mM dGTP, 10mM dCTP, 10mM dTTP). This was incubated for 2 minutes at 40°C. 200U of Superscript[™]II was added and the reaction incubated at 42°C for a further 50 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. The RNA strand was hydrolysed by adding 0.5M NaOH and incubating at 50°C for 30 minutes. This reaction was neutralised with 1M Tris HCl pH 8.0, and the single stranded (ss)DNA isolated by phenol chloroform extraction, chloroform extraction and ethanol precipitation before resuspension in 10µl water.

2.2.3 Isolation of DNA by Phenol Chloroform Extraction and Ethanol Precipitation

Where required DNA was purified by the addition of an equal volume of phenol/chloroform 50% v/v phenol equilibrated with 1x TE buffer (1mM EDTA, 10mM Tris HCl pH 8.0)/48% v/v chloroform, 2% v/v isoamyl alcohol, the emulsion was vortexed and centrifuged at 13,000xg for 10 minutes at room temperature. The aqueous layer was then aspirated and precipitated on ice for 10 minutes with 0.1 volumes of 3M sodium

acetate pH 5.2 and 2.5 volumes of neat ethanol. After centrifugation the pellet was washed in 70% v/v ethanol twice and resuspended in water.

2.2.4 Oligonucleotide Design

Oligonucleotide primers were designed to amplify the CD23 DNA sequence based on the published human CD23 gene sequence (Yokota *et al.*, 1988). Primer sequences are given in table 2.1. All were synthesised by the Protein and Nucleic Acid Chemistry Laboratory (PNACL, Leicester University, Leicester, UK). Restriction enzyme sites *Xho* I and *Xba* I, were added to the CD23 primer sequences of the sense and antisense oligos respectively, to aid ligation.

Table 2.1 CD23 Specific Primers used to Amplify the CD23 Coding Sequence

Primer	Sequence 5'-3'	Optimal
		Annealing
		Temp
CD23 5' Sense Primer	CCGCTCGAGCAAGCAGGACCGCCATGGAG	60°C
CD23 3' Antisense Primer	GCTCTAGACTGGGCCTGGCTGTATCCATG	58.9°C

Restriction enzyme sites shown in **bold: CTCGAG** Xho I, **TCTAGA** Xba I.

Optimal annealing temperature is calculated based upon melting temperature (T_m) of the primers.

2.2.5 Generation of CD23 cDNA by PCR

CD23 cDNA was generated by polymerase chain reaction (PCR). A variety of polymerases and reaction conditions were used. All PCR procedures were based on a standard method as outlined below using recombinant (r)Taq DNA polymerase. PCR reactions were carried out in a final reaction volume of 100µl. Each reaction mixture was set up on ice in the following order: water to a final volume of 100µl; 10µl 10x PCR buffer, 1.5mM MgCl₂, 0.2mM dNTP mix, 0.5µM CD23 5' sense primer, 0.5µM CD23 3' antisense primer and 0.1 to 0.5µg of ssDNA template from the reverse transcription reaction. DNA reaction mixes were amplified using a Techne PHC-3 Dri-Bloc thermal cycler (Techne Ltd, Cambridge, UK). Reactions were heated to 94°C for 10 minutes, after 5 minutes the reactions were 'hot started' by adding 2.5U rTaq DNA polymerase, mixed

with a pipette tip before mineral oil layered on top. This was followed by 30 cycles of PCR, each cycle consisting of 1 minute denaturation at 94°C, 1 minute annealing at 60°C and 1 minute extension at 72°C before a final 10 minutes extension at 72°C and cooling to 4°C (unless otherwise stated). 10% of the PCR product was analysed by gel electrophoresis (method 2.2.6).

Initially PCR reactions were set up using proof reading polymerase enzymes to amplify the CD23 coding sequence. These were to be used to ensure accurate sequence amplification, this was considered important as the product of this reaction was to be used to express recombinant CD23. The proof reading polymerase enzymes used were ULTmaTM (3U), PfU a thermostable polymerase (5U) and a mixture of PfU and taq 2000, at a ratio of 1:12 or 1:20 respectively (total of 5U per reaction) with specific reaction buffers as supplied with each of these enzymes. For rTaq DNA polymerase the reaction buffer contained 20mM Tris HCl pH 8.4, 50mM KCl. For ULTmaTM taq the reaction buffer contained 100mM Tris HCl, 500mM KCl, 15mM MgCl₂, 0.1% gelatin. For PfU and Taq 2000 the reaction buffer contained 200mM Tris HCl, 500mM KCl, 100mM Tris-HCl pH8.8, 20mM MgSO₄, 100mM KCl, 100mM (NH₄)₂SO₄, 1% Triton X-100, 1000µg/ml nuclease-free BSA. These reactions were amplified by PCR as described above, with a longer extension time of 2 minutes due to the reduced amplification efficiency of proof reading polymerases.

2.2.6 Agarose Gel Electrophoresis

Agarose (0.4g) was melted in 40ml Tris acetate EDTA (TAE) buffer (40mM Tris-acetate, 1mM EDTA) and cooled to 60°C. Ethidium bromide was added to a final concentration of 500ng/ml and the gel cast in the apparatus. Once set, the gel was submerged in 1x TAE containing 500ng/ml ethidium bromide. Samples were diluted with 6x loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 15% w/v Ficoll 400). Gels were loaded and run at 110 volts for 20 minutes.

2.3 Cloning of CD23 cDNA into pBluescript[®]KS II

2.3.1 Preparation of Cloning Vector pBluescript[®] KS II and CD23 cDNA

The CD23 cDNA product and the vector pBluescript[®] KS II (a 2961 base pair phagemid derived from pUC19) were digested with restriction enzymes *Xho* I and *Xba* I. Reactions consisted of 1U of each enzyme per μ g of DNA in 1x REact 2 buffer (50mM Tris HCl pH

8.0, 50mM NaCl, 10mM MgCl₂) in a final volume of 50µl water. Reactions were incubated at 37°C overnight. Digested vector DNA was extracted with phenol/chloroform and ethanol precipitated (as described in 2.2.3), then washed twice in 70% v/v ethanol. The vector was dephosphorylated using calf intestinal alkaline phosphatase (CIAP) as follows: DNA was resuspended in 1x CIAP buffer (10mM ZnCl₂, 10mM MgCl₂, 100mM Tris HCl pH8.3) in a final volume of 100µl, CIAP was added at a concentration equal to the number of pmole ends per µg DNA and incubated for 30 minutes at 37°C. 0.5% w/v SDS, 5mM EDTA and 100µg/ml proteinase K were added to the reaction mix and incubated at 56°C for 30 minutes. The digested cDNA and dephosphorylated vector were electrophoresed at 110 volts for 40 minutes on a 1% w/v agarose TAE gel (section 2.2.6). The DNA band of equivalent mobility to the known insert and vector fragment size (as compared to a DNA marker, 1 Kb ladder), were cut out of the gel and isolated using a SephaglasTM Bandprep kit according to manufacturer's directions.

2.3.2 Ligation of CD23 into pBluescript® KS II

A sample of Sephaglas[™] Bandprep purified plasmid DNA containing CD23 was electrophoresed at 110 volts for 20 minutes on a 1% w/v agarose TAE gel and the relative intensity of each band compared to known standards was used to estimate the concentration of DNA recovered. The molar ratios of vector/insert were calculated for optimal ligation. Ligation reactions were set up in the following order: water to a final volume of 10µl, vector DNA, insert DNA, 1x T4 DNA ligase dilution buffer (250mM Tris HCl pH7.6, 50mM MgCl₂, 5mM ATP, 5mM DTT, 25% w/v polyethylene glycol-8000), 0.1U T4 DNA ligase. Ligations were incubated overnight at 16°C.

2.3.3 Preparation of Competent Cells for Transformation with Plasmid DNA

Escherichia coli (*E.coli*) strain XL-1 Blue were made competent for transformation with plasmid containing the CD23 cDNA. *E. coli* XL-1 Blue cells were streaked onto a Luria-agar (L-agar) plate (Luria-broth; 1% w/v bacto-tryptone, 0.5% w/v yeast extract, 1% w/v NaCl with 1.5% agar added, autoclaved at 121°C for 15 minutes before use) to produce single colonies. A single colony was picked and used to inoculate 10ml Luria-broth (L-broth). The culture was grown at 37°C with shaking to an optical density of 0.5 at a wavelength of 600nm (OD₆₀₀ 0.5). The culture was then added to 100ml pre-warmed L-broth and grown at 37°C with shaking until it reached an OD₆₀₀ 0.6. A further 380ml pre-warmed L-broth was added and the culture grown again to an OD₆₀₀ 0.6. The culture was

then cooled on ice for 5-7 minutes and pelleted by centrifugation at 1500xg for 10 minutes at 4°C. The cells were then gently resuspended in 50ml ice cold TFB1 (30mM potassium acetate, 50mM MnCl₂, 100mM KCl, 10mM CaCl₂, 15% v/v Glycerol), re-pelleted as above, then resuspended gently in 10ml ice cold TFB2 (10mM MOPS, 75mM CaCl₂, 10mM KCl, 15% v/v Glycerol). 200 μ l cells were then aliquoted per vial and snap frozen on liquid nitrogen ready for storage at -70°C.

2.3.4 Transformation of Competent Cells

E. coli strain XL-1 Blue were used for growth and maintenance of plasmids. Stocks of competent cells (stored at -70°C) were thawed on ice, mixed gently and 100µl of cells added to each ligation mixture, these were then incubated on ice for 1 hour. After which the transformation mixture was heat-shocked for 2 minutes at 42°C, and then returned to the ice for 2 minutes before the addition of 0.9ml of pre-warmed L-broth. The cells were then incubated with shaking for 1 hour at 37°C, plated out onto L-agar plates supplemented with 100µg/ml ampicillin and incubated overnight at 37°C. Colonies were picked from plates and grown in L-broth supplemented with 100µg/ml ampicillin. Transformation of these colonies with recombinant plasmid was confirmed by insert typing (see section 2.3.5).

2.3.5 Insert Typing of Plasmid DNA

To determine which colonies contained recombinant vector, a rapid PCR based assay was developed. Bacterial colonies were grown in 5ml of L-broth containing $100\mu g/ml$ ampicillin for 5 hours. 1.5ml of cells were pelleted at 1500xg for 10 minutes, the supernatant was discarded and the pellet gently resuspended in $50\mu l$ of solution I (10mM EDTA pH 8.0, 25mM Tris HCl pH 8.0) and $100\mu l$ of solution II (0.2M NaOH, 1% w/v SDS), $50\mu l$ of solution III (3M potassium acetate, 11.5% glacial acetic acid) was then added and the mixture vortexed. An equal volume of phenol/chloroform was added and the solution vortexed and microcentrifuged for 5 minutes. The supernatant was aspirated and ethanol precipitated. The pellet was then washed in 70% v/v ethanol and air-dried, before resuspending in $20\mu l$ of water. The plasmid DNA was assayed for by PCR. The PCR reaction mixture was set up in separate top and bottom halves. For a single miniprep the reaction mixture was prepared as follows:

Bottom half:	Top half:
water to 12.5µl,	water to 12.5µl,
1.25µl 10x PCR buffer (method 2.2.5),	12.5µl 10x PCR buffer (method 2.2.5),
0.25µM CD23 5' sense primer,	0.1 mM of dNTP mix,
0.25µM CD23 3' antisense primer,	$1.5 \mathrm{mM} \mathrm{MgCl}_2,$
0.25µl template DNA	0.125µl rTaq DNA polymerase (5U/µl)

The bottom half mix was added to 0.5ml sterile eppendorf tubes, covered with one drop of mineral oil and run on the PCR programme detailed above (2.2.5). After 5 minutes the reaction was 'hot started' by the addition of 12.5 μ l of top half mix, added underneath the mineral oil and mixed briefly with a pipette tip. Once PCR was complete 5 μ l of each reaction was analysed on a 1% w/v agarose TAE gel (method 2.2.6).

2.4 Sequence Analysis and Mutagenesis of the Cloned CD23 cDNA

2.4.1 Wizard[™] Plus SV Miniprep kit for Plasmid DNA Purification

The Wizard[™] Plus SV Miniprep kit was used for rapid isolation of automated sequencing grade DNA. Bacterial colonies which were positive for CD23 inserts were grown up overnight in 5ml L-Broth containing ampicillin ($100\mu g/ml$), in a shaking incubator at 37°C. The cells were then centrifuged at 1500xg for 10 minutes and resuspended in 250µl of cell resuspension solution (50mM Tris-HCl pH7.5, 10mM EDTA, 100µg/ml RNAase A) in a 1.5ml sterile eppendorf. After vortexing, 250µl of cell lysis solution (0.2M NaOH, 1% w/v SDS) was added and the solution mixed by inverting the tube four times. The suspension was then incubated for 5 minutes to allow the solution to clear before 10µl of alkaline protease solution (supplied by Promega) was added. The solution was then mixed by inverting the tube four times and incubated at room temperature. After 5 minutes, 350µl of neutralisation solution (4M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid pH4.2) was added and mixed by inverting the tube four times. The bacterial lysate was then pellet by centrifuged at 13,000xg for 10 minutes at room temperature. The cleared lysate was transferred to a spin column, taking care to avoid transferring any of the white precipitate, and centrifuged at 13,000xg for 1 minute at room temperature. The column was then washed with 750µl of column wash solution (60mM potassium acetate, 10mM Tris-HCl pH7.5, 60% v/v ethanol) by centrifugation at 13,000xg

for 1 minute, then rewashed with 250μ l column wash solution by centrifugation at 13,000xg for 2 minutes. The spin column was then transferred to a fresh sterile eppendorf tube and the plasmid DNA eluted by the addition of 100μ l water to the spin column, followed by centrifugation at 13,000xg for 1 minute. The plasmid DNA was then stored at -20°C.

2.4.2 Sequencing Primer Design

To optimise sequencing PCR reactions, sequencing primers were designed with an optimal G+C content of between 40-50% that allowed an annealing temperature of between 50-60°C. Primers were designed along the length of the CD23 coding sequence. These were used in combination, along with primers designed to the T7 and T3 promoter sequences within the vector pBluescript® II KS (table 2.2). All primers were synthesised by PNACL (method 2.2.4).

pBluescript Sequencing Primers	5'-3'
T3 (pBluescript primer)	AATTAACCCTCACTAAAGGG
T7 (pBluescript primer)	GTAATACGACTCACTATAGGGC
CD23 Sequencing Primers	5'-3'
Primer 1 (sense)	CAAGCAGATCTGAGCAGCTTCAAG
Primer 2 (antisense)	CTTGAAGCTGCTCAGATCTGCTTG
Primer 3 (sense)	CTACTACTTCGGCAAGGGCACCAAG
Primer 4 (antisense)	CTTGGTGCCCTTGCCGAAGTAGTAG
Primer 5 (sense)	GAGTTTATCTGGGTGGATGGGAGC
Primer 6 (antisense)	GCTCCCATCCACCCAGATAAACTC

Table 2.2 Sequencing Primers

2.4.3 Automated Double-Stranded DNA Sequencing

Automated sequencing reactions were set up on ice in 0.5ml sterile eppendorf tubes. Reactions were set up in the following order: 8µl terminator ready reaction mix (1.85µM A-Dyedeoxy, 47.37µM C-Dyedeoxy, 0.42µM G-Dyedeoxy, 94.74µM T-Dyedeoxy, 94.74µM dITP, 15.79µM dATP, 15.79µM dCTP, 15.79µM dGTP, 15.79µM dTTP, 168.42mM Tris HCl pH 9.0, 42.10mM MgCl₂, 4.21mM (NH₄)SO₄, 0.42 units µl⁻¹ AmpliTaq DNA polymerase), 0.4µg template dsDNA, 32pM sequencing primer. Samples were amplified by PCR for 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 minutes at 60°C followed by cooling to 4°C. The PCR products were then purified for sequencing by precipitation with 0.1 volumes of 3M sodium acetate at pH4.6, and 2.5 volumes of 95% v/v ethanol. The solution was vortexed and incubated on ice for 10 minutes, then microcentrifuged at 13,000xg for 30 minutes. The supernatant was removed and the pellet washed in 70% v/v ethanol, before drying in a speed vac for 3 minutes. The dried DNA samples were then taken to PNACL for sequencing. Sequences were reported in electronic format and analysed using Mac Vector (IBI LTD, Cambridge, UK) and AutoAssembler (Applied Biosystems: Perkin Elmer) software.

2.4.4 Lithium Chloride Maxi-Preparation for Plasmid DNA Purification

Bacterial colonies successfully transformed with CD23/pBluescript® KS II plasmid were grown up overnight in 100ml of L-Broth containing 100µg/ml ampicillin in a shaking incubator at 37°C. The cells were then centrifuged at 1500xg for 10 minutes and resuspended in 5ml of solution I. Whilst the mixture was being swirled 10ml of solution II and 5ml of solution III were added, and the mixture vortexed and centrifuged at 1500xg for 10 minutes (Solutions I, II and III as used in 2.3.5). The supernatant was then filtered through a J-cloth and precipitated on ice with an equal volume of isopropanol for 20 minutes, then centrifuged at 1500xg for 10 minutes. After which, the supernatant was discarded and the pellet resuspended in 1.5ml of 1x TE (section 2.2.3), to which 2ml of 5M lithium chloride (chilled at -20°C) was added. The solution was then incubated on ice for 10 minutes, followed by centrifugation at 1500xg for 10 minutes. The RNA pellet was discarded and the supernatant precipitated with 2 volumes of neat ethanol (chilled at -20°C), re-centrifuged and washed with 95% v/v ethanol. The pellet was dissolved in 0.6ml of 1x TE containing boiled RNAase at a final concentration 40µg/ml and incubated for 15 minutes at 37°C. DNA was then precipitated on ice with 0.6ml of 20% w/v PEG 8000, 2.5M sodium chloride for 5 minutes. The precipitate was microcentrifuged for 10 minutes and the supernatant removed. The pellet was respun to remove traces of PEG, dissolved in 0.6ml of 1x TE and phenol/chloroform extracted twice, followed by ethanol precipitation and two washes in 70% v/v ethanol (see section 2.2.3). The DNA was resuspended in water at a concentration of 1mg/ml. DNA concentration was calculated based on 50µg/ml double stranded (ds)DNA is equal to 1 optical density unit at a wavelength of 260nm.

2.4.5 Site Directed Mutagenesis

The cloned CD23 sequence contained several point mutations, two of which would have created amino acid changes in the expressed protein as compared to the published sequence. Hence a site directed mutagenesis method was employed to alter these base pair mutations. Two complementary oligonucleotides containing a mutation encoding the correct, published codon and flanking sequence were synthesised by PNACL to cover each of the two point mutations within the CD23 cDNA sequence (table 2.3). These oligonucleotides were used to prime PCR reactions with the proof reading polymerase; cloned PfU. This polymerase was used as it has the lowest error rate $(1:1.3 \times 10^{-6} \text{ bases})$ of those commercially available. The PCR reaction mixture was set up on ice in the following order: water to a final volume of 50µl, 5µl 10x PfU PCR buffer (see method 2.2.5), 0.2mM dNTP mix (see method 2.2.5), 125ng of 5' primer, 125ng of 3' primer, 5-50ng dsDNA template, 2.5U cloned PfU DNA polymerase. Reactions were overlaid with 30µl mineral oil and heated to 95°C for 30 seconds, followed by 12 cycles of 30 seconds at 95°C, 1 minute at 55°C and 2 minutes at 68°C before cooling on ice for 2 minutes. The PCR product was then digested with 10U Dpn I, a methylation sensitive enzyme that only digests methylated DNA. The Dpn I was added directly to the reaction mixture, mixed thoroughly and microcentrifuged for 1 minute. The reaction was then incubated at 37°C for 1 hour to digest the parental (methylated) supercoiled dsDNA. After digestion the newly synthesised DNA was transformed into 100µl competent XL-1 Blue cells (method 2.3.4).

CD23 Mutagenesis Primers	Sequence 5'-3'
Mutagenesis Primer Set 1 (sense)	GAGCCATGTGGACTACAGCAACTGGGC
	CCCAGG
Mutagenesis Primer Set 1 (antisense)	CCTGGGGCCCAGTTGCTGTAGTCCACAT
	GGCTC
Mutagenesis Primer Set 2 (sense)	CCTGGGTCTGTGACCGGCTGGCCACATG
	CAC
Mutagenesis Primer Set 2 (antisense)	GTGCATGTGGCCAGCCGGTCACAGACCCA
	GG

Table 2.3 Primers used in Site Directed Mutagenesis of CD23

2.5 Expression of the CD23 Protein

2.5.1 Preparation of the CD23 Expression Cassettes by PCR

CD23 cDNA expression cassettes were prepared to express both membrane and truncated forms of the CD23 protein by PCR. Primers were designed to amplify CD23 cDNA with or without the N-terminal cytosol and transmembrane regions, to produce both membrane and truncated forms respectively. Included within the primer sequence for each cassette was a KOZAK sequence and expression tag sequence(s), incorporated to increase transcription via promoter recognition in mammalian cell lines and to aid the subsequent analysis of the expressed protein respectively (see table 2.4, and figure 2.1).

CD23 Expression Cassettes



Figure 2.1. CD23 Expression Cassettes

CD23 expression constructs encode for the membrane and truncated forms of the CD23 protein.

Key: RE Restriction enzyme site, enables cloning into a variety of constructs.

KOZAK consensus sequence for initiation of translation by eukaryotic ribosomes.

ATG start codon.

myc and 6x His expression tags for identification and purification of the protein. TGA stop codon.

CD23 Cloning Primers	Sequence 5' - 3'
Membrane CD23 (sense)	CCGGAATTCGCCACCATGGAGGAAGGTCAATATTCAG
	AG
Truncated CD23 (sense)	CCGGAATTCGCCACCATGGAACAAAAACTCATCTCA
	GAAGAGGATCTGAATGACACCACACAGAGTCTAAAA
	CAG
Membrane and Truncated	ATAGTTTAGCGGCCGCTCA <u>ATGATGATGATGATGAT</u>
CD23 (anti-sense)	<u>G</u> AGAGTGGAGAGGGGCAGAGGG

Table 2.4 Primers used to Clone CD23 into the Expression Vector pEV3

Restriction enzyme sites in **bold**: GAATTC EcoR I, GCGGCCGC Not I.

Kozak sequence <u>underlined</u>.

myc (sCD23 sense primer) and $[His]_6$ (mCD23 and sCD23 antisense primer) tags in **bold** and <u>underlined</u>.

All primers were synthesised by AstraZeneca Pharmaceuticals, Alderley Park. The cloned CD23 cDNA (in vector pBluescript® KS II) was linearised by digestion with the restriction enzyme Sca I. Digestion reactions were set up on ice in the following order: water to a final volume of 100µl, 10µl 10x buffer (100mM NaCl, 10mM Tris HCl, 10mM MgCl₂, 1mM DTT pH7.4), 5µg plasmid DNA, 6U enzyme per µg DNA. Reaction mixtures were digested for 2 hours at 37°C. The linearised plasmids were used as a template to amplify CD23 expression cassettes by PCR as outlined in section 2.2.5 using 100ng plasmid DNA as a template. The reactions were then amplified as follows; four minutes denaturation at 94°C, followed by 20 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 3 minutes extension at 72°C, before a final extension time of 10 minutes at 72°C and cooling to 4°C. PCR products were phenol chloroform extracted and ethanol precipitated (section 2.2.3) before resuspending in water. Amplified PCR products of the membrane and truncated CD23 expression cassettes were digested with the restriction enzymes Eco RI and Not I (digested in 1xREact 3 buffer; 50mM Tris HCl pH 8.0, 10mM MgCl₂, 100mM NaCl, as outlined in 2.3.1). Digested products were then electrophoresed at 110 volts for 40 minutes on a 1% w/v agarose TAE gel (method 2.2.6). The DNA bands equivalent to the predicted length of the CD23 membrane and truncated constructs were isolated from the gel using a Qiaex II gel extraction kit according to manufacturer's directions. The DNA expression cassettes were then ligated into pBluescript® KS II using a rapid DNA ligation kit ready for sequencing, to check that no further base mutations had occurred. The ligated constructs were then transformed into competent *E. coli* strain DH5 α (as outlined in section 2.3.4). Plasmids containing the membrane and truncated CD23 expression cassettes were identified by insert typing and then sequenced as described in section 2.4.1 and 2.4.3.

2.5.2 Caesium Chloride Maxi-Preparation for Plasmid DNA Purification

Caesium chloride density gradient was used to produce ultra pure expression vector DNA. The expression vector pEV3 (donated by AstraZeneca Pharmaceuticals) was transformed into 50µl competent DH5a. Bacterial cultures were grown up in 400ml of L-broth supplemented with 100µg/ml ampicillin overnight at 37°C with shaking. The cell cultures were then centrifuged for 10 minutes at 1500xg and the pellet resuspended in 20ml of lysis buffer A (50mM glucose, 250mM Tris HCl pH 8.0, 10mM EDTA) and incubated for 10 minutes at room temperature. 40ml of freshly prepared lysis buffer B (200mM NaOH, 1% w/v SDS) was then added, the solution mixed gently and incubated on ice for 5 minutes to denature the protein. Potassium acetate pH4.8 (final concentration 1.25M) was added and the mixture incubated on ice. After 15 minutes the mixture was centrifuged at 2500xg for 15 minutes at 4°C and the supernatant filtered through medical gauze into a clean vessel. The supernatant was precipitated with 0.6 volume of neat isopropanol for 2 minutes and then centrifuged at 2500xg for 10 minutes at 25°C. The pelleted DNA was resuspended in 26ml TE buffer (section 2.2.3) before the addition of 0.6mls of 0.5M EDTA pH 7.5 and 100µl of 2M Tris Base. The DNA was then added to 31g caesium chloride and mixed by inverting. Once dissolved 300µls of ethidium bromide (10mg/ml) was added and the solution transferred to 35ml ultracentrifuge tubes and balanced to within 0.1g of a partner tube. The tops were sealed by crimping and ultra-centrifuged (Beckman) at 45K overnight in a vertical TV850 rotor at 20°C. The following day 2.5ml of the stained DNA band was extracted using a sterile needle. The ethidium bromide was removed from the DNA by adding an equal volume of caesium chloride saturated isopropanol (caesium chloride is added to isopropanol until two layers are formed, the top layer is caesium chloride saturated isopropanol while the bottom layer is over saturated and the caesium chloride precipitates out of solution), mixing and then aspirating the aqueous layer. This was repeated 3-4 times until all visible ethidium bromide had been removed. The plasmid DNA was then diluted with 5ml of water and precipitated with 2 volumes of neat ethanol at room temperature for 15 minutes followed by centrifugation 13,000xg for 15 minutes at
4°C. Plasmid DNA was washed twice with chilled 70% v/v ethanol and resuspended at a final concentration of 1mg/ml in water.

2.5.3 Cloning of the CD23 Expression Cassettes into Expression Vectors

The membrane and truncated CD23 expression cassettes with correct sequence were excised from pBluescript® KS II by restriction enzyme digestion with *Eco R*I and *Not* I (as outlined in section 2.3.1). The isolated DNA fragments were then ligated into the expression vector pEV3 using a rapid DNA ligation kit. The pEV3 plasmid containing the CD23 expression cassettes were then linearised using *Pvu* II restriction enzyme (as outlined in 2.3.1 using 10x REact 6 buffer; 50mM Tris HCl pH 7.4, 6mM MgCl₂, 50mM NaCl, 50mM KCl). The DNA was then extracted by phenol/chloroform and ethanol precipitation before resuspending at 1mg/ml in sterile TE (see section 2.2.3).

2.5.4 Transfection of pEV3 into MEL cells by Electroporation

The CD23 expression cassettes (containing membrane and truncated CD23 cDNA) cloned into pEV3 were transfected into Murine Erythroleukemia (MEL)-C88 cells (donated by Mr. M. Needham, AstraZeneca Pharmaceuticals, Alderley Park, Macclesfield) for expression. MEL cells were grown in log phase four days before transfection by electroporation. This was achieved by splitting and re-seeding the cells at a density of $2x10^{5}$ /ml every 24 hours, 4 days prior to the transfection experiment (see method 2.6.2). MEL cells grown in log phase should have a doubling time of approximately 12 hours. Twenty-four hours before transfection the cells were centrifuged and re-seeded at a concentration of 2×10^5 /ml. 50µg linear plasmid DNA was added to an electroporation cuvette on ice. MEL cells were pelleted by centrifugation at 250xg for 7 minutes at room temperature. Cells were washed twice in electroshock buffer (140mM NaCl, 25mM HEPES pH7.5, 4M NaOH, 0.75mM Na₂HPO₄) and resuspended at 1×10^{7} /ml. 1×10^{7} cells were then added to the cuvette and incubated on ice for 10 minutes. The cells were quickly resuspended by flicking the electroporation cuvette before electroporation at 960µF, 250 volts using a Biorad Gene pulser (Biorad, Hemel Hempstead, Herts, UK). The cells were then left for 10 minutes to recover before diluting at two concentrations 1x10⁵/ml and $1x10^{4}$ /ml, each concentration suspension was plated at 1ml aliquots in 24 well plates. The transfected cells were incubated at 37°C with 5% CO₂, 95% air for 24 hours before the addition of selective media (growth media containing G418 Geneticin at a final concentration of 1mg/ml).

2.6 Cell Growth, Expression and Storage

2.6.1 Cell Growth and Culture Media Conditions

MEL cells were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% v/v foetal bovine serum (FBS), 1x Non Essential Amino Acids, 100U/ml Penicillin G, 100 μ g/ml Streptomycin Sulphate, 1x Glutamax. Geneticin G418 selection (1mg/ml) was added to cells transfected with the pEV3 plasmid containing CD23 DNA. RPMI 8866 cells, RPMI 8226 cells and EBV-DR B cells were all cultured in RPMI 1640 medium supplemented with 10% v/v FBS and 1x glutamax. All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂, 95% air.

2.6.2 Protein Expression by Transfected MEL Cells

Cells were grown in log phase by splitting and re-seeding at $2x10^5$ every 24 hours, for 3-4 days prior to induction of rCD23 expression. The number of viable cells was calculated using a hemacytometer based on trypan blue exclusion. Once viable cells were growing in log phase, dimethyl sulphoxide (DMSO) was added to a final concentration of 2% v/v. The cells were then cultured for four days at 37°C in a humidified atmosphere with 5% CO_2 95% air.

2.6.3 Long Term Storage of Mammalian Cells

Cells to be stored long term were prepared by centrifuging cell suspension at 250xg for 10 minutes at 20°C and resuspended at $1x10^{6}$ /ml in normal growth media supplemented with 10% v/v DMSO. The cells were aliquoted into 2ml cryotubes (Nunc), packed inside a padded polystyrene box. Aliquots were frozen for 24 hours at -70°C, in order to freeze the cells slowly to prevent ice crystal formation. Finally cells were then transferred to liquid nitrogen for long term storage. MEL cells were grown in log phase as described above (2.6.2) before preparing for long term storage.

2.7 Generation of Antibodies to Synthetic Peptides Representing CD23 Neo-Epitopes

2.7.1 Production of Neo-Epitope Peptides

Neo-epitope peptides were designed to the CD23 endogenous cleavage sites (producing 37 and 25 kDa sCD23 products) based on the published sequence (table 2.5). Peptides were synthesised by AstraZeneca Pharmaceuticals and conjugated to a carrier protein before injection into rabbits to produce an antibody response.

Anti-NeoEpitope Peptides Designed to:	Amino Acid Sequence
37kDa Cleavage Site (5' acetyl end)	NH ₃ ,Gln,Lys,Ser,Gln,Ser,Thr,Gln,Ile,Ser,Cys,
ZENECA Reference No. 802124	NH ₂ (C-terminal amide)
37kDa Cleavage site (3' carboxy end)	(N-terminal acetyl)
ZENECA Reference No. 802105	ac.Cys,Glu,Ser,His,His,Gly,Asp,Gln,Met,Ala.
25kDa Cleavage Site (5' acetyl end)	NH ₃ ,Met,Glu,Leu,Gln,Val,Ser,Ser,Gly,Phe,Cys
ZENECA Reference No. 802104	,NH ₂ (C-terminal amide)
25kDa Cleavage site (3' carboxy end)	(N-terminal acetyl)
ZENECA Reference No. 802106	ac.Cys,Leu,Arg,Glu.Glu,Val,Thr,Lys,Leu,Arg.

