Caspase 3 attenuates XIAP (X-linked inhibitor of apoptosis protein)-mediated inhibition of caspase 9

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During apoptosis, the initiator caspase 9 is activated at the apoptosome after which it activates the executioner caspases 3 and 7 by proteolysis. During this process, caspase 9 is cleaved by caspase 3 at Asp^{330}, and it is often inferred that this proteolytic event represents a feedback amplification loop to accelerate apoptosis. However, there is substantial evidence that proteolysis *per se* does not activate caspase 9, so an alternative mechanism for amplification must be considered. Cleavage at Asp^{330} removes a short peptide motif that allows caspase 9 to interact with IAPs (inhibitors of apoptotic proteases), and this event may control the amplification process. We show that, under physiologically relevant conditions, caspase 3, but not caspase 7, can cleave caspase 9, and this does not result in the activation of caspase 9. An IAP antagonist disrupts the inhibitory interaction between XIAP (X-linked IAP) and caspase 9, thereby enhancing activity. We demonstrate that the N-terminal peptide of caspase 9 exposed upon cleavage at Asp^{330} cannot bind XIAP, whereas the peptide generated by autolytic cleavage of caspase 9 at Asp^{315} binds XIAP with substantial affinity. Consistent with this, we found that XIAP antagonists were only capable of promoting the activity of caspase 9 when it was cleaved at Asp^{315}, suggesting that only this form is regulated by XIAP. Our results demonstrate that cleavage by caspase 3 does not activate caspase 9, but enhances apoptosis by alleviating XIAP inhibition of the apical caspase.

Key words: apoptosis, caspase 3, caspase 9, feedback loop, second mitochondrial activator of caspases (Smac), X-linked inhibitor of apoptosis protein (XIAP).

INTRODUCTION

Apoptosis is the main route by which animals eliminate unwanted cells. It is co-ordinated by the activity of a family of proteases named caspases [1]. The activation of apoptotic caspases is, at minimum, a two-step signalling pathway in which a sub-group of caspases (the initiators; caspases 8, 9 and 10) is activated by dimerization on oligomeric complexes [1] and subsequently cleaves and activates the executioner caspases 3 and 7 [2]. Executioner caspases exert their activity on a limited range of substrates [3,4] including initiator caspases. For example, during apoptosis, caspase 6 cleaves the initiator caspase 8 [5], whereas caspase 3 cleaves caspase 9 [6].

Cleavage of initiator caspases by the executioners has led to a state of confusion in the field where feedback/amplification mechanisms were proposed without a clear explanation as to their nature and role. Although cleavage of initiator caspases 8 and 9 does not result in their activation *in vitro* [7], it could provide a greater stability of the activated dimer [8]. Importantly, cleavage of initiator caspases by executioner caspases is often interpreted as a direct activating event, suggesting that executioner caspases could be an initiating point of an apoptotic process [5,9].

One of the pathways leading to the activation of executioner caspases, the intrinsic pathway, is integrated at the mitochondrion by the release of cyt *c* (cytochrome *c*) [10]. Cytosolic cyt *c* evokes the oligomerization of Apaf-1 (apoptotic protease activating factor 1) into a heptameric apoptosome that recruits monomeric caspase 9 and dimerizes it [11,12]. Once active, caspase 9 cleaves itself at Asp^{315}, generating a neo-N-terminal sequence: ATPF-. In the crystal structure of caspase 9 in complex with its endogenous inhibitor, XIAP (X-linked inhibitor of apoptosis protein), this neo-N-terminal extension makes contact with a groove found on the surface of the BIR3, the third BIR (baculovirus inhibitory repeat) domain of XIAP [13]. This interaction is crucial to anchor the inhibitor on to the caspase. Many members of the IAP (inhibitor of apoptosis protein) family are capable of binding amino acid sequences similar to that of the neo-N-terminus of caspase 9, most of which are characterized as having an alanine residue in the first position and often a proline residue in the third position. Thus these sequences have been commonly denoted as IBMs (IAP-binding motifs; reviewed in [14,15]). Smac (second mitochondrial activator of caspases)/DIABLO (direct IAP-binding protein with low pI) [16–18] possesses an IBM at its mature N-terminus [AVPI- (Ala-Val-Pro-Ile-)]. The mature forms of these proteins are released from the mitochondrion during the initiation phase of apoptosis. Once in the cytosol, these proteins are thought to relieve the inhibition of caspase 9 by XIAP–BIR3 domains; this, in turn, allows this caspase to cleave and activate the executioner caspases. In addition, Smac is capable of targeting the BIR2 domain of XIAP which inhibits the executioner caspases 3 and 7. It is clear that Smac derepresses caspase inhibition by XIAP in *in vitro* assays and cell-based systems [16–18], and Smac mimetics potentiate death stimuli [19–21]. Intriguingly, Smac [22] knockout mice lack an overt anti-apoptotic phenotype, a point that remains puzzling.

Abbreviations used: ABP, affinity-based probe; Afc, 7-amido-4-fluoromethylcoumarin; Apaf-1, apoptotic protease activating factor 1; AVPI, Ala-Val-Pro-Ile; bEVD-aomk, biotinylhexanoyl-Asp-Glu-Val-acyloxymethane; BIR, baculoviral inhibitory repeat; CARD, caspase recruitment domain; cyt *c*, cytochrome *c*; DTT, dithiothreitol; His_6, hexahistidine; HRP, horseradish peroxidase; IAP, inhibitor of apoptosis protein; IBM, IAP-binding motif; ICAD, inhibitor-of-caspase-activated DNAse; ITC, isothermal titration calorimetry; MVPI, Met-Val-Pro-Ile; pNA, *p*-nitroanilide; Smac, second mitochondrial activator of caspases; XIAP, X-linked IAP; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethane.

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Early reports by Srinivasula and colleagues [23] showed, using pull-down assays and deficient mouse cell-free extracts reconstituted with Apaf-1, caspase 9 and XIAP–BIR3, that caspase 9 interacts with XIAP-BIR3 by virtue of its small subunit processed at Asp^{315} but not at Asp^{330}. Later, Zou and colleagues [24] concluded the contrary, using a purely recombinant system, proposing that cleavage of caspase 9 by caspase 3 does not abrogate the inhibition by XIAP's BIR3 domain. This was quite surprising, because the neoepitope generated (AISS-) lacks the critical proline residue common to most IBM-containing proteins, although the crucial N-terminal alanine residue is conserved. Hence the effect of proteolytic processing at the second site in the caspase 9 linker region remains elusive.

In the light of these two contradictory studies, we sought to dissect the consequences of cleavage of caspase 9 by the executioner caspases and how this affects the regulation of the former by XIAP and Smac. Our working hypothesis is that cleavage of caspase 9 by caspase 3 does not directly activate it, but rather removes the peptide required for tight XIAP binding and thereby prevents inhibition of caspase 9. Thus we postulate that caspase 3 can mediate feedback amplification by removing inhibition and not by direct caspase 9 activation. We tested this hypothesis through a combination of biophysical measurements of protein interactions and by reconstituting caspase 9 activation in a cell-free system incorporating specific recombinant proteins.

