Regulation of the Apaf-1/Caspase-9 Apoptosome by Caspase-3 and XIAP*

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The apoptosome is a multiprotein complex comprising Apaf-1, cytochrome c, and caspase-9 that functions to activate caspase-3 downstream of mitochondria in response to apoptotic signals. Binding of cytochrome c and dATP to Apaf-1 in the cytosol leads to the assembly of a heptamer complex in which each Apaf-1 subunit is bound noncovalently to a procaspase-9 subunit via their respective CARD domains. Assembly of the apoptosome results in the proteolytic cleavage of procaspase-9 at the cleavage site PEPD to yield the large (p35) and small (p12) caspase-9 subunits. In addition to the PEPD site, caspase-9 contains a caspase-3 cleavage site (DQLD330), which when cleaved, produces a smaller p10 subunit in which the NH2-terminal 15 amino acids of p12, including the XIAP BIR3 binding motif, are removed. Using purified proteins in a reconstituted reaction in vitro, we have assessed the relative impact of Asp315 and Asp330 cleavage on caspase-9 activity within the apoptosome. In addition, we characterized the effect of caspase-3 feedback cleavage of caspase-9 on the rate of caspase-3 activation, and the potential ramifications of Asp330 cleavage on XIAP-mediated inhibition of the apoptosome. We have found that cleavage of procaspase-9 at Asp330 to generate p35, p10 or p37, p10 forms resulted in a significant increase (up to 8-fold) in apoptosome activity compared with p35/p12. The significance of this increase was demonstrated by the near complete loss of apoptosome-mediated caspase-3 activity when a point mutant (D330A) of procaspase-9 was substituted for wild-type procaspase-9 in the apoptosome. In addition, cleavage at Asp330 exposed a novel p10 NH2-terminal peptide motif (A1SS) that retained the ability to mediate XIAP inhibition of caspase-9. Thus, whereas feedback cleavage of caspase-9 by caspase-3 significantly increases the activity of the apoptosome, it does little to attenuate its sensitivity to inhibition by XIAP.

Caspases are a unique class of cysteine proteases that function as effectors of apoptosis, or programmed cell death (1, 2). Caspases are expressed in virtually all metazoan cells as catalytically inactive zymogens known as procaspases. Two mechanisms have been described for activating caspases: noncovalent association with caspase activating proteins (e.g. Fadd, Apaf-1) leading to autocatalytic cleavage of the procaspase polypeptide at specific aspartic acid residues, or cleavage at specific aspartic acid residues within the zymogen by other activated caspases. Caspases have been divided into initiators (caspases-2, -8, -9, and -10) and effectors (caspases-3, -6, and -7) based on their relative position within a caspase cascade (3–7). Initiator caspases are activated by the former mechanism, effector caspases by the latter. The combined proteolytic activities of initiator and effector caspases cleave a variety of vital protein substrates, including DFF45/ICAD, lamin B, gelsolin, Bid, and FAK2, leading to the morphological and biochemical characteristics of apoptosis (8–11).

Caspase-9 is the initiator caspase in the “intrinsic” or mitochondrial caspase pathway. Interaction of caspase-9 with Apaf-1, a human homologue of the Caenorhabditis elegans CED4 protein, to form the apoptosome occurs in response to cytochrome c release from the mitochondria of pre-apoptotic cells (12). The apoptosome is a multiprotein complex comprised of Apaf-1, cytochrome c, and caspase-9 in a 1:1:1 molar ratio (12, 13). The function of the apoptosome is to cleave and activate the apoptosis effector caspases-3, -6, and -7 (14, 29). The apoptosome is assembled when seven Apaf-1:cytochrome c heterodimers oligomerize to form a symmetrical “wheel” and pro-caspase-9 molecules become associated noncovalently to Apaf-1 via caspase-9 CARD/Apaf-1 CARD heterophilic interaction (15, 16). Binding of procaspase-9 to Apaf-1 is important for two reasons. First, it increases the intrinsic catalytic activity of the caspase-9 protease leading to the autolytic cleavage of pro-caspase-9 at Asp315 to yield a large (p35) and a small (p12) subunit (14, 17, 18). And second, cleavage exposes a neoepitope comprising the NH2-terminal four amino acids (ATPF) of the small p12 subunit that has been shown to be both necessary and sufficient for binding to the BIR3 domain of XIAP, leading to inhibition of caspase-9 (19). Once activated in the apoptosome, caspase-9 cleaves procaspase-3 at Asp175 and activates caspase-3. Studies in vitro (14, 29) and in intact cells (20) have shown that caspase-3 is capable of feedback cleavage of caspase-9 at Asp330, and that this cleavage is associated with an increase in apoptosome activity. Caspase-3-directed feedback cleavage of caspase-9 p35/p12 at Asp330 would remove the BIR3 recognition motif of caspase-9 (19), creating a caspase-9 species, p35/p10, which may be insensitive to XIAP inhibition. Using purified, recombinant Apaf-1, caspase-9, and caspase-3 the present study has addressed the regulation of the apoptosome at three levels. 1) Does caspase-9 cleavage affect apoptosome activity? 2) What is the impact of caspase-3-mediated feedback cleavage of caspase-9 on apoptosome activity? And 3) following feedback cleavage of caspase-9, what impact does the loss of the BIR3 binding motif from the linker region have on XIAP inhibition. Our results show that recombinant proteins can combine in a dATP- and cytochrome c-dependent manner to yield a catalytically active apoptosome.