Table 2.5 NeoEpitope Peptide Sequences

2.7.2 Carrier Protein Activation and Conjugation to Neo-Epitope Peptides

Bovine thyroglobulin or ovalbumin (25mg) used as carrier proteins, were dissolved in 1ml 0.02M sodium phosphate (Na,HPO, pH7.0). 1.25mg m-maleimeidobenzoyl-N-hydroxy succinimide ester (MBS) was dissolved in dimethyl formamide and added to the carrier protein dropwise and stirred for 1 hour at room temperature. Meanwhile a Tris acryl GF50 column was washed three times with 0.1M Na₂HPO₄ pH6.0 (phosphate buffer). The dissolved thyroglobulin/ovalbumin (in a final volume of 1ml) was applied to the column and allowed to pass through under gravity at a rate of 1 ml/minute. A further 5ml of phosphate buffer was added to the column and the fractions collected in 1ml aliquots. The concentration of the carrier protein was measured at 280nm. The first three fractions with a concentration greater than OD_{280} 3.0 were pooled and added to the immunogenic peptides of CD23 (prepared as described below 2.8.3). 0.2M Tris pH8.2 (3ml) was immediately added to neutralise the solution and stirred overnight at room temperature in the dark. A sample from the reaction was sent for amino acid analysis (at AstraZeneca Pharmaceuticals). Successful conjugation was determined by the change of the N-terminal cysteine to a succinyl-cys molecule. The protein-peptide conjugates were then aliquoted and stored at 4°C ready for immunisation.

2.7.3 Preparation of Immunogenic Peptides for Conjugation to a Protein Carrier 10mg of each CD23 immunogenic peptide was dissolved in 2ml 0.05M Na_2HPO_4 pH7.4, to which 4mg DTT was added and stirred for an hour at room temperature in the dark. The samples were washed 4 times with 4ml ethyl acetate, after the final wash trace elements of ethyl acetate were removed using nitrogen and the peptides were added to the 'activated' carrier protein.

2.7.4 Immunisation of Rabbits with Protein-Peptide Conjugates

Immunisation of rabbits and the collection of antiserum was performed by trained staff at AstraZeneca Pharmaceuticals. Female New Zealand white rabbits were immunised with the protein-peptide conjugates at monthly intervals. 200µg protein-peptide conjugate was diluted 1:1 with Freund's Complete adjuvant in the first instance (to help boost an immune response) and then with Freund's Incomplete adjuvant on subsequent immunisations. The mixture was split into four aliquots and each injected subcutaneously at a separate site. Ten days after immunisation 50ml blood was collected and mixed with 0.38% (final concentration) tri-sodium citrate and serum extracted by centrifugation.

2.8 Protein Analysis: Methods Used to Characterise Recombinant Protein Expression and Clarify Anti-Neo-Epitope Antibody Specificity

2.8.1 Enzyme Linked Immunoabsorbent Assay

Enzyme Linked Immunoabsorbent Assays (ELISAs) were used to determine if a specific antibody response had been raised to the peptide conjugates. Maxisorp 96 well plates (Nunc) were coated with 100µl of 20µg/ml of each peptide conjugate diluted in carbonate/bicarbonate buffer pH9.6 (35mM NaHCO₃, 15mM Na₂CO₃). ELISA plates were incubated with antigen for 2hrs at room temperature with shaking. The plates were then washed three times with phosphate buffered saline (PBS), Tween 20 (0.05%) and tapped dry onto absorbent towels on the bench. Plates were then incubated with 100µl blocking buffer per well for 15 minutes at room temperature to block non-specific antibody binding. The blocking buffer was then removed and 100µl of rabbit serum diluted over a range of concentrations in blocking buffer (PBS, 0.05% v/v Tween 20, 1% w/v Marvel), was added to each well. The plates were then incubated for a further two hours at room temperature with shaking. Serum was removed by washing three times with blocking buffer and the plates were tapped dry, before the addition of the secondary goat anti-rabbit IgG peroxidase conjugate at a dilution of 1/2000. Plates were incubated for a further 2 hours at room temperature with shaking. After extensive washes with PBS and Tween 20 (0.05% v/v) the plates were tapped dry and 50µl O-Phenylenediamine Dihydrochloride (OPD) substrate (1 OPD tablet dissolved in 20ml Phosphate Citrate buffer) was added. Once a yellow coloured solution was observed the reaction was stopped by the addition of 25μ l 2M H₂SO₄ and the reaction analysed on a spectrophotometer at a wavelength of 492nm.

2.8.2 Immunofluorescent Cell Staining for Analysis by Flow Cytometry or Confocal

Cells were washed twice by centrifugation at 250xg for 10 minutes and resuspended in assay buffer (1X PBS, 1% w/v BSA) at $1x10^6$ per sample. Cell suspensions were then transferred into 4ml Falcon tubes. To these 50µl of diluted primary antibody was added and incubated on ice for 30 minutes. After which the cells were washed twice (as above), resuspended at $1x10^6$ ml and 50µl of diluted FITC-conjugated secondary antibody (specific for the primary antibody) added. The cells were then incubated on ice in the dark for 30 minutes, before washing twice (as above) and finally resuspending in 500µl assay buffer ready for analysis by flow cytometry using a FACScan and Lysis II software (Becton Dickinson), or in 100µl assay buffer for analysis by confocal microscopy, Kalman 6 analysis.

2.8.3 IgE Binding to Recombinant CD23

Recombinant CD23 expressed by MEL cells was analysed for its IgE binding capacity in comparison with that expressed by RPMI 8866 cells. MEL cells induced to express CD23 were washed in DMEM plus 1% w/v BSA. RPMI 8866 cells were washed in RPMI 1640 medium plus 1% w/v BSA. Both cell cultures were resuspended at $1x10^{6}$ /ml and then incubated with human myeloma IgE (0.5mg/ml) (donated by Dr. Roger Drew, The Binding Site, Birmingham, UK) or polyclonal human IgG (1mg/ml) for 1 hour at 37°C in a 5% CO₂, 95% air, 100% humidity incubator. Cells were then washed twice in DMEM or RPMI 1640 plus 1% w/v BSA as appropriate, and resuspended at $1x10^{6}$ /ml. IgE binding by CD23 on the cells was then determined by flow cytometry (see section 2.8.2) using a sheep anti-human IgE and anti-sheep IgG FITC as the secondary and tertiary detection antibodies.

2.8.4 Intracellular Cell Staining for Confocal Analysis

Cells were washed in PBS and resuspended at 1×10^6 /ml. To each ml of cells, 1ml fixative (4% w/v formaldehyde diluted in ice-cold PBS) buffer was added and the solution was incubated on ice for 20 minutes, shaking occasionally. The cells were then pelleted at 250xg for 10 minutes and washed once in wash buffer (1xPBS, 0.1% w/v BSA), before

resuspending at 1x10⁶/ml in permeabilisation buffer (1xPBS, 0.1% w/v Saponin, 1% w/v BSA), and incubating at room temperature. After 10 minutes diluted primary antibody was added and the cell solution incubated for a further 45 minutes. The cells were then washed in fresh permeabilisation buffer, resuspended in diluted secondary antibody solution and incubated at room temperature in the dark. After 45 minutes the cells were washed again, once in permeabilisation buffer and once in wash buffer, before resuspending in 100µl wash buffer ready for confocal microscopy, Kalman 6 analysis.

2.8.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse recombinant CD23 protein expression and cleavage. All gels were run under reducing conditions. A 12.5% separating gel (12.5% w/v acrylamide, 0.4% w/v bisacrylamide, 375mM Tris HCl pH8.8, 0.1% w/v SDS, 0.25% w/v APS, 0.025% v/v TEMED) was cast into a BioRad protein gel system over which a 4.5% stacking gel (4.5% w/v acrylamide, 0.15% w/v bisacrylamide, 130mM Tris HCl pH6.8, 0.1% w/v SDS, 3.75% w/v APS, 0.004% v/v TEMED) was carefully layered to prevent mixing of the interphase. A toothed comb was inserted and the gel was left to polymerise overnight at 4°C. Samples were prepared by the addition of 3x SDS sample buffer (4% w/v SDS, 20% v/v glycerol, 100mM Tris HCl pH6.8, 0.2% w/v bromophenol blue, 100mM DTT) and incubated at 65°C for 30 minutes to fully denature the protein, followed by a brief centrifugation at 13,000xg. The comb was removed from the gel and the samples loaded into the wells. Gels (x2) were electrophoresed in SDS running buffer (25mM Tris, 0.25M glycine, 0.1% w/v SDS) at 75mA for approximately 4.5 hours.

2.8.6 Silver Staining of SDS-PAGE Gels

The SDS-PAGE gel was stained in 45% v/v methanol, 10% v/v acetic acid for 25 minutes with gentle shaking, then in 5% v/v methanol, 2.5% v/v acetic acid for 15 minutes. Gels were fixed by incubation in 10% v/v glutaraldehyde for 20 minutes followed by washing in 1L milliQ water overnight again with gentle shaking. Gels were then incubated in 5 μ g/ml DTT for 15 minutes followed by 0.1% w/v silver nitrate for 20 minutes. The stain was developed by incubating in a solution of 384mM sodium carbonate, 0.00064% formaldehyde until protein bands could be seen. The developing solution was then quickly removed by washing briefly in milliQ water and the reaction stopped by incubating in 1% v/v acetic acid. The gels were then washed 3x 30 minutes in milliQ water and the results photographed by the reprographics department (Leicester University).

2.8.7 Western Blotting / Immunoblotting

Proteins separated by SDS-PAGE were transferred to Immobilon-P membranes electrophoretically. SDS-PAGE gels were soaked in transfer buffer (25mM Tris, 192mM glycine, 10% v/v methanol) for 10 minutes. Immobilon-P membrane was cut to the size of the SDS-PAGE gel to be blotted and wet with neat methanol, then rinsed in milliQ water for 2 minutes and finally soaked in transfer buffer for 5 minutes. The gel was laid upon 2 sheets of pre-soaked Whatman[®] 3MM paper orientated so that lane 1 of the gel was on the right hand side of the 3MM paper. The Immobilon-P membrane was placed on top of the gel followed by another 2 sheets of pre-soaked 3MM paper. Air bubbles were removed by rolling a sterile stripette gently over the top. The sandwiched gel was then placed inside the transfer tank with the Immobilon-P membrane next to the anode. The tank was then filled with transfer buffer and electrophoresed at 300mA overnight at 4°C. If Immobilon-P membranes could not be used in immunodetection methods straight away, they were removed from the Western blot electrophoresis tank and left to air dry for two hours before storing at 4°C.

2.8.8 Immunodetection of Western Blots using ECL Chemiluminescence

If Immobilon-P blots had been left to dry and stored at 4°C then they were re-wet with neat methanol, followed by soaking in milliQ water for 2 minutes and then soaked in blocking buffer (10mM Tris pH 7.4, 0.15NaCl, 0.1% w/v Tween 20) for 5 minutes. Otherwise Immobilon-P blots were removed from electrophoresis apparatus and soaked in blocking buffer for 5 minutes. The membrane was then placed into fresh blocking buffer containing 8% (w/v) non-fat dry milk (Marvel) to block non-specific binding of antibodies. After 15 minutes the membrane was removed from the blocking solution and incubated in diluted primary antibody solution (with blocking buffer containing 8% w/v Marvel) for 1-2 hours depending on the affinity/avidity of the antibody. The membrane was then washed 3 times for 10 minutes with fresh changes of blocking buffer containing 8% w/v Marvel and incubated for 1 hour in diluted horseradish peroxidase (HRP)-labelled second antibody solution (diluted with blocking buffer containing 8% w/v Marvel). Finally the membrane was washed 3 times for 10 minutes in blocking buffer then rinsed in milliQ water, before developing with ECL[™] luminescence substrate. The membrane was then heat-sealed within a clear plastic cover (nylon polyethylene vacuum pouch) to prevent the luminescent substrate from leaking onto the film and placed against three sheets of Kodak autoradiographic film and left overnight to ensure full exposure before developing.

2.8.9 Extraction of Membrane Proteins from MEL Cells

1 litre induced MEL cells expressing recombinant CD23 and untransfected controls (see section 2.6.2) were centrifuged for 8 minutes at 250xg and resuspended in 50ml F-10 Ham's nutrient media. The cells were re-pelleted at 300xg then resuspended in 4x pack cell volume hypertonic medium (3 parts water, 1 part media) containing protease inhibitors (see section 2.8.10) on ice. The cells were then mechanically lysed three times using a polytron (Kinematica) up to 22000rpm on ice, allowing the cells to rest for 30 seconds between each round. The lysed cells were layered on top of 10ml 41% (w/v) sucrose in ultracentrifuge tubes, being careful not to mix the interphase and the tube filled to the top with media. The tubes were then centrifuged at 28K under vacuum in an ultracentrifuge, rotor SW28 (Beckman) for 1 hour, after which the interphase was aspirated and added to fresh media in a new ultracentrifuge tube. The cells and media were mixed to dilute any sucrose contamination and Ultracentrifuged at 23000 rpm for 20 minutes. The cells were finally resuspended in 1×10^8 cell membranes/ml in PBS, aliquoted into 1ml freezing vials and stored at -70° C until required for analysis.

2.8.10 Protease Inhibitors

The following protease inhibitors were added to cells and corresponding supernatant fractions lysed by freeze-thawing and mechanical lysis methods.

Inhibitor Name	Target Proteases	Concentration
E64	Cysteine	5μΜ
EDTA	Metallo-	10mM
Leupeptin	Trypsin-like serine and	100µM
	some cysteine proteases	
Pepstatin	Aspartic	1µM
1/10-Phenanthroline	Metallo-	10mM
PMSF	Serine	1mM
TLCK	Trypsin-like serine	100µM

2.8.11 Column Purification of 6x His Tagged Proteins using TalonTM Metal Affinity Resin Talon metal affinity resin is supplied as a 50% (v/v) slurry in non-buffered 20% v/v ethanol, this was removed before binding of 6x His tagged protein by centrifuging Talon resin at 700xg for 2 minutes at 4°C and replacing non-buffered ethanol with an equal volume of PBS. Protein samples purified by NP-40 lysis (see section 2.8.12) from induced MEL cells expressing rCD23 or MEL cell controls and corresponding supernatant samples, were incubated with 500µl to 1ml resuspended Talon resin in PBS overnight at 4°C. Bound protein was eluted by a gravity flow column purification method under native conditions. The samples were added to a 2ml gravity flow column and unbound protein allowed to drain through. The column was then washed twice with 10 bed volumes of wash buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 10mM Imidazole), the bound protein was then eluted with elution buffer (50mM NaH₂PO₄ pH 8.0, 300mM NaCl, 250mM Imidazole) and collected in 1ml aliquots.

2.8.12 Protein Purification by NP-40 Lysis

 $10x10^{6}$ cell were washed in PBS, resuspended in 1ml fresh PBS and transferred to 1.5ml eppendorf tubes. Cells were briefly centrifuged for 20 seconds, resuspended in 200µl lysis buffer (0.14M NaCl, 1.5mM MgCl2, 10mM Tris HCl pH8.6, 0.5% v/v NP-40) and incubated on ice for 1 minute, after which 200µl lysis buffer containing 1% v/v NP-40 and 24% w/v sucrose was added underneath the lysed cells. The sample was then centrifuged at 13000xg for 10 minutes and the supernatant aspirated (containing the cytoplasmic and membrane proteins). Protein samples were stored at -70° C until required.

2.8.13 Biosynthetic Labelling of Protein

Cells were washed and resuspended at $2x10^6$ /ml in methionine/cysteine free growth media (depending on cell type) supplemented with 2% v/v FBS, 1x Non-essential amino acids and 1x L-glutamax and incubated at 37°C to deplete intracellular pools of methionine and cysteine. After 2 hours the cells were pelleted at 250xg for 10 minutes at 20°C, resuspended at $5x10^6$ /ml in warm methionine/cysteine free media plus 0.1mCi/ml Trans ³⁵S labelTM (³⁵S labelled methionine and cysteine). After 30 minutes at 37°C the cells were repelleted at 250xg for 10 minutes at 4°C, before resuspending at 1x10⁶/ml in normal growth media (containing cysteine and methionine) and incubated at 37°C. After a chase period of between 2-24 hours, cells were finally centrifuged at 250xg for 10 minutes, the supernatant aspirated and the cells lysed by NP40 (see section 2.8.12), both supernatant and cell lysates

were kept for analysis of rCD23 labelled protein expression by immunoprecipitation and SDS-PAGE.

2.8.14 Immunoprecipitation of CD23 using rProtein A Sepharose® Fast Flow

Recombinant (r)protein A Sepharose® fast flow was used to precipitate anti-CD23 antibody/rCD23 protein complexes from cell lysates and supernatant samples. Protein A specifically binds the Fc region of IgG and was therefore used to purify anti-CD23 IgG antibody/CD23 complexes. rProtein A Sepharose is supplied as a slurry in 20% v/v ethanol, this was removed by centrifugation at 700xg for 2 minutes at 4°C and replaced with an equal volume of PBS. Before immunoprecipitation with anti-CD23 antibody, cell lysates were pre-cleared with pre-immune rabbit serum (obtained from rabbits used in method 2.7.4). rProtein A Sepharose resuspended in PBS (50µl), diluted rabbit serum and cell lysate samples were incubated on ice for 2 hours, samples were then centrifuged at 4°C and the unbound cell lysate aspirated and used in the immunoprecipitation experiment. rProtein A Sepharose resuspended in PBS (500µl), diluted anti-CD23 IgG antibody and either lysate or supernatant samples were incubated overnight at 4°C whilst rotating. Bound protein was eluted by gravity flow using a column purification method. The samples were added to a 2ml gravity flow column (separately) and unbound protein allowed to drain through under gravity. The column was then washed twice with 10 bed volumes of wash buffer (0.15M NaCl, 20mM Tris HCl pH 7.4) and the bound protein eluted with elution buffer (0.1M glycine HCl pH 3.0) and collected in 1ml aliquots containing 10µl 2M Trizma base used to neutralise the pH of the solution to around pH7.5. The samples were then analysed by SDS-PAGE.

2.9. Miscellaneous

2.9.1 Long Term Storage of Bacterial Cells

Bacterial cells/clones positive for CD23 were grown in L-broth plus antibiotic selection overnight. Cells were then pelleted by centrifugation at 1500xg for 10 minutes at 4°C and resuspended in L-broth, 850µl resuspended cells were aliquoted into 2ml cryotubes (Nunc), to which 150µl sterile glycerol was added before storage at -70°C.

2.9.2 Preparation of Oligonucleotides for PCR

Oligonucleotide primers were supplied in concentrated ammonia from Protein and Nucleic Acid Chemistry Laboratory (PNACL) (Leicester University), these were purified by two rounds of ethanol precipitation and resuspended in water before use in PCR.

2.9.3 Peripheral Blood Mononuclear Cell Extraction from Human Blood

Whole blood was diluted with an equal volume of F^+ media (RPMI 1640, 2mM Lgultamine, 10% v/v FCS) supplemented with heparin to a final concentration of 10U/ml. The diluted blood (25ml) was layered slowly onto 20ml of Lymphoprep density gradient media (to prevent mixing of the interphase) and centrifuged at 800xg for 30 minutes at 20°C. Peripheral blood mononuclear cells (PBMCs) were aspirated from the interphase and diluted with an equal volume of F⁺ media. The cells were then washed twice in PBS and used as a source of RNA (as outlined in 2.2.1).

2.9.4 Analysis of RNA by Northern Hybridisation

Northern blotting was used to analyse transcription levels of CD23 mRNA. Between 10-20µg of total RNA per sample was adjusted to 10µl total volume with water, to which 30µl of denaturing buffer (0.5ml 10x Northern buffer, 2.5ml deionised formamide, 0.3ml formaldehyde, 1.7ml water) was added, mixed and heated at 60°C for 5 minutes. The samples were then loaded into an agarose/formaldehyde gel (0.95g agarose melted in 80ml 1x Northern buffer (20mM MOPS pH7.2, 1mM EDTA pH 8.0, 8mM sodium acetate) and allowed to cool to 60°C before the addition of 15ml of formaldehyde) and run at 100mAmps for five hours, until the bromophenol blue had migrated two-thirds of the length of the gel. The denatured RNA was then transferred to a nylon membrane (Hybond N) by capillary blotting overnight. After transfer the RNA was cross-linked to the membrane by baking for 2 hours at 80°C in a vacuum oven. The membrane was then prehybridised for 2 hours at 65°C in Church-Gilbert buffer (0.5M Na₂HPO₄ pH 7.4, 7% w/v SDS, 1mM EDTA) plus 1mg boiled and sonicated ssDNA. A CD23 probe (100ng ds DNA) or internal control either β -actin for human primary cells or cell lines or β -globin for murine cell lines was radiolabelled using a multiprime DNA labelling kit, following manufacturers instructions. The radiolabelled probe was added to the Church-Gilbert buffer and the membrane hybridised overnight at 42°C. After hybridisation the membrane washed in 0.1% w/v SDS, 2x SSC at room temperature for 5 minutes, 0.1% w/v SDS, 0.2x SSC at 50°C for 15 minutes and finally in 0.1% w/v SDS, 0.1x SSC at 50°C for 15 minutes. The membrane was then wrapped in Saran Wrap and exposed to X-ray film (Kodak) and incubated at -70°C for 7 days before developing.

Chapter 3: Development of a Cell Based Assay to Elucidate Processing of CD23

3.1 Introduction

In order to assay the cleavage events in CD23 a model system was required. Several options were available. (1) Resting primary B cells; CD23 expression is tightly regulated on primary cells, with constitutive CD23 (a form) being found only on a subset of IgM⁺/IgD⁺ double bearing B lymphocytes (Kikutani et al., 1986). (2) Activation of CD23 expression on primary B cells; expression levels of both CD23 a and b forms can be upregulated on many other cell types (including B cells) by a variety of mediators, such as IL-4, IL-13 (Punnon et al., 1993 and McKenzie et al., 1993), IL-2, IFN-α (Delespesse et al., 1991) and phorbol esters (Gordon et al., 1986). (3) Transformed B cells; transformation of B cells by Epstein-Barr virus (EBV) induces permanent expression of CD23 at relatively high levels. However, despite this relatively high level of membrane CD23 expression, only a small fraction is cleaved from the cell surface to form soluble CD23 products. The concentration of soluble CD23 shed from EBV-transformed B cells is in the range of 70-300ng/10⁶ cells/ml every 24 hours (Cairns and Gordon 1990). This includes the release of all sizes of sCD23 fragments, comprised of 35-50% 37kDa sCD23, 17-25% 33kDa sCD23, 15% 29kDa sCD23 and 17-25% 25kDa sCD23 (Marolewski et al., 1998). This is a relatively small amount of material which is not easily detected by standard protein detection methods (e.g. Coomassie blue staining, which detects ≤50ng of protein, silver staining, which detects ≤5ng of protein and immunodetection, where sensitivity depends on affinity/avidity of the antibody but is usually the most sensitive of the three).

Therefore, in order to analyse CD23 processing using these cell systems as a model, the material needs to be concentrated or analysed using highly sensitive methods such as radiolabelling protein with ¹²⁵I or ³⁵S. Both these strategies are expensive and labour intensive and pose problems of their own (which are discussed in greater detail in chapter 4). On the other hand immunodetection presents an inexpensive, sensitive and quantitative detection method but relies on the availability of specific immunoreagents for each of the cleaved fragments. In order to overcome these problems and provide a relatively inexpensive source of CD23, CD23 was cloned and transfected into murine

erythroleukaemia (MEL) cells, using a mammalian expression system known to produce high level expression of recombinant protein (described below). In addition antineoepitope antibodies, specific for the amino and carboxy termini of cleaved CD23 were generated to provide a mechanism for detecting the different sized sCD23 molecules (the generation and characterisation of these antibodies are described in detail in chapters 4 and 5).

3.1.1 Advantages of Recombinant Proteins

Both membrane and soluble CD23 fragments have been expressed previously as recombinant proteins in mammalian and insect cell expression systems (Ludin *et al.*, 1987, Lui *et al.*, 1991 and Graber *et al.*, 1992). This approach provides many advantages in that CD23 is produced in workable quantities, relatively inexpensively and quickly once the system has been developed. It also provides the opportunity to incorporate affinity tags (to the N- and C-terminus of the recombinant protein) to which commercial antibodies are available. The addition of these tags to the recombinant protein provides a mechanism by which the cleaved fragments can be easily identified and purified from the culture media/supernatant. The production of a recombinant protein also allows the researcher to mutate the protein to explore structure/function relationships. Hence, recombinant full-length CD23 was cloned and expressed in MEL cells.

Recombinant protein expression is a method often used to produce truncated or mutated forms of the protein of interest to investigate its biological function or regulation. Therefore a truncated (t)CD23 molecule, representing the extracellular portion of the membrane molecule (i.e. without the intracellular and transmembrane domains see figure 1.1) was also cloned and expressed by MEL cells. The production of a truncated form of CD23 would provide a tool to assay proteolytic cleavage events in the absence of the cell membrane and to test CD23 for any associated autoproteolytic activity (as described by Letellier *et al.*, 1990).

3.1.2 Anti-CD23 Antibodies

The majority of anti-CD23 antibodies that are commercially available are monoclonal antibodies that recognise epitopes in the lectin domain of the native molecule (Bonnefoy *et al.*, 1990). These antibodies are particularly suited to assaying CD23 under native conditions but are unable to recognise denatured CD23 in immunoblots. The lectin

domain is present in all sCD23 fragments as well as membrane CD23, therefore these antibodies can be used to detect both membrane and soluble CD23. However, the limitations of these antibodies are that the majority of them do not distinguish between the sizes of sCD23 fragments detected (this limitation is due to the research method to which the antibodies are suited e.g. flow cytometry and ELISA). Several researchers (Cairns and Gordon 1990, Marolewski *et al.*, 1998) have created polyclonal antibodies that recognise both the membrane and soluble CD23 fragments, and because these antibodies recognise denatured CD23 they can be used in immunoblot detection methods to determine the fragment size. However, these antibodies to not differentiate between fragments of the same size, i.e. a 25kDa fragment could represent a sCD23 molecule cleaved between residue 149 and 150 containing the lectin domain and the C-terminal tail. Alternatively it may represent a sCD23 molecule, cleaved firstly between residues 80 and 81, and secondly within the C-terminal tail. Therefore, the addition of affinity tags not only provides a means of identifying rCD23, they also allow determination of which fragment of the molecule is present and facilitate tracking of the cleavage events in relation to structure.

3.2 Cloning CD23

Detailed studies of the CD23 gene sequence are published (Suter *et al.*, 1987, Yokota *et al.*, 1988). Hence, CD23 (a form) was cloned by reverse transcription-polymerase chain reaction (RT-PCR), using oligonucleotide primers complementary to the published CD23 DNA sequence. RNA isolated from EBV-DR B cells (kindly donated by Dr. Wendy Lawley, Leicester University, Leicester, UK) was used as a source of messenger (m)RNA, to provide a template for the amplification of the CD23 coding sequence. Cloning of the resulting DNA fragment into a high copy number vector pBluescript®II KS and the subsequent transformation into bacterial cells (*E.coli*; XL-1Blue) provided an unlimited source of the CD23 cDNA construct for further manipulation.

3.2.1 Oligonucleotide Primer Design

Oligonucleotide primer sequences, and their location within the CD23 gene sequence are shown in figure 3.1. Oligonucleotide primers were designed with similar optimal annealing temperatures to contain approximately 60% G+C content and include the

(a) Full length mRNA Sequence.

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	non-coding region	ATG	cytoplasmic domain	transmembrane region	coiled-coil stalk	lectin head	C-teminal tail	non-coding region
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(b)

CD23 5' Sense Primer

Primer5' CCG CTC GAG CA AGC AGG ACC GCC ATG GAG3'CD23 Sequence5' AAT CCA AGC AGG ACC GCC ATG GAG GAA GGT ... 3'

CD23 3' Anti-Sense Primer

3'	ACT C GTA CCT ATG TCG GTC CGG GTC 5'	CD23	Sequence
	3' GTA CCT ATG TCG GTC CGG GTC AGA TCT GC	5'	Primer

Figure 3.1 CD23 Primers and their Locations within the CD23 mRNA Sequence.

(a) Primers were designed to be complementary to the non-coding (flanking RNA sequence up- and down-stream) and the coding sequence for CD23 (a form). Primers were designed with similar optimal annealing temperatures (60° C sense primer and 58.9° C antisense primer). (b) Actual primer sequences used to amplify CD23, aligned against the non-coding and coding (**bold**) mRNA sequence for CD23. The restriction enzyme sequences *Xho* I and *Xba* I (*bold italics*) were incorporated into the 5' sense and 3' antisense oligonucleotide primers respectively, to aid cloning of the resulting PCR product.

restriction enzyme recognition sequences *Xho* I and *Xba* I (within the 5' and 3' oligonucleotide primers respectively). The restriction enzyme sites were included to aid ligation of the resulting cDNA fragment into the multiple cloning site (MCS) of the vector pBluescript®II KS.

3.2.2 Choice of Cloning Vector

The pBluescript®II KS vector (figure 3.2) contains several important features useful in cloning. It is a high copy number vector, thus resulting in relatively high plasmid DNA yields. Copy number is determined by the region of DNA surrounding and including the origin of replication in the plasmid. This region is known as the replicon, it controls replication of plasmid DNA by bacterial enzyme complexes, all pBluescript vectors (from Stratagene) contain the ColE1 origin of replication. Other useful elements within this vector include the ampicillin resistance gene (Amp^r), this allows for antibiotic selection of transformed bacterial colonies containing the vector. The vector also contains regulatory sequences and the coding information for the first 146 amino acids (amino terminal) of the β -galactosidase gene (*lac Z*). When this vector is transformed into a host cell encoding the carboxy protein of the β -galactosidase gene, the plasmid- and host-encoded proteins can associate to form an enzymatically active protein. This type of complementation is called α -complementation (Ullmann et al., 1967). The Lac⁺ bacteria that result from α complementation are easily recognised as they form blue colonies in the presence of a chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Horwitz et al., 1964). The multiple cloning sites within this vector are contained within the β galactosidase gene segment, successful ligation of the cDNA insert into this site results in the production of an amino terminal fragment that is not capable of α -complementation and colonies remain white in colour.

3.2.3 DNA Polymerases

As the cloned CD23 DNA sequence was to be used in the development of a cell based assay to elucidate proteolytic cleavage of CD23, a proof reading polymerase was used in the RT-PCR strategy to ensure an error free product. It was considered important to clone CD23 with a sequence identical to that published, as the resulting product was to be used to produce a recombinant protein. Any variation in the amino acid sequence may have affected recognition and cleavage of this protein by endogenous or exogenous protease(s).

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Figure 3.2 Vector pBluescript®II KS (+/-) Phagemid, and the Location of the CD23 Insert, Cloned into the Multiple Cloning Site (MCS). (a) Map of vector pBluescript®II KS, a 2961bp phagemid derived from pUC19. This vector contains the ColE1 origin of replication which is used in the absence of helper phage; a *lacZ* gene promotor that provides for α -complementation of blue/white colour selection of recombinant phagemids; a multiple cloning site (MCS) flanked by the T3 and T7 RNA promoters; and an ampicillin resistance gene for antibiotic selection of bacteria containing the phagemid vector. (b) Location of the CD23 cDNA cloned into the *Xho* I and *Xba* I sites of the MCS within the vector pBluescript®II KS.

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PfU polymerase was chosen to amplify the CD23 sequence, as this polymerase has the lowest error rate of any thermostable DNA polymerase studied (Flaman *et al.*, 1994). DNA polymerases that possess a proof reading activity generate blunt ended PCR products. This may pose problems in subsequent manipulations such as cloning of the product into a vector. For this reason restriction enzyme recognition sequences were built into the oligonucleotide primers and added to the CD23 sequence by PCR, to aid ligation of the cDNA product into the vector. Digestion of the cDNA product and vector by these restriction enzymes leaves protruding 5' and 3' termini which allow ligation of the cDNA product into the vector. However, digestion of PCR products by restriction enzymes near to the end of cDNA sequence is inefficient. The majority of restriction enzymes require flanking DNA around the recognition site. Efficiency is not only affected by the presence of this additional DNA, but the number of bases and their sequence is important. For these reasons, 3 nucleotides that have been shown to improve restriction enzyme digestion efficiency (as determined by New England Biolabs, Hitchin, Hertfordshire, UK) were added to the end of each primer.

A reported disadvantage of proof reading enzymes is their slower amplification and extension rates, e.g. the nucleotide incorporation rate of PfU polymerase is fivefold lower than Taq DNA polymerase (Hogrefe 1996). This may result in a reduced quantity of amplified template when compared to non-proof reading polymerase enzymes. Optimisation of PCR has to be determined empirically, parameters which may affect this reaction include; annealing temperature, extension time, magnesium chloride concentration, primer concentrations, dNTPs and most importantly the quantity and quality of template. These were all titrated to produce a CD23 cDNA product.