EXPERIMENTAL

Cell culture and reagents

The human embryonic kidney 293A cell line (293Ad; MP Biomedicals, Solon, OH, U.S.A.) was propagated in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA, U.S.A.) supplemented with 10 % (v/v) fetal bovine serum (Atlas Biologicals, Fort Collins, CO, U.S.A.), 2 mM L-glutamine (Invitrogen Corp., Carlsbad CA), and antibiotics. Horse cyt *c*, dATP, and various other chemicals were from Sigma–Aldrich (St. Louis, MO, U.S.A.). AcDEVD-pNA (acetyl-Asp-Glu-Val-Asp *p*-nitroanilide) was from Bachem (King of Prussia, PA, U.S.A.) whereas AcDEVD-Afc (AcDEVD-7-amido-4-fluoromethylcoumarin) and AcLEHD-Afc (acetyl-Leu-Glu-His-Asp-Afc) were from MP Biomedicals.

bEVD-aomk (biotinylhexanoyl-Asp-Glu-Val-acyloxymethane; KMB01) was generously provided by Dr Matthew Bogyo (Stanford Comprehensive Cancer Center, Stanford, CA, U.S.A.). Smac heptamer peptide (AVPIAQK) was a gift of Dr J. Reed at this Institute, whereas caspase 9 linker peptides were synthesized, then purified by HPLC by Dr Fernando Ferrer, also of this Institute.

Recombinant protein expression and purification

Full-length caspase 9, the single mutants D315A and D330A and the double mutants (DD → AA) were described elsewhere [7]. AVPI-Smac (Ala-Val-Pro-Ile-Smac) and MVPI-Smac (Met-Val-Pro-Ile-Smac) were described previously [25]. Wild-type caspase 3 and caspase 7 and all other hexahistidine (His_6)-tagged proteins were expressed in *Escherichia coli* BL21(DE3) strain (Novagen, San Diego, CA, U.S.A.), purified on Ni^{2+}-charged chelating Sepharose (GE Healthcare, Piscataway, NJ, U.S.A.), and eluted using an imidazole gradient. To produce caspase 9 processed at Asp^{330}, we expressed the full-length caspase 9 D315A mutant [7] and mixed the initial bacterial lysate with 0.5 vol.-equiv. of lysate from bacteria expressing untagged wild-type caspase 7. The lysate was made 4 mM with respect to 2-mercaptoethanol to promote caspase activity and the His_6-tagged caspase 9 protein was purified as described above. The caspase 9 mutant D315A/caspase 7 ratio was optimized so that greater-than-95 % processing at Asp^{330}, and no undesired processing event, occurred. XIAP–BIR3 (residues 252–348) was expressed as an N-terminal His_6-tagged protein. All protein concentrations were initially determined using the Edelhoch relationship [26], and active enzymes were active-site-titrated to determine the exact active enzyme concentration.

Caspase titration and enzymatic assays

All caspases were active-site-titrated using the irreversible caspase inhibitor Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethane). Caspases 3 and 7 were titrated as described in [27]. Caspase 9 was titrated as followed. The enzyme was diluted to ~200 nM (based on protein) in 1 M sodium citrate assay buffer [50 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 1.0 M sodium citrate, 1 % sucrose, 0.05 % CHAPS and 10 mM DTT (dithiothreitol)] and incubated at 22 °C for 30 min to allow complete dimerization. An equal volume of Z-VAD-fmk (0–1000 nM) diluted in the same buffer was added to the enzyme and incubated for an additional 30 min at 37 °C. The residual enzymatic activity was measured in 1 M sodium citrate assay buffer at a final protein concentration ~40 nM by the addition of the fluorescent substrate AcLEHD-Afc. Assays for caspases 3 or 7 were done in low-salt buffer [10 mM Pipes, pH 7.2, 100 mM NaCl, 10 % (w/v) sucrose, 0.1 % CHAPS, 10 mM DTT, and 1 mM EDTA] and activity was monitored with 100 μM AcDEVD-Afc ($\lambda_{excitation} = 405$ nm; $\lambda_{emission} = 510$ nm) in an FMAX fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). All caspases titrated between 50 and 100 % of the estimated protein concentration, suggesting that most, if not all, the caspase was active in each preparation.

Cell-free extracts preparation, depletion and activation

The 293A cell extracts were prepared in a hypo-osmotic buffer (20 mM Pipes, pH 7.4, 20 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$ and 2 mM DTT) as described previously [10,28] and kept at −80 °C. Caspase 9 depletion was done using rabbit anti-(caspase 9) antibodies coupled to Protein A–agarose beads using the dimethyl pimelimidate method [29]. The depletion efficiency was tested in an activation assay for the lack of AcDEVD-pNA hydrolysis above the background level. All added proteins were diluted in 50 mM Tris base, pH 7.4, and 100 mM NaCl unless otherwise mentioned. A typical activation reaction was assembled as followed: 40 μl of cell-free extract, 2 μl of caspase proteins, 1.5 μl of Smac or XIAP–BIR3, 2 μl of 10 mM AcDEVD-pNA and 1.5 μl of cyt *c*/dATP mixture, always added last (0.45 μl of 100 μM cyt *c* and 0.45 μl of 100 mM dATP in water). AcDEVD-pNA hydrolysis was monitored in a SpectraMax 390 microplate reader (Molecular Devices) at 405 nm. For the time course of caspase processing, 5 μl of the reaction mixture was transferred to a new tube containing 5 μl of labelling mixture (20 μM bEVD-aomk in hypo-osmotic buffer) and was further incubated for 30 min at 37 °C. All reactions were stopped at the indicated time point by the addition of a 0.5 vol. of a 3-fold-concentrated solution of SDS/PAGE loading buffer.

ITC (isothermal titration calorimetry)

The Micro Calorimetry System (MicroCal, Northampton, MA, U.S.A.) was used to perform the ITC measurements to follow the protein–protein interactions. All purified protein and peptides were dialysed or prepared in 50 mM sodium phosphate buffer, pH 7.5, and titration experiments were performed at 23 °C. Each peptide ligand was injected into the 1.5 ml sample cell containing

either protein or buffer alone in 2–6 μl volumes at 4 min intervals. Approx. 20–45 injections were titrated for each measurement. The titration data were analysed using the ORIGIN data analysis software (MicroCal). The heat of dilution obtained from injecting a ligand into buffer was subtracted before the fitting process. Non-constraint fitting was performed where the directly measured heat changes occurring after addition of small volumes of each ligand permit extraction of the enthalpy (ΔH), the binding affinity (K_a) and the stoichiometry of the interaction (N). The accuracy of each ΔH, K_a and N determination was estimated from the fitting error of non-linear least squares. The remaining thermodynamic parameters, including ΔG (change in Gibbs free energy) and ΔS (entropy) of the interaction were calculated using the relationship:

$$\Delta G = \Delta H - T\Delta S = -RT \cdot ln\, K_a \quad (1)$$

where T is the absolute temperature and R is the gas constant.

