COMMENTARY

Caspase-9 cleavage, do you need it ?

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Caspase-9 which is activated by association with the Apaf-1 apoptosome complex cleaves and activates the downstream effector caspases-3 and -7, thereby executing the caspase-cascade and cell death programme. Although, caspase-9 does not need to be cleaved to be active, apoptotic cell death is always accompanied by autocatalytic cleavage and by further downstream effector caspase-dependent cleavage of caspase-9. In this issue of the *Biochemical Journal,* Denault and co-workers evaluate the role of caspase-3-dependent cleavage of caspase-9 and conclude that this mechanism mainly serves to enhance apoptosis by alleviating XIAP inhibition of the apical caspase.

Key words: caspase-9, caspase-3, Apaf-1 apoptosome, XIAP (X-linked inhibitor of apoptosis), Smac (Second mitochondrial activator of caspases).

ACTIVATING THE CASPASE CASCADE AND THE ROLE OF XIAP

Caspases are present in healthy cells as zymogens, which are usually activated by proteolytic cleavage of an interlinker peptide sequence that allows rearrangement of peptide loops to form a fully functional active site. Caspases-3 and -7 are the apical effector caspases and are activated, by either caspase-9 or caspase-8. The inappropriate activation of this essentially irreversible caspase cascade requires an independent and tightly regulated failsafe mechanism. To this end, the cell has evolved altogether different mechanisms for activating the initiator caspases which are responsible for cleaving and activating procaspases-3 and -7. In the case of the intrinsic pathway which can be triggered by chemical or radiation-induced damage, the execution phase of cell death involves an efflux of cytochrome *c* from the mitochondria, which binds to and

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activates Apaf-1 (apoptotic protease-activating factor-1). This large (~130-140 kD) protein, is a mammalian homologue of CED-4, an essential protein involved in *C.elegans* programmed cell death. Apaf-1 oligomerizes to form the seven-spoked, wheel-like Apaf-1 apoptosome complex which recruits caspase-9 to form the active holo-enzyme caspase-activating apoptosome complex. This complex then efficiently recruits and directly cleaves procaspase-3 or -7 with high efficiency [1]. However, caspase-9 (like caspase-8 in the DISC complex) is an unusual caspase in that in addition to its CARD (caspase recruiting domain) it has a long interlinker peptide separating the small and large subunit domains. Crystallographic studies show that this flexible linker peptide allows formation of the active site pocket in the zymogen but further conformational changes are required to fully activate its active site [2]. The mechanism for this is controversial: one theory suggests that the apoptosome activates caspase-9 by facilitating dimerization [3], whilst an alternative hypothesis suggests that binding to the apoptosome is sufficient to induce the necessary conformational changes to activate caspase-9 [4]. The proform of caspase-9 has a low but measurable cleavage activity, but when associated with the apoptosome is stimulated 2000-fold. Significantly a non-cleavable caspase-9 mutant is also activated in an apoptosome-dependent manner with no loss of activity [5-7]. This is the essential characteristic of caspase-9, in that irrespective of its cleavage state, it has to be associated with the Apaf-1 apoptosome in order to achieve its full proteolytic activity. Significantly, the monomeric cleaved form of the enzyme on its own (i.e., without the apoptosome) at physiological concentrations is not active and will not process procaspase-3.

Although procaspase-9 cleavage is not essential for its proteolytic activation, numerous cellular studies have shown that intrinsic cell death is always accompanied by caspase-9 cleavage to yield two large subunits. These are the p35 and p37 subunits which are formed *via* the initial auto-catalytic (apoptosome-dependent) at Asp^{315} (PEPD↓ATPF) and subsequent caspase-3-dependent (DQLD↓AISS) cleavage at Asp^{330} , respectively. Given that procaspase-9 does not requires cleavage to be active, then a key question is; what is the significance (if any) of these cleavage events in the execution of the caspase cascade? Current theories have focussed on the concept that caspase-9 cleavage enables the caspase to be regulated by the endogenous XIAP (X-linked inhibitor of apoptosis) protein. A seminal paper by Alnemri and colleagues showed that caspase-9 cleavage at Asp^{315} not only generates the p35 subunit, but also produces

the small subunit (p12) with a neo-N-terminal ATPF motif. [8]. This sequence shares significant homology with the N-terminal (AVPI) amino acids of Smac (second mitochondrial activator of caspases)/DIABLO and the *Drosophila* cell death proteins Hid/Grim/Reaper, thereby defining a new class of proteins with a conserved IAP binding motif (IBM). The IBM of caspase-9 binds to a groove on the surface of the third BIR (baculovirus inhibitory repeat) domain of XIAP. This IBM hook stabilizes a further interaction between the caspase-9 dimer interface and a second binding patch on the BIR3 domain of XIAP [5]. These interactions result in a catalytically incompetent conformation at the active site of caspase-9 which inhibits the caspase processing activity of the apoptosome holoenzyme complex. SMAC which is also released from the mitochondria has a higher binding affinity for the BIR3 IBM groove and competitively displaces XIAP from caspase-9 [9]. These studies have led to the idea that caspase-3 cleavage of procaspase-9 is a positive feedback mechanism whereby endogenous XIAP inhibition of apoptosome-activated caspase-9 is relieved by active caspase-3, which is produced as a result of the caspase processing activity of the apoptosome.