3.2.4 Ligation of PCR Product into Plasmid Vector

Both vector and PCR product were digested with the restriction enzymes *Xho* I and *Xba* I. To prevent the vector from self-ligating, due to the similarity in overhang sequence produced by restriction digestion with these two enzymes, the vector was treated with calf intestinal alkaline phosphatase to remove the phosphate groups from the 5' ends of the vector DNA (Sambrook *et al.*, 1989). The resulting vector and DNA inserts were then ligated, transformed into competent *E.coli* (XL-1 Blue) cells, and plated onto L-agar plates containing ampicillin, IPTG and X-gal. White colonies were picked and insert typed by PCR to check that they contained an insert of the size expected for the cloned CD23 DNA

sequence. Plasmid DNA from these clones were then sequenced using an array of primers complementary to the published CD23 DNA sequence, and to the T3 and T7 primer sequences contained within the MCS of the pBluescript®II KS vector (figure 3.3).

3.3 Expression of Recombinant CD23

Recombinant CD23 was expressed in a mammalian expression system, using cells that do not naturally express the CD23 protein. It was hoped that this would provide several advantages over studying the CD23 protein expressed on EBV-B cells (see section 3.1.1). The addition of an affinity tag(s) incorporated into the recombinant CD23 protein provided an additional tool for cleavage analysis, using commercially available antibodies in immunoblotting methodologies and for purification via the 6xHis tag using Talon metal affinity resin (see method 2.8.11).

The structure of CD23 is relatively complex, being N-glycosylated at asparagine residue 63 and trimerised at the cell surface. Therefore expression in a bacterial or yeast cell system was considered unlikely to make the post-translational modifications required to produce a recombinant protein representative of the native molecule. Instead expression in mammalian or insect cell systems was considered to be more appropriate. These cell systems (insect cell/baculovirus expression system and Chinese Hamster Ovary cells) have already been shown to produce recombinant CD23 with a structure and function comparable to the native molecule (Letellier *et al.*, 1990, Lui *et al.*, 1991, Graber *et al.*, 1992). The murine erythroleukaemia (MEL) cell expression system was chosen to express both recombinant mCD23 and a truncated (t)CD23 form representing the extracellular domain (without the intracellular and transmembrane domains). This system shares the utility and advantages of an insect cell/baculovirus system but without the disadvantages of needing to prepare virus or control infection and lysis.

3.3.1 Recombinant Protein Expression by Murine Erythroleukaemia Cells

The MEL cell expression system has been used previously to express both membrane (human calcitonin receptor, Needham *et al.*, 1995) and secreted soluble proteins (type I and II human tumour necrosis factor receptors, Newton *et al.*, 1994). This expression system provides several advantages over other mammalian and insect cell systems: (1) This





pBluescript Sequencing Primers	5'-3'
T3 (pBluescript primer)	AATTAACCCTCACTAAAGGG
T7 (pBluescript primer)	GTAATACGACTCACTATAGGGC

CD23 Sequencing Primers	5'-3'
Primer 1 (sense)	CAAGCAGATCTGAGCAGCTTCAAG
Primer 2 (antisense)	CTTGAAGCTGCTCAGATCTGCTTG
Primer 3 (sense)	CTACTACTTCGGCAAGGGCACCAAG
Primer 4 (antisense)	CTTGGTGCCCTTGCCGAAGTAGTAG
Primer 5 (sense)	GAGTTTATCTGGGTGGATGGGAGC
Primer 6 (antisense)	GCTCCCATCCACCCAGATAAACTC

'igure 3.3 CD23 Sequencing Primers and Locations within the CD23 Coding Sequence. equencing primers used to check the DNA sequence of the cloned CD23 product and their scation within the CD23 gene sequence. All primers were designed to have an optimal nnealing temperature of 55°C.

system offers the ability to obtain stable clones, with high expression levels shortly after transfection (3-4 weeks). This is achieved by inducing differentiation of the MEL cells to express the transfected protein. (2) Recombinant protein expression in MEL cells is not affected by positional integration effects of the plasmid DNA. This is a problem in the production of many stable transfectants, in which the DNA surrounding the site of inserted DNA has a major effect upon recombinant protein expression levels. Positional integration effects have been overcome by the use of a beta globin locus control region (LCR) sequence, integrated into the pEV3 vector to direct high level expression in cells of erythroid lineage. The globin LCR has been shown to confer insertion site independent expression on stably transfected genes when these are placed adjacent to the globin LCR sequence (Talbot et al., 1989, Blom van Assendelft et al., 1989). (3) The MEL expression system can be induced to undergo terminal differentiation in vitro; accompanied by high levels of expression of the beta globin proteins, such that the recombinant protein accounts for up to 25% of the total cellular proteins produced (Kabat et al., 1975). (4) MEL cells have a very short doubling time (10-16 hours), they grow to high numbers in suspension culture and are easy to transfect with plasmid DNA. These features therefore result in the production of stable clones with high levels of expression from low copy number transfected DNA within weeks.

3.3.2 Construction of CD23 Expression Cassettes

The antigenic tags myc and 6xHis, plus the restriction enzyme sites Eco RI and Not I were incorporated into CD23 expression subcloning primers and added to the 5' and 3' ends of the CD23 cDNA sequence respectively by PCR (figure 3.4). The antigenic tag myc was only added to the tCD23 construct, as the intracellular expression of this tag may have affected processing, expression and/or intracellular signalling of the recombinant membrane protein. Vertebrate mRNAs contain the consensus sequence ⁹GCCGCC^A/_GCCAUGG immediately upstream of the initiation of translation site. Kozak (1989) has shown that altering of this sequence affects the efficiency of translation. Hence this sequence, known as the Kozak sequence, was included within the expression primers in an attempt to maximise transcription efficiency.

Bacterial plasmids are supercoiled within a covalently closed circular form, which may reduce sequence recognition and annealing of the primers. Therefore in order to assist

(a) CD23 Expression Cassettes



(b) Expression Primer Sequences

5' sense primer for the production of membrane CD23 expression cassette:

5'CCGGAATTCGCCACCATGGAGGAAGGTCAATATTCAGAG3'

5' sense primer for the production of truncated CD23 expression cassette:

5' CGGAATTCGCCACCATGGAACAAAAACTCATCTCAGAAGAGGATCT

GAATGACACCACACAGAGTCTAAAACAG 3'

3' antisense primer for the production of both mCD23 and tCD23 expression cassettes:

5'ATAGTTTAGCGGCCGCTCAATGATGATGATGATGATGATGATGAGAGAGTGGAG

AGGGGCAGAGGG 3'

Figure 3.4 Construction of CD23 Expression Cassettes. (a) CD23 expression cassettes used for subcloning into the expression vector pEV3. The addition of allergenic tag sequences (myc and 6xHis), KOZAK sequence and restriction enzyme sequences were all added to the cloned DNA sequence by PCR. (b) Primer sequences used to create mCD23 and tCD23 expression cassettes. Antigenic taq sequence(s) 6xHis (**bold** and <u>underlined</u>) and myc (**bold** and *italics*), the Kozak sequence (<u>underlined</u>) and restriction enzyme sites *Eco* RI and *Not* I (**bold**) were incorporated within the primer sequences to aid detection and purification of the recombinant protein, promote transcription and assist subcloning into the expression vector pEV3 respectively.

amplification by PCR, the vector was first linearised by restriction enzyme digestion using *Sca* I. PCRs were amplified using the proof reading polymerase PfU and the resulting PCR products were digested with *Eco* RI and *Not* I then purified using Qiaex II gel extraction kit. Purified products were religated back into (1) pBluescript®II KS for further sequence analysis, to ensure no base mutations had been incorporated that may lead to altered amino acid expression and (2) into the expression vector pEV3 (figure 3.5). These vector constructs were then transformed back into bacterial cells (*E. coli* DH5 α) to increase DNA yield. Finally the vector constructs were purified by caesium chloride method 2.5.2, to purify the plasmid DNA from bacterial DNA, RNA, protein, endotoxins and chemical contaminants which may inhibit successful transformation of the plasmid into MEL cells.

3.3.3 CD23/pEV3 Transfection and Induced Protein Expression by MEL Cells

Plasmid pEV3, containing the mCD23 and tCD23 expression cassettes were transfection into MEL-C88 cells (Deisseroth and Hendrick 1978) by electroporation. Antibiotic G418 was added to the cells 24 hours after transfection to select for stable transfectants. Although this antibiotic is toxic to both prokaryotic and eukaryotic cells, the vector pEV3 contains the neo-mycin phosphotransferase sequence conferring resistance to stably transfected clones, which enables them to grow in its presence. Before expression of the recombinant CD23 protein was induced, cells were maintained in exponential growth for a period of four days. Cells were then induced to differentiate and express rCD23 by the addition of DMSO at a final concentration of 2% (v/v). Messenger (m)RNA and recombinant protein expression was analysed by Northern blotting, flow cytometry, SDS-PAGE and immunoblotting.

3.4 Results

3.4.1 Development of the Cloning Strategy

In an attempt to clone CD23 from EBV-B cells by RT-PCR, several proof reading polymerase enzymes were used including PfU and ULTmaTM Taq polymerase. Initial reactions were unsuccessful and no product was produced for either CD23 specific reactions or the positive control β -actin, which was included to check for successful reverse transcription of the RNA template (see figure 3.6). The absence of a β -actin product cast doubts on the efficiency of the reverse transcription reactions. To check

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Figure 3.5 Vector pEV3. Vector pEV3 contains: a β -globin promoter, a multiple cloning site (MCS) located between the β -globin promoter and β -globin gene, the neomycin phosphotransferase sequence for selection of stable transfectants with the antibiotic G418, and the globin locus control region, which has been shown to confer insertion site independent expression of recombinant protein at high levels of stably transfected genes.



Marker 1 2 3 4 5 6 7 8 9

1018bp -506bp -

Figure 3.6 CD23 cDNA Cloned from EBV-B Cells. Amplification of CD23 cDNA using Taq DNA polymerase but not proof-reading polymerases. CD23 was amplified by PCR from reverse transcribed (RT) template from total RNA isolated from EBV-B cells using CD23 specific primers for 30 cycles in total, annealing at 61°C, with an extension time of 5 minutes for the first 5 cycles then reduced to 2 minutes for a further 25 cycles. Products separated on a 1% agarose TAE gel. Lane 1: 1Kb ladder, DNA size markers. Lane 2 and 3: 1µg and 2µg RNA amplified by RT-PCR using PfU. Lane 4 and 5: 1µg and 2µg RNA amplified by PCR using Taq DNA polymerase. Lane 8: negative control, no template added. Lane 9: positive control, 1µg RNA amplified by PCR using Taq DNA polymerase with β-actin primers.

efficiency, the reverse transcribed samples were amplified with recombinant Taq DNA polymerase. Both CD23 and β -actin cDNA were amplified using recombinant Taq DNA polymerase (figure 3.6), and CD23 was visualised as a defined band of approximately 1Kb (lanes 6 & 7). Specificity of the product is indicated by a negative control containing no PCR product (lane 8), while the positive control (lane 9) shows the amplification of β -actin confirming successful reverse transcription of the purified RNA. The β -actin band is not as prominent as the CD23 product due to the lower amount of starting material in the reverse transcription reaction and the use of a higher than optimal annealing temperature (61°C, above the optimal annealing temperature of 55°C) during amplification.

The results with recombinant Taq DNA polymerase suggested that the problems in CD23 amplification using proof-reading polymerases may be due to the low level of CD23 mRNA expression resulting in low template concentrations combined with reduced nucleotide incorporation rate of proof-reading polymerases. In an attempt to overcome this problem a mixture of Taq 2000 and PfU was used at a ratio of 12:1 and 20:1. This strategy has been shown to overcome the problems of reduced nucleotide incorporation rates (personal communication Stratagene technical support service), although fidelity is reduced in relation to the ratio of the enzymes. Despite continued effort to optimise the RT-PCR reaction; including assessing RNA quantity and quality after purification by spectrophotometry, no CD23 PCR product was obtained, even though β -actin (positive control) was amplified using a mixture of PfU and taq 2000 polymerase at a ratio of 20:1 (figure 3.7). Hence CD23 was cloned using recombinant Taq DNA polymerase.

Sequencing of the cloned CD23 product identified several single base mutations (figure 3.8). Two of these mutations would have resulted in the synthesis of two altered amino acids within the recombinant protein. The serine residue at position 243 would have been changed to a glycine residue, both uncharged polar amino acids, a conservative substitution. While the arginine residue at position 284 would have been changed to a glutamine, arginine is a positively charged amino acid with a basic R group, whereas glutamine has an uncharged polar R group, resulting in a semi-conservative amino acid substitution (figure 3.9). However, if left unaltered these changes in amino acid sequence may have caused structural and conformational alterations in the recombinant protein.



Figure 3.7 Increasing Amounts of Proof Reading Polymerase Failed to Generate a CD23 PCR Product. RT-PCR reactions (lanes 2-6) from 1µg total RNA template (lane 2) and 2µg total RNA template (lanes 3-6). PCR reactions as described in figure 3.6, using a combinantion of PfU and Taq 2000 polymerase (ratio 20:1). Lane 2-4: show the results of amplification with CD23 specific primers, Lane 5: negative control reaction (no template), Lane 6: positive control using β -actin primers (a faint band can be seen in this lane showing positive amplification), lanes 1 and 7: 1Kb ladder, DNA size markers. Products were separated on a 1% agarose TAE gel. β -actin product concentration was low due to the use of a higher annealing temperture (61°C), than that optimal for the β -actin primers (55°C).

779 826 PUB GAG GAC TGC GTG ATG ATG CGG GGC TCC GGT CGC TGG AAC GAC GCC TTC CON GAG GAC TGC GTG ATG ATG CGG GGC TCC GGT CGC TGG AAC GAC GCC TTC * * * 827 874 PUB TGC GAC CGT AAG CTG GGC GCC TGG GTG TGC GAC CGG CTG GCC ACA TGC CON TGC GAC CGT AAG CTG GGC GCC TGG GTc TGt GAC CaG CTG GCC ACA TGC 875 922 PUB ACG CCG CCA GCC AGC GAA GGT TCC GCG GAG TCC ATG GGA CCT GAT TCA CON ACG CCG CCA GCC AGC GAA GGT TCC GCG GAG TCC ATG GGA CCT GAT TCA 923 970 PUB AGA CCA GAC CCT GAC GGC CGC CTG CCC ACC CCC TCT GCC CCT CTC CAC CON AGA CCA GAC CCT GAC GGC CGC CTG CCC ACC CCC TCT GCC CCT CTC CAC 971 PUB TCT TGA CON TCT TGA

Figure 3.8 Nucleotide Sequence of Cloned CD23 DNA, Compared to the Published Sequence by Yokota *et al.*, (1988). Consensus (CON) sequence of cloned CD23 DNA, compared to the published (PUB) sequence by Yokota *et al.*, (1988), Medline accession number 89028672. Cloned CD23 DNA was sequenced at least three times using oligonucleotide primers shown in figure 3.3 and a consensus sequence constructed. Vector sequence and restriction sites are not shown. Base differences are shown in lower case (bold) and highlighted by a * symbol.

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Cloned cDNA	MEEGQYSEIEELPRRRCCRRGTQIVLLGLVTAALWAGLLTLLLWHWDTT
Published	MEEGQYSEIEELPRRRCCRRGTQIVLLGLVTAALWAGLLTLLLWHWDTT
Cloned cDNA	QSLKQLEERAARNVSQVSKNLESHHGDQMAQKSQSTQISQELEELRAEQQ
Published	QSLKQLEERAARNVSQVSKNLESHHGDQMAQKSQSTQISQELEELRAEQQ
Cloned cDNA	RLKSQDLELSWNLNGLQADLSSFKSQELNERNEASDLLERLREEVTKLRM
Published	RLKSQDLELSWNLNGLQADLSSFKSQELNERNEASDLLERLREEVTKLRM
Cloned cDNA	ELQVSSGFVCNTCPEKWINFQRKCYYFGKGTKQWVHARYACDDMEGQV
Published	ELQVSSGFVCNTCPEKWINFQRKCYYFGKGTKQWVHARYACDDMEGQV
Cloned cDNA	SIHSPEEQDFLTKHASHTGSWIGLRNLDLKGEFIWVDGSHVDY
Published	SIHSPEEQDFLTKHASHTGSWIGLRNLDLKGEFIWVDGSHVDYSNWAPGE
Cloned cDNA	SRSQGEDCVMMRGSGRWNDAFCDRKLGAWVCDLATCTPPASEGSAES
Published	SRSQGEDCVMMRGSGRWNDAFCDRKLGAWVCDRLATCTPPASEGSAES
Cloned cDNA	MGPDSRPDPDGRLPTPSAPLHS

Published MGPDSRPDPDGRLPTPSAPLHS

Figure 3.9 Predicted Amino Acid Sequence of Cloned CD23 DNA, Compared to the Published Sequence by Yokota *et al.*, (1988). Medline accession number 89028672. The boxed amino acids highlight the differences between the cloned CD23 sequence and the published sequence.



Figure 3.10 Diagram Showing the Location of the Predicted Amino Acid Alterations, Resulting from DNA Mutations in the Cloned Sequence.

Analysis of the cloned CD23 sequence showed two base mutations that would have resulted in an altered amino acid sequence expressed in the resulting recombinant protein, changing a serine to a glycine at position 243 and an arginine to a glutamine at position 284. in their size, shape and properties of the R group. The location of these altered amino acids is shown in figure 3.10. The first is located at the base of the lectin head between the conserved cysteine residues and the second within the lectin head domain, in a region where two ligands, IgE (Vercelli *et al.*, 1989) and CD21 (Mossalayi *et al.*, 1992) bind CD23. As little is known about the endogenous or exogenous proteases that cleave CD23, their interaction with CD23 during cleavage or their mechanism of recognition, it was considered important to repair the cloned sequence to match the published sequence. Therefore two base pair mutations were altered by site directed mutagenesis to match the published sequence (figure 3.11).

3.4.2 Site Directed Mutagenesis

The DNA mutagenesis strategy followed was based on the principle used in Stratagene's QuikChange[™] Site-Directed Mutagenesis Kit. This strategy was chosen, as it is a simple and efficient method that allows site-specific mutation of double stranded (ds) plasmid DNA. Almost all other strategies require single stranded DNA as a template and therefore require subcloning into M13-based bacteriophage vectors and single stranded (ss)DNA rescue. Oligonucleotide primers containing the desired base mutation were designed complementary to opposite strands of the vector around the site to be mutated (figure 3.11/2). These primers were extended by PCR (as outlined in 2.4.5) using PfU polymerase and the CD23 cDNA cloned into pBluescript®II KS as a template, this generates a mutated copy with staggered nicks (figure 3.11/3). The PCR product was then digested with Dpn I. This endonuclease is specific for methylated and hemimethylated DNA and was used to digest the parental plasmid DNA template, as almost all DNA isolated from E. coli strains is *dam* methylated. The nicked vector DNA (incorporating the mutation) was then transformed back into competent XL-1 Blue E. coli cells, where the nicks are repaired by the host cell's enzymes. PfU DNA polymerase was chosen for these experiments as it contains the lowest mutation rate of those available and was unlikely to be susceptible to the problems encountered in the original cloning strategy, as the template was not limited. Two rounds of mutagenesis were undertaken to repair the two-nucleotide bases separately. Positive bacterial colonies were sequenced after each round of mutagenesis to check for successful inclusion of the mutated base. Sequencing results showed that this strategy had a high success rate, with 75% of clones sequenced containing the mutated base.

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CD23 cloned into pBluescript® II KS. Target site for mutation



Parental methylated DNA template digested with Dpn I



Plasmid denatured and oligonucleotide primers containing the desired mutation annealed.



ds nicked DNA containing mutated base transformed into *E. coli* (XL-1 Blue).



PfU DNA polymerase used to extend and incorporate the mutagenic primer. Resulting in nicked circular strands. E. coli repair the nicks in the mutated plasmid.

Figure 3.11 Schematic Overview of the Site Directed Mutagenesis Method Used. Parental DNA shown in red, new DNA (incorporating the mutation) shown in black. The corrected CD23 cDNA was then amplified with subcloning primers (figure 3.4b) to produce expression cassettes representing both membrane and truncated forms of CD23 incorporating immunogenic motifs, the Kozak sequence and restriction enzyme sites (figure 3.4a). PCR products were ligated into both the expression vector pEV3 and then back into pBluescript®II KS for sequence analysis. Sequencing results showed no further base mutations had occurred.

3.4.3 Expression and Characterisation of Recombinant Human CD23

3.4.3.1 Expression of Recombinant mCD23

Selected MEL cell clones transfected with membrane CD23 were analysed by flow cytometry for expression of induced recombinant protein using a commercially available anti-CD23 antibody BU38 (The Binding Site). Figure 3.12 shows increased expression of CD23 on CD23 transfected MEL cell lines assessed by flow cytometry, as compared to the untransfected MEL cell controls (CD23 negative cells), indicating the expression of recombinant mCD23 on the surface of these cells. Recombinant mCD23 expression was studied in 12 cell lines established from single clones, grown in media containing the selection antibiotic G418. Seven of these lines were shown to express the recombinant protein as detected by flow cytometry techniques; one sample was shown to be of mixed population, containing both CD23⁺ and CD23⁻ cells, and four cell lines were CD23⁻.

3.4.3.2 Binding of IgE to Recombinant mCD23

Further characterisation of recombinant mCD23 was assessed by the ability of the recombinant protein to bind myeloma IgE (kindly donated by Professor D. Pritchard, University of Nottingham). Native CD23 binds to IgE within the CD23 lectin domain. Molecular binding studies indicate that only oligomeric forms of CD23 have sufficient affinity/avidity to bind IgE (Shi *et al.*, 1997). Therefore binding of myeloma IgE by recombinant mCD23 would indicate oligomerisation of the recombinant protein at the cell surface. Purity of the myeloma IgE sample used in this experiment was assessed by SDS-PAGE protein separation and silver staining (figure 3.13). This figure shows the heavy and light chain fragments of myeloma IgE cleaved by the denaturing conditions used in SDS-PAGE gel electrophoresis, producing two distinct bands representing two heavy

-Events 1021 100 256 101 102 103 104 Fluorescence Fluorescence **MFI=296** M ∞ Events 100 100 256 10¹ 10² 10³ 10⁴
Fluorescence Fluorescence MFI=277 M 10.4 9 Events 0100 100 256 Fluorescence Fluorescence MFI=344 M 104



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Figure 3.12 Expression of Recombinant CD23 on MEL Cell Lines Assayed by Flow Cytometry. $1x10^6$ MEL cells were incubated with anti-CD23 monoclonal antibody BU38 (diluted 1/100) for 45 minutes. Binding of the antibody was detected using a secondary rabbit anti-mouse IgG FITC (Serotech) followed by flow cytometic analysis (unless otherwise stated). MFI= Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) Clone 4, control no antibodies, (2) Clone 4, BU38 alone, (3) Clone 4, seconday antibody alone, (4) Clone 1, (5) Clone 2, (6) Clone 3, (7) Clone 4, (8) Clone 5, (9) Clone 6, (10) Clone 7, (11) Clone 8, (12) Clone 10, (13) Clone 11, (14) Clone 12.
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antibudy 9510 (figure 3.15). These teachs indicated that the recombinant (CD2) protein expected by contratected AIEL cells was not securited, as the recombinant protein was detected in the MRL cell lyaster but not in the corresponding prowth media/inpermitant simplet. The ICD21 was confirmed to user in blackholder between, as cell surface

Figure 3.13 Purity of Myeloma IgE used in Recombinant Membrane CD23 Binding Studies. Myeloma IgE (donated by Prof. D. Pritchard, Nottingham University) was denatured using SDS sample buffer (lane 2), then analysed by 12% SDS-PAGE gel electrophoresis and Silver Staining techniques. The migration of IgE was compared to SeeBlue[™] (Novex) protein molecular weight size makers (lane 1). Denaturation of immunoglobulins cleaves the disulphide bond producing a polypeptide chain of approximately 50kDa, termed the heavy (H) chain, and a 25kDa chain termed the light (L) chain (sizes based on those for IgG molecules).

munications all viry was observed in the growth model covernetation of granticeum only out not next introductions MEL cell controls (figure 3.16). The training of recombinant of mCTV's from the cell surface resplits if the production of several subficle flagments of opportinging 37, 33, 30, 25, 25, 22,5 and 35 cDs in size (figure 1.16 kms 2). polypeptide fragments of approximately 50kDa and two light chain polypeptide fragments of approximately 25kDa.

Flow cytometry results showed recombinant mCD23 bound myeloma IgE (figure 3.14, panel 5), as indicated by the increase in mean fluorescence intensity. RPMI 8866 cells (B lymphoblastoid cells positive for CD23), which constitutively express membrane CD23 (were used as a positive control for this experiment), were also shown to bind IgE (figure 3.14, panel 2). Binding was shown to be specific for IgE as cells expressing recombinant mCD23 were unable to bind IgG (figure 3.14, panel 6). The ability of recombinant mCD23 to bind myeloma IgE indicates that the recombinant protein represents both antigenic and functional properties of native CD23.

3.4.3.3 Expression of Recombinant tCD23

The expression of tCD23 by transfected MEL cells was confirmed using an anti-myc antibody 9E10 (figure 3.15). These results indicated that the recombinant tCD23 protein expressed by transfected MEL cells was not secreted, as the recombinant protein was detected in the MEL cell lysates but not in the corresponding growth media/supernatant samples. The tCD23 was confirmed to have an intracellular location, as cell surface detection was not detected by flow cytometry analysis (data not shown). Expression of recombinant tCD23 was studied in 12 cell lines established from single cell transfectants grown in G418 selective medium, eight of these lines were shown to express the recombinant protein by immunoblotting techniques.

3.4.4 Cleavage Analysis of the Expressed Recombinant CD23 Protein

3.4.4.1 Recombinant mCD23

SDS-PAGE/immunoblot analysis of recombinant mCD23 expression by transfected MEL cells, suggests that recombinant mCD23 is cleaved from the cell surface as CD23 immunoreactivity was observed in the growth media/supernatants of transfected cells but not from untransfected MEL cell controls (figure 3.16). The release of recombinant mCD23 from the cell surface results in the production of several soluble fragments of approximately 37, 33, 30, 28, 25, 22.5 and 15kDa in size (figure 3.16 lane 2).

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Figure 3.15 Expression of Recombinant Truncated CD23. MEL cells transfected with tCD23/pEV3 vector and untransfected control cells were grown in log phase for 4 days then induced with 2% DMSO. Four days after the induction of protein expression, samples were centrifuged, lysed by freeze thawing in the presence of protease inhibitors (see section 2.8.10) then analysed by denaturing 12% SDS-PAGE gel electrophoresis and immunoblotting. Recombinant tCD23 was detected using an anti c-myc monoclonal antibody 9E10 (diluted 1/1000, donated by AstraZeneca Pharmaceuticals). Lane 1: induced untransfected MEL cell control, Lane 2-13: induced MEL cell clones 1-12 (respectively) transfected with tCD23 expression constructs, Lane 14: pT-cell receptor containing the antigenic epitope c-mvc, (a recombinant c-myc containing antigen control donated by Dr. Colin Hewitt, Leicester University, Leicester). Corresponding supernatant samples were also analysed for recombinant tCD23, however no immunoreactivity was detected. Therefore it appears that recombinant tCD23 is not secreted from transfected MEL cells but is expressed intracellularly. Protein band sizes were determined by comparison to SeeBlue™ protein molecular weight markers (Novex).

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Figure 3.16 MEL Cells Shed Recombinant mCD23. MEL cells transfected with mCD23/pEV3 vector (lanes 2 and 4) or untransfected control cells (lanes 1 and 3) were grown in log phase for 4 days then induced with 2% DMSO. Four days after the induction of protein expression samples were centrifuged, the supernatant aspirated and analysed directly (lanes 1 and 2) or after TALONTM metal affinity resin chromatography (Clontech) which purifies proteins containing (6x) His tags (lanes 3 and 4), by denaturing 12% SDS-PAGE gel electrophoresis and immunoblotting. Soluble CD23 products shed from recombinant membrane CD23 were detected using a sheep anti-CD23 polyclonal antibody (10 μ g/ml). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

After 4 days the 37 and 33kDa fragments were seen in greatest abundance. In order to analyse the release of these fragments further, CD23 products released from recombinant mCD23 were purified from the culture supernatant by their 6xHis affinity tags using 6xHis Talon[™] metal affinity resin. The results of this experiment are shown in figure 3.16, lane 4. Here three predominant fragments can be seen of approximately 37, 33 and 25kDa in size and two fainter bands of approximately 30 and 28kDa, however the two smaller bands of 15 and 22.5kDa are missing. It is therefore possible that these two smaller fragments are sCD23 species that have lost the C-terminal tail, the end of which contains the 6xHis affinity tag, and therefore were not purified by the Talon[™] metal affinity resin.

The corresponding cell lysate samples show four predominant bands of approximately 46, 43, 37 and 15kDa and a faint band of approximately 33kDa (figure 3.17 lane 2). Based on calculated molecular weights, the 46kDa band is probably full-length mCD23. The 37 and 33kDa bands may represent cleaved CD23 fragments. However, as these fragments are found associated with the cell membrane (see figure 3.17, lane 4), it is possible that they are retained on the cell surface associated with other full-length mCD23 molecules, within the trimeric structure brought about by oligomerisation (figure 3.18). If this is true, this result suggests that the endogenous protease(s) is capable of cleaving just one CD23 molecule from within the trimeric.

The 15kDa band may represent the cleaved stump of CD23 retained on the membrane after cleavage and release of the C-terminus. Alternatively, this fragment may represent an internally cleaved fragment as described by Grenier-Brossette *et al.*, (1992), who demonstrated that surface CD23 is endocytosed and subsequently cleaved to form a 16kDa fragment. It is possible that the 43kDa band represents synthesised full-length mCD23, which has not yet been processed and is missing the N-linked carbohydrate chain, as described by Letellier *et al.*, (1988). Analysis of the N-linked glycoslylation of CD23 by this group showed that treatment of RPMI 8866 cells (CD23⁺ cells) with N-glycanase, an enzyme that cleaves all N-linked oligosaccharides, or tunicamycin, which inhibits N-glycoslylation of glycoproteins, reduced 45kDa mCD23 to a 43kDa fragment.

Purification of membranes from transfected MEL cells that had been induced to express recombinant mCD23 (figure 3.17, lane 4) showed the presence of an additional dominant

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Figure 3.17 Cleavage of Recombinant Membrane CD23 from the Cell Surface. MEL cells transfected with mCD23/pEV3 vector (clone 4) or untransfected controls were grown in log phase for 4 days then induced with 2% DMSO. Four days after the induction of protein expression, samples were centrifuged, and the cells lysed by freeze thawing in the presence of protease inhibitors (see section 2.8.10). These samples were then analysed by denaturing 12% SDS-PAGE gel electrophoresis and immunoblotting, using a sheep anti-CD23 polyclonal antibody (10μ g/ml). Lanes 1 and 2 show the results from untransfected MEL cell controls and transfected MEL cell clone 4 respectively. Lanes 3 and 4 show membrane protein samples from induced untansfected MEL cells and transfected MEL cells clone 4 samples respectively, extracted from the cell membrane as described in method 2.8.9. Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

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Figure 3.18 Soluble CD23 Fragments may be Retained at the Cell Surface Through an Interaction with the Cleaved CD23 Molecule and Other Membrane CD23 Fragments. It is possible that smaller CD23 fragments seen within the cell lysate samples represent cleaved recombinant membrane CD23 fragments. These fragments may be retained at the cell surface through an interaction between the coiled coil stalk domains, which are predicted to encourage oligomerisation. band at approximately 24kDa, it is likely that this band represents the 25kDa sCD23, tethered to the membrane by the coiled-coil structure of mCD23 monomer. Several other faint bands can be seen with respective molecular weights, 32, 29 and 27kDa approximately. Soluble CD23 fragments of 33kDa, 29kDa, 27kDa and 25kDa have all been previously described as sCD23 fragments derived from full-length mCD23 by proteolytic cleavage (Letellier *et al.*, 1989 and Kikutani *et al.*, 1986 respectively). It is possible that the 32, 29 and 27kDa fragments represent these sCD23 fragments, retained on the cell surface by interaction with full-length mCD23 as described above.

3.4.4.2 Recombinant tCD23

In contrast to the recombinant mCD23 molecule, recombinant tCD23 remains intact. SDS-PAGE/immunoblot analysis of recombinant tCD23 shows a single band of approximately 36kDa as detected with anti-CD23 polyclonal antibody $(10\mu g/ml)$ and the monoclonal antibody 9E10 (diluted 1/1000) specific for the myc epitope (figure 3.19). Recombinant tCD23, therefore, provides a useful reagent to assay the specificity of anti-neoepitope antibodies (described in chapter 4). This protein could also be purified via the 6xHis tag that had been added to the carboxy terminus and used to assay the properties of the endogenous and exogenous proteases that cleave CD23.