SDS/PAGE, immunoblotting and biotin detection

SDS/PAGE was peformed using gradient (8–18 %, w/v) acrylamide gels in the 2-amino-2-methylpropan-1,3-diol/glycine buffer system [30]. Gels were either stained using GelCode Blue (Pierce Chemical Co., Rockford, IL, U.S.A.) or electroblotted to Immobilon-P membrane (Millipore, Billerica, MA, U.S.A.) using 10 mM Caps, pH 11, and 10 % (v/v) methanol for 45 min at 0.4 A and at 4 °C. Immunoblots were carried out using standard protocols with the following antibodies: anti-(caspase 3) antibody (0.02 μg/ml; H-277; Santa Cruz), anti-(caspase 7) (1:2500; 9492; Cell Signaling), anti-(caspase 9) (1:10000; a gift from Dr Douglas R. Green, Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, U.S.A.), and ICADs (inhibitor-of-caspase-activated DNAses; 1:6000; PX023 and PX024; Cell Science). Streptavidin–HRP (horseradish peroxidase, 0.2 μg/ml; Sigma–Aldrich) was used to reveal biotinylated proteins. All blots were revealed with WestPico SuperSignal (Pierce Chemical Co.).

RESULTS

Caspase 3-mediated cleavage of caspase 9 does not result in its activation

Recombinant caspase 9 is not activated by proteolysis, but rather by dimerization [14,31]. We explored the consequences of caspase 9 cleavage by employing cell-free extracts that can be programmed to recapitulate apoptosis *in vitro* by adding cyt *c* and dATP [10,28]. Induction of cell-free apoptosis causes a fast, robust and reproducible activation of caspase 9, followed by executioner caspase 3. Cleavage of caspase 9 from its full-length form is observed after the addition of cyt *c* or a low concentration of recombinant caspase 3, and the sizes of resultant caspase 9 fragments are in agreement with autoprocessing at Asp^{315} (p35) or cleavage at Asp^{330} (p37) by caspase 3 (Figure 1). Further trimming of caspase 9's CARD (caspase recruitment domain) occurs later and is seen only after all executioner caspases are activated (results not shown). Therefore, caspase 9 lacking the CARD cannot be recruited at the apoptosome and it remains inactive. If caspase 3 participated in a feedback/amplification loop by cleaving caspase 9, we should detect active caspase 9 after the addition of caspase 3 to the extract. We used the ABP (affinity-based probe) bEVD-aomk [32] to selectively label the active caspases. Labelled proteins were captured using streptavidin beads and their identity was revealed by immunoblotting. We found that only the addition of cyt *c*/dATP, and not caspase 3, resulted in the labelling of caspase 9 (and downstream caspase

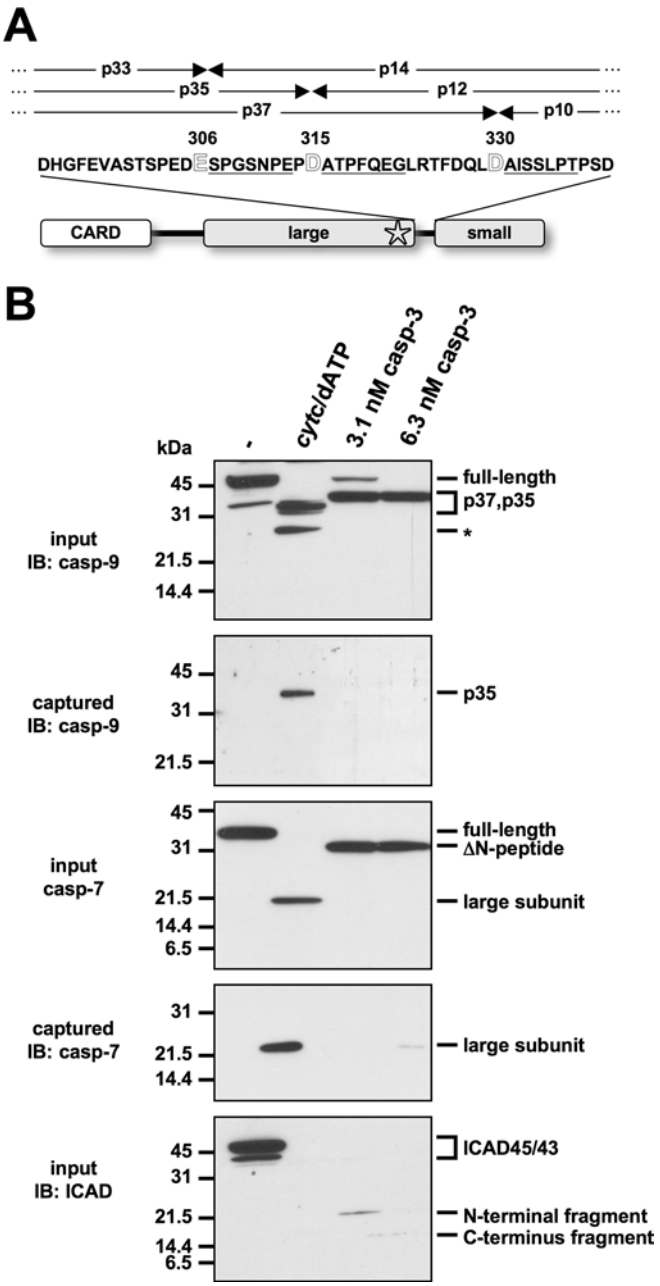


Figure 1 Cleavage by caspase 3 does not activate caspase 9

(**A**) Schematic representation of human caspase 9 with the cleavage sites (Asp^{315} and Asp^{330}) and the atypical cleavage site Glu^{306}. The various forms of the large and small subunits produced by those cleavage sites are presented. (**B**) Cell-free extracts were left untreated or treated for 1 h with cyt *c*/dATP or the indicated concentration of active-site-titrated recombinant caspase 3. Samples were then incubated with 10 μM bEVD-aomk for 30 min to label active caspases. Labelled proteins were captured with streptavidin–agarose beads. The input (10 %) and captured material (equivalent to 20 % of input) was analysed by immunoblotting (IB) using anti-(caspase 9), anti-(caspase 7) or anti-ICAD antibodies. Caspase 7 processing was used to monitor caspase 9 activity. The asterisk most likely indicates the large subunit of caspase 9 lacking the CARD domain. The poor detection of ICAD cleavage products is most likely due to proteasomal degradation of the caspase cleavage products in the extract and/or the transfer conditions used.

