COMMENTARY

Caspase-9 cleavage, do you need it?

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Caspase-9, which is activated by association with the Apaf-1 (apoptotic protease-activating factor-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 (X-linked inhibitor of apoptosis) inhibition of the apical caspase.

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

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 that 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 activates Apaf-1 (apoptotic protease-activating factor-1). This large (~130–140 kDa) protein is a mammalian homologue of CED-4, an essential protein involved in *Caenorhabditis 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 holoenzyme 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 (death-inducing signalling 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], whereas 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, this 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 autocatalytic (apoptosome-dependent) cleavage at Asp³¹⁵ (PEPD ↓ ATPF) and subsequent caspase-3-dependent (DQLD ↓ AISS) cleavage at Asp³³⁰ respectively. Given that procaspase-9 does not require 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 focused 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 [8] showed that caspase-9 cleavage at Asp³¹⁵ not only generates the p35 subunit, but also produces the small subunit (p12) with a neo-N-terminal ATPF (Ala-Thr-Pro-Phe) motif. This sequence shares significant homology with the N-terminal (AVPI) amino acids of Smac (second mitochondrial activator of caspases)/DIABLO [direct IAP (inhibitor of apoptosis protein)-binding protein with low pI] and the *Drosophila* cell death proteins Hid/Grim/Reaper, thereby defining a new class of proteins with a conserved IBM (IAP-binding motif). 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

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p37/p10 form of caspase-9 could also be inhibited by XIAP via a novel N-terminal IBM motif (AISS), which is exposed on the p10 subunit [10]. This study also reported that caspase-9 had to be cleaved at Asp³³⁰ for maximum activity, and thus suggested that caspase-9 activity is regulated by XIAP at both cleavage sites. The paper by Denault et al. [11] which appears in this issue of the *Biochemical Journal* describes experiments which are aimed at resolving these apparent contradictions. They have used bEVD-aomk (biotinylhexanoyl-Glu-Val-Asp-acyloxymethane) as an active-site label to trap and to immunoprecipitate active caspases-9 and -7. The rationale behind this approach is that bEVD-aomk will covalently label and tag the active-site cysteine residue of the large subunit of a 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. [11] 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 study by Denault et al. [11] 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 emphasized in the study by Denault et al. [11], which used isothermal calorimetry to show that XIAP has low affinity (K_d approx. 80 μ M) for an AISS-containing peptide, but high affinity for ATPFQEG (K_d = 322 nM) and AVPIAQK (Smac-like; K_d = 308 nM) peptides. These results 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 [11] 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 are more active at processing procaspase-3, but are only minimally stimulated by the presence of Smac. Thus the authors [11] have convincingly shown that the 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 results 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 recognize 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 the p35/p12 and/or p35/p10 form. Studies with recombinant proteins have shown that apoptosome-bound p37/p10 and p35/p10 forms of caspase-9 efficiently cleave Ac-LEHD-AMC (*N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin), 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. [11] 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 study by Denault et al. [11] 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 Asp³¹⁵ enables XIAP to inhibit the caspase-processing activity of the apoptosome. XIAP inhibition is relieved by anti-apoptotic proteins such as Smac, which allow caspase-9 to process procaspase-3, which in turn cleaves caspase-9 at Asp³³⁰, 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 study by Denault et al. [11] and other work in the literature clearly show that caspase-9 cleavage is not a 'bystander event', but is an integral and essential mechanism for initiating and regulating the caspase cascade.

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