3.4.5 Messenger RNA Expression Levels of Recombinant CD23

The purpose of cloning and expressing recombinant membrane and truncated versions of CD23, was to provide high level expression of CD23 with which proteolytic shedding could be assayed. CD23 messenger (m)RNA expression levels between transfected MEL cells induced to express either recombinant mCD23 or tCD23, untransfected MEL cell controls, RPMI 8866 cells and PBMC's isolated from whole blood +/- IL-4 stimulation were assayed by Northern blotting. RNA yields between these cells and cell lines varies enormously, therefore total RNA concentrations ($\approx 20\mu g$ total), extracted from 1×10^7 PBMCs was used to standardise the experiment. CD23 mRNA was only detected in transfected MEL cells induced to express membrane and truncated CD23, while levels of CD23 mRNA expression in RPMI 8866 cells and PBMC's, even those stimulated with IL-4 were below the threshold level of detection of this technique, suggesting very low levels of expression (figure 3.20). Comparison of total RNA subunit (figure 3.20).



Figure 3.19 Intact Recombinant Truncated CD23. MEL cells transfected with tCD23/pEV3 vector and untransfected controls were grown in log phase for 4 days then induced with 2% DMSO. Four days after induction of protein expression, samples were centrifuged, and lysed by freeze thawing in the presence of protease inhibitors (see section 2.8.10). These samples were then analysed by denaturing 12% SDS-PAGE gel electrophoresis and immunoblotting, using an anti *c-myc* monoclonal antibody 9E10 (1/1000, lanes 1 and 2) or anti-CD23 polyclonal antobody ($10\mu g/ml$, lanes 3 and 4). Lane 1 and 3: tCD23 transfected MEL cell clone 5, Lanes 2 and 4: untransfected MEL cell controls. Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).



Figure 3.20 CD23 mRNA Expression Levels. RNA (approximately 20µg per sample) extracted from; PBMCs cultured for 0, 3, 6, and 12 hours after extraction (lanes 1-4 respectively), PBMC's stimulated with 10ng/ml recombinant human IL-4 (Sigma), cultured for 0, 3, 6 and 12 hours after extraction (lanes 5-8 respectively), induced untransfected MEL cell control (lane 10), induced MEL cells transfected with mCD23/pEV3 (lane 11), induced MEL cells transfected with tCD23/pEV3 (lane 12) and RPMI 8866 cells (lane 13). Samples were separated on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane by northern blotting. (a) CD23 mRNA expression analysed using radiolabelled (³²P) CD23 cDNA probe. (b) Equal loading of lanes was shown by the cross-hybridisation to the 18S ribosomal (r)RNA fragment.

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3.5 Discussion

3.5.1 Cloning CD23

Amplification of the CD23 coding sequence proved more difficult than first anticipated. As neither CD23 nor β -actin were amplified by either of the proof reading polymerases (PfU and ULTma Taq) it is unlikely that, low template concentrations from the reverse transcription reaction were the only problem. Instead it appears that these enzymes are incompatible with this type of strategy, possibly due to the carry over of contaminant(s) that inhibit enzyme reactions from the reverse transcription reaction or due to secondary structure of the mRNA. Amplification of both CD23 and β -actin using recombinant Taq polymerase suggests the problem is not due to either the reverse transcription reaction or other components of the PCR reaction e.g. concentrations of the individual components, annealing temperature or non-specific binding/secondary structure.

Northern analysis of CD23 mRNA expression levels shows that even in induced PBMC's and RPMI 8866 cells (where CD23 expression is expressed at relatively high levels) CD23 mRNA levels were low. Hence, it is likely that the combination of these; low efficiency of proof reading polymerase activity and low template concentrations both contributed to the problems of CD23 amplification from a reverse transcribed template, using proof reading polymerases. A typical mammalian cell contains about 10pg of RNA, of this only 1-5% is mRNA. The majority of total RNA consists of ribosomal RNA (80-85%) (Sambrook *et al.*, 1988). Purification of mRNA from total RNA for use in the reverse transcription reaction may have aided in the amplification of CD23. However, CD23 had already been cloned by RT-PCR using recombinant Taq DNA polymerases. Therefore this was used as a template for recombinant protein production. Sequence analysis revealed several base changes within the cloned sequence, two of which would have resulted in the incorporation of altered amino acids. These were mutated to match the published sequence by a mutagenesis protocol based on Stratagene's QuikChange™ mutagenesis strategy.

3.5.2 Recombinant mCD23 Mimics Native CD23

Recombinant mCD23 appeared to be expressed in a form representing the native molecule based on several methods of analysis. Firstly the recombinant protein is recognised by anti-CD23 monoclonal antibody BU38 (The Binding Site) specific for the lectin domain, in

flow cytometry analysis. Secondly recombinant mCD23 is capable of binding myeloma IgE, with respect to native CD23/IgE binding studies, this suggests that the recombinant mCD23 is able to form dimers or trimers at the cell surface. Finally mCD23 is cleaved from the cell surface forming soluble products that represent those found shed from the native molecule. The only fragment found shed from recombinant mCD23 not seen cleaved from native CD23 is the 22.5kDa fragment (based on published data). This product could possibly represent a sCD23 fragment cleaved firstly within the coiled coil stalk and secondly within the C-terminal tail, similar to the smaller 16kDa sCD23 molecule that has been described by Sarfati *et al.*, (1992). This is supported by the purification of sCD23 fragments via the 6xHis tag added during cloning, as neither the 15 nor 22.5kDa fragments were detected.

3.5.3 Proteases that Cleave mCD23 are Expressed Across the Species Barrier

Recombinant mCD23 expressed by induced transfected MEL cells is cleaved from the cell surface producing soluble products similar in size, and proportional to those found cleaved from human B cells and B cell lines transfected with EBV (Kikutani et al., 1986, Ludin et al., 1987, Letellier et al., 1989). This indicates that the endogenous protease(s) responsible for these cleavage events is/are not species specific. The question remains though, whether the same protease(s), which produce sCD23 fragments from human cells also produce murine sCD23, as the amino acid sequence flanking the cleavage sites within human and murine CD23 differ (figure 3.21). Implicating that the proteolytic cleavage of CD23 within these two species is via different proteases. Alternatively, proteolytic shedding by this/these protease(s) may be determined by factors other than amino acid sequence such as conformational or topological requirements. This later hypothesis is supported by mutation studies by Zong et al., (1994), and Ehlers et al., (1996). Studies by these two groups have suggest that proteolytic cleavage by metalloproteinases appears to be governed by the distance of the cleavage site from the transmembrane domain, rather than sequence identification (this will be discussed in greater detail in chapter 6).

Hu	MEEGQYSEIEELPRRRCCRRGTQIVLLGLVTAALWAGLLTLLLLWHWDTTQSL
Mu	MEENEYSGYWEPPRKRCCCARRGTQLMLVGLLSTAMWAGLLALLLLWHWETEKNL
	* *
Hu	KQLEERAARNVSQVSKNLESHHGDQMA QKS QSTQISQELEELRAEQQR LKS QDLE
Mu	KQLGDTAIQNVSHVTKDLQKFQSNQLAQKSQVVQMSQN LQE LQAEQKQMKAQDSR
Hu Mu	LSWNLNGLQADLSSFKSQELNERNEASDLLERLR LSQNLTGLQEDLRNAQSQNSKLSQNLNRLQDDLVNIKSLGLN EKR TASDSLEKLQ * *
Hu	EEVTK LRMEL QVSSGFVCNTCPEKWINFQRKCYYFGKGTKQWVHARYACDDMEGQ
Mu	EEVAKLWIEILISKGTACNICPKNWLHFQQKCYYFGKGSKQWIQARFACSDLQGR
Hu	LVSIHSPEEQDFLTKHASHTGSWIGLRNLDLKGEFIWVDGSHVDYSNWAPGEPTS
Mu	LVSIHSQKEQDFLMQHINKKDSWIGLQDLNMEGEFVWSDGSPVGYSNWNPGEPNN
Hu	RSQGEDCVMMRGSGRWNDAFCDRKLGAWVCDRLATCTPPASEGSAESMGPDSRPD
Mu	GGQGEDCVMMRGSGQWNDAFCRSYLDAWVCEQLATCEISAPLASVTPTRPTPKSE
Hu	PDGRLPTPSAPLHS
Mu	P

Figure 3.21 Comparison of Human and Murine CD23 Amino Acid Sequences (a form) and Cleavage Site Locations.

The published cleavage sites within CD23 are shown in bold. Cleavage sites within human CD23 are highlighted with a ε , while cleavage sites within the murine sequence are highlighted with a \blacklozenge . Cleavage occurs N-terminal of the highlighted section.

Both human and murine CD23 are glycosylated between the transmembrane domain and the lectin head. Human CD23 contains one site of N-glycosylation (Letellier *et al.*, 1988) while the murine protein contains two (Keegan and Conrad 1987), in addition to O-linked glycosides (both human and murine). The N-linked oligosaccharides appear to provide partial protection against proteolysis (Letellier *et al.*, 1988). Therefore it is possible that the location and number of glycosylation sites influence protease activity and location of cleavage within CD23.

3.5.4 Cleavage of a Single CD23 Molecule within a Trimeric Structure

The presence of recombinant CD23 fragments of approximately 37, 33, 29, 27 and 24kDa in transfected, induced MEL cell lysate and cell membrane extractions (figure 3.17), suggests that the protease(s) responsible for cleaving these fragments may be able to cleave a single CD23 molecule from within the trimer. It is possible that the association of these soluble fragments, through the interactions of their coiled-coil stalks and lectin domains, with the remaining full-length mCD23 molecules within the trimeric structure prevents them from dissociating. However, these bands could be due to proteolytic degradation of full-length mCD23 during purification and analysis, as denatured proteins are especially susceptible to proteolytic degradation, although a cocktail of protease inhibitors were added to prevent further degradation during experimental procedures. Further analysis would be required to determine whether these recombinant CD23 fragments are indeed sCD23 fragments.

3.5.5 Intact Recombinant Truncated CD23

Transfected MEL cells successfully expressed recombinant tCD23. The recombinant tCD23 was expressed intracellularly, where it was not susceptible to proteolytic cleavage. The production of an intact recombinant tCD23 proved useful in the determination of the specificity's of the anti-neoepitope antibodies (described in chapter 4). Furthermore, the ability to purify tCD23 via the addition of a 6xHis tag may have provided a useful tool to study proteolytic processing events, especially those of exogenous proteases such as Der p1, in the absence of exogenous cleavage events.

The transfected MEL cells did not secrete tCD23 in detectable quantities (as assessed by immunoblotting experiments). It is not surprising that transfected MEL cells do not secrete tCD23, as sCD23 fragments found within the culture media/supernatant are cleaved

products from mCD23 and not soluble proteins in their own right. The construct used to encode the recombinant tCD23 protein contains no identifiable secretory sequence. In addition, processing sequences contained at the 5' end of the CD23 coding DNA sequence that may facilitate the transport of protein out of the endoplasmic reticulum, may have been lost by the deletion of the coding sequence which results in the intracellular and transmembrane domains. Several researchers have produced recombinant sCD23 proteins by adding known secretory sequences taken from the IL-2 gene sequence (Jensen *et al.*, 1991, Graber *et al.*, 1992). This approach was not pursued as recombinant mCD23 expression had already shown that MEL cells expressed the protease(s) responsible for cleaving CD23, therefore the secreted recombinant protein may have been susceptible to proteolytic cleavage and would be of no advantage to the study.

4.1 Introduction

It has been hypothesised that the various soluble fragments of CD23 have functional activities different to those of membrane CD23 and to those of each other. The underlying basis of this hypothesis stems from the findings that the 25kDa sCD23 has a reduced affinity for IgE. Binding of CD23 to IgE is thought to be dependent on oligomerisation of CD23. Oligomerisation of CD23 is encouraged by the α helical coiled-coil stalk (Drieks *et al.*, 1993, Beavil *et al.*, 1995), a region that is lost in the 25kDa sCD23 molecule. Hence, it was speculated that the reduced affinity of the 25kDa sCD23 molecule for IgE was due to the loss of the α helical coiled-coil stalk. It has since been shown that oligomerisation is important for orientating the lectin heads of CD23 to enable them to interact with IgE (Kelly *et al.*, 1998). Despite the loss of the coiled-coil stalk the 25kDa sCD23 still retains the cytokine like properties, attributed to soluble CD23 through an interaction with CD21 (Mossalayi *et al.*, 1992). This was demonstrated by the ability of recombinant 25kDa sCD23 to promote; (1) thymocyte differentiation (Mossalayi *et al.*, 1990a), (2) growth and differentiation of early myeloid cells (Mossalayi *et al.*, 1990b, Bertho *et al.*, 1991) and (3) aid survival of germinal centre B cells in synergy with IL-1 α (Lui *et al.*, 1991).

Dissecting the specific properties of each of the soluble CD23 molecules has posed several problems for workers in this field. As discussed above, the production of a recombinant CD23 molecule expressing the domains, encompassed by the 25kDa sCD23 fragment used in studies by Lui *et al.*, (1991) demonstrated a role for this fragment in the rescue of germinal B cells from apoptosis. However, this does not rule of the possibility that the 37, 33, and 29kDa fragments have a role in this function, as all contain the CD21 binding domain. Indeed the production of a recombinant 37kDa sCD23 fragment was also shown to be able to rescue germinal centre B cells from apoptosis (Graber *et al.*, 1992). Problems in dissecting the specific properties of each of the soluble CD23 molecules, stem from the fact that many of the

original antibodies specific for CD23 only recognised native CD23 within the lectin domain and therefore detected all soluble fragments (Bonnefoy *et al.*, 1990). In addition many of these antibodies did not detect denatured sCD23 fragments separated by SDS-PAGE/Immunoblotting (especially monoclonal antibodies). It is possible that these antibodies recognised epitopes that either become buried or denatured when the antigen is bound to nitrocellulose or PVDF membranes. The demonstration that the antibody EBVCS 1 bound to CD23 outside the IgE binding domain within the lectin head (Bonnefoy *et al.*, 1990), first identified a possible tool to differentiate the IgE binding properties of CD23. The potential of this antibody to facilitate investigation of the biological properties of sCD23 was demonstrated by Katira and Gordon (1995). These studies showed that EBVCS 1 could be used to capture 29, 33 and 37 kDa sCD23 products in an enzyme linked assays (ELISAs) but was unable to capture the 25kDa sCD23 fragments. Therefore, this antibody could be used to differentiate between CD23 fragments > 25kDa or \leq 25kDa.

The production of antibodies that can distinguish between the different sCD23 fragments and the mCD23 molecule, in both native and denatured forms, would provide a useful tool by which the different sCD23 molecules could be assigned to a function. As research within this field focuses on the mechanism of CD23 shedding and the identification of endogenous and exogenous proteinase(s) responsible for cleavage, the importance of assigning functional properties to the different sCD23 and mCD23 molecules becomes more prominent. Several studies have now shown that inhibition of CD23 cleavage by metalloprotease inhibitors prevents the shedding of the 37 and 33kDa sCD23 molecules and down-regulates IgE responses (Christie *et al.*, 1997 and Wheeler *et al.*, 1998). However, if the production of smaller sCD23 molecules (>33kDa) is independent of this cleavage event, then these smaller fragments (29, 25 and 16kDa) may be capable of promoting IgE synthesis through interactions with IgE and CD21. Conversely, if production of the smaller sCD23 molecules are dependent upon cleavage of the 37 and 33kDa sCD23 molecules, then inhibition of this cleavage event may have adverse consequential effects on thymocyte cell differentiation, myeloid cell differentiation and on the rescue of germinal centre B cells from apoptosis.

Anti-neoepitope antibodies that detect newly created amino (NH₂-) or carboxy (COOH-) terminal sequences, resulting from specific proteolytic events have been used successfully in the study of aggrecanase mediated cleavage of aggrecan (Hughes *et al.*, 1992, Hughes *et al.*, 1995 and Hutton *et al.*, 1996), and collagenase mediated cleavage of collagen (Vankemmelbeke *et al.*, 1997). It was suggested that the production of anti-neoepitope antibodies to the cleavage sites within CD23 would confer similar analytical advantages; assisting in the analysis of proteolytic processing events within CD23, and helping elucidate the functional properties of sCD23 molecules *in vitro* and *in vivo*. An increased understanding of the proteolytic processing events and functional properties of membrane and soluble CD23 molecules, would also aid prediction of the possible outcomes of preventing CD23 from being shed.

4.2 Production of Anti-Neoepitope Antibodies

Anti-neoepitope antibodies were produced by coupling peptides representing the amino and carboxy termini of two of the cleavage locations within CD23, to a carrier protein followed by immunisation of rabbits to raise antipeptide antibodies.

4.2.1 Selection of Amino Acid Sequence used to Produce Anti-Neoepitope Antibodies

In order to produce anti-neoepitope antibodies reactive with the amino (N) and carboxy (C) termini of the cleavage sites within CD23, 10-amino acid long peptides corresponding to the sequence of the N- and C-termini at these sites were synthesised by AstraZeneca Pharmaceuticals (figure 4.1). Peptides were synthesised representing the amino- and carboxy-terminus of two predominant cleavage sites within CD23, i.e. those that result in the production of sCD23 molecules of approximately 37 and 25kDa's (Letellier *et al.*, 1989 and Nakajima *et al.*, 1987 respectively). Neoepitope peptide sequences were screened against the protein sequence libraries available from the Human Genome Mapping Project (HGMP) using the Basic Logical Alignment Search Tool (BLAST) to identify proteins with matching amino acid sequences that may potentially cross-react with the antibody.





(Figure adapted from an original kindly supplied by Dr. R. Beavil, Kings College, London.)

There are many recommended guidelines for the selection of peptide sequences to create an antipeptide antiserum. Generally peptide sequences should be between 10-15 amino acids in length and contain a high number of hydrophilic, charged amino acids, as these are usually located in externally exposed locations within the native molecule (Hopp and Woods 1981). The amino and carboxy- terminal regions of a protein are often chosen to generate antibodies that detect native proteins. These regions are often exposed on the protein surface and/or are more mobile than the rest of the protein, making them more accessible to antibody recognition (Westhof *et al.*, 1984). However, as the aim of this exercise was to generate anti-neoepitope antibodies, the emphasis was shifted away from the recognition of the native (whole) molecule. Hence the choice of amino acid sequence used to create these antibodies was restricted to the terminal amino acid sequence produced after cleavage. It was hoped that antibodies produced to these locations within CD23 would only recognise the newly produced termini after proteolytic cleavage. Prior to proteolytic cleavage these sites are incorporated within the coiled-coil stalk of the CD23 molecule, a location that is predicted to be inaccessible and unlikely to be reactive to the anti-neoepitope antibodies.

4.2.2 Selection of a Carrier Protein and Coupling Strategy

Haptenic peptides alone are often poorly immunogenic due to their relatively small size, hence the peptide sequences were coupled to larger immunogenic molecule called a carrier protein. Proteins that are commonly used as carrier molecules contain a number of amino acid residues with reactive side chains that can be used for coupling. Each peptide was coupled (separately) to the carrier proteins bovine thyroglobulin and ovalbumin. Bovine thyroglobulin has a molecular mass of 660kDa (Mercken *et al.*, 1985) which makes it highly immunogenic; in addition this protein is relatively soluble which eases handling of the protein in solution. This is an advantage over other common carrier proteins such as Keyhole Limpet Haemocyanin (KLH) which is relatively insoluble, making it difficult to determine the solubilised protein concentrations, required for effective conjugation. The thyroglobulin amino acid sequence was screened against the BLAST database to eliminate the potential for antibodies produced against the carrier protein to cross-react against CD23 or known MEL cell proteins. Ovalbumin is far smaller in size at 42kDa (Nisbet *et al.*, 1981). Peptides were conjugated to ovalbumin to be used as a non-relevant neoepitope peptide-carrier protein substrate in enzyme

immunoassays, designed to measure the response of the anti-peptide antibodies. Only peptides coupled to thyroglobulin were used to immunise rabbits, peptides conjugated to ovalbumin were used as controls in ELISA to facilitate absorption of the peptide to the microtitre plate during screening assays.

Each peptide was coupled to the carrier protein by a terminal cysteine residue. As there were no cysteine residues present in the peptide sequences, a cysteine residue was added during synthesis to the opposite end to that of the neoepitope. That is peptides corresponding to the amino terminus of cleavage sites within CD23 were coupled through the addition of a cysteine residue to the carboxy terminus and visa versa for carboxy termini. The addition of a cysteine residue to the peptide sequence provides a free sulfhydryl on the synthetic peptide for coupling to free amino groups on the carrier protein accomplished via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). MBS cross-links thiol groups with amino groups at neutral pH, this was achieved in a two stage reaction firstly, cross-linking the carrier protein to MBS producing MB/carrier protein, then cross-linking of the MB/carrier protein conjugate to the cysteine resin of the peptide (figure 4.2).

4.2.3 Production of an Antibody Response

Rabbits were immunised with peptide/thyroglobulin conjugates. Before injection the peptide thyroglobulin conjugate was mixed with either Freund's complete adjuvant, for the first round of injections, or Freund's incomplete adjuvant for subsequent injections. Adjuvant has two roles, firstly it converts soluble protein antigens into particulate material which are more readily ingested by antigen presenting cells, and secondly it prolongs the half-life of the antigen by protecting it from degradation (Audibert and Lise 1993). As antibody responses directed against peptide/carrier protein conjugates can vary between individual rabbits, three rabbits were inoculated for each peptide/thyroglobulin conjugate. Rabbits were first inoculated with peptide/thyroglobulin conjugates mixed with Freund's complete adjuvant, and then given booster injections every 4-5 weeks. In total, the rabbits were given three boosters injections. The presence of mycobacteria in Freund's complete adjuvant activates the T cell



Figure 4.2 Coupling of Neoepitope Peptides via a Terminal Cysteine (Cys) Residue to the Carrier Protein Thyroglobulin (or Ovalbumin) using *M*-Maleimidobenzoyl-*N*-Hydroxysuccinimide Ester. The above diagram schematically depicts coupling of a C-terminal peptide. N-terminal peptides where coupled via the addition of a cysteine residue at the C-terminus (not shown). Thy, thyroglobulin (carrier protein)

(Figure adapted from an original published in Current Protocols in Immunology 1991)

population providing the necessary lymphokines for B cell stimulation and maturation. However, Freund's complete adjuvant can cause granuloma and necrotic abscesses, therefore its use is limited to the first injection only (Harlow and Lane 1988). Test bleeds were taken after seven days and sera assayed by enzyme-linked immunoabsorbent assay (ELISA) for specific antibody responses to the neoepitope peptides.

4.2.4 Development of an ELISA to Screen Antisera

ELISA is a versatile, quantitative and sensitive technique that is useful for evaluating antibody activity of polyclonal antisera (Engvall and Perlman 1971). An indirect ELISA strategy was developed to screen antiserum samples for the presence of neoepitope specific antibodies. Each antiserum was assayed against three capture antigens; firstly the peptide-thyroglobulin carrier protein conjugate, with which the rabbit was immunised, secondly the peptide used to immunise the rabbit but conjugated to ovalbumin, and thirdly an unrelated peptide, not used for immunisation that was conjugated to ovalbumin. The amount of specific antibody captured was detected using a goat anti-rabbit IgG peroxidase conjugate antibody quantified by measurement of the conversion of a colourless substrate (OPD, see section 2.8.1) to a coloured reaction product at a wavelength of 492nm using a spectrophotometer.

4.2.5 CD23 Antigen Used to Screen Antibodies Produced to the Neoepitope Sequences

In order to screen for antibodies produced to the neoepitope sequences within CD23, both recombinant CD23 expressed by MEL cells (described in chapter 3) and native CD23 expressed by the EBV-transformed B cell line RPMI 8866 were used. Each of these cell lines offers different advantages for screening but also possess disadvantages that should be taken into account when analysing the results. RPMI 8866 cells express native CD23 that is cleaved by an endogenous protease(s) *in vitro*. However, the comparatively low cleavage rate and low level production of sCD23 may limit detection of these molecules by neoepitope specific antisera. Concentrating the released material is complicated by the presence of 10% foetal calf serum in the growth medium, and any reduction in the serum component of medium reduces cell growth and may change the morphology of the cells and their state of differentiation as the cells become adherent to the plastic tissue culture flasks (personal observation). Although recombinant CD23 expressed by transfected MEL cells overcomes

some of the difficulties experienced when using RPMI 8866 cells the location of the recombinant mCD23 cleavage sites have not been sequenced.

4.3 Results

4.3.1 Neoepitope Peptide Sequences

Peptide sequences used to generate anti-neoepitope antibodies and their location within the CD23 amino acid sequence are shown in figure 4.1. These peptide sequences have been termed neoC₃₇ (ac-CESHHGDQMA), neoN₃₇ (QKSQSTQISC-NH₂), neoC₂₅ (ac-CLREEVTKLR) and neoN₂₅ (MELQVSSGFC-NH₂) indicating which terminus the peptide represents (C- or N-) and which sCD23 fragment (37 or 25kDa) is generated after cleavage at this site. BLAST sequence searches showed that peptide neoN₃₇, neoC₂₅ and neoN₂₅ were only present in CD23 and did not match any other known amino acid/protein sequence. The neoC₃₇ peptide sequences (see below). There are 7-13 different mammalian myosin heavy chain genes, the products of which are expressed mainly in cardiac and skeletal muscle, smooth muscle and foetal muscle (Edwards *et al.*, 1985) and not in MEL or B cells. Therefore the potential for antibodies produced to neoC₃₇ to cross-react with the myosin heavy chain was considered to be negligible in these cells.

Query: Neoepitope Peptide NeoC37NeoC37 SequenceCESHHGDQMAMyosin Heavy ChainLESDHMatch 3/5 amino acids 60%

It should be noted that the peptide $neoN_{37}$ contains an N-terminal glutamine (Gln) residue. Once reconstituted in a neutral pH7 buffer the Gln residue of this peptide is likely to slowly eliminate ammonia and form a pyroglutamyl (Glp) residue (Abraham and Podell *et al.*, 1981). Therefore any antibody raised against this peptide is likely to recognise Glp rather than Gln. This may affect the pattern of recognition of this antibody, as the N-terminal Gln in the newly cleaved 37kDa sCD23 molecule may have not had sufficient time to change to a Glp residue.

4.3.2 Successful Conjugation of Peptide Sequences to the Carrier Protein

Successful conjugation of the neoepitope peptides to the carrier protein (either thyroglobulin or ovalbumin) was determined by the conversion of the end terminal cysteine residue to a succinyl cysteine, this was identified by chromatographic amino acid analysis. Amino acid analysis performed by AstraZeneca Pharmaceuticals showed that all conjugations were successful (figure 4.3.1-4.3.4), by the presence of an additional succinyl cysteine peak eluted after 10-11 minutes.

4.3.3 Analysis of Anti-Neoepitope Antibodies by ELISA

Serum samples collected from each of the immunised rabbits were screened for specific antibody responses created to the immunising neoepitope peptide (carrier protein conjugate) by ELISA. The specificity of the antibody response was determined by comparing the binding characteristics of each antiserum sample with pre-immune sera, and the reactivity of the antiserum against an unrelated neoepitope peptide-ovalbumin conjugate (i.e. not the peptide used to produce the antibody response being measured). The results gained for antiserum samples taken after the second round of injection are shown in figure 4.4a and figure 4.4b (second bleed results are shown as these antisera proved most reliable in subsequent experiments). Results were plotted for specific antibody responses to (1) an unrelated necepitope peptide conjugated to ovalbumin, (2) the immunising necepitope peptide conjugated to ovalbumin and (3) the immunising neoepitope peptide conjugated to thyroglobulin. The specific antibody response to the neoepitope sequence used to immunise the rabbit was represented by the difference between the binding to the neoepitope peptide conjugated to ovalbumin, and the binding of the unrelated neoepitope peptide conjugated to ovalbumin. Figures 4.4.1 and 4.4.2, represent the average values of three experiments. Preimmune antisera did not bind to the antigen (figure 4.4.3), indicating that the antibody responses measured were specific for the peptide-carrier conjugate. Although all of the rabbits produced an antibody response against the injected peptide-thyroglobulin conjugate, the titre of the antibody response varied considerably between each rabbit. Hence, antisera from



a) Amino acid analysis of peptide neoN₃₇ conjugated to thyroglobulin

b) Amino acid analysis of peptide neoN₃₇ conjugated to ovalbumin



Figure 4.3.1 Successful Conjugation of Neoepitope Peptide $NeoN_{37}$ to the Carrier Protein. Successful conjugation of the neoepitope peptide $neoN_{37}$ to the carrier protein (either thyroglobulin or ovalbumin) was determined by the conversion of the end terminal cysteine to a succinyl cysteine (eluted after 11 minutes), this was identified by chromatographic amino acid analysis. Amino acid analysis performed by AstraZeneca Pharmaceuticals.



a) Amino acid analysis of peptide $neoC_{37}$ conjugated to thyroglobulin

b) Amino acid analysis of peptide neoC37 conjugated to ovalbumin



Figure 4.3.2 Successful Conjugation of Neoepitope Peptide NeoC₃₇ to the Carrier Protein. Successful conjugation of the neoepitope peptide $neoC_{37}$ to the carrier protein (either thyroglobulin or ovalbumin) was determined by the conversion of the end terminal cysteine to a succinyl cysteine (eluted after 11 minutes), this was identified by chromatographic amino acid analysis. Amino acid analysis performed by AstraZeneca Pharmaceuticals.



a) Amino acid analysis of peptide neoN25 conjugated to thyroglobulin

b) Amino acid analysis of peptide neoN25 conjugated to ovalbumin



Figure 4.3.3 Successful Conjugation of Neoepitope Peptide $NeoN_{25}$ to the Carrier Protein. Successful conjugation of the neoepitope peptide $neoN_{25}$ to the carrier protein (either thyroglobulin or ovalbumin) was determined by the conversion of the end terminal cysteine to a succinyl cysteine (eluted after 11 minutes), this was identified by chromatographic amino acid analysis. Amino acid analysis performed by AstraZeneca Pharmaceuticals.



a) Amino acid analysis of peptide $neoC_{25}$ conjugated to thyroglobulin





Figure 4.3.4 Successful Conjugation of Neoepitope Peptide NeoC₂₅ to the Carrier Protein. Successful conjugation of the neoepitope peptide $neoC_{25}$ to the carrier protein (either thyroglobulin or ovalbumin) was determined by the conversion of the end terminal cysteine to a succinyl cysteine (eluted after 11 minutes), this was identified by chromatographic amino acid analysis. Amino acid analysis performed by AstraZeneca Pharmaceuticals.





Figure 4.4.1 Antibodies Raised to Neoepitope Peptides NeoC₃₇ and NeoN₃₇ Screened by ELISA. Rabbit antisera were screened by ELISA for the presence of antibodies raised to the neoepitope peptide conjugates, (a) antibody response generated to $neoC_{37}$, (b) antibody response generated to $neoN_{37}$. Antisera were screened against the following peptide-carrier protein conjugates: (1) immunising peptide-thyroglobulin conjugate, (2) immunising peptide-ovalbumin conjugate, and (3) alternative peptide-ovalbumin conjugate.

thy; thyroglobulin, oval = ovalbumin.





Figure 4.4.2 Antibodies Raised to Neoepitope Peptides NeoC₂₅ and NeoN₂₅ Screened by ELISA. Rabbit antisera were screened by ELISA for the presence of antibodies raised to the neoepitope peptide conjugates, (a) antibody response generated to $neoC_{25}$, (b) antibody response generated to $neoN_{25}$. Antisera were screened against the following peptide-carrier protein conjugates: (1) immunising peptide-thyroglobulin conjugate, (2) immunising peptide-ovalbumin conjugate.

thy; thyroglobulin, oval = ovalbumin.



Binding of Pre-Immune Sera to Immunising Peptide-Thyroglobulin Conjugate

Figure 4.4.3 Binding of Pre-Immune Rabbit Antisera to Immunising Peptide-Thyroglobulin Conjugates, Screened by ELISA. Pre-Immune rabbit antisera were screened by ELISA for the presence of antibodies to the immunising peptide-thyroglobulin conjugates. Antisera were screened against the following peptide-carrier protein conjugates: NeoC₃₇-thyroglobulin, NeoN₃₇thyroglobulin, NeoC₂₅-thyroglobulin and NeoN₃₇-thyroglobulin. **thy**; thyroglobulin.

rabbits shown to produce the highest titre of antibody were chosen for subsequent experiments. ELISA results also confirm the immunogenicity of the four peptides, with the highest titre antibody responses being against the peptides $neoC_{37}$ and $neoC_{25}$.