7 activation), despite the complete cleavage of caspase 9 by 6.3 nM active caspase 3 (Figure 1). CARD-less caspase 9 is not labelled by the ABP, in agreement with its generation outside the apoptosome, where it is inactive. At the concentration of caspase 3 used, the N-peptide of caspase 7, which is a *bona fide* substrate of caspase 3 [33], is completely cleaved. Removal of this segment

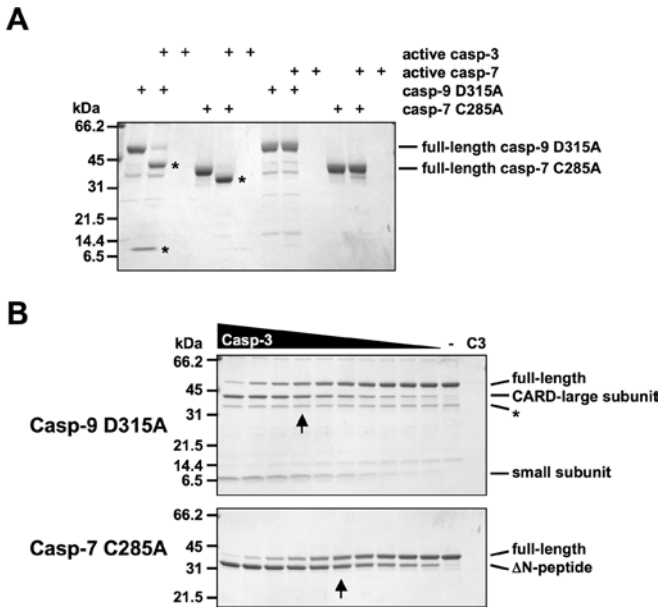


Figure 2 Caspase 3, but not caspase 7, cleaves caspase 9

(**A**) Recombinant caspase 9 D315A or caspase 7 C285A at 2 μM was incubated in assay buffer alone or with 100 nM of active-site-titrated caspase 3 or 7 for 1 h at 37 °C. The asterisk indicates cleavage products. (**B**) Recombinant caspase 9 D315A (top panel) or caspase 7 catalytic mutant (C285A, bottom panel) at 2 μM was incubated with serial 2/3-fold dilutions (100 nM down to 2.6 nM) of active-site-titrated caspase 3 as in (**A**). A sample without caspase 3 (–) and one with caspase 3 only (C3) at the highest concentration used was also included as controls. Approximately twice as much caspase 3 was required to cleave caspase 9 D315A compared with caspase 7 C285A. The asterisk indicates inactive caspase 9 processed at Glu^{306}. Gels were stained with GelCode Blue.

does not result in the activation of the protease, as demonstrated by the lack of labelling by the ABP (Figure 1). Nevertheless, this event is a prerequisite for the efficient activation of caspase 7 in some cell lines [33]. In contrast with the addition of caspase 3 to the extract, cyt *c* addition resulted in strong labelling of caspase 7's large subunit, in accordance with the ability of apoptosome-bound caspase 9 to activate caspase 7 in this cell-free system [10,28]. Finally, ICAD, another putative caspase 3 substrate, is readily cleaved under both conditions. This demonstrates that the proteolytic activity of caspase 3 on caspase 9 in the absence of apoptosome formation does not result in activation of the apical caspase.

Caspase 3, but not caspase 7, cleaves caspase 9

Because caspases 3 and 7 share the same preference for synthetic substrates [34,35], we compared the ability of each enzyme to cleave caspase 9 in a purified system. The removal of the caspase 7 N-peptide by caspase 3 is a critical event for caspase 7 activation *in vivo* [33,36], and we employed this event to compare the efficacy of cleavage. We found that, for the same concentration of active caspase (50 nM), only caspase 3 was capable of cleaving caspase 9 and caspase 7's N-peptide (Figure 2A). These assays were performed at a caspase 7 concentration that is higher than the maximal expected cytosolic concentration [37], demonstrating that the two substrates are specific to caspase 3.

For the removal of the caspase 9 IBM to be a biologically relevant event, it must be cleaved with an efficacy similar to, or greater than, that of other death substrates found in the cytosol. We sought to determine the efficiency of cleavage of caspase 9 by caspase 3. The D315A caspase 9 mutant or the C285A caspase 7 mutant was incubated with a range of caspase 3 concentrations and the cleavage products were observed by SDS/PAGE (Figure 2B). Importantly, caspase 3 cleaved caspase 9 with an efficiency similar to that of the N-peptide of caspase 7, a known physiological caspase 3 substrate. This suggests that the generation of caspase 9 cleaved at Asp^{330} is highly probable during apoptosis.

Smac relieves the XIAP-mediated inhibition of endogenous caspase 9

We implemented the use of the IAP antagonist Smac to determine whether XIAP was inhibiting endogenous caspase 9 in cell-free extracts. We added recombinant mature (AVPI-Smac) or a mutant IAP-binding and incompetent (MVPI-Smac) Smac protein [25] to the cell-free extracts (Figure 3A). The Smac concentration used (50 nM) was optimized to maximize XIAP derepression and minimize non-specific effects of the mutant control Smac (see Supplementary Figure 2C at http://www.BiochemJ.org/bj/405/bj4050011add.htm) [38]. This concentration is below the cellular levels found in 293A cells (Supplementary Figures 2A and 2B).

We monitored the cleavage of pro-(caspase 3) and pro-(caspase 7) over time after the addition of cyt *c*/dATP (Figure 3B). Relieving XIAP inhibition of caspase 9 should result in faster activation of executioner caspase zymogens. Indeed, executioner caspase activation was hastened in the presence of AVPI-Smac, as demonstrated by ABP labelling (Figure 3B) and by the hydrolysis of AcDEVD-pNA chromogenic substrate (Figure 3C). In the latter case the control MVPI-Smac was identical with the sample without added Smac protein, whereas AVPI-Smac shifted the trace leftward, indicating enhanced caspase activity. In the absence of cyt *c*/dATP, Smac proteins did not generate a significant amount of executioner caspase activity (Figure 3C), although some cleavage of caspase 9 was observed after 4 h of incubation (Figure 3B), probably reflecting the presence of a trace amount of processed caspase 3. These studies demonstrate that endogenous XIAP regulates caspase 9 in our cell-free system.

Caspase 9 cleaved at Asp^{330} is not inhibited by XIAP–BIR3

XIAP–BIR3 binds caspase 9 using two sites [13], one of which is the N-terminus of the caspase 9 small subunit. The second site is the dimerization interface of caspase 9. The major conflict between the studies of Srinivasula and colleagues [23] and Zou and colleagues [24] is whether this N-terminus retains affinity for XIAP–BIR3 when produced by the cleavage at Asp^{330}. If it were true, there would not be a stark difference in affinity for short peptides derived from the respective cleavage sites. To test this we employed ITC to measure the binding affinities of the caspase 9 linker-derived peptides and Smac peptide towards XIAP–BIR3 (residues 252–348) (Figure 4). We tested heptameric peptides corresponding to P1′–P7′ from the cleavage site at Asp^{315} (ATPFQEG), Asp^{330} (AISSLPT) and also from the aberrant cleavage site at Glu^{306} (SPGSNPE) and compared the thermodynamic parameters with those ontained with the well-characterized Smac peptide Smac7 [16]. The heat released during the titration of peptides into a XIAP–BIR3 solution exhibited properties consistent with a single-site binding interaction as determined by stoichiometric (*N*) analysis. Table 1 summarizes the thermodynamic parameters of the interaction between XIAP–BIR3 with the various peptides.