However, a study using recombinant proteins to reconstitute the apoptosome complex provided controversial evidence that the p37/p10 form of caspase-9 could also be inhibited by XIAP, *via* a novel AISS IBM motif which is exposed on the p10 subunit [10]. This study also reported that caspase-9 had to be cleaved at Asp^{330} for maximum activity and thus suggests that caspase-9 activity is regulated by XIAP at both cleavage sites. The paper by *Denault et al* which appears in this issue of the *Biochemical Journal* describes experiments which are aimed at resolving these apparent contradictions. They have used biotinylated-EVD-aomk (bEVD-aomk) as an active site label to trap and to immuno-precipitate active caspases-9 and -7. The rationale behind this approach is that bEVD-aomk will covalently label and tag the active site cysteine of the large subunit of an caspase. Thus, the authors show that in dATP/cytochrome c activated cell free lysates the large subunit of caspase-7 is only generated when the p35 subunit of caspase-9 is labelled (i.e., catalytically active). The p37 form of caspase-9, as prepared by incubating the cell lysates with recombinant active caspase-3 is not labelled with bEVD-aomk and significantly this form of caspase-9 in the absence of apoptosome formation does not process procaspase-7 to the large subunit.

Taken together the findings of *Denault et al* agree with other studies, for example with caspase-3 null MCF-7 cell lysates and recombinant apoptosome complexes which show that only

caspase-3 and not caspase-7 produces the p37 form of caspase-9 [1]. However, the current study clearly consolidates the view that the most active form of apoptosome bound caspase-9 contains either the p35 or p37 subunit in conjunction with the p10 subunit. Caspase-3 by removing the ATPF containing linker peptide facilitates the activation of caspase-9, thereby allowing more procaspase-3 to be cleaved and activated. This point is emphasised in the current study which used isothermal calorimetry to show that XIAP has low affinity (K_D approx 80 μM) for an AISS containing peptide, but high affinity for an ATPFQEG (K_D = 322 nM) and AVPIAQK (Smac-like, K_D = 308 nM) peptides. These data indicate that XIAP preferentially targets the p12 (ATPF containing) and not the p10 (AISS containing) subunit of caspase-9. This was confirmed by depleting cell free lysates of endogenous caspase-9 and then adding back various cleavage mutants of caspase-9. The authors showed that although the $C9^{ATPF}$ mutant supports a reduced apoptosome caspase-activating activity, it needs Smac (XIAP binding protein) to reach maximal activity. Conversely, $C9^{AISS}$ and double cleavage mutants on are more active at processing procaspase-3, but are only minimally stimulated by the presence of Smac. Thus, the authors have convincingly shown that p35/p12 form of caspase-9 is more potently inhibited by XIAP, which must be displaced from caspase-9 to enable the apoptosome holoenzyme complex to achieve full activity. The data also show that although, the p35/p10 form of caspase-9 is very active it binds XIAP less efficiently.

A fundamental question; is what is the optimal or predominant catalytic form of caspase-9 in an apoptotic cell? Unfortunately, this question has not been resolved unequivocally as the currently available antibodies only recognise the p37 and p35 forms of caspase-9 and do not detect the p10 and p12 small subunits. Typically, cells undergoing apoptosis via the mitochondrial (intrinsic) pathway contain both the p37 and p35 subunits. Thus, caspase-9 must be present either as the p37/p10 or as either the p35/p12 and/or p35/p10 form. Studies with recombinant proteins have shown that apoptosome bound p37/p10 and the p35/p10 forms of caspase-9 efficiently cleave Ac-LEHD.amc, a fluorescent peptide often used to measure apoptosome activity [10]. Thus, both forms of caspase-9 can contribute to the caspase-activating activity of the apoptosome complex and although this conclusion is based on studies with a small peptide substrate and not the intact procaspase-3 protein, it does suggest that the p10 subunit is the key factor. In this context, *Denault et al* argue that the major if not the sole purpose of caspase-3-mediated cleavage of caspase-9 is to remove the IBM and thereby relieve XIAP

inhibition. Furthermore, they also suggest that this is not a feedback amplification mechanism and would be better classified as a derepression, rather than an amplification mechanism. This is a moot point, but the current study is valuable in that it consolidates the original suggestions of Shiozaki et al [5] which highlighted the importance of the ATPF motif.

So in answer to the question, 'caspase-9 cleavage, do you need it ? the answer is most certainly yes. Caspase-9 cleavage allows the initiation of the caspase cascade to be very tightly controlled and both cleavage events are important. The first cut at Asp^{315} enables XIAP to inhibit the caspase processing activity of the apoptosome. XIAP inhibition is relieved by anti-apoptotic proteins like Smac which allows caspase-9 to process procaspase-3, which in turn cleaves caspase-9 at Asp^{330} , removing the IBM motif from the small subunit. The resultant form of caspase-9 is now essentially insensitive to XIAP inhibition and thus the second cleavage removes the last brake on the caspase cascade. Finally, it is often stated that caspase-9 cleavage is not required for its activation, and undoubtedly this is correct. However, the current study and other work in the literature clearly show that caspase-9 cleavage is not by-stander event but is an integral and essential mechanism for initiating and regulating the caspase cascade.

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