4.3.4 Analysis of Anti-Neoepitope Antibody Specificity by Immunoblotting

Antisera shown to possess the highest titre of anti-neoepitope specific antibodies for each of the four immunising peptides, were then assayed for their ability to detect the neoepitope sequences within cleaved CD23 fragments by immunoblotting. RPMI 8866 cells, mCD23 transfected MEL cells and untransfected MEL cell lysates and their corresponding supernatants were separated by SDS-PAGE and transferred to PVDF membrane by electroblotting. The presence of mCD23 and cleaved sCD23 fragments was then assessed by immunoblotting, using an anti-CD23 polyclonal antibody as a positive control and each of the anti-neoepitope antisera (diluted over a range of 1/100 to 1/10,000).

Antisera raised to neoepitope peptides $neoC_{37}$, $neoC_{25}$ and $neoN_{25}$ were unable to detect denatured mCD23 or shed sCD23 fragments. However, the antiserum raised against the neoepitope peptide $neoN_{37}$ was shown to react with a protein of approximately 37kDa from RPMI 8866 and mCD23 transfected MEL cell supernatants but not untransfected MEL cell supernatants (figure 4.5, lanes 3, 4 and 5). This band corresponds in size to the 37kDa sCD23 fragment detected with the anti-CD23 polyclonal antibody and is probably the 37kDa sCD23 fragment (figure 4.5 lane 7). To assess the specificity of each of the anti-neoepitope antisera, the secondary antibody (Goat anti-rabbit HRP) alone and the pre-immune serum samples were tested for the possibility that they could be binding directly to proteins contained within these samples. Both were shown not to detect denatured mCD23 or shed sCD23 fragments by immunoblotting methods.

The inability of anti-neoN₃₇ antiserum to recognise full length CD23 from either RPMI 8866 or mCD23 transfected MEL cell lysate samples indicates that this anti-serum contains a true neoepitope antibody, recognising the N-terminus of the 37kDa sCD23 fragment. In order to determine the specificity of this antibody for the N-terminus of the 37kDa sCD23 fragment the

Supernatants



Figure 4.5 Anti-NeoN₃₇ Antiserum Detects a 37kDa Protein in the Supernatants of CD23 transfected Cells. MEL cells transfected with mCD23/pEV3 vector (clone 4) and untransfected controls were grown in log phase for 4 days and then induced with 2% DMSO. Four days after induction of protein expression, samples were centrifuged and the supernatant aspirated. RPMI 8866 cells were grown optimally (between 2-8x10⁵/ml) before allowing cells to overgrow, thus allowing the sCD23 fragments to accumulate in the growth media/supernatant. The cells were pelleted and the supernatant collected, then concentrated 5 fold using Centricon-10 microconcentrators. Supernantants were separated by denaturing 12% SDS-PAGE gel electrophoresis, transferred to Immobilon-P membrane, and then used to screen anti-neoepitope antisera by immunoblotting. The above figure shows supernatant samples from; (1) untransfected MEL cell controls (UT lanes 1, 3 & 6), (2) mCD23 transfected MEL cells (T lanes 2, 4 & 7), (3) RPMI 8866 cells (lane 5). All prepared as described above, then immunoblotted with (a) pre-immune serum (lanes 1 & 2), (b) antineoN₃₇ antiserum (diluted 1/500, lanes 3-5), (c) anti-CD23 polyclonal antibody (10µg/ml). Protein band sizes were determined by comparison to Rainbow™ protein molecular weight markers (Amersham Life Science).

neoN₃₇ antiserum was screened against the recombinant tCD23 cell lysate sample by SDS-PAGE/Immunoblotting as described above. The neoN₃₇ epitope is contained within the stalk region of this recombinant protein and not at the N-terminus, therefore the neoN₃₇ epitope should not be detected by anti-neoepitope antibodies. The result of this experiment is shown in figure 4.6. Anti-neoN₃₇ antiserum was unable to bind recombinant tCD23, supporting the specificity of this antibody for sCD23 molecules containing the neoN₃₇ epitope at the N-terminus. However, despite strong evidence to suggest that this antiserum binds to the 37kDa sCD23 molecule, further evidence is required to show that the protein is indeed sCD23 (this is discussed in greater detail in chapter 5).

There are several reasons why the anti-neoepitope antisera $neoC_{37}$, $neoC_{25}$ and $neoN_{25}$ may not have recognised the corresponding epitopes in sCD23 fragments released from either RPMI 8866 cells or MEL cells expressing recombinant mCD23. (1) The production of the 25kDa sCD23 fragment found within the supernatants of RPMI 8866 cells and mCD23 transfected MEL cells, appears to be far lower than that of the 37 and 33kDa fragments (shown in figure 3.16). Therefore it is possible that the concentration of this sCD23 fragment is below the detection range of the anti-neoN₂₅ and anti-neoC₂₅ antisera. The polyclonal antibody used as a positive control for these experiments was raised to the whole CD23 molecule. Hence the avidity (the sum total of the strength of binding to multiple sites) is likely to be greater than that of an anti-necepitope antibody whose specificity is based only on a single epitope at the N- or C-terminus. (2) Once mCD23 is cleaved from the cell surface, releasing sCD23 into the supernatant, the remaining stump left on the cell surface may be quickly internalised and degraded. (3) After cleavage by an endogenous protease(s) the newly produced amino and carboxy termini may be susceptible to degradation by a carboxypeptidase or an aminopeptidase. The loss of one or two amino acids may be enough to prevent recognition of the neoepitope sequence by the antibody. (4) The antibody response shown by ELISA was due to recognition of the coupling region that cross-links the peptide to the carrier protein.



Figure 4.6 Anti-NeoN₃₇ Antiserum Does Not Detect the Recombinant Truncated CD23. NeoN₃₇ antiserum was screened against tCD23 transfected MEL cell lysate samples to assess whether the anti-neoN₃₇ neoepitope antibody recognised the neoN₃₇ epitope within tCD23. This epitope is contained within the coiled-coil stalk of the recombinant tCD23 protein. Untransfected MEL cells (UT lanes 1 & 3) and tCD23 transfected MEL cells, clone 5 (T lanes 2 & 4), were grown in log phase for 4 days then induced with 2% DMSO. Four days after the induction of protein expression, samples were centrifuged, the cells lysed by freeze thawing in the presence of protease inhibitors (2.8.10) and then analysed by denaturing 12% SDS-PAGE gel electrophoresis and immunoblotting. Recombinant tCD23 was screened with a) neoN₃₇ antiserum; diluted 1/500 (lanes 1 & 2), b) anti *c-myc* monoclonal antibody 9E10; diluted 1/500 (lane 3 & 4). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).
4.3.5 Analysis of Anti-Neoepitope Antibody Specificity by Flow Cytometry

Anti-neoC₃₇ and anti-neoC₂₅ antisera were used in flow cytometry experiments, to analyse their ability to detect the corresponding epitopes in cleaved CD23 stumps retained on the cell surface. Flow cytometry offers the ability to detect the presence of these stumps on the surface of a greater number of cells quickly, without the problems of concentrating samples for SDS-PAGE analysis and the possible degradation of the protein caused by cell lysis techniques. As this method allows quick and sensitive analysis of a large number of cells, CD23 expressed on RPMI 8866 cells was used as a substrate to screen antisera produced to the $neoC_{37}$ and $neoC_{25}$ peptide conjugates. Figure 4.7 shows that antiserum raised to the peptide neoC₂₅ clearly recognises an epitope expressed on the cell surface of RPMI 8866 cells (figure 4.7, no.5). The corresponding pre-immune serum control (figure 4.7, no.6) does not bind. Antiserum raised to the peptide $neoC_{37}$, also appears to detect an epitope expressed on the surface of RPMI 8866 cells (figure 4.7, no.3). However, the mean fluorescence intensity (MFI) of cells stained with this antiserum is lower than that of the anti-neoC₂₅ antiserum (MFI $neoC_{37}=5.8$, MFI $neoC_{25}=10.8$). The MFI for cells stained with the anti-neoC₃₇ is lower than expected when compared to the estimated proportions of 37kDa sCD23 fragment (35-50%) released from the cell surface, relative to the proportion of 25kDa sCD23 fragment (17-25%) (Marolewski et al., 1998). Several reasons may account for the lower mean fluorescence intensity of this antiserum. Firstly, cleavage at this site could lead to rapid internalisation of the cleaved stump (as predicted above). Secondly, due to the close proximity of this epitope to the cell surface, the antiserum may be unable to bind due to conformational limitations. Thirdly, the epitope could be further degraded, losing one or more of the carboxy terminal amino acids (alanine or methionine), and thus destroying the epitope.

4.3.6 Confocal Microscopy Analysis of Intracellular CD23 Cleaved Stumps using Anti-Neoepitope Antibodies

To ascertain if the $neoC_{37}$ epitope is internalised after proteolytic cleavage of the 37kDa sCD23 fragment, RPMI 8866 cells, induced mCD23 transfected MEL cells and induced untransfected MEL cells were permeabilised to allow the antibodies to intracellular antigens. It was considered possible that the epitope recognised by the anti-neoC₃₇ antiserum is





Figure 4.7 Anti-NeoC₃₇ and Anti-NeoC₂₅ Antisera Recognise Antigens on the Surface of RPMI 8866 Cells. RPMI 8866 cells were used to screen anti-neoepitope antisera by flow cytometry. 1×10^6 RPMI 8866 cells were incubated in the presence of anti-neoC₃₇ and anti-neoC₂₅ antisera or pre-immune serum (all diluted 1/500) for 60 minutes. Binding of the antisera was detected using a secondary anti-rabbit Ig FITC labelled antibody (Sigma). MFI = Mean Fluorescence Intensity, calculated for the area defined by the marker M1. (1) control; no antibodies, (2) secondary anti-rabbit Ig FITC only, (3) anti-neoC₃₇ antiserum, (4) pre-immune serum (before immunising with peptide neoC₃₇), (5) anti-neoC₂₅ antiserum, (6) pre-immune serum (before immunising with peptide neoC₂₅).

inhibited by the proximity of this epitope to the cell surface, hindering antibody binding. It was therefore speculated that disruption of the cell membrane with a non-ionic detergent - saponin, prior to antibody treatment may allow the antibody access to the internalised epitope. The results of these experiments are shown in figures 4.8.1 and 4.8.2. CD23 monoclonal antibody BU38 FITC (diluted 1/100) binds to CD23 either on the cell surface of induced mCD23 transfected MEL cells or intracellularly (figure 4.8.1, A). However anti-neoC₃₇ antiserum does not appear to bind to induced mCD23 MEL cells (figure 4.8.1, B), or untransfected MEL cells (figure 4.8.2, A and B). The results gained using RPMI 8866 cells were also negative for anti-neoC₃₇ antiserum binding (data not shown).

4.3.7 Purification of Cell Membrane Proteins and Detection of Cleaved CD23 Products using Anti-Neoepitope Antisera

One possible reason why the antisera raised to the anti-neoepitope peptides $neoC_{37}$ and $neoC_{25}$ do not recognise the corresponding epitopes in cleaved CD23 fragments, may be due to the low-level of expression of these fragments at the cell surface. The purification and analysis of cell membrane proteins by SDS-PAGE and immunoblotting using an anti-CD23 polyclonal antibody has identified a number of CD23 fragments, not seen previously in the analysis of cell lysate samples by the same method (described in chapter 3, section 3.4.4). The purification of membrane proteins expressed by MEL cells transfected with mCD23 was therefore repeated. Membrane CD23 transfected MEL cells and untransfected controls were grown in log phase, after four days, cells were induced to differentiate with 2% DMSO and left growing. After four days the cells were centrifuged and the membrane proteins extracted. MEL cell membranes from cells transfected with mCD23 and untransfected controls were separated by SDS-PAGE and Western blotted onto PVDF membrane. These samples were used to screen antisera raised to neoepitope peptides $neoC_{37}$ and $neoC_{25}$ for their ability to detect cleaved CD23 fragments. The results of this experiment are shown in figure 4.9.

The antiserum raised to neoepitope peptide $neoC_{25}$ detected a band of approximately 15kDa but did not recognise full-length membrane CD23, this is consistent with the theory that antineoC₂₅ antiserum detects mCD23 stumps on the cell surface but not full-length mCD23. However, the detection of a 15kDa band caused some concern, as the predicted size of the

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Figure 4.8.1 Analysis of Intracellular and Cell Surface Binding of Anti-NeoC₃₇ **Antiserum by Confocal Microscopy.** MEL cells transfected with mCD23/pEV3 vector or untransfected controls were grown in log phase for 4 days then induced with 2% DMSO. Four days after induction of protein expression, cells were used to screen anti-neoC₃₇ antiserum for intracellular or cell surface binding by confocal microscopy (2.8.2). Binding of antisera was detected using a secondary antibody; anti-rabbit Ig FITC (Sigma). (A) MEL cells transfected with mCD23 incubated with BU38-FITC (1/100), (B) MEL cells transfected with mCD23 incubated with anti-neoC₃₇ antiserum (1/500). Cells positively stained with the antisera/antirabbit Ig FITC or BU38 FITC were viewed by confocal microscopy, Kalman 6 analysis (images captured every 0.5 micron through cell profile, these are then filtered, of which 6 are over laid to form the right hand image above). This image is shown in comparison to cells viewed by phase contrast (left hand image).

Chapter 4: Generation of Immunological Tools to Study the Proteolytic Processing of CD23





Henry 4.1.1 Analysis of himsenhoist and Cell Surface Binding of Anti-NeoCo Antiberent by Contour Philemeson. MEL cell manhend with mC003/pEV3 were a menufactul controls wavegation in lag phase for 4 days first totanol with 2% DMSG. For data and induction of protein expression cells wave until is arrefu estimated with 2% DMSG. For antidectatur or out pathers binding by analysis antionized (2.8.2). Etading of antisets wa datased rating a separatery antibudy an erabul is HTC (Signat). (A) MEL cells monitorial incolored with universe and BU03-FTTC (1960). (B) MEL cells mentioned with mC003 incolored with universe and BU03-FTTC (1960). (B) MEL cells mentioned with mC003 incolored with universe and BU03-FTTC (1960). (B) MEL cells mentioned with mC003 incolored with universe and BU03-FTTC (1960). (B) MEL cells mentioned with mC003 incolored with universe and bU03-FTTC (1960). (B) MEL cells mentioned with mC003 incolored with anti-meeC₁ antiserium (1.500). (Cells postively drived with the antiserium incolored in firm the right field intege of postively drived with the antiserium intervected in firm the right field intege with the sector of the intervection intervected in firm the right field intege show). This image is show in comparison in cells intervected by duals control ([e]) high intege is show in comparison in cells



Figure 4.8.2 Analysis of Intracellular and Cell Surface Binding of Anti-NeoC₃₇ Antiserum by Confocal Microscopy. MEL cells transfected with mCD23/pEV3 vector or untransfected controls were grown in log phase for 4 days then induced with 2% DMSO. Four days after induction of protein expression, cells were used to screen anti-neoC₃₇ antiserum for intracellular or cell surface binding by confocal microscopy (2.8.2). Binding of antisera was detected using a secondary antibody; anti-rabbit Ig FITC (Sigma). (A) Untransfected MEL cells incubated with anti-neoC₃₇ antiserum (1/500), (B) Untransfected MEL cells incubated with pre-immune serum (1/500). Cells positively stained with the antisera or pre-immune sera/anti-rabbit Ig FITC were viewed by confocal microscopy, Kalman 6 analysis (images captured every 0.5 micron through cell profile, these are then filtered, of which 6 are over laid to form the right hand image above). This image is shown in comparison to cells viewed by phase contrast (left hand image).





Antibody (a) anti-CD23 (b) anti-neo C_{37} (c) anti-neo C_{25}

Figure 4.9 Membrane CD23 Transfected MEL Cell Membranes Screened with Anti-NeoC₃₇ and Anti-NeoC₂₅ Antisera. Membrane proteins extracted from induced MEL cells transfected with mCD23 and untransfected controls separated by denaturing 12% SDS-PAGE gel electrophoresis, transferred to Immobilon-P membrane then screened with anti-neoepitope antisera by immunoblotting. The above figure shows membrane protein samples extracted from untransfected MEL cell controls (UT lanes 1, 3 & 5) and mCD23 transfected MEL cells (T lanes 2, 4 & 6). Samples were then immunoblotted with (a) anti-CD23 polyclonal antibody $(10\mu g/ml; lanes 1 \& 2)$ and (b) anti-neoC₃₇ antiserum (diluted 1/500; lanes 3 & 4), (c) anti-neoC₂₅ antiserum (diluted 1/500; lanes 5 & 6). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

membrane stump after cleavage of the 25kDa sCD23 molecule was 22kDa. No protein bands were detected by the anti-neoC₃₇ antiserum. Detection of a 15kDa band by anti-neoC₂₅ antiserum strongly supports results shown by flow cytometry (see section 4.3.5 and figure 4.7). This result also suggests that cleavage of the 25kDa sCD23 fragment is independent of the 37kDa sCD23 shedding event, and that prior cleavage of the 37kDa sCD23 molecule is not required for the cleavage of the 25kDa molecule i.e. the two events are not necessarily sequential.

4.3.8 Metabolic Labelling of Cell Surface Proteins and Immunoprecipitation with Anti-Neoepitope Antisera

Characterisation of the anti-neoepitope antisera was hampered by the limited amount of antigen in the cell systems. In order to assess the binding properties of the anti-neoepitope antisera further, cell proteins were metabolically labelled with 35 S cysteine and methionine (Trans 35 S labelTM), the labelled protein was then immunoprecipitated using protein-A (detailed in 2.8.13 and 2.8.14). Recombinant Protein-A Sepharose® Fast Flow (Pharmaceia Biotech) was used as a support for the purification of antiserum bound protein extraction. Recombinant Protein-A Sepharose® Fast Flow (Pharmaceia Biotech) was used as a support for the purification of antiserum bound protein extraction. Recombinant Protein-A for the Fc region of IgG. Although the antibody isotypes produced to the neoepitope peptide carrier conjugates were not determined, it was speculated that these would be predominantly IgG to which protein-A binds. Bound material was eluted from the rProtein-A Sepharose matrix by altering the pH of the buffer. This alters the degree of ionisation of charged groups on both the ligand and absorbed protein, affecting the binding properties of the protein-A Sepharose for the absorbed protein, reducing their affinity, or causing indirect changes in their affinity by alterations in conformation, resulting in elution of the bound protein.

RPMI 8866 cells, induced MEL cells transfected with mCD23 and untransfected controls were used as a source of cleaved CD23. Cells were grown in medium in the absence of cysteine and methionine for 2 hours to deplete internal stores of these amino acids. Cells were then incubated for 30 minutes with radiolabelled ³⁵S cysteine and methionine, followed by growth in normal medium. Cell lysates and corresponding supernatant samples were collected 2 and

24 hours after labelling. Labelled protein was purified by binding to the antiserum/protein-A Sepharose overnight at 4°C and then eluted. Eluted proteins were separated by SDS-PAGE and transferred to PVDF membrane by electroblotting then exposed to autoradiographic film. The CD23 specific monoclonal antibody MHM6 (kindly donated by Prof. J. Gordon, University of Birmingham, Birmingham, UK) was used as a positive control. This antibody specifically binds an epitope in the lectin head of CD23. The results from this experiment (shown in figure 4.10) show two prominent bands of approximately 37 and 33kDa, and a faint band of approximately 25kDa, immunoprecipitated from MEL cell supernatants transfected with mCD23 by the monoclonal antibody MHM6 (lane 5). However, antisera raised to the necepitope peptides were unable to selectively immunoprecipitate protein corresponding in size to either cleaved mCD23 stumps or sCD23 fragments. Binding by the anti-neoepitope antisera was shown to be non-specific, as proteins eluted from the anti-neoepitope antiserum/protein-A conjugates were also found to be eluted from the pre-immune controls. The absence of smaller sCD23 bands from immunoprecipitation experiments with monoclonal antibody MHM6 may reveal a time delay in the production of these fragments requiring a longer time (i.e. >24 hours) for the shedding of smaller soluble CD23 molecules to take place.

The inability of the anti-neoN₃₇ antiserum to detect the 37kDa fragment, by immunoblotting suggests that the anti-neoepitope antisera are poor antibodies for immunoprecipitation. This has also been the experience of others who commonly use antibodies raised to peptides (personal communication Mr. R. Forder, AstraZeneca Pharmaceuticals, Macclesfield. UK). Although there may be several alternative reasons for the failure to detect bound CD23 fragments to the anti-neoepitope antisera, this strategy was not pursued further.

4.3.9 Purification of a 25kDa Recombinant sCD23 Protein used to Characterise Specificity of Antiserum Raised to Neoepitope Peptide NeoN₂₅

Characterisation of the binding properties of the anti-neoepitope antisera by SDS-PAGE/Immunoblotting proved to be difficult. As outlined throughout this section, one of the reasons, believed to be a factor contributing to these problems is the unknown quantity of each of the epitopes present in the recombinant CD23 expression system used for analysis. That is, it is not known if each of the mCD23 stumps and sCD23 fragments containing the neoepitopes





Immunoprecipitates (a) untransfected MEL cells (b) mCD23 transfected MEL cells

Metabolic Labelling of MEL **Cell Surface Proteins** Figure 4.10 and Immunoprecipitation of CD23 using Anti-Neoepitope and Monoclonal Anti-CD23 MEL cells transfected with mCD23 and untransfected controls were Antibodies. metabolically labelled with Trans ³⁵S label[™] (ICN) (2.8.13), then incubated for a further 24 hours before immunoprecipitating with the anti-neoepitope antisera and the anti-CD23 monoclonal antibody MHM6. The above figure shows immunoprecipitated supernatant samples collected from (a) untransfected MEL cells, lanes 1-4, and (b) mCD23 transfected MEL cells, lanes 5-8. Immunoprecipitated with antibodies: anti-CD23 monoclonal antibody MHM6 (lanes 1 & 5); anti-neoN₃₇ antiserum (lanes 2 & 6); anti-neoN₂₅ antiserum (lanes 3 & 7); and pre-immune antiserum (lanes 4 & 8). Protein band sizes were determined by comparison to SeeBlue[™] protein molecular weight marker (Novex).

to which the antisera were raised, are present at detectable levels by Immunoblotting. Purification of shed recombinant CD23 via the 6xHis tag added to the C-terminus using TALON[™] metal affinity resin, was shown to detect three bands believed to represent 37, 33 and 25kDa sCD23 fragments (as described in section 3.4.4). This procedure was repeated to provide a source of 25kDa sCD23, against which the binding properties of the anti-neoN₂₅ antiserum could be characterised. His (x6) tagged proteins from the culture supernatants of induced MEL cells transfected with recombinant mCD23 and untransfected controls were purified using TALON[™] metal affinity resin. Purified protein samples were then separated by SDS-PAGE gel electrophoresis, transferred to Immobilon-P membrane and screened with anti-neoN₂₅ antiserum. The results of this experiment are shown in figure 4.11. Anti-neoN₃₇ antiserum and the anti-CD23 polyclonal antibody were used as positive controls. The antiserum raised to neoepitope peptide neoN25 did not recognise the 25kDa sCD23 fragment (figure 4.11, lane 6). It could be argued that the recombinant mCD23 is not cleaved at the same location as the native molecule and therefore does not contain the $neoN_{25}$ epitope. However, recognition of a membrane protein by antiserum raised to neoepitope peptide neoC₂₅, indicates that recombinant mCD23 is either cleaved at the same location as the native molecule or within close proximity. Characterisation of this antiserum was not pursued further.



Figure 4.11 Detection of rCD23 (Purified by the 6xHis Tag) with Anti-Neoepitope Antisera. His (6x) tagged proteins from the culture supernatants of induced mCD23 transfected MEL cells and untransfected controls were purified using TALONTM metal affinity resin (2.8.11), then separated by 12% SDS-PAGE gel electrophoresis, transferred to Immobilon-P membrane and screened against anti-neoepitope antisera by immunoblotting. The figure above shows His (6x) proteins isolated from untransfected MEL cells (UT), lanes 1, 3 & 5, and mCD23 transfected MEL cells (T), lanes 2, 4 & 6. Immunoblotted with: (a) anti-CD23 polyclonal antibody 10μ g/ml (lane 1 & 2); (b) anti-neoN₃₇ antiserum (lanes 3 & 4); (c) anti-neoN₂₅ antiserum (lane 5 & 6). Protein band sizes were determined by comparison to RainbowTM protein molecular weight marker (Amersham Life Science).

4.4 Discussion

4.4.1 Antigenicity and Immunogenicity of Immunising Peptides

Antisera were raised against all neoepitope peptides, as determined by ELISA. It is interesting to note that the antibody titre responses against neoepitope peptides $neoC_{37}$ and $neoC_{25}$ were greater than those shown for peptides $neoN_{37}$ and $neoN_{25}$. A possible reason for this may be due to the properties of the amino acids contained within the neoepitope peptide sequences. Amino acids are commonly classified based on the polarity of their R-groups. The four main classes are defined as (1) nonpolar or hydrophobic, (2) polar but uncharged, (3) polar positively charged, and (4) polar negatively charged. The classification of each of the amino acids within the four neoepitope peptide sequences are shown in table 4.1. This table clearly demonstrates that the neoepitope peptide $neoC_{37}$ and $neoC_{25}$ sequences contain more amino acids that are classified as polar and charged (either positively or negatively) than the other two peptides ($neoN_{37}$ and $neoN_{25}$). It is possible that charged amino acids are more antigenic than uncharged and nonpolar amino acids.

Within each antiserum it is possible that there are antibodies present, that are specific for the immunising peptide, the carrier protein thyroglobulin and the peptide-carrier conjugate. Therefore subsequent experimental procedures were needed to control for the possible binding of antibodies to these latter two groups of molecules. Results of the antisera screening are only shown for antiserum samples collected after the second round of immunisation and for those rabbits shown to produce the highest titres of anti-peptide antibodies, as these samples were found to produce the strongest, clearest results. The average affinity and specificity of antibodies raised to immunising antigen has been shown to increase with repeated immunisations (Klinman and Press 1975). However, antisera taken after the third and fourth rounds of immunisation were found to result in higher levels of non-specific binding, which obscured the anti-peptide (neoC₃₇, neoN₃₇, neoC₂₅ and neoN₂₅) responses. Therefore antisera taken after the second round of immunisation were used in all subsequent experiments.

4.4.2 Antisera Raised to Neoepitope Peptides $NeoC_{25}$ and $NeoN_{37}$ Appear to Recognise CD23 Antisera raised to the neoepitope peptides $neoN_{37}$ and $neoC_{25}$ appear to recognise the 37kDa sCD23 and the remaining CD23 stump after cleavage of the 25kDa sCD23 fragment

	Neoepitope Peptide Sequence			
Amino Acid Classification	NeoC ₃₇	NeoN ₃₇	NeoC ₂₅	NeoN ₂₅
	Glu – P ^{-ve}	Gln – P	Leu – N	Met – N
	Ser – P	Lys – P ^{+ ve}	$Arg - P^{+ve}$	$Glu - P^{+ve}$
	$His - P^{+ve}$	Ser – P	$Glu - P^{+ve}$	Leu – N
	$His - P^{+ve}$	Gln – P	$Glu - P^{+ve}$	Gln – P
	Gly – P	Ser – P	Val – N	Val – N
	Asp – P ^{-ve}	Thr – P	Thr – P	Ser – P
	Gln – P	Gln – P	Lys – P ^{+ ve}	Ser – P
	Met – N	Ile – N	Leu – N	Gly – P
	Ala – N	Ser – P	$Arg - P^{+ve}$	Phe – N

Table 4.1 Neoepitope Peptide Amino Acid Sequences and Classification. Amino Acidsrepresented in three letter codes. Classification:- N – Nonpolar, P – Polar uncharged, P^{+ve} –Polar positively charged, P^{-ve} – Polar negatively charged.

respectively. Antiserum raised to neoepitope peptide neoN₃₇ repeatedly bound a 37kDa band detected by Immunoblotting, from the supernatant of CD23 positive cells (RPMI 8866 cells and MEL cells transfected with mCD23) that was not seen in samples extracted from CD23 negative cells (untransfected MEL cells). This band was also detected in samples purified using TALON[™] metal affinity resin, indicating that this fragment contained the whole Cterminal tail (including the 6xHis tag) and the lectin domain. While antiserum raised to the neoepitope peptide neoC₂₅ bound to a protein present on the surface of RPMI 8866 cells detected by flow cytometry, and to a 15kDa band purified from the membrane of MEL cells transfected with mCD23, but not untransfected MEL cells by Immunoblotting. Neither antisera appeared to recognise full-length recombinant mCD23 and are therefore thought to be specific for the neoepitopes generated after cleavage. Antibodies produced to the neoepitope peptide neoN₃₇ appear to be of a lower titre than those produced to neoC₃₇ or neoC₂₅ (according to ELISA results figure 4.4a and b). However, the higher concentration of this cleaved molecule in the supernatant may account for the specific binding of this antiserum as detected by Western blotting and immunodetection. Characterisation of the antiserum raised to the neoepitope peptide $neoC_{25}$, first required the concentration of material containing the neoC₂₅ epitope.

4.4.3 Characterisation of Antisera Raised to the Neoepitope Peptides NeoC₃₇ and NeoN₂₅ Flow cytometry experiments using anti-neoC₃₇ antiserum indicated that antibodies contained within this serum sample recognised a specific epitope present on the surface of RPMI 8866 cells that was not detected by the pre-immune serum. The mean fluorescence intensity value for anti-neoC₃₇ antiserum staining was consistently higher than that of pre-immune serum controls for repeat experiments, suggesting that this result was significant. The low mean fluorescence intensity result seen when using anti-neoC₃₇ antiserum indicates low level detection of this epitope. This result contrasts with those predicted, based on those previously reported showing a higher proportion of 37kDa sCD23 fragments than 25kDa sCD23 levels detected in the supernatant (Marolowski *et al.*, 1998). Hence the mean fluorescence intensity value gained using anti-neoC₃₇ antiserum was expected to be greater than that for anti-neoC₂₅ antiserum. Although this could be accounted for by the possible rapid internalisation and degradation of the stump after proteolytic shedding of the 37kDa sCD23. Unfortunately this

was not seen in permeabilisation experiments, where MEL cells transfected with mCD23 were permeabilised using saponin to allow detection of internalised fragments with the anti-neo C_{37} antiserum (viewed by confocal microscopy).

Despite positive ELISA results, antibodies created to neoepitope peptide neoN₂₅ do not appear to recognise the 25kDa soluble CD23 molecule cleaved from mCD23 transfected MEL cells. It is possible that the antibody binding affinity for this peptide (conjugated to ovalbumin) measured by ELISA, results from recognition of the linker MBS, or alternatively the C-terminus of the 25kDa sCD23 molecule may be particularly susceptible to proteolytic degradation upon cleavage.

4.4.4 Summary

Difficulties in characterising the properties of the neoepitope antibodies generated to $neoC_{37}$, $neoN_{37}$, $neoC_{25}$ and $neoN_{25}$ peptides, were believed to be largely due to the low concentration of antigen containing these newly created epitopes. However, despite the problems encountered, it appears that three of the antisera contain anti-neoepitope antibodies. Antisera raised to the neoepitope peptides $neoC_{37}$, $neoN_{37}$ and $neoC_{25}$ all recognise an epitope expressed by CD23 positive cells that is not detected by pre-immune serum samples or present on CD23 negative untransfected MEL cells. The finding that the anti-neoC₂₅ antiserum binds to RPMI 8866 cells as seen in flow cytometry experiments and that this antiserum detects a band isolated from MEL cell membranes transfected with mCD23, indicates that cleavage of the 25kDa sCD23 molecule is independent of that of the 37kDa molecule. This is particularly important as the production of these smaller fragments were previously thought to be dependent upon prior cleavage of the larger 37kDa sCD23 fragment. Identification of the epitopes recognised by each antiserum and determination of the specificity for CD23 is discussed in greater detail in Chapter 5.

5.1 Introduction

The experiments discussed in chapter 4, illustrated that the anti-neoepitope antisera generated to peptides $neoC_{37}$, $neoN_{37}$ and $neoC_{25}$, all recognised epitopes that were expressed on RPMI 8866 cells and MEL cells transfected with mCD23, but were not present on untransfected MEL cells. However, these experiments alone to do not prove that the epitopes recognised by the anti-neoepitope antisera are located within CD23. Therefore, before the anti-neoepitope antisera could be used to map the proteolytic cleavage events within CD23, their specificity had to be confirmed. N-terminal amino acid sequencing of immunoreactive bands on Immunoblots would have shown the epitope specificity of the antisera. Unfortunately, this strategy could not be used due to insufficient amounts of material. Concentration and purification of the material recognised by the anti-neoepitope antisera by affinity chromatography was attempted but was unsuccessful due to the poor immunoprecipitation properties of the antisera. For these reasons, alternative strategies to determine the specificity of the anti-neoepitope antisera were employed.