The affinities of the caspase 9 ATPFQEG peptide and the Smac peptide towards XIAP–BIR3 were comparable (ATPFQEG, $K_d = 322$ nM; Smac, $K_d = 308$ nM) (Figures 4A and 4D). On the other hand, the AISSLPT peptide ($K_d = 80$ μM) exhibited an affinity to XIAP–BIR3 that is approx. 250-fold weaker than that

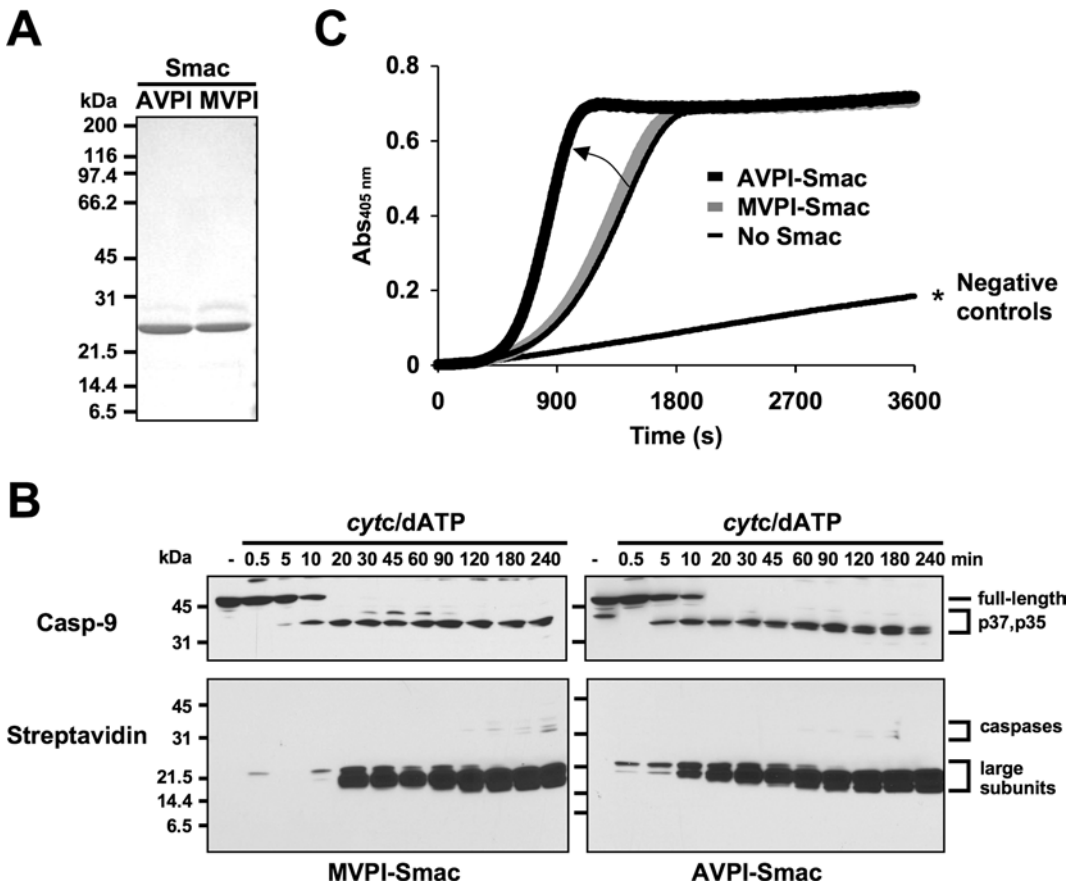


Figure 3 Smac de-represses XIAP inhibition in the cell-free extract assay

(**A**) GelCode Blue-stained gel showing the purified AVPI-Smac (mature) and MVPI-Smac (control) proteins used. (**B**) Time course of cell-free extracts activation in the presence of 50 nM MVPI-Smac (left panels) or AVPI-Smac (right panels). Cell-free extracts were left untreated or were activated for the indicated period of time with cyt *c*/dATP. Ensuing samples were either directly analysed by immunoblotting with the anti-(caspase 9) antiserum or further incubated for 30 min with 10 μM bEVD-aomk then analysed with streptavidin–HRP (lower panels). Each pair of immunoblots were performed, processed and developed simultaneously. (**C**) AVPI-Smac, MVPI-Smac, or buffer alone was added to cell-free extracts and caspase activation was triggered with cyt *c*/dATP. The activation of caspase 3 was monitored continuously with the chromogenic substrate AcDEVD-pNA (200 μM). Negative control (asterisk) samples comprised non-activated extracts with or without the individual Smac proteins (not all traces are presented, but all overlapped with the presented trace). The arrow indicates the effect that AVPI-Smac has on caspase activation.

for the ATPFQEG peptide (Figure 4C). Finally, the SPGSNPE peptide displayed the lowest affinity measured of all, with a K_d above 138 μM (Figure 4C). The ITC measurements showed a variable range of enthalpy (between -0.66 and -7.0 kcal/mol; 1 kcal $\equiv$ 4.181 kJ) and entropy components ($-T\Delta S$ between 1.6 and 4.2 kcal/mol) in the interaction of XIAP–BIR3 with peptides. The observed favourable enthalpies and unfavorable entropies indicate that the interaction is energetically driven by exothermic enthalpy, indicating a preponderance of polar interactions [39]. Our findings imply that interaction between XIAP–BIR3 and caspase 9 involves residues exposed after autocleavage at residue Asp^{315}. Processing at either Glu^{306} or Asp^{330} does not produce an interaction motif that would enforce caspase 9 inhibition by XIAP–BIR3. This implies that cleavage of caspase 9 by caspase 3 would indeed remove one of the two interaction sites and mitigate XIAP-mediated inhibition of caspase 9.