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capable of cleaving and activating recombinant procaspase-3. The data further demonstrate that association of procaspase-9 with Apaf-1 leads to a partial active apoptosome containing p35/p12 caspase-9. Activation of caspase-3 by the p35/p12-containing apoptosomes leads to the initiation of a feedback loop whereby caspase-3 cleaves caspase-9 at Asp-163. Our data further demonstrate that p10-containing apoptosomes have enhanced catalytic properties relative to p12-containing species. Finally, our study identifies a novel motif at the NH2 terminus of the p10 subunit capable of mediating XIAP inhibition. This, together with our recent studies and the p10 and p12 NOEs by caspase-3, canently increases the proteolytic activity of the apoptosome, it does little to attenuate its sensitivity to inhibition by the endogenous caspase-9 inhibitor, XIAP.

EXPERIMENTAL PROCEDURES

General Methods and Materials—Tris glycine gels, molecular weight standards for SDS-PAGE, and 10× Tris glycine gel running buffer were from Invitrogen, dATP was obtained from Amersham Biosciences. Horse heart cytochrome c was purchased from Sigma and further purified by passing through the ion exchange column (Mono S). The fluorogenic tetrapeptide substrates DEVD-AMC and LEHD-AMC were synthesized at Idun Pharmaceuticals as previously described (21). General molecular biology methods were used as described in Sambrook et al. (22).

Production of Recombinant Proteins—Recombinant Apaf-1, procaspase-3, and procaspase-9 proteins were produced in a baculovirus expression system. Expression plasmids for Apaf-1 and wild-type procaspase-9 were constructed as described previously (12) and kindly provided by Dr. Xiaodong Wang (University of Texas, Dallas, TX). Caspase-3 with an in-frame His6 coding sequence at the 3′-end was subcloned into pfastBacI vector at BamHI and NotI sites. Caspase-9 active site (C297A) mutant, cleavage site mutants (D315A, D330A, and D315A/D330D) cells (Novagen), and p10 dissolved in Tween-20 were prepared as described (23). These mutants, engineered to express a His6-tag at the COOH termini, were subcloned into pfastBac1 vector at BamHI and EcoRI sites. Expression plasmids were transformed into DHi10Bac Escherichia coli cells (Invitrogen), recombinant bacmid were purified as recommended by the manufacturer (Invitrogen), and their identity were confirmed by PCR amplification analysis. The DNA was then used to transfect SF21 cells, and virus was amplified as described (12). The virus stocks were amplified to 200 ml and used to transfect Sf21 cells, and virus was amplified by the PCR-SOEing method (23). These mutants, engineered to express a His6-tag at the COOH termini, were subcloned into pfastBacI vector at BamHI and EcoRI sites. Expression plasmids were transformed into DHi10Bac Escherichia coli cells (Invitrogen), recombinant bacmid were purified as recommended by the manufacturer (Invitrogen), and their identity were confirmed by PCR amplification analysis. The DNA was then used to transfect SF21 cells, and virus was amplified as described (12). The virus stocks were amplified to 200 ml and used to transfect 1 liter of SF21 cells at a density of 1 × 10^6 cells/ml. The infected cells were harvested after 38 h for Apaf-1, 20 h for procaspase-9 and procaspase-3, and 24 h for the procaspase-9 mutants and processed caspase-9 (p35/p12). Recombinant proteins were purified by nickel affinity chromatography as described (12), followed by ion exchange chromatography (Mono Q). The eluted protein was dialyzed with buffer A (20 mM Hepes-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM NaEDTA, 1 mM NaEGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and stored in multiple aliquots with 20% glycerol at −80°C. To obtain the p35/p10 and p37/p10 forms of caspase-9, 2 × 10^5 units of recombinant human caspase-3 was incubated with 17 mg of either caspase-9 p35/p12 or the D315A mutant of caspase-9 at 30°C for 30 min in the presence of 200 μM dATP and 600 nm cytochrome c to a total volume of 10 μl in buffer A to allow the formation of Apaf-1/caspase-9 apoptosome. Recombinant Apaf-1, procaspase-9, and procaspase-3 at the desired concentration in a 30- or 40-μl reaction mixture in buffer A containing both dATP and cytochrome c. The active site mutants of procaspase-3, or its active site mutant (C163A) were combined with increasing amounts of either DEVD-AMC (50 μM, pH 7.5, for caspase-3) or LEHD-AMC (100 μM, pH 6.5, for caspase-9). Caspase activity was monitored as the release of the AMC product over 60 min at room temperature using a Cytoflour fluorometer. Caspase activity was expressed as percent change in fluorescence over time derived from the linear phase of the reaction.