5.2 Determination of Anti-Neoepitope Antisera Specificity

Two methods were employed to determine the specificity of the anti-neoepitope antisera. Firstly soluble CD23 fragments were purified by affinity chromatography using a monoclonal antibody specific for the lectin domain of CD23 (MHM6). These purified fragments were then used in SDS-PAGE gel electrophoresis and immunoblotting experiments to screen anti-neoepitope antibodies, to show specificity for cleaved sCD23. However, this strategy was unsuitable for the purification of proteolytically cleaved CD23 fragments that do not contain the lectin domain (e.g. the CD23 stump left after cleavage of the 37 and 25kDa soluble CD23 molecules).

Instead a second strategy was developed to determine the specificity of the anti-neo C_{37} and anti-neo C_{25} antisa (the antisera raised to the new neoepitopes which were retained at the cell surface after cleavage). Anti-neoepitope antisera were incubated with increasing concentrations of the immunising peptide to which they were raised. The antiserum-peptide mixtures were then used to screen CD23 expressing cells for neoepitope expression by flow cytometry and immunoblotting. Inhibition of anti-neoepitope binding would indicate the specificity of the anti-neoepitope antisera for the immunising peptide and the corresponding epitope found in cleaved CD23 fragments.

5.3 Results

5.3.1 Immunoprecipitation of Soluble CD23 Fragments Followed by Detection with Anti-NeoN₃₇ Antiserum

Supernatants from MEL cells transfected with mCD23 (containing shed recombinant sCD23 fragments), were immunoprecipitated with monoclonal antibody MHM6. Bound protein was eluted using 0.1M glycine HCl pH 3.0. The eluted material was then separated by SDS-PAGE gel electrophoresis under reducing conditions, transferred to Immobilon-P membrane and immunoblotted with either anti-neoN₃₇ antiserum or the anti-CD23 polyclonal antibody. The procedure was repeated using supernatant collected from untransfected MEL cells to control for any cross-reactivity of the antibody with a protein other than CD23.

The results of this experiment are shown in figure 5.1. This figure shows that the anti-neoN₃₇ antiserum recognises a single major band of approximately 37kDa, immunoprecipitated from mCD23 transfected MEL cell supernatant samples (figure 5.1, lane 6). This fragment is not present in the immunoprecipitated sample extracted from untransfected MEL cell supernatants (figure 5.1, lane 5). This band corresponds in size to that detected using anti-CD23 polyclonal antibody (figure 5.1, lane 2, shown for comparison). In figure 5.1, lanes 1-4 supernatant samples that have not been immunprecipitated. MEL cell supernatants (from both untransfected MEL cells and MEL cells transfected with mCD23) were then immunoprecipitated with an unrelated antibody of the same isotype. The results of



Figure 5.1 Characterisation of Anti-NeoN₃₇ Antiserum using Immunoprecipitated Soluble **CD23.** Membrane CD23 transfected MEL cells and untransfected controls were grown in log phase for four days then induced with 2% DMSO. Four days after the induction of protein expression, samples were centrifuged and the supernatant aspirated. Supernatants were then immunoprecipitated (IP) using a monoclonal anti-CD23 antibody MHM6 (a gift from Dr. J. Gordon, University of Birmingham, Birmingham, UK) as described in section 2.8.14. Immunoprecipitated eluents and untreated supernatant controls were then separated by 12% SDS-PAGE gel electrophoresis (under denaturing conditions), transferred to Immobilon-P membrane and used to screen anti-neoN₃₇ antiserum. The above figure shows supernatant samples from untransfected MEL cells (lanes 1 & 3), mCD23 transfected MEL cells (lanes 2 & 4), untransfected MEL cells immunoprecipitated with MHM6 (lanes 5), mCD23 transfected MEL cells immunoprecipitated with MHM6 (lane 6), untransfected MEL cells immunoprecipitated with mouse anti-HN3 (an antibody specific for the β -subunit of the 20S proteasome, a gift from Dr Klaus Hendil, Openhagen University, Denmark) (lanes 7) and mCD23 transfected MEL cells immunoprecipitated with anti-HN3 (lane 8). Immunblots were probed with either anti-CD23 polyclonal antibody (PAb) 10µg/ml (lanes 1&2), or anti-neoN₃₇ antiserum diluted 1/500 (lanes 3-8). Protein band sizes were determined by comparison to Rainbow[™] protein molecular weight markers (Amersham Life Science). Supernatant samples in lanes 1-4 have not been immunoprecipitated.

this experiment were negative (figure 5.1, lanes 7 and 8). This experiment illustrates that the antisera raised to the neoepitope peptide neoN₃₇, recognises an epitope that is present on a 37kDa protein, that can be purified using a CD23 lectin head specific antibody. The size of the protein detected using anti-neoN₃₇ antiserum is consistent with that detected by the anti-CD23 polyclonal antibody. This 37kDa band is not detected in supernatants from cells that do not express CD23. Together this evidence supports the suggestion that the anti-neoN₃₇ antiserum is specific for the amino neoepitope generated by cleavage of the 37kDa sCD23 fragment from the cell surface.

5.3.2 Competitive Inhibition of the Binding of the Anti-Neoepitope Antiserum to the NeoC₃₇ and NeoC₂₅ Epitopes (Assayed by Flow Cytometry)

5.3.2.1 Competitive Inhibition with Immunising Peptides

Peptide blocking experiments were performed to confirm the specificity of anti-neoepitope antisera raised against peptides neoC₃₇ and neoC₂₅. Anti-neoepitope serum was incubated overnight at 4°C, in the presence of the peptide used for immunisation. The antiserum/peptide mixtures were then used to assay CD23 neoepitope expression on RPMI 8866 cells by flow cytometry. The results of this experiment are shown in figure 5.2. The flow cytometer was set up using unstained cells (panel 1) and the secondary FITC labelled antibody (panel 2). As a positive control, BU38 a CD23 lectin domain binding antibody was used to establish that the cells were expressing CD23 (panel 3). When the cells were stained with the anti-neo C_{37} antiserum, there was a shift in the mean fluorescence intensity (MFI) from 2.97, obtained with the secondary antibody alone, to 10.38 (panel 4). Since the pre-immune serum and secondary antibody together stained the cells with a MFI of 3.98, this data suggests that the anti-neo C_{37} antiserum specifically recognises an epitope expressed by RPMI 8866 cells. To demonstrate whether the epitope recognised by anti-neoC₃₇ antiserum was the same as that represented by the peptide neoC₃₇, the immunising peptide neoC₃₇ was used to inhibit the interaction between the anti-neoepitope antibody and the epitope present on RPMI 8866 cells. Figure 5.2, panel 5 shows that $10\mu gml^{-1}$ of peptide neoC₃₇ reduced the MFI of cells stained with anti-neoC₃₇ antiserum by over 50%. However, the inhibition was not specific to peptide neo C_{37} , as a second unrelated peptide neoC₂₅, also inhibited the reaction of the anti-neoepitope antibody $neoC_{37}$ with the cells by an equivalent amount (panel 6). This result suggests that although



Figure 5.2 Competitive Inhibition of Antiserum Reactivity to RPMI 8866 Cells by Immunising Peptides, Assayed by Flow Cytometry. $1x10^6$ RPMI 8866 cells were incubated for 60 minutes in the presence of anti-neoC₃₇ or anti-neoC₂₅ antiserum, or pre-immune serum (all diluted 1/500) with or without immunising peptides neoC₃₇ or neoC₂₅. Binding of antisera was detected using secondary antibody anti-rabbit Ig FITC (Sigma) and assayed by flow cytometry. **MFI=**Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) Control; no antibodies, (2) secondary antibody Ig FITC only, (3) BU38-FITC (1/100), anti-CD23 monoclonal antibody (The Binding Site), (4) anti-neoC₃₇ antiserum, (5) anti-neoC₃₇ antiserum; pre-incubated with neoC₃₇ peptide (10µg/ml), (6) anti-neoC₃₇ antiserum pre-incubated with the unrelated peptide neoC₂₅ (10µg/ml), (7) anti-neoC₂₅ antiserum, (8) anti-neoC₂₅ antiserum pre-incubated with the unrelated peptide neoC₃₇ (10µg/ml), (9) pre-immune serum. Antiserum/peptide solutions were incubated overnight at 4°C before use in flow cytometry experiments.

the anti-neoC₃₇ antiserum recognises an epitope on RPMI 8866 cells, the epitope recognised may not be the neoC₃₇ terminus of CD23. Similar experiments with anti-neoC₂₅ antiserum showed that although the anti-neoC₂₅ antibody bound to the cells with a MFI of 25.11, significantly greater than that of the pre-immune serum, this interaction was not inhibited by the peptide neoC₂₅. Again this result suggests also that the anti-neoepitope antibody neoC₂₅ does not bind an epitope at the neoC₂₅ terminus of CD23.

There are several reasons which could account why the immunising peptides were unable to specifically inhibit neoepitope antisera binding to RPMI 8866 cells. (1) The antibodies present in the anti-neoepitope antisera are directed against either the carrier protein thyroglobulin or the peptide-carrier conjugate. (2) Anti-neoepitope antisera cross-react with, or recognise a protein unrelated to CD23 that is expressed on the surface of RPMI 8866 cells. (3) The concentration of the competing neoC₂₅ peptide used in these experiments was too low such that the binding of the anti-neoepitope antiserum was not visibly affected. (4) Anti-neoepitope antisera may be recognising the full-length native CD23 molecule. However, as the MFI of the positive control (figure 5.2, panel 3) is greater than that for either the anti-neoC₃₇ (panel 4) or the anti-neoC₂₅ antiserum (panel 7), suggests that this is not the case.

5.3.2.2 Competitive Inhibition with Thyroglobulin and Immunising Peptide-Thyroglobulin Conjugates

In order to assess whether the cross reactivity observed was due to a cross-reaction of an antibody specific for either the carrier protein thyroglobulin or the peptide-carrier protein conjugate, thyroglobulin and peptide-thyroglobulin conjugates were tested as blocking reagents. Neoepitope peptides, $neoC_{37}$ and $neoC_{25}$ conjugated to thyroglobulin and thyroglobulin alone were incubated with the anti- $neoC_{37}$ and $anti-neoC_{25}$ antiserum overnight at 4°C. The antiserum/peptide conjugates or thyroglobulin mixtures were then used to assay CD23 expression on RPMI 8866 cells by flow cytometry. The results of these experiments are shown in figures 5.3.1 and 5.3.2. Again, the flow cytometer was set up using unstained cells (figure 5.3.1 and 5.3.2, panel 1) and the secondary FITC labelled antibody (figures 5.3.1 and 5.3.2, panel 2). The cells are shown to be positive for CD23 by the staining of a CD23 lectin head antibody BU38 (figures 5.3.1 and 5.3.2, panel 3). When the cells were stained with the



Figure 5.3.1 Competitive Inhibition of Antiserum Reactivity to RPMI 8866 Cells by Immunising Peptide-Thyroglobulin Conjugates, Assayed by Flow Cytometry. 1×10^6 RPMI 8866 cells were incubated for 60 minutes in the presence of anti-neoC₃₇ or anti-neoC₂₅ antiserum (all diluted 1/500) with or without peptide-thyroglobulin conjugates. Binding of antisera was detected using secondary antibody anti-rabbit Ig FITC (Sigma) and assayed by flow cytometry. **MFI=**Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) Control; no antibodies, (2) secondary antibody Ig FITC only, (3) BU38-FITC (1/100), anti-CD23 monoclonal antibody (The Binding Site), (4) anti-neoC₃₇ antiserum, (5) anti-neoC₃₇ antiserum; pre-incubated with neoC₃₇-thyroglobulin (100µg/ml), (6) anti-neoC₃₇ antiserum pre-incubated with neoC₂₅thyroglobulin (100µg/ml), (7) anti-neoC₂₅ antiserum, (8) anti-neoC₂₅ antiserum pre-incubated with neoC₂₅-thyroglobulin (100µg/ml), (9) anti-neoC₂₅ antiserum pre-incubated with neoC₃₇thyroglobulin (100µg/ml). Antiserum/peptide solutions were incubated overnight at 4°C before use in flow cytometry experiments.



Figure 5.3.2 Competitive Inhibition of Antiserum Reactivity to RPMI 8866 Cells by Thyroglobulin, Assayed by Flow Cytometry. 1×10^6 RPMI 8866 cells were incubated for 60 minutes in the presence of anti-neoC₃₇ or anti-neoC₂₅ antiserum (all diluted 1/500) with or without thyroglobulin. Binding of antisera was detected using secondary antibody anti-rabbit Ig FITC (Sigma) and assayed by flow cytometry. **MFI=**Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) Control; no antibodies, (2) secondary antibody Ig FITC only, (3) BU38-FITC (1/100), anti-CD23 monoclonal antibody (The Binding Site), (4) anti-neoC₃₇ antiserum, (5) anti-neoC₃₇ antiserum; pre-incubated with thyroglobulin (10µg/ml), (6) antineoC₃₇ antiserum pre-incubated with thyroglobulin (10µg/ml), (7) anti-neoC₂₅ antiserum, (8) anti-neoC₂₅ antiserum pre-incubated with thyroglobulin (10µg/ml), (9) anti-neoC₂₅ antiserum preincubated with thyroglobulin (100µg/ml). Antiserum/peptide solutions were incubated overnight at 4°C before use in flow cytometry experiments.

anti-neoepitope antibody C_{37} a shift in the MFI of over 100% was detected (figures 5.3.1 and 5.3.2, panel 4). In order to determine whether antibodies present in the anti-neo C_{37} antiserum were specific for either the peptide-thyroglobulin conjugate or thyroglobulin alone, the preincubated mixtures were used to stain RPMI 8866 cells. Although, incubation of the antineoN₃₇ antiserum with either 100µgml⁻¹ peptide-thyroglobulin conjugate (figure 5.3.1 panels 5 and 6) or thyroglobulin alone (figure 5.3.2 panels 5 and 6) slightly reduced MFI, inhibition was not seen to the same extent of that seen with peptide alone (figure 5.2, panels 5 and 6).

These experiments were repeated using the anti-neoC₂₅ antiserum. The results of this experiment are shown in figures 5.3.1 and 5.3.2 (panels 7, 8 and 9). These results demonstrate that although the anti-neoC₂₅ antiserum clearly bound to the RPMI 8866 cells (Figures 5.3.1 and 5.3.2, panel 7), the binding of the anti-neoC₂₅ antiserum was not inhibited by the thyroglobulin-peptide conjugate (figure 5.3.1, panels 8 and 9) or the addition of thyroglobulin alone (figure 5.3.2, panels 7 and 8). Together these results suggest that neither anti-neoC₃₇ nor anti-neoC₂₅ antiserum bind to an epitope present on thyroglobulin, or an epitope generated by the conjugation of the peptide to thyroglobulin.

5.3.2.3 Titration of Anti-Neoepitope Antisera Concentrations in Competitive Inhibition Experiments

Finally to assess the possibility that the anti-neoepitope antisera concentrations exceeded those of the peptide-carrier conjugate or thyroglobulin inhibition, the above experiments were repeated with reduced concentrations of the anti-neoepitope antisera (diluted 1/1000 and 1/2000, figure 5.4.1 and 5.4.2). Serial dilution experiments described in section 4.3.5, had previously shown that below this concentration antiserum-antigen interactions on the surface of RPMI 8866 cells were not detected by flow cytometry (especially those of anti-neoC₃₇ antiserum).

The flow cytometer was set up using unstained cells and the secondary FITC labelled antibody (figures 5.4.1 and 5.4.2, panel 1). When the cells were stained with the anti-neoepitope antibody C_{37} there was a shift in the MFI by over 100% (figure 5.4.1, panels 4 and 7). In order



Figure 5.4.1 Titration of Anti-NeoC₃₇ Antiserum in Competitive Inhibition Experiments, Assayed by Flow Cytometry. 1×10^6 RPMI 8866 cells were incubated for 60 minutes in the presence of anti-neoC₃₇ antiserum, with or without immunising peptides neoC₃₇ or neoC₂₅ conjugated to thyroglobulin. Binding of antisera was detected using secondary antibody antirabbit Ig FITC (Sigma) and assayed by flow cytometry. **MFI=**Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) secondary antibody Ig FITC only, (2) antineoC₃₇ antiserum (1/2000) + thyroglobulin (1mg/ml), (3) anti-neoC₃₇ antiserum (1/2000) + neoC₂₅-thyroglobulin (100µg/ml), (4) anti-neoC₃₇ antiserum (1/1000) (5) anti-neoC₃₇ antiserum (1/1000) + neoC₃₇-thyroglobulin (10µg/ml), (6) anti-neoC₃₇ antiserum (1/1000) + neoC₃₇thyroglobulin (100µg/ml), (7) anti-neoC₃₇ antiserum (1/2000), (8) anti-neoC₃₇ antiserum (1/2000) + neoC₃₇-thyroglobulin (10µg/ml), (9) anti-neoC₃₇ antiserum (1/2000) + neoC₃₇-thyroglobulin (100µg/ml). Antiserum/peptide/thyroglobulin solutions were incubated overnight at 4°C before use in flow cytometry experiments.



Figure 5.4.2 Titration of Anti-NeoC₂₅ Antiserum in Competitive Inhibition Experiments, Assayed by Flow Cytometry. 1×10^6 RPMI 8866 cells were incubated for 60 minutes in the presence of anti-neoC₂₅ antiserum, or pre-immune serum with or without immunising peptides neoC₃₇ or neoC₂₅ conjugated to thyroglobulin. Binding of antisera was detected using secondary antibody anti-rabbit Ig FITC (Sigma) and assayed by flow cytometry. **MFI=**Mean Fluorescence Intensity, calculated for the area defined in region M1. (1) secondary antibody Ig FITC only, (2) anti-neoC₂₅ antiserum (1/2000) + thyroglobulin (1mg/ml), (3) anti-neoC₂₅ antiserum (1/2000) + neoC₃₇-thyroglobulin (100µg/ml), (4) anti-neoC₂₅ antiserum (1/1000) (5) anti-neoC₂₅ antiserum (1/1000) + neoC₂₅-thyroglobulin (10µg/ml), (6) anti-neoC₂₅ antiserum (1/1000) + neoC₂₅thyroglobulin (100µg/ml), (7) anti-neoC₂₅ antiserum (1/2000), (8) anti-neoC₂₅ antiserum (1/2000) + neoC₂₅-thyroglobulin (10µg/ml), (9) anti-neoC₂₅ antiserum (1/2000) + neoC₂₅-thyroglobulin (100µg/ml). Antiserum/peptide/thyroglobulin solutions were incubated overnight at 4°C before use in flow cytometry experiments.

to determine whether the anti-neoC₃₇ antibody was recognising an epitope present in either the peptide-thyroglobulin conjugate or thyroglobulin alone, the pre-incubated mixtures were used to stain RPMI 8866 cells. Thyroglobulin (panel 2) was unable to inhibit anti-neoC₃₇ antibody binding, showing a comparable MFI of 7.92 compared with that of anti-neoC₃₇ antiserum alone (MFI=6.44, panel 7). Again no effect was seen when the anti-neoepitope antiserum anti-neoC₃₇ was incubated in the presence of either 10μ gml⁻¹ (panels 5 and 8) or 100μ gml⁻¹ peptide-thyroglobulin conjugate (panels 6 and 9). Titration of the anti-neoC₃₇ antiserum did not enable either the peptide-thyroglobulin conjugate or thyroglobulin alone inhibit antibody binding.

These experiments were repeated with anti-neo C_{25} antiserum shown in figure 5.4.2. The panels in this figure are arrange similarly to those in figure 5.4.1, except that anti-neo C_{25} antiserum was used as the detection antibody and peptide neo C_{25} -thyroglobulin conjugate was used to inhibit antibody binding. Titration of the anti-neo C_{25} antiserum concentration did not enable either the peptide-thyroglobulin conjugate or thyroglobulin alone to inhibit antibody binding. Taken together the experiments described in section 5.3.2 suggest that the antisera raised against neo C_{37} and neo C_{25} peptide conjugates appear not to recognise cleaved CD23 neoepitopes on the surface of RPMI 8866 cells. However, nor do they appear to recognise epitopes present in thyroglobulin or formed by the peptide-carrier conjugate.

5.3.3 Competitive Inhibition of Anti-Neoepitope Antiserum (Assayed by Immunoblotting) Despite the problems encountered with competitive inhibition experiments assayed by flow cytometry, the specificity of the anti-neo N_{37} and anti-neo C_{25} antisera were determined by peptide competition experiments screened by immunoblotting. Anti-neoepitope antisera were incubated overnight at 4°C, in the presence of corresponding immunising peptides over a range of concentrations. The antiserum/peptide mixtures were then used to detect CD23 cleavage fragments from MEL cell lysates transfected with mCD23 and corresponding media/supernatants extracts. MEL cell lysates and supernatants from untransfected MEL cells were also included to exclude the possibility of any cross-reactivity of a native protein expressed by MEL cells with either the anti-neo N_{37} or the anti-neo C_{25} antiserum.

The results from these experiments are shown in figures 5.5 and 5.6. Anti-neoN₃₇ antiserum recognises a single major immunoreactive band of approximately 37kDa in the media/supernatant extracted from MEL cells transfected with mCD23 (figure 5.5 A and B lane 2). This band is not seen in the media/supernatant extracted from untransfected MEL cells and is believed to represent the 37kD sCD23 fragment. Incubation of the anti-neoN₃₇ antiserum with increasing concentrations of the peptide neoN₃₇, increasingly inhibits binding of the neoepitope antiserum to the 37kDa fragment from the media/supernatant of MEL cells transfected with mCD23 (figure 5.5 A lanes 6, 8 and 10). Inhibition was detected at peptide concentrations as low as 1µg/ml (figure 5.5B lane 6), and complete inhibition is seen at 40µg/ml (figure 5.5A lane 10). Immunoreactivity was not inhibited by the unrelated neoN₂₅ peptide (figure 5.5A lane 4). These results support those described in section 5.3.1 and demonstrate that the anti-neoN₃₇ antiserum recognises the neoN₃₇ epitope.

Anti-neoC₂₅ antiserum recognises a single major immunoreactive band of approximately 15kDa in the lysates of mCD23 transfected MEL cells (figure 5.6 A and B lane 2). This band is not seen in untransfected MEL cell lysates (figure 5.6 A and B lane 1) and is believed to represent mCD23 cleaved at the 25kDa sCD23 cleavage site (figure 4.9). Incubation of the anti-neoC₂₅ antiserum with increasing concentrations of the peptide neoC₂₅, increasingly inhibits binding of the neoepitope antiserum to the 15kDa fragment from the mCD23 transfected MEL cell lysates (figure 5.6 A lanes 4, 6, 8 and 10). Again inhibition was detected at peptide concentrations as low as 1µg/ml (figure 5.6B lane 6), and almost complete inhibition is seen at 20µg/ml (figure 5.6A lane 8). Immunoreactivity was not inhibited by the unrelated neoC₃₇ peptide (figure 5.6B lane 8). This result shows that the anti-neoC₂₅ antiserum recognises the epitope neoC₂₅ in CD23 positive cell lysates (untransfected MEL cells). Unfortunately the specificity of the anti-neoC₃₇ antiserum could not be assessed by this method, as this antiserum does not detect cleaved CD23 by Immunoblotting.



Figure 5.5 Competitive Inhibition of Anti-NeoN₃₇ Antiserum, Assayed by Immunoblotting. Membrane CD23 transfected MEL cells and untransfected controls were grown in log phase for 4 days then induced with 2% DMSO. Four days after the induction of protein expression, samples were centrifuged and the supernatant collected. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P Supernatant from induced untransfected MEL cells lanes 1, 3, 5, 7 and 9. membrane. Supernatants from induced MEL cells transfected with mCD23 lanes 2, 4, 6, 8 and 10. Protein expression was analysed by immunoblotting using the following antiserum/peptide solutions: (A) Lanes 1-2) anti-neoN₃₇ antiserum, 3-4) anti-neoN₃₇ antiserum/peptide neoN₂₅ (20µg/ml), 5-6) anti-neoN₃₇ antiserum/peptide neoN₃₇ (10µg/ml), 7-8) anti-neoN₃₇ antiserum/peptide neoN₃₇ $(20\mu g/ml)$, 9-10) anti-neoN₃₇ antiserum/peptide neoN₃₇ (40 $\mu g/ml$). (B) Lanes 1-2) anti-neoN₃₇ antiserum, 3-4) anti-neoN₃₇ antiserum/peptide neoN₃₇ (0.5µg/ml), 5-6) anti-neoN₃₇ antiserum/peptide neoN₃₇ (1µg/ml), 7-8) anti-neoN₃₇ antiserum/peptide neoN₃₇ (5µg/ml), 9-10) anti-neoN₃₇ antiserum/peptide neoN₃₇ (10µg/ml). Anti-neoN₃₇ antiserum diluted 1/500. Antiserum/peptide solutions were incubated at 4°C overnight before use in immunoblotting experiments. Protein band sizes were determined by comparison to Rainbow™ protein molecular weight markers (Amersham Life Science).



Figure 5.6 Competitive Inhibition of Anti-NeoC₂₅ Antiserum, Assayed by Immunoblotting. Membrane proteins extracted from induced MEL cells transfected with mCD23 and untransfected controls (method 2.8.9) were separated by denaturing 12% SDS-PAGE gel electrophoresis then transferred to Immobilon-P membrane. Membrane proteins from induced untransfected MEL cells lanes 1, 3, 5, 7 and 9 and mCD23 transfected MEL cells lanes 2, 4, 6, 8 and 10. Protein expression was analysed by immunoblotting using the following antiserum/peptide solutions, (A) Lanes 1-2) anti-neoC₂₅ antiserum, 3-4) anti-neoC₂₅ antiserum/peptide neoC₂₅ (5μ g/ml), 5-6) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml), 7-8) anti-neoC₂₅ antiserum/peptide neoC₂₅ (20μ g/ml), 9-10) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml), 7-8) anti-neoC₂₅ (0.5μ g/ml), 5-6) anti-neoC₂₅ antiserum/peptide neoC₂₅ (1μ g/ml) 7-8) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml). (B) Lanes 1-2) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml). 7-8) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml). 7-8) anti-neoC₂₅ antiserum/peptide neoC₂₅ (10μ g/ml). Anti-neoC₂₅ antiserum diluted 1/500. Antiserum/peptide solutions were incubated at 4°C overnight before use in immunoblotting experiments. Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers, the 14.3kDa band can be seen between lanes A2-3, 4-5, 6-7 and 8-9 and B2-3, 4-5 and 6-7 (Amersham Life Science).

5.4 Discussion

5.4.1 Anti-NeoN₃₇ and –NeoC₂₅ Antisera are Specific for CD23

The experiments described above show that the anti-neoN₃₇ antiserum recognises a 37kDa protein, which shares homology with the lectin domain of CD23 (recognised by the monoclonal antibody MHM6). Furthermore binding of this antiserum to a 37kDa protein present in supernatants extracted from CD23 positive cells is inhibited by the immunising peptide neoN₃₇, to which the antisera was raised. Together these experiments provide strong evidence that the anti-neoN₃₇ antisera, specifically recognises the amino terminus of 37kDa sCD23. Similar experimental evidence supports the specificity of the anti-neoC₂₅ antiserum for the new mCD23 carboxy terminus, created after shedding of the 25kDa sCD23 fragment. This antiserum recognises a 15kDa fragment present in the cell lysates of CD23 positive cells and is inhibited by prior incubation of the anti-neoC₂₅ antiserum with the immunising peptide neoC₂₅.

Unfortunately the specificity of the anti-neoC₃₇ antiserum could not be determined.

5.4.2 Failure to Competitively Inhibit Binding of the Anti-Neoepitope Antisera in a Flow Cytometric Assay

The competitive inhibition of the anti-neoC₂₅ antiserum by the immunising peptide neoC₂₅ in immunoblotting contrasts with the results gained using flow cytometric analysis. The difference between these results is most likely to be accounted for by the different experimental conditions of the two techniques. Specificity and sensitivity of the techniques is reliant upon antibody-antigen interactions. Here the format of the antigen differs between the two systems. In flow cytometry experiments using viable cells, the antigen is present in a native form. In immunoblots, however, the SDS used in electrophoresis denatures the protein before immunoblotting. Also the mobility of the peptide is constrained on a solid support during immunoblotting. It is possible that the anti-neoC₂₅ antiserum may have a higher affinity for the native protein, and therefore dissociated from the immunising (linear) peptide and binds to the native antigen (neoC₂₅ epitope present in cleaved CD23 stumps) in flow cytometry experiments. During immunoblotting experiments the cleaved CD23 protein is denatured, therefore the immunising peptide is better able to compete for binding of the anti-

neoepitope antibody. The counter argument to this hypothesis is that anti-neoepitope antibodies were originally raised to linear neoepitope peptides, therefore they are more likely to be specific for the linear peptide. It is possible that in immunoblotting experiments the mobility of the epitope (cleaved CD23) was constrained by the solid support (the membrane), whereas in the flow cytometry experiments the epitope is not constrained, possibly reducing the ability of the neoepitope peptide to compete against the epitope for antisera binding.

5.4.3 Cleavage of 25kDa sCD23 is not Dependent on Prior Cleavage of 37kDa sCD23

Determination of the specificity of the $neoC_{25}$ antiserum by immunoblotting has important implications for the processing of CD23. The reactivity of this antiserum with the $neoC_{25}$ epitope from membrane extracts of mCD23 transfected MEL cells (shown in figure 5.6), implies that proteolytic shedding of the 25kDa soluble CD23 does not require prior cleavage of the 37kDa molecule. This supports findings by Moulder *et al.*, (1993), research by this group observed a 25kDa sCD23 fragment released from RPMI 8866 cells grown in serum free media in the absence of a 37kDa sCD23 fragment.

Although these results indicate that the protease responsible for cleavage of the 25kDa soluble CD23 molecule recognises and cleaves membrane CD23 from the cell surface, this may not be the sole means by which the 25kDa soluble CD23 product is produced. Letellier *et al.*, (1988) suggested that the 25kDa soluble CD23 fragment is formed as a result of proteolytic degradation of the 37kDa fragment. Their research showed that the molecular mass of sCD23 shifts from 27/25kDa to 37kDa when incubated with a cocktail of protease inhibitors (PMSF, benzamidine, idoacetamide, ϵ -amino caproic acid, leupeptin, pepstatin and soybean trypsin inhibitor).

5.4.4 Implications for the Biological Activity of Soluble CD23 Molecules

CD23 transgenic and knockout models in mice, both support a role for membrane CD23 in the inhibition of IgE production (Texido *et al.*, 1994, Lamers and Yu 1995, Haczu *et al.*, 1997, Payet *et al.*, 1999). Thus inhibition of CD23 cleavage may reduce IgE production due to the proposed negative feedback mechanism controlling IgE synthesis of full-length membrane

CD23 retained on the cell surface though IgE binding (Sherr et al., 1989, Bonnefoy et al., 1990).

The independent generation of the 37 and 25kDa soluble CD23 fragments, implies that more than one protease is responsible for CD23 cleavage and that inhibition of the metalloproteinase activity responsible for the cleavage of the 37kDa sCD23 molecule (Christie *et al.*, 1997) may not inhibit the generation of the 25kDa sCD23 fragment. The significance of this is that, inhibition of only one proteolytic event may not be enough to prevent the adverse effects of IgE production. Although IgE synthesis appears to be inhibited in the presence of metalloproteinase inhibitors, proteolytic cleavage of the 25kDa sCD23 molecule could effect this equilibrium between IgE synthesis and control.

In vitro studies support a direct role for sCD23 in the induction of IgE synthesis through an interaction with CD21 (Aubry *et al.*, 1992). Although 25kDa sCD23 is thought to have a reduced affinity for IgE (as compared to membrane CD23), recognition of CD21 is not impaired (Mossalayi *et al.*, 1992, Bonnefoy *et al.*, 1993). This raises the possibility that the 25kDa sCD23 could enhance IgE production directly through an interaction with CD21. Although all of these interactions are hypothetically possible and supported *in vitro*, there is no experimental evidence *in vivo* to support a role for sCD23 in the induction of IgE synthesis through CD21 ligation. Therefore one can envisage situations where the different sCD23 fragments behold individual functional properties and are produced in response to different environmental conditions and stimuli.