A direct prediction from our ITC results is that executioner caspase activation should be slower if caspase 9 retains its IBM. To test this hypothesis, we used caspase 9-depleted cell-free extracts that we reconstituted with the desired form of caspase 9 (Figure 5D). Addition of recombinant caspase 9 to depleted extracts reconstituted cyt *c*-dependent cell-free apoptosis, as the activation profile is similar to the one from undepleted extracts (Figure 5C). To avoid confusion, we define caspase 9 cleaved at Asp^{315} as caspase 9^{ATPF}, signifying that the small subunit begins with the ATPF- epitope. Similarly, caspase 9 processed at Asp^{330} is identified as caspase 9^{AISS}, with the AISS- epitope being displayed. Using active-site-titrated caspase 9 proteins (see Supplementary Figure 1), we compared caspase 9^{AISS} with caspase 9^{ATPF}. Caspase 9^{AISS} activated downstream caspases more rapidly than did caspase 9^{ATPF} (Figure 5A). We questioned why the former is a better enzyme than the latter, and we resolved this by observing the effect of the IAP antagonist Smac. Addition of AVPI-Smac shifted the activation rate of the 'slower' caspase 9^{ATPF} to the same profile as caspase 9^{AISS}. The control protein, MVPI-Smac, was unable to significantly enhance activation at concentrations up to 250 nM (see Supplementary Figure 2C). This strongly suggests that XIAP is only capable of inhibiting the autoprocessed form of caspase 9 and that this inhibition is relieved by capase-3 cleavage. When both cleavage sites were ablated by mutation (DD $\rightarrow$ AA), we observed only a minimal shift in the activation profile, whereas wild-type caspase 9 that is mainly cleaved at Asp^{315} during preparation (see Figure 5D) demonstrated a considerable shift characteristic of XIAP-mediated inhibition. However, this shift was not as dramatic as for caspase 9^{ATPF}, owing to the removal of its IBM by caspase 3 (Figure 5B). The inhibition of executioner caspases by XIAP was also likely relieved by Smac, but is assumed to be identical for all assays.

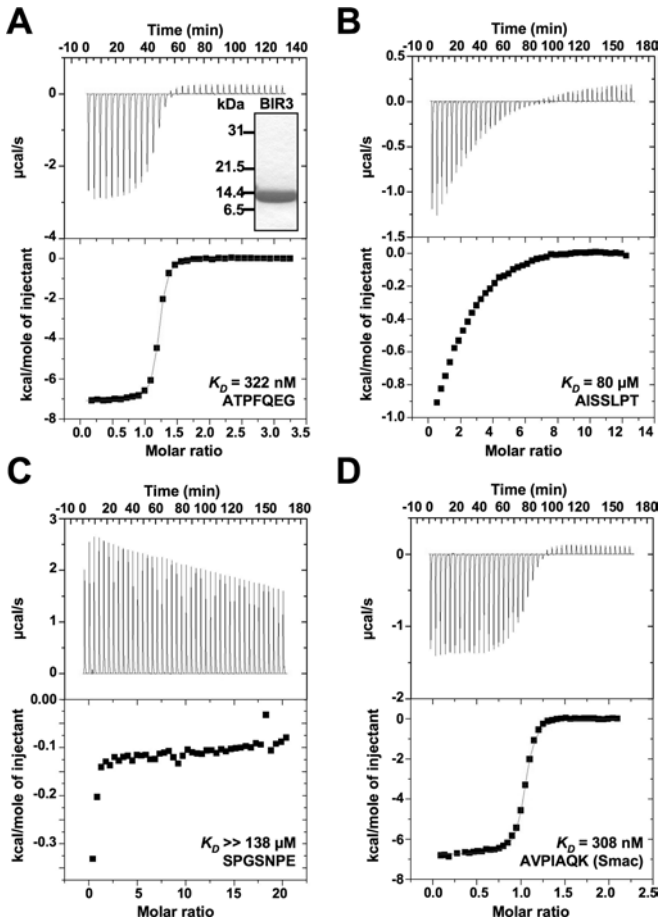


Figure 4 The new N-terminal epitope of caspase 9 generated by caspase 3 has no significant affinity for XIAP–BIR3

Representative ITC datasets of three heptameric peptides derived from the interdomain linker of caspase 9 (**A**–**C**) or Smac peptide (**D**) binding to XIAP's BIR3. Each peak corresponds to the heat release for each peptide injection (upper panels) and all peaks translate into a curve (lower panels) used to determine to thermodynamic parameters of the interaction reported in Table 1. See the Experimental section for technical details. The inset in (**A**) shows the integrity of the XIAP–BIR3 protein used.

A corollary of the above experiment is that XIAP–BIR3 should slow the activation of executioner caspases with greater potency for caspase 9^{ATPF} versus caspase 9^{AISS}. However, because caspase 9^{AISS} still has an intact dimerization interface (which constitutes the other XIAP–BIR3 binding site), we do not expect a complete resistance of caspase 9^{AISS} towards inhibition. To test this, we repeated the assay described in Figure 5(A), but we used recombinant XIAP–BIR3 instead of Smac (Figure 6). As expected, caspase 9^{ATPF} was less efficient at supporting caspase activation than caspase 9^{AISS} and the former was almost completely inhibited by 150 nM XIAP–BIR3, whereas caspase 9^{AISS} still retained a significant activation capacity. Up to 250 nM XIAP–BIR3 did not completely abolish the ability of caspase 9^{AISS} to activate downstream caspases (results not shown). Our results are consistent with the loss of one of the two binding sites of XIAP–BIR3. The dimerization interface of caspase 9 provides the remaining inhibitory activity by XIAP–BIR3.

DISCUSSION

Several hypotheses have been raised regarding the role of caspase 9 cleavage by caspase 3. These include the fact that cleavage at Asp^{330}: (1) could activate the enzyme [2]; (2) could enhance caspase 9 activity [24]; and (3) could remove the inhibition by the endogenous inhibitor XIAP [23]. As a result of this confusion, this cleavage event was purposefully ignored from recent mathematical modelling studies [40]. In the present study we designed experiments aimed at refining our understanding of the consequences of this cleavage event.

Cleavage of caspase 9

Many researchers have equated cleavage of the apical caspase 9, and of caspase 8 for that matter [5,9], to an activation event. The confusion arises from: (1) the fact that the cleavage of initiator caspases always occurs following caspase activation; (2) initial work on caspase 3 clearly demonstrated cleavage as the driving force for activation, and it was assumed that this applies to all caspases (reviewed in [41–43]). Our results demonstrated that endogenous caspase 9, in a system able to recapitulate caspase activation and death-substrate cleavage, is not activated by caspase 3 cleavage.

We demonstrated that cleavage of caspase 9 at Asp^{330} cannot be performed by caspase 7, the closest homologue to caspase 3, despite the complete overlapping substrate preference as determined using positional scanning substrate libraries [34,35], confirming previously obtained results based on caspase 7 immunodepletion experiments [2]. This exemplifies the requirement for additional determinants for substrate recognition [3]. The opposite discrimination exists for the poly(ADP-ribose) polymerase that is cleaved with much greater efficacy by caspase 7, despite the higher catalytic capacity of caspase 3 as measured on small peptidic substrates [44].