Active Site Titration of Caspase-9—To determine accurately the relative kinetic properties of the various caspase-9 species, two irreversible caspase-9 inhibitors were used to determine the concentration of caspase-9 active sites after association with Apaf-1. Briefly, serial dilutions of the inhibitors were incubated with Apaf-1, dATP, cytochrome c, and the various caspase-9 species for 2 h at room temperature to allow complete inactivation. Aliquots were removed, mixed with 60 μM procaspase-9, and subsequently incubated for an additional 30-min period for the addition of 50 μM DEVD-AMC. Caspase-3 activity assay was monitored as above. The active enzyme concentration was defined as the minimal inhibitor concentration that completely depleted the caspase-9 activities. Titration with either the two inhibitors resulted in identical results. Caspase-9 and caspase-9 mutant concentrations used in Figs. 3 and 4 and Tables I and II, refer to active site concentrations.

Determination of Caspase-9 Kinetic Parameters—The K_m for LEHD-AMC was determined for each form of Apaf-1/caspase-9 apoptosome. Aliquots containing 100 μM Apaf-1 and 100 μM caspase-3 (concentration determined by active site titration) were incubated at 30°C for 30 min and procaspase-9 activities. Titration with either the two inhibitors resulted in identical results. Caspase-9 and caspase-9 mutant concentrations used in Figs. 3 and 4 and Tables I and II, refer to active site concentrations.

RESULTS

Recombinant Apaf-1, Procaspase-9, and Procaspase-3 Reconstitute dATP/Cytochrome c-regulated Caspase-3 Activation—To characterize the biochemical events leading to Apaf-1/caspase-9 apoptosome-mediated cleavage of procaspase-3, we expressed recombinant Apaf-1, procaspase-9, and procaspase-3 using a baculovirus expression system as described under “Experimental Procedures.” Each protein was purified to apparent homogeneity using nickel affinity chromatography followed by ion exchange chromatography (Fig. 1B). To demonstrate that these recombinant proteins could support dATP/cytochrome c-dependent caspase activation, we monitored the cleavage of procaspase-9 and -3 after incubation with Apaf-1, dATP, and cytochrome c. As expected, procaspase-9 and procaspase-3 were processed in a time-dependent manner yielding products that were consistent with previously published reports (Fig. 1, A and C) (26, 27). The abbreviation used is: Chaps, 3-[3-cholamidopropyl]dimethyl-ammonio]-1-propanesulfonic acid.
Caspase-9 cleavage by caspase-9 at Asp315, caspase-3 cleavage of caspase-9 schematic diagram showing procaspase-9 and products obtained from caspase-3, (25.5 nM) were incubated with cytochrome c and incubated with different amounts of Apaf-1 and procaspase-9 or procaspase-9 mutants with Apaf-1 and dATP. 

Recombinant wild-type procaspase-9 and its cleavage-site mutants (D315A, D330A, and D315A/D330A) were purified as described under “Experimental Procedures.” A, 5 μg of procaspase-9 or procaspase-9 mutants were directly subjected to 16% SDS-PAGE (left), or incubated with 10 ng of caspase-3 at 30 °C for 30 min and then subjected to 16% SDS-PAGE (right). Caspase-9 species were detected with Coomassie Blue staining. B, procaspase-9 or procaspase-9 mutants (35.2 nm) were incubated with Apaf-1 (4.7 nm), dATP (200 μM), and cytochrome c (300 nm) at 30 °C for 30 min. The reaction mixtures were subjected to 16% SDS-PAGE followed by immunoblotting for caspase-9 (top) or caspase-3 (middle). Duplicate aliquots were analyzed for caspase-3 activity using the fluorogenic tetrapeptide substrate DEVD-AMC (bottom panel). Fu, fluorescence units.