Chapter 6: Proteolytic Processing of CD23

6.1 Introduction

At the start of this research project little was known about the protease(s) responsible for cleaving CD23 or the molecular mechanisms of its activity. Early studies by Letellier *et al.*, (1990) indicated that CD23 shedding was possibly due to autoproteolytic activity of the membrane CD23 protein itself. However, similarities between the membrane processing of CD23 and the proteolytic release of a diverse range of other membrane proteins were soon observed. The proteolytic shedding mechanism of these membrane proteins is now recognised as an important regulation mechanism in cell-cell interactions and cell function. The proteolytic shedding of membrane proteins can convert membrane receptors into competitive soluble receptors for their own ligands and result in the loss of membrane protein interactions (reviewed in Ehlers and Riordan 1991). Examples of proteins shed in this manner include the angiotension converting enzyme (ACE), β -amyloid precursor protein (APP), L-selectin, interleukin-6 receptor (IL-6R), transforming growth factor (TGF)- α , Kit ligand (KL), tumour necrosis factor (TNF)- α and the TNF receptors 1 and 2.

Der p1 is the group 1 allergen of the house dust mite *Dermatophagoides pteronyssinus*. This allergen has been shown to cleave CD23 from EBV-transformed B cells (Hewitt *et al.*, 1995, Schulz *et al.*, 1995). More recently Der p1 has been shown to cleave CD23 at two locations between Serine residues 155 and 156 and Glutamic acid 298 and Serine 299, to produce a 17kDa soluble fragment containing the lectin domain and part of the C-terminal tail (Schulz *et al.*, 1997). These findings have led to the speculation that the high IgE levels found in patients allergic to Der p1, may be due to the disruption of the regulatory mechanism of CD23 on IgE synthesis, as a direct result of CD23 shedding induced by the proteolytic activity of Der p1. Indeed, Schulz *et al.*, (1997) observed a time dependent decrease in IgE binding by RPMI 8866 B cells treated with Der p1, that correlated with the cleavage of CD23. This supported suggestions that the loss of CD23 from the cell surface may disrupt the negative feedback regulation of IgE synthesis thought to be delivered by the binding of IgE to membrane CD23. The release of a
sCD23 molecule by Der p1 also has the potential to promote IgE synthesis through an interaction between sCD23 and CD21 (as demonstrated in principle by Aubry *et al.*, 1992 *in vitro*). However, as yet the effects of the exogenous protease activity of Der p1 has not been characterised with respect to endogenous proteolytic activity. The aim of this chapter is to characterise the endogenous proteolytic mechanistic class(es) responsible for cleavage of CD23 and to assay these proteolytic events in relation to the activity of an exogenous protease Der p1.

6.1.1 Membrane Protein Secretases

An increasing number of serum/plasma proteins are now recognised as soluble derivatives of their membrane counterparts. Proteins shown to be released in this fashion include cytokine receptors, membrane bound growth factors, cell adhesion molecules, ectoenzymes (Massagué 1990, Ehlers and Riordan 1991, Bazil 1995 and Hopper *et al.*, 1997), and many others which remain to be identified in terms of sequence or function (Arribas and Massagué 1995 and Arribas *et al.*, 1996). The proteases responsible for the proteolytic cleavage of these membrane anchored proteins have been termed secretases, sheddases or convertases, and the mechanism by which these proteins are released from the cell surface has been called shedding.

Detailed studies investigating the mechanisms of proteolytic shedding have shown that a wide diversity of cleavage site sequences, exist within this group of proteins. This led to initial suggestions that a diverse group of unrelated proteases were responsible for cleaving each of the molecules. Despite this, there appears to be an array of common factors that indicate a conserved underlying mechanism may be responsible for the proteolytic shedding of all these proteins. Firstly, shedding appears to occur at or near to the cell surface within a stalk region that is accessible to proteases (Bosenberg *et al.*, 1993). Secondly, many proteolytic shedding events are activated by protein kinase C activators such as phorbol 12-myrisate 13-acetate (PMA) (reviewed in Arribas and Massagué 1995 and Bazil 1995). Thirdly, although there appears to be a weak preference for lysine or arginine residues at the site of cleavage, many secretases have been shown to tolerate broad amino acid substitutions within the cleavage site (reviewed in Hopper *et al.*, 1997). Finally, early reports suggested that proteolytic shedding events were sensitive to serine and metalloproteinase inhibitors, leading to suggestions that there may be two families of secretases with different mechanisms of activity, or alternatively a cascade of proteolytic

enzymes involved (Robache-Gallea *et al.*, 1995). However, proteolytic cleavage of all identified membrane proteins from the cell surface, has now been shown to be completely blocked by hydroxamic-acid metalloproteinase inhibitors. Initial sensitivities of these cleavage events to serine protease inhibitors are suggested to be due to their effects on the trafficking of the substrate proteins to the site of secretase action (transport of newly synthesised proteins to the membrane), rather than inhibition of proteolytic cleavage (Arribas *et al.*, 1996). Alternatively i is possible that these inhibitors prevent the activation of metalloproteases.

Given the diversity of amino acid sequences cleaved, and the findings that many amino acids can be tolerated within the cleavage sites, most attention has been directed towards identifying othe factors that govern these cleavage events. The results of these experiments have led to speculation that topological requirements, such as cleavage within an open stalk, with either minimum distance from the transmembrane domain or an extracellular proximal domain, may direct cleavage at a particular site (Brakebusch *et al.*, 1994, Migaki *et al.*, 1995, Chen *et al.* 1995, Arribas *et al.*, 1996). A second governing factor is the possible location of the proteas and the substrate within the membrane. The identification of TNF- α converting enzyme (TACE (Black *et al.*, 1997, Moss *et al.*, 1997) and the localisation of other secretase enzymes a transmembrane proteins (the APP α -secretase; Roberts *et al.*, 1994, ACE secretase; Oppong an Hopper 1993), has led to speculation that processing is facilitated as well as controlled by th relative spatial location of the protein substrate and the protease within the membrane.

TACE was purified from the cell membrane of a lipopolysaccharide (LPS) stimulated huma monocytic cell line, THP-1. The amino acid sequence was deduced from peptide fragments an by PCR analysis of a THP-1 cDNA cell library. Sequence analysis indicated that TACE is member of the adamalysin family of zinc binding metalloproteinases, called the ADAMs (disintegrin and metalloproteinase domain), and showed that the primary sequence had 299 homology with the bovine mammalian disintegrin-metalloproteinase (MADM/ADAM 10) (Blacl *et al.*, 1997 and Moss *et al.*, 1997). Amino acid sequence analysis indicates that TACE (also called ADAM 17) contains 8 distinct domains common to members of ADAM family proteins (1) a signal sequence, (2) a pro-domain with a cysteine switch at residue 184, (3) a catalytic domain (metalloprotease) preceded by a potential furin cleavage site (residues 211-214), (4) a

disintegrin domain, (5) an epidermal growth factor (EGF) like domain, (6) a crambin like domain followed by the (7) transmembrane and (8) cytoplasmic domains (Black *et al.*, 1997).

ADAMs are all type I transmembrane proteins (except ADAMTS-1 a cellular disintegrin and metalloproteinase with a thrombospondin type-1 motif, Kuno *et al.*, 1997). The function of TACE is to cleave TNF- α (a 26kDa, type II transmembrane protein) releasing a 17kDa soluble TNF- α product, MADM may also process pro-TNF- α (Lunn *et al.*, 1997). TACE has also been demonstrated to contain α -secretase activity, cleaving the β -amyloid precursor protein (Buxbaum *et al.*, 1998). However, without purification and sequencing of additional secretase proteins it is difficult to predict if any one protease is responsible for a group of shedding events, or whether each protein is shed by a different secretase and to what extent their activities overlap. Although it is worth noting that the ADAMs are a large family of related proteins, thus supporting the possibility that a different protease may be responsible for a each shedding event.

6.1.2 CD23 Sheddases

The characterisation of neoepitope antiserum antibody properties provides strong evidence that cleavage at the two predominant cleavage sites within CD23 (i.e. resulting in soluble fragments of 37 and 25kDa) are independent. This raises the possibility that two different proteases are responsible for CD23 shedding, or alternatively that a single protease is able to cleave at numerous sites within the membrane molecule. To aid the following discussion the protease that releases 37kDa sCD23 fragment will be referred to as CD23 sheddase 1, while the protease that releases 25kDa sCD23 fragment will be referred to as CD23 sheddase 2.

6.1.3 Exogenous Proteinases that Cleave CD23

Der p1 is the group 1 allergen of the house dust mite *Dermatophagoides pteronyssinus*. It is recognised by most T and IgE bearing cells from mite allergic individuals and has been implicated as a causative agent in a number of allergic disorders such as asthma, rhinitis, dermatitis and conjunctivitis (Platts-Mills *et al.*, 1987). However, the question remains why should such an innocuous, un-invasive molecule provoke an allergic response? One suggestion is that allergenicity is enhanced by the biochemical properties (e.g. proteolytic activity) of such

allergens (Stewart *et al.*, 1996), in particular, proteolytic activity of Der p1 and its effects on the innate and adaptive immune system.

The exogenous proteinase Der p1 has been shown to cleave CD23 from EBV-transformed B cell surface and this proteolytic cleavage event could compromise the IgE-regulating functions of membrane CD23. Der p1 has been shown to cleave CD23 at two locations, at the top of the coiled-coil stalk domain beneath the lectin domain (between serine residues 155 and 156) and within the C terminal tail (between glutamic acid 298 and serine 299) (figure 6.1), releasing a 17kDa sCD23 fragment (Schulz *et al.*, 1997). Cleavage within the lectin domain is considered unlikely as the serine residues 155 and 156 lie buried within the coiled-coil stalk of CD23. Hence, it was speculated that Der p1 cleaves CD23 in a sequential manner, cleaving the C-terminal tail (Glu298-Ser299) destabilising the trimer and allowing access to the serine residues (155-156). Cleavage of CD23 by Der p1 was shown to be inhibited by the cysteine protease inhibitor E64, however there appears to be some debate about the proteolytic activity of Der p1, depending on the source of house dust mite extract. Der p1 is considered to behold a cysteine protease activity (Chua *et al.*, 1988), however Der p1 purified from mixed (northern and southern Europe) mite populations appear to exhibit both cysteine and serine proteolytic activities (Hewitt *et al.*, 1997).

Cleavage of CD23 by Der p1 releases a 17kDa soluble CD23 fragment by cleavage within CD23 at a location close to that resulting in the release of the 16kDa sCD23 molecule by an endogenous protease. The native 16kDa fragment is produced by a cleavage event between the cysteine residue at position 160 and the asparagine residue at 161 and a second cleavage event within the C-terminal tail (not determined). As the CD23 specific monoclonal antibody 3.5, which is specific for the C-terminal tail domain does not react with the native 16kDa sCD23 fragment (Sarfati *et al.*, 1992). The production of a native 16kDa sCD23 product cleaved from the cell surface, has been shown to be independent of the 25kDa cleavage event, this fragment has also been shown to suppress IgE synthesis (Sarfati *et al.*, 1992). Correlating these findings with that of the 17kDa sCD23 produced as a result of proteolytic activity by Der p1, implies that this fragment will also be unable to promote IgE synthesis directly and instead may have an inhibitory effect on IgE production. Therefore any possible effect of Der p1 at promoting IgE



Figure 6.1 Der p1 Cleavage Sites within CD23. Der p1 cleaves CD23 between Serine residues (155 and 156) and between Glutamic acid residue 298 and Serine residue 299 (Schulz *et al.*, 1997). The first cleavage site lies close to the endogenous cleavage locations producing sCD23 molecules of 25kDa (Kikutani *et al.*, 1986) and 16kDa (Sarfati *et al.*, 1992). The native 16kDa sCD23 molecule is formed by cleavage between cysteine residue 160 and aspargine161, and a second cleavage event within the C-terminal tail (not determined).

(Figure adapted from an original kindly supplied by Dr. R. Beavil, Kings College, London.)

synthesis may be through the loss of the negative feedback regulation mechanism through IgE binding to membrane CD23.

6.1.4 Identification of Endogenous Protease Activity Responsible for Cleaving CD23

Proteases are classified into broad mechanistic classes; serine, cysteine, threonine, aspartic and metallo- proteases. In order to elucidate the mechanistic activity of the endogenous protease(s) responsible for cleaving CD23 a range of protease inhibitors were used (outlined in table 6.1), protease activity was determined based upon their susceptibility to these inhibitors. Protease inhibitors can be toxic to cells at high concentrations therefore all protease inhibitors were titrated corresponding to recommended concentrations (Beynon and Bond 1990), and cell viability (\geq 90%) was checked by trypan blue exclusion. It should be noted that PMSF was found to be particularly toxic to MEL cells, decreasing cell viability over time at recommended working concentrations.

Table 6.1 Protease Inhibitors, Specificity and Concentrations used to Screen EndogenousCD23 Protease Shedding Mechanism

Name of Inhibitor	Target Proteases	Final Concentration	
E64	Cysteine	1-10µM	
EDTA	Metalloprotease	1mM	
Leupeptin	Cysteine-serine	100µM	
Pepstatin A	Aspartic	1-10µM	
Marimastat	Metalloprotease	10-30µM	
PMSF	Serine/Cysteine	1mM	

6.2 Results

6.2.1 Analysis of the Proteolytic Activities of CD23 Sheddase 1 and 2

6.2.1.1 Detection of Shed sCD23 Cleaved by CD23 Sheddase 1

The proteolytic mechanism(s) of CD23 sheddase 1 and 2 (figure 6.1) were assessed by monitoring the release of recombinant membrane CD23 expressed by MEL cells over a period of 24 hours, in the presence and absence of protease inhibitors (listed in table 6.1). Cleaved CD23 fragments were separated by SDS-PAGE gel electrophoresis and detected by immunoblotting, using either a sheep anti-human CD23 polyclonal antibody (figure 6.2) or the anti-neoN₃₇ antiserum (figure 6.3). Experimental results show three bands present in the media/supernatant extracts of mCD23 transfected MEL cells which react with the anti-CD23 polyclonal antibody (figure 6.2, lane 1). The 46kDa band seen in figure 6.2, lane 1, is also seen in the supernatant extracts from untransfected MEL cells (lane 7) and is therefore not believed to be CD23, while the 37 and 33kDa bands are thought to represent the 37 and 33kDa sCD23, cleaved from recombinant mCD23. Incubation of the mCD23 transfected MEL cells with the protease inhibitors E64 (lane 2) and Pepstatin A (lane 5), do not effect the release of these products into the culture media/supernatant. Whereas, addition of EDTA (lane 3) a broad metalloproteinase inhibitor to the culture medium, appears to promote shedding of the 33kDa product (this is especially evident at early time points of 3 and 6 hours). In contrast, shedding of the 37 and 33kDa soluble CD23 fragments were slightly inhibited by the addition of Leupeptin (lane 4) a cysteine/serine protease inhibitor and dramatically inhibited by Marimastat (lane 6) a peptidic hydroxamic-acid metalloproteinase inhibitor donated by AstraZeneca Pharmaceuticals. In samples treated with Marimastat, a 45kDa fragment was detected. This band may represent fulllength membrane CD23 present on contaminating MEL cells that could have been aspirated with the cell media/supernatant and is seen as a faint band in all samples after 24 hours incubation. A second band of approximately 44kDa is also detected after 24 hours incubation (figure 6.2), however, this band is seen in the untransfected MEL cell control and is therefore, not thought to be related to mCD23. The 25kDa soluble CD23 band was not detected in any of the cell culture supernatants, therefore the effects of the protease inhibitors on CD23 sheddase 2 activity could not be determined at this point.

Cell supernatants



The above experiment samples were also analysed using the anti-neoN₃₇ antiserum to specifically define the effects of the panel of protease inhibitors on CD23 sheddase 1. Figure 6.3 shows the detection of a single band of approximately 37kDa in the supernatant sample aspirated from mCD23 transfected MEL cells (lane 1: 6 and 24 hours). Evidence to support that this band is the 37kDa sCD23 fragment was detailed in chapter 5. Faint bands above and below the 37kDa sCD23 band are believed to be a result of non-specific binding of the antiserum and are seen in the untransfected MEL cell supernatant control (lane 7: 3, 6 and 24 hours). The addition of E64 (lane 2), Pepstatin A (lane 5), and Leupeptin (lane 4) do not appear to effect the release of this protein into the culture media. While the addition of EDTA (lane 3) to the culture media appears to promote shedding of this protein. Whereas, incubation of the mCD23 transfected MEL cells with Marimastat (lane 6) completely inhibits the release of the 37kDa sCD23 product.

6.2.1.2 Analysis of CD23 Fragments Retained in the Cell Lysates

Analysis of mCD23 fragments retained on the cell surface from the above experiment, incubated in the presence/absence of a range of protease inhibitors over a period of 24 hours is shown in figure 6.4. Cleaved CD23 fragments retained on the cell surface were separated by SDS-PAGE gel electrophoresis and detected by immunoblotting with anti-CD23 polyclonal antibody. Experimental results show four bands of approximately 46, 44, 37 and 15kDa, present in the transfected mCD23 MEL cell lysates, which react with the anti-CD23 polyclonal antibody (figure 6.4, lanes 1 (3 hours), 7 (6 hours) and 13 (24 hours)). The 46 and 44kDa fragments may represent full-length recombinant mCD23 with and without the N-linked carbohydrate respectively (Letellier *et al.*, 1988). The 37kDa fragment may represent the 37kDa sCD23 molecule also seen in the corresponding supernatant samples, retained on the cell surface after cleavage through an interaction with a CD23 specific ligand, or by the interaction of cleaved CD23 with other mCD23 molecules present within a dimeric/trimeric conformation. The 15kDa fragment may represent either the CD23 stump retained on the cell surface after shedding of sCD23 shown in previous experiments detected with anti-neoC₂₅ antiserum (figure 5.6), or the internalised 16kDa fragment described by Grenier-Brosette *et al.*, (1992).

After 3 hours incubation with the panel of protease inhibitors, the addition of E64 (lane 2), EDTA (lane 3), and Pepstatin (lane 5) to the cells do not appear to effect the expression of the various



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Figure 6.4 The Effects of Protease Inhibitors on CD23 Shedding: Analysis of Cell Lysates with Anti-CD23. Induced MEL cells transfected with mCD23, were incubated in the presence of a range of protease inhibitors. Cell lysates were collected after 3, 6 and 24 hours and separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions, then transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10µg/ml). Lanes 1-6; samples taken after 3 hours incubation with protease inhibitors, 7-12; taken after 6 hours and 13-18 taken after 24 hours. Protease inhibitors added: (lanes 1,7 and 13) control, no inhibitors added, (2, 8 and 14) 10µM E64, (3, 9 and 15) 1mM EDTA, (4, 10 and 16) 100µM Leupeptin, (5, 11 and 17) 10µM Pepstatin A, (6, 12 and 18) 20µM Marimastat. This experiment was repeated with untransfected MEL cells, no bands were detected (data not shown). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

CD23 bands compared to the control (lane 1, without any protease inhibitors). Whereas, the concentration of the 37kDa fragment is reduced in cell lysates incubated with Marimastat (lane 6), while the 15kDa band appears to be enhanced. The addition of Marimastat to the culture supernatant was shown to inhibit the release of the 37kDa sCD23 fragment (figures 6.2 and 6.3) supporting the theory that the 37kDa fragments seen within the culture media/supernatant and cell lysates are the same. As cleavage of the 37kDa fragment is reduced by Marimastat, the enhanced 15kDa band seen in lane 6 may be due to the greater concentration of mCD23 available for cleavage by a second endogenous protease (CD23 sheddase 2). In contrast to the results seen in the culture media/supernatant above, when the mCD23 transfected MEL cells were incubated with Leupeptin a cysteine/serine protease inhibitor (lane 4), an additional band of approximately 33kDa can be seen.

After 6 hours incubation with/without protease inhibitors, the pattern of band expression is similar to that seen at 3 hours. However, an additional band of approximately 22kDa is seen in the cell lysates incubated with Leupeptin (lane 10) and the presence of the 15kDa band is enhanced in mCD23 transfected MEL cell lysate samples incubated with E64 a cysteine protease inhibitor (lane 8), Leupeptin (lane 10) and Marimastat (lane 12).

After 24 hours the presence of the 37kDa band is reduced in all cell samples. The 15kDa band is absent from the cell lysates of samples treated with no inhibitors (lane 13), EDTA (lane 15) and Pepstatin (lane 17). However, the 15kDa fragment is present at increased concentrations in cell lysate samples treated with E64 (lane 14) and Leupeptin (lane 16). The 33kDa and 22kDa bands are not seen in samples treated with Leupeptin at this time point. All band intensities are reduced in samples treated with Marimastat after 24 hours. The cell viability of this sample was 93% (as determined by trypan blue exclusion), therefore the results seen are not likely to be due to the toxic effects of the Marimastat on the cells and may be due to differences in loading. However, it may be possible that treatment with Marimastat for 24 hours affects the transcription of mCD23.

6.2.2 Effects of a Hydroxamic-Acid Inhibitor; Marimastat on CD23 Processing

In order to characterise further the effects of the metalloproteinase inhibitor Marimastat on CD23 processing, the concentration and time required for Marimastat to inhibit the release of sCD23 were investigated.

6.2.2.1 Effects of Marimastat on CD23 Processing Over Time

To determine how quickly Marimastat inhibits CD23 processing MEL cells where incubated in the presence of Marimastat, and the effects of this metalloprotease inhibitor on CD23 processing were assessed after 1, 3, 6 and 12 hours. The results of this experiment are shown in figures 6.5 and 6.6. CD23 was detected using a sheep anti-human CD23 polyclonal antibody. Figure 6.5 lane 1, shows the release of a 37kDa CD23 fragment into the culture media/supernatant, from mCD23 transfected MEL cells after just one hour. The release of this fragment was inhibited when mCD23 transfected MEL cells were incubated in the presence of Marimastat (figure 6.5 lanes 3 and 4). The effects of Marimastat on mCD23 at the cell surface are shown in figure 6.6. Three bands of approximately 46, 44 and 37kDa were seen to be expressed on the cell surface of MEL cells transfected with mCD23 after 1 hour (lane 1) and 3 hours (lane 6) incubation. The 25kDa band seen in these samples, is also seen in untransfected MEL cell lysates and is therefore not thought to be CD23 (lane 16). When mCD23 transfected MEL cells were incubated with Marimastat the concentration of the 37kDa band was reduced after 1 and 3 hours incubation, while the concentration of the 46kDa mCD23 band was increased (lanes 3, 4, 8 and 9). This result suggests that Marimastat inhibits the proteolytic cleavage of 37kDa sCD23, that leads to an increase in the concentration of 46kDa mCD23 at the cell surface. However, after 6 hours incubation the concentration of 46kDa mCD23 appears to be constant, although the 37kDa CD23 band remains reduced (lanes 11, 13 and 14). The 15kDa band corresponding to that described above is also seen in samples treated with Marimastat after 3 hours incubation and can be seen in all samples after 6 hours incubation. The production of this band is discussed in greater detail in section 6.2.2.2.

Figures 6.5 and 6.6 also show the effects of PMA treatment upon CD23 processing with respect to Marimastat treatment, these results will be addressed in sections 6.2.3 and 6.2.4.



Membrane CD23 transfected MEL cell supernatants

Figure 6.5 Characterisation of the CD23 Sheddase 1 in Response to Marimastat and PMA: Cell Supernatants. Induced MEL cells transfected with mCD23, were grown in the presence either PMA (10ng/ml), Marimastat (10 μ M or 20 μ M) or both PMA (10ng/ml) and Marimastat (20 μ M). Samples were collected after a 1, 3, 6 and 12 hour incubation, centrifuged and the supernatant collected. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10 μ g/ml). Lane (1) control, no inhibitors added, (2) PMA (10ng/ml), (3) 10 μ M Marimastat, (4) 20 μ M Marimastat, (5) PMA (10mg/ml) and 20 μ M Marimastat. This experiment was repeated using untransfected MEL cells, no bands were detected (data not shown). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

6.2.2.2 Titrating the Dose Effects of Marimastat on CD23 Processing

The concentration of Marimastat added to the cell samples was titrated to assay the sensitivity of CD23 sheddase 1 to the hydroxamic-acid inhibitor. The results of this experiment are shown in figures 6.7 and 6.8. Figure 6.7 shows that as little as 1μ M Marimastat (lane 2) was enough to visibly inhibit the proteolytic release of the 37kDa sCD23 fragment into the culture media/supernatant. Detection was completely inhibited by the addition of 10μ M Marimastat (lane 5).

The corresponding effects of Marimastat on surface mCD23 expression are shown in cell lysate samples in figure 6.8. Although the addition of increasing concentrations of Marimastat do not appear significantly to effect the presence of the 46kDa, 44kDa or the 37kDa bands, the presence of a 15kDa band increased in intensity with the addition of increasing concentrations of Marimastat (lanes 3-7). As suggested previously it is possible that this band represents the mCD23 cleaved stump retained at the cell surface after shedding of the 25kDa sCD23 fragment. It is possible that the protease activity responsible for cleaving this fragment increases, when the cells are incubated with increasing concentrations of Marimastat. As Marimastat has been shown to inhibit endogenous CD23 sheddase 1 activity, this may increase the concentration of mCD23 available for cleavage by CD23 sheddase 2.

6.2.2.3 The Effects of Marimastat on Membrane CD23 Messenger RNA Transcription

To ascertain if Marimastat was inhibiting the release of the 37kDa sCD23 from full-length membrane CD23 and not effecting the transcription rate of membrane CD23, RNA extracted from induced MEL cells transfected with mCD23 that had been treated with Marimastat were analysed by agarose electrophoresis and transferred to nylon membrane. Messenger (m)RNA was then detected by hybridisation of a ³²P labelled CD23 probe (figure 6.9). These results show that the effects of Marimastat are probably post-transcriptional, as Marimastat does not appear to affect CD23 mRNA levels. This implies that the effects of Marimastat on CD23 shedding are likely to be due to inhibition of the proteolytic processing of CD23 on the cell surface. Reactivity of the 18S ribosomal RNA fraction is included to show equal loading of total RNA.



Membrane CD23 transfected MEL cell lysates

MEL cells



Membrane CD23 transfected MEL cell supernatants

Figure 6.7 Analysis of the Effects of Marimastat on the Shedding of 37kDa sCD23: Cell Supernatants. Induced MEL cells transfected with mCD23, grown in the presence of Marimastat at various concentrations. After 3 hours incubation, samples were centrifuged and the supernatant collected. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10 μ g/ml). Lane (1) control, no Marimastat added, (2) 1 μ M Marimastat, (3) 3 μ M Marimastat, (4) 6 μ M Marimastat, (5) 10 μ M Marimastat, (6) 20 μ M Marimastat. This experiment was repeated using untransfected MEL cells, no bands were detected (data not shown). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).



Membrane CD23 transfected MEL cell lysates

Figure 6.8 Analysis of the Effects of Marimastat on the Shedding of 37kDa sCD23: Cell Lysates. Induced MEL cells transfected with mCD23 and untransfected controls, grown in the presence of Marimastat at various concentrations. After 3 hours incubation, samples were centrifuged and the supernatant removed. Cells were resuspended in PBS, freeze-thawed in the presence of protease inhibitors (2.8.10), then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10 μ g/ml). Lane 1 Induced untransfected MEL cell control (no Marimastat added), lanes 2-8 Induced MEL cells transfected with mCD23, incubated with: (2) control, no Marimastat added, (3) 1 μ M Marimastat, (4) 3 μ M Marimastat, (5) 6 μ M Marimastat, (6) 10 μ M Marimastat, (7) 20 μ M Marimastat. Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

6.2.3 Effects of the Protein Kinase C Activator PMA on CD23 Processing

The secretion of soluble proteins by post-translation hydrolysis of membrane forms has been widely recognised as a secretory mechanism that appears to result from a common processing action. Two common factors found in examples of such secreted proteins, is that they are a) in general, inhibited by hydroxamic-acid metalloproteinase inhibitors and b) secretion is induced by protein kinase C (PKC) activators, such as PMA. To elucidate if the CD23 shedding mechanism occurs as a result of a similar process, induced MEL cells transfected with mCD23 were incubated with PMA. The result of this experiment is shown in figure 6.5, here PMA was shown to induce shedding of the 37kDa sCD23 fragment into the culture media/supernatant (lane 2) above that seen in untreated controls (lane 1).

6.2.3.1 Titrating the Dose Effects of PMA on CD23 Processing

The addition of increasing amounts of PMA to mCD23 transfected MEL cells cultures increased the shedding of both the 37 and 33kDa soluble CD23 fragments in a dose dependent fashion (figure 6.10). The PMA induced CD23 cleavage appears to be saturated with the addition of 6ng/ml PMA (lane 4), as the concentration of the 37kDa and 33kDa fragments do not increase above those seen in lane 4 with increasing PMA concentrations (lanes 5 and 6). Comparison of these results with the CD23 fragments retained at the cell surface (in cell lysates, figure 6.11), shows no obvious increase in the 37kDa band. However, the relative concentrations of the 46kDa (full-length membrane CD23), 44kDa and 15kDa fractions are shown to decrease with increasing concentrations of PMA (lanes 3-7).

6.2.3.2 The Effects of PMA on Membrane CD23 Messenger RNA Transcription

In order to establish if this response was possibly due to effects of PMA on CD23 mRNA transcription levels, RNA samples treated with PMA taken at different time points were analysed by northern blotting, probed with ³²P labelled mCD23 probe (figure 6.12). CD23 messenger RNA transcription levels do not appear to be affected by the addition of PMA (figure 6.12, lanes 1-3 RNA isolated from CD23 transfected MEL cells incubated without PMA, lane 4-6 RNA isolated from CD23 transfected MEL cells incubated with PMA). These results suggest that the effects seen in the immunoblotting experiments described above, are due to post-



Figure 6.9 Analysis of the Effects of Marimastat on CD23 mRNA Steady-State Expression Levels. RNA (approximately 20µg per sample) was extracted from induced MEL cells transfected with mCD23 (lanes 1-6) and untransfected MEL cell controls (lanes 7-10), incubated with 20µM Marimastat (lanes 4-6 and 10) or without Marimastat (lanes 1-3 and 7-9) for 1, 3 and 6 hours. Samples were separated on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane by Northern blotting. RNA expression was analysed using radiolabelled (³²P) probes (a) CD23 (b) murine β-globin which cross-hybridised with ribosomal RNA, included to show equal loading of total RNA. RNA band sizes were estimated by comparison to 1Kb DNA ladder loaded alongside the samples and stained with ethidium bromide.

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Figure 6.10 Analysis of the Effects of PMA on the Shedding of 37 and 33kDa sCD23 Fragments Released into the Supernatant. Induced MEL cells transfected with mCD23, were grown in the presence of PMA (at various concentrations). After 12 hours incubation, samples were centrifuged and the supernatant collected. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody $(10\mu g/ml)$. Lane (1) control, no PMA added, (2) lng/ml PMA, (3) 3ng/ml PMA, (4) 6ng/ml PMA, (5) 10ng/ml PMA, (6) 20ng/ml PMA. This experiment was repeated using untransfected MEL cells, no bands were detected (data not shown). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

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Figure 6.11 Analysis of the Effects of PMA on the Shedding of 37 and 33kDa sCD23 Fragments in Cell Lysates. Induced MEL cells transfected with mCD23 and untransfected controls, were grown in the presence of PMA (at various concentrations). After 12 hours incubation, samples were centrifuged and the supernatant removed. Cells were resuspended in PBS, freeze-thawed in the presence of protease inhibitors (2.8.10), then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10µg/ml). Lane (1) Induced untransfected MEL cell control, lanes (2-8) Induced MEL cells transfected with mCD23, incubated with: (2) control (no PMA added), (3) 1ng/ml PMA, (4) 3ng/ml PMA, (5) 6ng/ml PMA, (6) 10ng/ml PMA, (7) 20ng/ml PMA. Protein band sizes were determined by comparison to Rainbow[™] protein molecular weight markers (Amersham Life Science).

transcriptional effects, possibly on proteolysis at the cell surface. Again, reactivity of the 18S ribosomal RNA fraction is included to show equal loading of total RNA.

6.2.4 Analysis of the Combined Effects of the Protease Inhibitor Marimastat and the PKC Activator PMA

MEL cells transfected with mCD23 were incubated with PMA and Marimastat together to show that the induced shedding of the 37kDa sCD23 product after incubation of PMA was related to endogenous metalloproteinase activity of CD23 sheddase 1. The addition of Marimastat and PMA together, to induced MEL cells transfected with mCD23 inhibited shedding of the 37kDa sCD23 fragment from the cell surface into the culture media/supernatant (figure 6.5 lane 5). This result suggests that PMA upregulates metalloproteinase activity, either by upregulating protease synthesis, increasing activation, decreasing endogenous protease inhibitors or through increasing the interaction between membrane CD23 and CD23 sheddase 1. PMA does not appear to significantly increase synthesis of membrane CD23.