Caspase 3 removes an IBM

Using a reconstituted apoptosome-driven caspase 9 activation system, Zou and colleagues [24] found that the cleavage of caspase 9 by caspase 3 still generates a functional IBM. The neo-epitope generated as a result of cleavage at Asp^{330} is AISS, and although an alanine residue is present at the N-terminus, this epitope lacks the proline residue that is found in the third position of many IBM-containing proteins. This study also reported that

Table 1 Thermodynamic parameters in the interaction between XIAP's BIR3 with Smac and caspase 9 linker peptides as measured by ITC

N is the stoichiometry of the interaction (that is, the ligand/protein ratio).

Peptide sequence	$10^{-6} \times K_a$ (M)	K_D (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	*N*	$-T\Delta S$ (kcal/mol)	[Ligand] (mM)	[Protein] (μM)
AVPIAQK (Smac)	3.099	0.308	−8.648	−6.768	1.025	1.88	2	110
SPGSNPE	0.00723	»138	−2.258	−0.6579	1.671	1.6	10	110
ATPFQEG	3.24	0.322	−8.234	−7.059	1.175	3.78	2	110
AISSLPT	0.01156	80	−3.774	−1.421	2.353	4.155	6	110

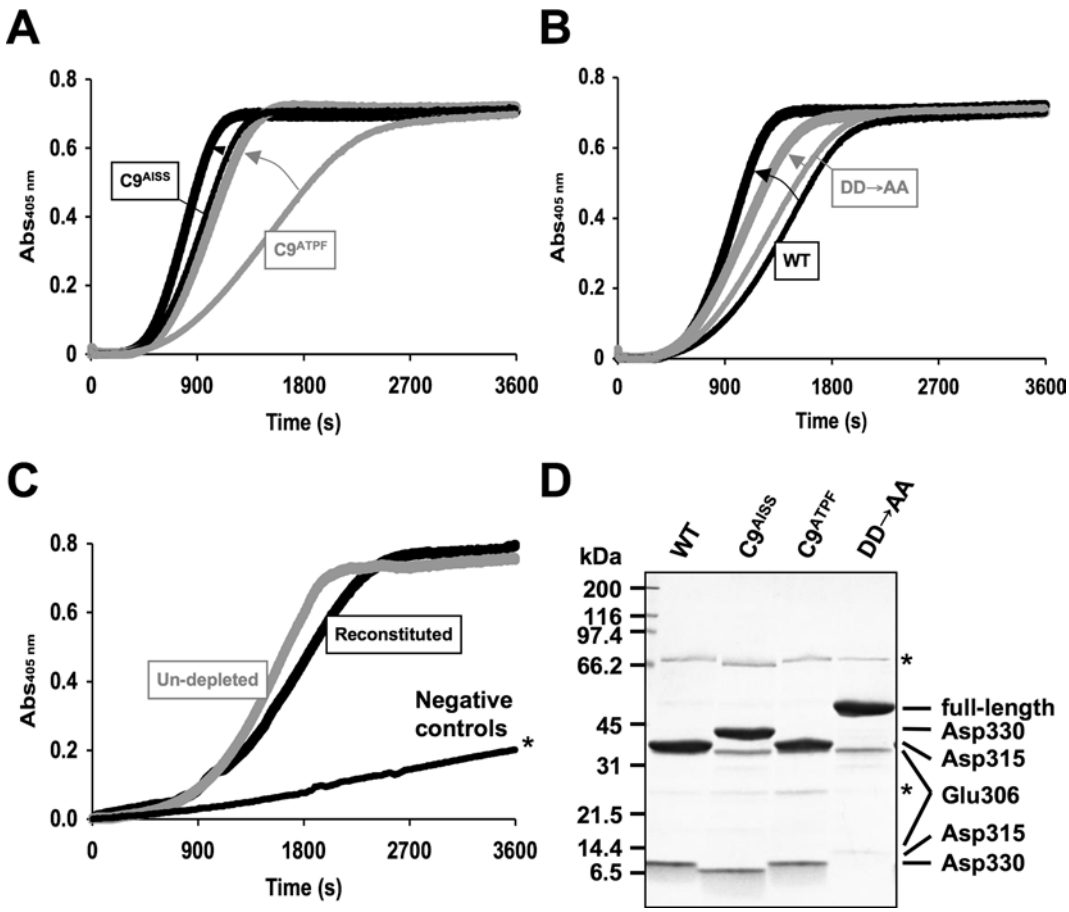


Figure 5 Smac efficiently relieves Caspase 9^{ATPF}

(**A**) Cell-free extracts depleted in caspase 9 were reconstituted (40 nM of titrated caspase 9) with recombinant caspase 9^{ATPF} (grey traces) or caspase 9^{AISS} (black traces) and activated with cyt *c*/dATP in the presence of mature Smac (AVPI-Smac; thick traces) or the control protein MVPI-Smac (thin traces). The activation of caspase 3 was monitored continuously with the chromogenic substrate AcDEVD-pNA (200 μM). See the Experimental section for technical details. (**B**) Same as in (**A**) with extracts reconstituted with wild-type caspase 9 (mainly processed at Asp^{315}) or uncleavable caspase 9 (double mutant, DD → AA). (**C**) Control for the experiment described in (**A**) and (**B**). Positive controls (thick traces): depleted extracts reconstituted with wild-type caspase 9 (grey trace) or the corresponding non-depleted extract (black trace) both activated with cyt *c*/dATP. Negative controls (asterisk, not all traces are presented but all overlapped with the presented trace): non-activated extracts (normal or depleted), non-activated extracts with Smac proteins (mature or MVPI-Smac), non-activated depleted extract with caspase 9 added, and activated depleted extract without added caspase 9. (**D**) GelCode Blue-stained gel showing the caspase 9 proteins used. Fragments are identified by the amino acid of the cleavage site that generated them. The asterisk indicates non-specific proteins. Arrows indicate the effect that AVPI-Smac has on caspase activation.

mutation of the AISS- epitope to GGSS ablated inhibition of caspase 9, demonstrating the importance of the alanine residue and suggesting that the AISS epitope is a functional IBM. These findings were in sharp contrast with the findings of earlier work demonstrating that caspase 9^{AISS} was not inhibited by XIAP–BIR3 [23]. The conflict between these previous studies was most likely due to kinetic issues. We attempted to determine the inhibitory constants (K_i) of XIAP–BIR3 on caspase 9 and realized that conditions that allow reliable kinetic analysis ($I \gg E$, where I is inhibitor concentration and E is enzyme concentration) are difficult to attain with caspase 9 because of its low intrinsic activity. Furthermore, these assays necessitated the use of micromolar amounts of caspase 9, which is well above the cellular concentration. ITC-based analysis allowed us to measure the contribution of the peptides representing the differentially processed caspase 9 linker region and thus dissect the tethering interaction of the small subunit of caspase 9 with the IBM groove of XIAP–BIR3. Our ITC results unambiguously demonstrate that the peptide corresponding to cleavage at Asp^{315} (ATPFQEG) has a much higher affinity for XIAP–BIR3 than does the peptide corresponding to cleavage at Asp^{330} (AISSLPT). Because XIAP inhibits caspases via a two-site binding mechanism [13,45], loss of one of the two binding sites (the IBM) is sufficient to weaken the interaction with caspase 9 enough to promote the activation of executioner caspases. Because the binding of XIAP–BIR3 to caspase 9's dimerization interface is the inactivating event, the IBM [13,45] acts as a tethering site, not as an inhibitor *per se*. Tethering of XIAP–BIR3 increases its affinity for caspase 9 and, by inference, the potency of the inhibitor at the dimerization interface. We conclude that caspase 3 removes the IBM (the tethering site) of caspase 9 by proteolysis at Asp^{330}, thereby denying XIAP the ability to efficiently inhibit caspase 9.

In our reconstitution assays, only caspase 9 was depleted from the extract, while the rest of the apoptotic machinery remained intact at the endogenous protein ratios (Apaf-1, XIAP and caspase 3). We used this set-up to test two direct predictions from the ITC results: (1) Smac should efficiently de-repress XIAP-mediated inhibition of caspase 9^{ATPF}, but not of caspase 9^{AISS}, as the latter should be poorly regulated by XIAP; and (2) XIAP–BIR3 should dramatically reduce the activity of caspase 9^{ATPF} only. This is effectively what we observed. Caspase 9^{ATPF} was more sensitive to both mature Smac and XIAP–BIR3 than caspase 9^{AISS}.

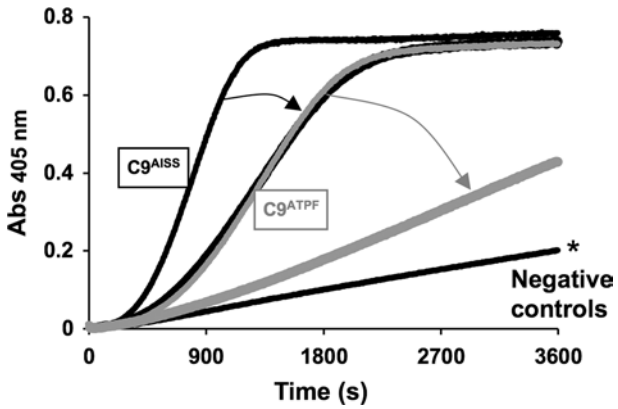
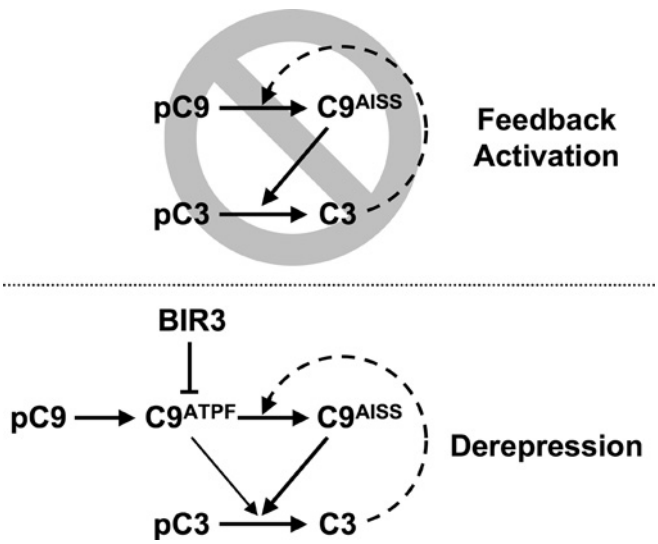


Figure 6 Caspase 9^{AISS} is poorly inhibited by XIAP's BIR3

Cell-free extracts depleted in caspase 9 were reconstituted (40 nM of titrated caspase 9) with recombinant caspase 9^{ATPF} (grey traces) or caspase 9^{AISS} (black traces) and activated with cyt *c*/dATP in the presence of 150 nM XIAP–BIR3 domain (thick traces) or buffer alone (thin traces). The activation of caspase 3 was monitored continuously with the chromogenic substrate AcDEVD-pNA (200 μM). Controls (asterisk) include non-activated and activated depleted extract (non-reconstituted). See the Experimental section for technical details. Arrows indicate the effect that XIAP–BIR3 has on caspase activation.



Scheme 1 A representation of the derepression mechanism

We contrast two models for an amplification/feedback loop created by the same cleavage of caspase 9 (C9) at Asp^{330} by caspase 3 (C3). In the top model, which we call 'Feedback activation', caspase 3 cleaves pro-caspase 9 (pC9) and directly activates it. Our data refute this model. In the lower-panel model, named 'Derepression', the cleavage of caspase 9 by caspase 3 removes the peptide containing the IBM that interacts with IAPs, thus preventing IAP-mediated inhibition of caspase 9. Our data support the latter model.

Feedback mechanisms

Our results demonstrate that caspase 3 cleavage of caspase 9 at Asp^{330} removes an IBM necessary for efficient inhibition by XIAP–BIR3, hence relieving a break from the initiator caspase and promoting further activation of executioner caspase 3 and 7. In Scheme 1 we depict the alternative mechanism described in the present study to the usually assumed feedback–amplification loop. Our data suggest that the sole role of caspase 3 cleavage of caspase 9 is to deny the ability of XIAP (or any other IAPs for that matter) to bind caspase 9, by removing its IBM. We propose the term 'derepression' to describe the removal of the IBM, because it has the same effect as the derepression exerted by Smac on XIAP [14,15], instead of the more generic term of 'amplification' that is often construed into an activation event.

Proteolytic pathways often employ feedback mechanisms to stop, modulate or accelerate the specific process in which proteases are involved, and caspase activation is no exception. In terms of the amplification of apoptotic pathways we now can recognize several levels of regulation. For example, caspase 3 can participate in an amplification loop by cleaving the proto-oncoprotein Bcl-2 (B-cell lymphoma 2) and removing its anti-apoptotic function [46,47] and possibly even by cleaving important mitochondrial proteins and driving forward a mitochondrially mediated death pathway [48]. In the present study we describe how a downstream protease within a pathway augments its activation by cleaving an upstream component of the same pathway. Amplification downstream of mitochondrial involvement does not occur by direct proteolytic activation of apical caspases. A recent theoretical model of amplification raised the hypothesis that XIAP mediates a positive feedback in which cleaved caspase 3 withdraws the inhibitor from caspase 9, hence allowing more caspase 3 activation [40]. This model was based on the assumption that binding of caspases 3 and 9 by XIAP was mutually exclusive [49]. However, the theoretical study omitted another report disproving the mutual exclusivity of XIAP binding [50], casting doubt on the validity of the model's assumptions. More likely, as we demonstrate here, amplification of apoptosis at the execution phase simply requires removal of an inhibitory interaction, and it is tempting to speculate that the Asp^{330} cleavage site, conserved in all higher vertebrates, has co-evolved with the caspase-binding IAPs as a way to regulate apoptosis.

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