6.2.5 Analysis of the Effects of the Exogenous Protease Der p1 on CD23 Processing

Der p1 has been shown to cleave CD23 from the cell surface (Hewitt *et al.*, 1995, Schulz *et al.*, 1995). Our aim was to study this cleavage event, with respect to endogenous shedding events traced by immunoblotting. Induced MEL cells transfected with mCD23 and untransfected controls were incubated in the presence of Der p1. The preparation of Der p1 used in these experiments has been shown to be enzymatically active by previous studies from our group (Hewitt *et al.*, 1995, Hewitt *et al.*, 1997). CD23 fragments were detected using a sheep antihuman CD23 polyclonal antibody. The results of this experiment (figure 6.13), show that the anti-CD23 polyclonal antibody was unable to detect any additional sCD23 cleaved products released into the supernatant of Der p1 treated cells (lanes 3 and 4), that are not already seen in untreated mCD23 transfected MEL cells (lane 2). The concentration of shed 37 and 33kDa sCD23 bands remained unaffected (lanes 2, 3 and 4), however, the presence of a 46kDa band, previously thought to represent full-length mCD23 expressed on MEL cells aspirated with the cell supernatants is decreased in samples treated with Der p1.



Figure 6.12 Analysis of the Effects of PMA on CD23 mRNA Steady-State Expression Levels. RNA (approximately 20µg per sample) was extracted from induced MEL cells transfected with mCD23 (lanes 1-6) and untransfected MEL cell controls (lanes 7-10), incubated with 10ng/ml PMA (lanes 4-6 and 10) or without PMA (lanes 1-3 and 7-9) for 1, 3 and 6 hours. Samples were separated on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane by Northern blotting. RNA expression analysed using radiolabelled (³²P) probes (a) CD23 (b) murine β -globin which cross-hybridised with ribosomal RNA, included to show equal loading of total RNA. RNA band sizes were estimated by comparison to 1Kb DNA ladder loaded alongside the samples and stained with ethidium bromide.

Analysis of corresponding cell lysate samples suggest that incubating mCD23 transfected MEL cells with Der p1 does not affect full-length CD23 (46kDa) expression levels, nor does it affect the concentration of the 44kDa unglycosylated precursor (figure 6.14, Letellier *et al.*, 1988). However, an additional band of approximately 15kDa (lanes 2 and 3) and a faint band of approximately 26kDa (lane 3) can be seen in cell lysate samples treated with Der p1, along with increased concentrations of the 37kDa band (lanes 2 and 3). This suggest Der p1 is cleaving recombinant CD23 and at least some of the cleaved fragments are being retained within the cell lysate, possibly at the cell surface through interaction with CD23 specific ligands or other CD23 molecules within a dimeric/trimeric conformation. The reason these fragments are not detected in the culture media/supernatant (figure 6.13) may be because their concentration levels are below the limit of detection by the anti-CD23 polyclonal antibody, or that they don't contain epitopes recognised by the antibody. It is possible that the 15kDa fragment represents the protein seen in samples treated with Marimastat (figure 6.8) and that detected with anti-neoC₂₅ antiserum (figure 5.6).

6.2.6 Analysis of the Effects of the Exogenous Protease Der p1 in Respect to CD23 Sheddase 1 Activity

Previous experiments demonstrated that CD23 sheddase 1 was inhibited by metalloproteinase inhibitors, therefore the effects of Der p1 on CD23 shedding were assessed in respect to this endogenous cleavage event by the addition of Marimastat, and/or PMA to cell cultures co-treated with Der p1. The results of this experiment are shown in figures 6.15 and 6.16. Der p1 alone does not affect the cleavage and subsequent release of either the 37kDa or the 33kDa sCD23 molecules into the culture media/supernatant (figure 6.15 lane 1 untreated samples, lanes 5 and 6 samples treated with Der p1). However, when mCD23 transfected MEL cells are incubated in the presence of PMA and Der p1, the addition of Der p1 reduces the concentration of full-length mCD23 (46kDa) and the 44kDa CD23 bands (lane 2 treated with PMA, lane 7 treated with PMA and Der p1). While, incubation of MEL cells transfected with mCD23 with both Marimastat and Der p1 increases the concentration of the 46kDa (full-length) CD23.



Figure 6.13 Analysis of the Effects of Der p1 an Exogenous Protease on CD23 Shedding; Released into the Supernatant. Induced MEL cells transfected with mCD23 (lane 2-4) and untransfected MEL cell controls (lane 1) were incubated for 60 minutes with or without Der p1. After 60 minutes incubation, samples were centrifuged and the supernatant aspirated. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis (under reducing conditions) and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10µg/ml). Supernatant samples extracted from: (1) Untransfected MEL cell control treated with 2µg/ml Der p1, (2) mCD23 MEL cells unstimulated, (3) MEL cells transfected with mCD23 treated with 1µg/ml Der p1, (4) MEL cells transfected with mCD23 treated with 2µg/ml Der p1. Protein band sizes were determined by comparison to Rainbow[™] protein molecular weight markers (Amersham Life Science).



Figure 6.14 Analysis of the Effects of Der p1 an Exogenous Protease on CD23 Shedding: Released in Cell Lysates. Induced MEL cells transfected with mCD23 (lanes 1-3) and untransfected MEL cells (lanes 4 and 5), were incubated for 60 minutes with or without Der p1. After 60 minutes incubation, samples were centrifuged and the supernatant removed. Cells were resuspended in PBS, freeze-thawed in the presence of protease inhibitors (2.8.10), then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10µg/ml). Membrane CD23 transfected MEL cells incubated with (1) control (no Der p1), (2) 1µg/ml Der p1, (3) 2µg/ml Der p1, untransfected MEL cells incubated with (4) control (no Der p1), (5) 1µg/ml Der p1. Protein band sizes were determined by comparison to Rainbow[™] protein molecular weight markers (Amersham Life Science).



Figure 6.15 Analysis of the Effects of Der p1 an Exogenous Protease, in Relation to the Proteolytic Shedding of 37kDa sCD23 Fragment Released into the Supernatant. Induced MEL cells transfected with mCD23 and untransfected MEL cell controls, were incubated for 60 minutes with or without Der p1, 20 μ M Marimastat and/or PMA (10ng/ml). After 60 minutes incubation, samples were centrifuged and the supernatant collected. Supernatants were then separated on a 12% SDS-PAGE gel by electrophoresis (under reducing conditions) and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10 μ g/ml). Supernatant samples extracted from induced MEL cells transfected with mCD23 incubated with: (1) control (no additives), (2) PMA, (3) Marimastat, (4) PMA and Marimastat, (5) 1 μ g/ml Der p1, (6) 2 μ g/ml Der p1, (7) PMA and 2 μ g/ml Der p1, (8) Marimastat and 2 μ g/ml Der p1, (9) PMA, Marimastat and 2 μ g/ml Der p1. Supernatant samples from induced untransfected MEL cells: (10) control (no additives), (11) PMA and Marimastat, (12) 2 μ g/ml Der p1. Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).



Figure 6.16 Analysis of the Effects of Der p1 an Exogenous Protease, in Relation to the Proteolytic Shedding of 37kDa sCD23 Fragment in Cell Lysates. Induced MEL cells transfected with mCD23, were incubated for 60 minutes with or without Der p1, 20 μ M Marimastat and/or PMA (10ng/ml). After 60 minutes incubation, samples were centrifuged and the supernatant removed. Cells were resuspended in PBS, freeze-thawed in the presence of protease inhibitors (2.8.10), then separated on a 12% SDS-PAGE gel by electrophoresis under reducing conditions and transferred to Immobilon-P membrane. Protein expression was analysed by immunoblotting with an anti-CD23 polyclonal antibody (10 μ g/ml). Cell lysate samples extracted from induced MEL cells transfected with mCD23 incubated with: (1) control (no additives), (2) 1 μ g/ml Der p1, (3) 2 μ g/ml Der p1, (4) PMA, (5) PMA and 1 μ g/ml Der p1, (6) PMA and 2 μ g/ml Der p1, (7) PMA and Marimastat, (8) Marimastat, (9) Marimastat and 1 μ g/ml Der p1, (10) Marimastat and 2 μ g/ml Der p1, (11) PMA, Marimastat and 1 μ g/ml Der p1, (12) PMA, Marimastat and 2 μ g/ml Der p1. This experiment was repeated with untransfected MEL cells, no bands were detected (data not shown). Protein band sizes were determined by comparison to RainbowTM protein molecular weight markers (Amersham Life Science).

Analysis of the cell lysate samples indicates that the addition of Der p1 increases production of a 37kDa band and results in the additional release of a 15kDa band (Figure 6.16 lanes 2 and 3). Der p1 could mediate this effect either by directly cleaving CD23 generating soluble CD23 fragments, or alternatively Der p1 may promote endogenous protease activity. The addition of Marimastat and Der p1 to the cells inhibits the production of the 37kDa band. This supports the latter hypothesis for the production of the 37kDa fragment i.e. Der p1 is promoting CD23 sheddase 1 activity. However, the addition of Marimastat either alone or in combination with Der p1, does not effect the production of a 15kDa band (lane 9) indicating that the production of this fragment is independent of CD23 sheddase 1. However, when CD23 sheddase 1 activity is promoted through the treatment of mCD23 transfected MEL cells with PMA the concentration of the 15kDa fragment is reduced (lane 4) even in the presence of Der p1 (lane 5) and Marimastat (lanes 7 and 11). This poses the question, are the 15kDa bands seen in samples treated with Der p1 and Marimastat the same or do they represent different fragments of cleaved CD23?

6.2.7 Characterisation of the 37 and 15kDa Bands Detected in mCD23 Transfected MEL Cell Lysates using Anti-Neoepitope Antibodies

To determine whether the 37kDa and 15kDa bands seen in mCD23 transfected MEL cell lysates in the above experiments are the result of endogenous proteases (i.e. contain the neoN₃₇ and neoC₂₅ epitopes respectively), cell lysates treated with protease inhibitors and/or Der p1 were probed with the anti-neoN₃₇ and the anti-neoC₂₅ antiserum. Unfortunately no binding was observed (data not shown). Possible reasons for this may be that: (1) the amount of material loaded was less than the limit of detection of the anti-neoepitope antibodies (compare figures 6.2 and 6.3), (2) it was possible that the fragments may have lost one or more amino or carboxy terminal amino acids, thereby decreasing the affinity of the anti-neoepitope antibodies (3) cleavage is at another site.

6.3 Discussion

6.3.1 CD23 Sheddase 1 is a Metalloproteinase

The hydoxamic-acid metalloprotease inhibitor Marimastat surpressed the activity of the endogenous proteinase CD23 sheddase 1, responsible for the generation of the soluble 37 and 33kDa CD23 fragments. The other three mechanistic classes (covered by a range of commercially available inhibitors) had no significant effect on this proteolytic event, although some reduction was seen in the concentration of these fractions in samples incubated with the typsin-like serine protease inhibitor leupeptin. These results support those published by Christie *et al.*, (1997) and Wheeler *et al.*, (1998). In addition, this proteolytic event is upregulated by the addition of the PKC activator PMA to the mCD23 transfected MEL cell cultures. The properties of the endogenous proteinase CD23 sheddase 1, appear to be similar to those previously described for the proteolytic cleavage of ACE, APP, L-selectin, IL-6R, TNF- α , TGF- β and TNFR 1 and 2 (table 6.2). A proteinase with the activities of CD23 sheddase 1 has been isolated from the plasma membrane of RPMI 8866 cells (Marolewski *et al.*, 1998), adding further support to the theory that a family of similar membrane metalloproteases may be responsible for ectodomain shedding.

6.3.2 CD23 Sheddase 2 Activity is Enhanced in the Presence of Cysteine, Serine and Metalloproteinase Inhibitors

The proteolytic mechanism of CD23 sheddase 2 was not determined. The soluble 25kDa CD23 fragment generated by CD23 sheddase 2 cleavage was not detected in the culture media/supernatant of mCD23 transfected MEL cells probably due to the low concentration of this fragment. The 15kDa fragment seen in mCD23 transfected MEL cell lysates co-migrates with the 15kDa fragment detected by the anti-neoC₂₅ antiserum (figure 5.6) and is therefore thought to represent the CD23 sheddase 2. Interestingly, treatment of mCD23 transfected MEL cells with the cysteine protease inhibitors (E64 and Leupeptin) increased accumulation of the 15kDa fragment in cell lysates. Several reasons may account for this observation: (1) The cysteine protease inhibitor blocked further degradation or internalisation of this fragment, and (2) the addition of cysteine protease inhibitors blocked the turnover of CD23 sheddase 2. Inhibition of

 Table 6.2 Properties of Membrane Protein Secretases (summary of those most commonly studied).

Substrate	Topology	Protease	Mechanism	Cleavage	Distance	Activated
			(class of	Site	from	by Phorbol
			protease)	(P ₁ √ P ₁')	Membrane	Ester?
ACE	Туре І	ACE	Metallo-	GQR↓LAT	93	Yes
		secretase				
	Туре І	α-secretase	Metallo-	HQK↓LVF	12	Yes
APP		β -secretase	Serine(?)	VKM↓DAE	28	No
		γ-secretase	n.d.	GVV↓IAT	within	n.d.
					membrane	
CD23	Type II	Sheddase I	Metallo-	QMA↓QKS	33	Yes
		Sheddase II	Serine(?)	KLR↓MEL	102	No(?)
IL-6R	Туре І	IL6R	Metallo-	AVQ↓DSS	1	Yes
		secretase				
L-Selectin	Туре І	L-selectin	Metallo-	LDK↓SFS	11	Yes
		secretase				
TGF-α	Type I	TGF-α	Metallo-	LLA↓VVA	9	Yes
		secretase	(serine?)			
TNF-α	Type II	TACE	Metallo-	AQA↓VRS	20	Yes
			(serine?)			
TNFR-I	Туре І	TNFR	Metallo-	IEN↓VKG	10	Yes
		secretase				
TNFR-II	Туре І	TNFR	Metallo-	GAV↓HLP	43	n.d.
		secretase				

n.d. not determined

? some experimental evidence but not yet unequivocally determined

CD23 sheddase 1 proteolytic activity by Marimastat also increased accumulation of the 15kDa CD23 fragment in cell lysates, possibly by enhancing the concentration of mCD23 available for cleavage by CD23 sheddase 2.

6.3.3 The Effect of PMA on CD23 Shedding is Dose Related

Shedding of the 37 and 33kDa sCD23 fragments in response to PMA is dose related. PMA does not appear to induce shedding of the 25kDa sCD23 molecule as this is not detected in the culture media/supernatant. Furthermore, the 15kDa fragment observed in cell lysate samples treated with Marimastat, E64 and Leupeptin is not present. The addition of PMA to mCD23 transfected MEL cell cultures appears to reduce the concentration of full-length mCD23 present in cell lysates, while not affecting steady-state mRNA expression (shown in figure 6.12). From these results it is possible to speculate that the increase in CD23 shedding activity induced by PMA is a result of (1) up-regulating transcription of the membrane protease responsible for cleavage or (2) enhancing the interaction between the membrane protease and membrane CD23.

Research by Arribas *et al.*, (1996) demonstrated that approximately 2% of all surface proteins present on CHO cells were shed in response to the addition of the PKC activator PMA. The biological effects of PMA are due exclusively to its ability to activate certain isoforms of PKC and the activation of NF- κ B (Baeuerle *et al.*, 1988). Hence it is possible that PMA induces protease gene expression through protein kinase C (PKC) activation and phosphorylation of the NF- κ B inhibitory proteins I κ B, disrupting NF- κ B-I κ B complex releasing NF κ B to enter the nucleus and activate gene expression (Verma *et al.*, 1995, Baldwin 1996).

6.3.4 Der p1 Cleaves CD23 Directly or Promotes Endogenous CD23 Sheddase Activity?

Der p1 appears to cleave mCD23 resulting in the increased amount of 37kDa CD23 and the presence of a 15kDa fragment in mCD23 transfected MEL cell lysates. The 15kDa fragment comigrates with the protein band detected when mCD23 transfected MEL cells are treated with Marimastat and the cysteine protease inhibitors E64 and Leupeptin (figure 6.4), and corresponds in size to that detected by the anti-neoC₂₅ antiserum (figure 5.6). No CD23 fragments cleaved by Der p1 were detected in the culture media/supernatant. It is possible that these were not detected because the amount of cleaved material present was below the limit of detection with the anti-CD23 polyclonal antibody.

These results suggest that Der p1 either promotes endogenous CD23 sheddase 1 and 2 proteolytic activity, or cleaves CD23 at or nearby the endogenous protease cleavage sites. The addition of Der p1 and Marimastat together to mCD23 transfected MEL cells inhibits the production of the 37kDa band seen in Der p1 alone treated cells, this result is therefore consistent with the theory that Der p1 promotes CD23 sheddase 1 metalloproteinase activity.

6.3.5 Summary

The results described above show that CD23 sheddase 1 is inhibited by the metalloproteinase inhibitor Marimastat, supporting findings by Christie *et al.*, (1997) and Wheeler *et al.*, (1998) that CD23 sheddase 1 is a membrane metalloproteinase. Cleavage of CD23 is induced upon incubation of the PKC activator PMA adding further support that this protease belongs to a family of proteases responsible for the cleavage of membrane proteins by a common mechanism. Incubation of mCD23 transfected MEL cells with Der p1 increases the concentration of 37kDa and 15kDa CD23 fragments observed in cell lysates, suggesting that Der p1 cleaves CD23. However, the mechanism of cleavage is not clear, it may be that Der p1 and Marimastat inhibits production of the 37kDa fragment.

Chapter 7: Discussion

7.1 Introduction

Inhibition of CD23 shedding may have the potential to alleviate both allergic and inflammatory diseases. Therefore the identification of the endogenous protease(s) responsible for these shedding events are of particular interest for therapeutic intervention. Since the work in this thesis was started, several groups have shown that hydroxamic acid metalloprotease inhibitors inhibit IgE synthesis. These inhibitors prevent the release of the 37 and 33kDa sCD23 fragments (Christie *et al.*, 1997, Wheeler *et al.*, 1998). However, none of these studies address the effects of this group of protease inhibitors on the production of 29, 27, 25 and 16kDa sCD23 molecules.

Experimental evidence suggests that these smaller fragments possess different biological properties to those of the larger 37 and 33kDa sCD23 molecules. It is hypothesised that the loss of the coiled-coil stalk domain in these smaller fragments reduces their affinity/avidity for IgE (Kelly *et al.*, 1998) and therefore it is unlikely that they compete with membrane CD23 for soluble IgE. However, the smaller soluble CD23 fragments (\leq 29kDa) still retain cytokine like properties and are capable of binding CD21 (Mossalaysi *et al.*, 1992). For instance recombinant 25kDa sCD23 has been shown to rescue germinal centre B cells from apoptosis (Lui *et al.*, 1991) and to promote the production of inflammatory cytokines via binding of sCD23 to membrane CD21 expressed on monocytes (Mossalayi *et al.*, 1990). However, these studies do not exclude the possibility that the larger sCD23 fragments (37 and 33kDa sCD23) play a part in these biological functions. In addition the identification of soluble CD21 indicates the potential for membrane and soluble CD23 molecules to have overlapping functions.

To date, there is no evidence to define specific biological properties to the various soluble CD23 molecules, however one would envisage a scenario where the different sized molecules perform different functions in response to differing stimuli, possibly in different tissue. Therefore it is clear that more research is required to study the mechanism(s) of formation of each of the soluble CD23 products and determine their specific biological activities.

7.2 Achievements

- Full length (error free) CD23 was cloned from RNA extracted from EBV-transformed B cells by RT-PCR.
- Recombinant membrane and truncated CD23 was expressed in transfected MEL cells, producing a cell based expression system with high level expression of recombinant CD23.
- Antibodies were raised to the amino and carboxy neoepitopes of the 37 and 25kDa sCD23 fragments produced after proteolytic degradation of full-length membrane bound CD23.
- The binding specificity of the neoepitope antisera was characterised and the anti-neoN₃₇ and the anti-neoC₂₅ antisera were shown to be specific for their respective epitopes.
- The anti-neoN₃₇ and the anti-neoC₂₅ antisera were used along side an anti-CD23 polyclonal antibody to assay cleavage events within CD23 with respect to (1) the mechanistic class of endogenous protease activity, (2) the temporal sequence of cleavage events, (3) assessment of the proteolytic activity in response to the addition of PMA, and (4) analysis of the effects of the exogenous protease Der p1.

7.2.1 Proteolytic Processing of Human CD23 Expressed in a Murine Cell System

Recombinant membrane bound CD23 expressed on MEL cells, is shed to produce soluble fragments similar to those observed with RPMI 8866 cells. This finding has two implications; firstly, MEL cells, which do not naturally express native CD23, express the endogenous protease(s) responsible for cleaving human CD23. Secondly the protease(s) responsible for cleaving human CD23 is (are) not species specific. Within this expression system murine homologues of the CD23 sheddase(s) recognise and cleave human CD23 despite several differences between the human and murine CD23 structure and amino acid sequence (figure 3.20). The possibility that the murine homologue to the human CD23 sheddase(s) is (are) able to cleave recombinant human CD23 poses the question, what are the defining properties that initiate cleavage of CD23 at such specific locations? Analysis of the amino acid sequence surrounding cleavage site locations, within human and murine CD23 show almost no sequence similarity (Bartlett *et al.*, 1995). However, both molecules are cleaved within the coiled-coil stalk domain, suggesting that the governing factor(s) underlying the location of the cleavage site is associated with this domain. It is possible that cleavage location is dependent upon either distance from the
membrane or distance from a proximal domain (such as the lectin domain), as described for other sheddases/secretases (Hooper *et al.*, 1997).

It was observed that recombinant truncated CD23 expressed by MEL cells was not secreted into the culture supernatant but retained within the cell. This was not unexpected as native soluble CD23 is not naturally secreted but cleaved from the cell surface and the full-length CD23 mRNA sequence does not contain a recognised secretory sequence. Interestingly recombinant truncated CD23 was not further degraded, this indicates that the protease(s) responsible for proteolytic cleavage are not present within the cytosol where the recombinant protein was synthesised.

7.2.2 Proteolytic Cleavage Events Responsible for Creating the 37 and 25kDa Soluble CD23 Fragments are Independent of Each Other

Characterisation of the anti-neoN₃₇ and the anti-neoC₂₅ neoepitope antisera showed that antibodies in these sera were specific for their immunising peptides. Identification of a 15kDa protein present within the membrane extract of mCD23 transfected MEL cells that reacts with the anti-neoC₂₅ antiserum, indicated that this cleavage event, which results in the release of a 25kDa sCD23 fragment was not dependent upon prior cleavage of the larger 37 and 33kDa sCD23 molecules. Cleavage of the 25kDa sCD23 molecule without the production of either the 37 or 33kDa sCD23 fragments has been shown in supernatants from RPMI 8866 cells grown in the absence of serum (Moulder *et al.*, 1993). However, since the 37kDa sCD23 product (detected with the anti-neoN₃₇ antiserum) is found in the culture media/supernatant of transfected MEL cells shown to contain the neoC₂₅ epitope, suggests that the two proteolytic events occur simultaneously. The finding that the two major cleavage events within CD23 are not dependent upon each other has major implications for targeting the proteolytic cleavage of CD23 to effect IgE production. This finding emphasises the importance of ascertaining the biological functions of mCD23 and each of the sCD23 molecules.

7.2.3 The 37kDa Soluble CD23 Fragment is Cleaved by a Metalloproteinase

Characterisation of the mechanistic class of the protease(s) responsible for the shedding of the 37 and 25kDa sCD23 fragments indicates that different proteases are responsible for the shedding of each of these two fragments. The addition of a range of protease inhibitors covering the four common mechanistic classes of protease activity show that the 37kDa sCD23 fragment is cleaved from the cell surface by a metalloproteinase, which I have termed CD23 sheddase 1. This result has been previously demonstrated by Christie *et al.*, (1997), who studied the proteolytic processing of CD23 from the RPMI 8866 lymphoblastoid cells, human monocytes and tonsillar B cells. Reduced sCD23 production from cells cultured with these inhibitors is associated with reduced IgE synthesis both *in vitro* from IL-4, anti-CD40 monoclonal antibody treated tonsillar B cells and *in vivo* from splenic B cells from ovalbumin-challenged mice (Wheeler *et al.*, 1998). However, neither of these studies addressed the effects of protease inhibitors on the cleavage of the 25kDa sCD23 fragment, nor assayed the effects of inhibiting production of the 37/33kDa sCD23 fragment on the production of the smaller sCD23 molecules.

Anti-neoepitope antibodies specific for the amino and carboxy termini produced after cleavage of the 25kDa sCD23 molecule were raised to address this question. Despite the production of antibodies specific for the neoC₂₅ epitope, we failed to identify the protease class responsible for cleavage of the 25kDa sCD23 molecule. Non-specific binding of the anti-neoC₂₅ antiserum to proteins present in the transfected MEL cell expression system, confounded the analysis of the proteolytic processing events responsible for the release of the 25kDa sCD23 fragment. However, results were obtained using a polyclonal anti-CD23 antibody. The identification of a 15kDa band in the membrane extracts from MEL cells transfected with mCD23, were shown to contain the neoC₂₅ epitope (figure 4.8 and 5.6). This indicates that the 15kDa band detected by the anti-CD23 polyclonal antibody, probably represents the CD23 stalk retained in the membrane after cleavage and release of the 25kDa sCD23 fragment. If this is true, then the addition of Marimastat does not effect the proteolytic cleavage of the 25kDa sCD23 molecule. Implying that the two fragments (the 37 and 25kDa sCD23 molecules) are cleaved by different proteases.

Analysis of the protease activity responsible for the shedding of a 25kDa sCD23 molecule termed CD23 sheddase 2, expressed by MEL cells transfected with mCD23 indicates that this sheddase

activity is enhanced by the addition of Marimastat to the cell cultures. This is possibly due to an increase in the amount of full-length CD23 available on the cell surface, due to inhibition of the proteolytic shedding of the 37 and 33kDa sCD23 fragments.

CD23 sheddase 2 activity is also enhanced by the addition of the cysteine protease inhibitors E64 and Leupeptin, implicating a cysteine protease is responsible for regulating the endogenous CD23 sheddase 2 activity or is preventing further degradation of cleaved CD23. This result contradicts findings by Letellier et al., (1989). Analysis of the proteolytic cleavage events by this group demonstrated that the addition of the cysteine protease inhibitor iodoacetamide, prevented degradation of 37kDa sCD23 precursors into 25kDa sCD23 fragments. However, the concentration of iodoacetamide used in this experiment was a 200-2000 fold greater than recommended working concentrations (Beynon and Bond 1990). The proteolytic mechanism of cleavage of this sheddase was then suggested to be that of a serine protease (Cairns and Gordon 1990). This paper demonstrates isolation of intact 45kDa membrane CD23 in the presence of a serine protease TLCK, which is reduced to a 25kDa fragment prepared in the absence of TLCK. This result suggests that a membrane associated serine protease is responsible for the proteolytic degradation of full-length membrane CD23 into a 25kDa product. However, it should be noted that sCD23 fragments of 25kDa and 16kDas have been described (Lee et al., 1989 and Grennier-Brossette et al., 1992), produced by internalisation of membrane CD23 and subsequent degradation within acidic endosomes and lysosmes. Therefore it is a possibility that smaller CD23 fragments isolated from cell lysates represent internally degraded mCD23 fractions rather than externally cleaved molecules.

7.2.4 PMA Induces Proteolytic Cleavage of 37 and 33kDa sCD23

Shedding of the 37 and 33kDa sCD23 fragments induced by the addition of PMA, suggests that the endogenous proteinase responsible for these cleavage events is related to a family of sheddases/secretases, that cause the release of a number of secreted proteins including ACE, APP, IL-6R, TGF- α , KL, TNF- α , TNFR1 and TNFR2. Within this group only the membrane proteinase responsible for cleaving TNF- α (TACE) has been isolated and sequenced. Sequence analysis indicated that TACE was a member of the adamalysin family of zinc binding metalloproteinases called ADAMs (Black *et al.*, 1997, Moss *et al.*, 1997). This proteinase has also been shown to cleave APP at same location as the α secretase (Buxbaum *et al.*, 1998) while MADM (bovine mammalian disintegrin-metalloproteinase) is capable of cleaving pro-TNF- α . Marolewski *et al.*, (1998) have recently isolated a membrane protein with an approximate molecular mass of 63kDa with CD23 processing activity. Further characterisation of this proteinase will provide further information about this family of metalloproteinases. The finding that more than one enzyme can cleave both pro-TNF- α and APP producing identical cleaved products, implies that more than one enzyme may cleave each protein at the same location or that single enzyme is responsible for cleaving several proteins.

7.2.5 Der p1 Promotes Proteolytic Shedding of CD23

Der p1 has been shown to cleave CD23 at two locations, between amino acid residues serine 155 and serine 156 and between glutamic acid 298 and serine 299 (Schulz et al., 1997). The serine residues 155 and 156 lie close to the top of the coiled-coil stalk domain, a region that is predicted to be stable and unsusceptible to proteolytic attack. It has therefore been proposed that Der p1 may first cleave CD23 within the C-terminal tail, between residues 298/299 and that the proteolytic loss of the C-terminal tail destabilises the molecule allowing Der p1 access to the second site between serine residues 155/156 (Schulz et al., 1997). Incubation of Der p1 with mCD23 transfected MEL cells results in the cleavage of membrane CD23 leaving a 15kDa fragment retained in the cell lysate. However, a sCD23 fragment corresponding to that released into the culture supernatant was not detected. The 15kDa fragment runs parallel to that produced when CD23 transfected MEL cells are incubated with the protease inhibitors Marimastat, E64 and Leupeptin and is of a similar molecular weight to that detected by anti-neoC₂₅ antiserum. This data is consistent with that reported by Schulz et al., (1997) in that Der p1 could be cleaving CD23 near to or at the serine residue 155/156. The endogenous protease responsible for cleaving the 25kDa sCD23 fragment and Der p1, both cleave CD23 within 5 amino acid residues at the top of the coiled coil stalk, suggesting that CD23 is susceptible to proteolytic activity at this site. Despite similar cleavage locations, it is unlikely that the two cleavage events are related as the addition of the cysteine protease inhibitor (E64) has previously been shown to inhibit cleavage of CD23 by Der p1 (Hewitt et al., 1995, Schulz et al., 1995). However, the same protease inhibitor appears to promote endogenous protease activity (CD23 sheddase 2).

7.3 Future Work

Despite limitations resulting from detection sensitivities of the anti-neoepitope (neoN₃₇ and C₂₅) antibodies further analysis of the cleavage events within CD23 in relation to endogenous and exogenous proteases may be possible. The limiting factor for analysing CD23 cleavage events throughout these experiments appears to be the low concentration of sCD23 fragments, with this concentration being below the sensitivity of the anti-neoepitope antibodies. However, these may possibly be overcome by purification and concentration of the antibodies from the immune sera or by concentration of the mCD23 molecule and its soluble components (where possible). I therefore believe that these antibodies have great potential to determine the biological functions carried out by membrane CD23 and its soluble counterparts, possibly resulting in the characterisation of the membrane and soluble CD23 expression under normal and diseased conditions.

Isolation of the membrane protease responsible for cleaving the 37 and 33kDa sCD23 fragments will provide further information for the design of inhibitors to prevent this proteolytic cleavage event. The hydroxamic acid metalloproteinase inhibitors used so far to inhibit CD23 shedding, has broad target specificity. This broad spectrum of inhibition makes the hydroxamic acid metalloproteinase inhibitors used so far to prevent CD23 shedding unsuitable for use *in vivo*. However identification of the factors governing cleavage of CD23 may provide information to design a more specific mechanism of control. The production of recombinant CD23 mutants, mutated within the stalk domain near to the location of cleavage, or within the lectin domain preventing CD23-ligand interactions or within the leuzine zipper motif shown to be responsible for the α helical coiled-coil structure (Beavil *et al.*, 1992), may provide further information on the governing factors controlling cleavage.

The effects of Der p1 in relation to endogenous cleavage events still remain undetermined and the implications of these cleavage events require further analysis to ascertain if Der p1 may promote an allergic response through the cleavage of CD23. The results discussed in chapter 6 are consistent with the finding that Der p1 cleaves CD23 at the top of the coiled-coil stalk close to the lectin domain. Incubation of CD23 with Der p1 also appears to promote susceptibility of the molecule to endogenous proteolytic cleavage by CD23 sheddase 1, releasing the 37 and 33kDa

sCD23 molecules into the culture supernatant. The rapid loss of membrane CD23 may interfere with the regulation of IgE synthesis through a negative feedback mechanism as described previously. However, this hypothesis is based on *in vitro* experimental evidence, Der p1 has not been shown to interact directly with CD23 *in vivo*. One strategy to analyse the cleavage of CD23 by Der p1 *in situ* is by the production of anti-neoepitope antibodies to the new amino and carboxy termini of CD23 created after cleavage by Der p1. These antibodies could then be used to probe for Der p1 cleaved CD23 fragments in samples donated from Der p1 allergic individuals.

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