To identify which cleavage events occur upon association of procaspase-9 with Apaf-1, we incubated either wild-type procaspase-9 or its cleavage-site mutants with Apaf-1, dATP, and cytochrome c and monitored procaspase-9 cleavage. Incubation of Apaf-1 with wild-type procaspase-9 or the D330A mutant yielded a p35 cleavage product (Fig. 2A), whereas reactions with cleavage-site mutants D315A or D330A yielded no detectable p35 cleavage product. These results confirm that Apaf-1 induces cleavage of procaspase-9 at Asp330. Thus, feedback cleavage of procaspase-9 (C287A) supports this proposal (Fig. 2A).

To determine the relative impact of each cleavage event on caspase-3 activity within the apoptosome, we preincubated procaspase-9 or the procaspase-9 mutants with Apaf-1 and procaspase-3 in the presence of dATP and cytochrome c. The reactions were then tested for caspase-3 activity using the fluorogenic tetrapeptide substrate DEVD-AMC. Addition of increasing amounts of wild-type procaspase-9 leads to an increase in caspase-3 activity as expected (Fig. 2C). In contrast to published work (17), reactions with the procaspase-9 mutant (D315A) generated nearly the same amount of caspase-3 activity as reactions containing the wild-type procaspase-9, whereas reactions with the procaspase-9 mutant (D330A) yielded no products. These results confirm that Apaf-1 induces cleavage of procaspase-9 at Asp330 preferentially to Asp315 (14).

To determine the degree of caspase-3 processing in each sample (Fig. 1C, lower panel). Cleavage Requirements for Procaspase-9 in the Apoptosome—Several publications have addressed the relationship between caspase-9 cleavage status and the activity of the apoptosome (17, 19). These studies, employing cell-free extracts with and without immunodepletion of endogenous caspase-9, disagree on the impact of the cleavage state of caspase-9 on apoptosome activity. The establishment of a completely recombinant system to study Apaf-1-mediated caspase activation allowed us to address this issue under more defined conditions. To determine the relationship between sites of procaspase-9 cleavage and apoptosome activity, we first expressed and purified several procaspase-9 proteins containing point mutations, including those containing point mutations, including D315A, D330A, and D315A/D330A and C287A (Fig. 2A). Each of these mutants were tested for their ability to be cleaved, in vitro, by recombinant active caspase-3. Consistent with previous reports (14), procaspase-9 mutants D330A and D315A/D330A were not processed by caspase-3, whereas the cleavage site mutant (D315A) and the procaspase-9 active site mutant (C287A) yielded the expected cleavage product, p37. Incubation of wild-type caspase-9 with caspase-3 led to the formation of a p35 product, suggesting that caspase-3 directed cleavage of caspase-9 yields an active caspase-9 (p37/p10) capable of cleaving p37 subunits at Asp315 to generate p35. The lack of p35 production in reactions containing caspase-9 (C287A) supports this proposal (Fig. 2A).

In Vitro Characterization of the Apoptosome

confirm that the cleaved caspase-3 products were catalytically active, we incubated aliquots with DEVD-AMC. The time-dependent appearance of DEVD-AMC cleaving activity parallels the degree of caspase-3 processing in each sample (Fig. 1C, lower panel).
The concentration ranged from 0.4 to 50 nM in 2-fold increments. Caspase-9 in the apoptosome was maintained at a 1:1 molar ratio of Apaf-1/caspase-9 (p35/p12) apoptosome. The molar ratio of Apaf-1 to caspase-9 was determined by Western blot analysis. Wild-type procaspase-9 or caspase-9 cleavage products of p37, p35 (note the p35/p37 doublet at 5 min), p12, and p10 were generated, whereas the majority of procaspase-3 was cleaved within 5 min (Fig. 3A). In the procaspase-3 (C163A) mutant reaction, the caspase-9 cleavage products were exclusively p35 and p12, and no processing of procaspase-3 was observed within the first 5 min (Fig. 3A, lower right panel). To rule out the possibility that the difference in procaspase-3 cleavage observed in Fig. 3A was because of cleavage of procaspase-3 by activated caspase-3 in the wild-type caspase-3 reactions, we incubated procaspase-3 with increasing amounts of active caspase-3. Fig. 3B demonstrates that up to 10 units of caspase-3 is insufficient to cleave procaspase-3, whereas as little as 2 units efficiently cleaves procaspase-9, indicating that all the procaspase-3 cleavage observed in Fig. 3A is apoptosis-mediated. In addition to the differences in caspase-3 cleavage rate, the rates for caspase-9 cleavage in each reaction were also different. In reactions containing wild-type procaspase-3, procaspase-9 cleavage was observed within 2 min and its cleavage was nearly complete by 10 min. In the reactions with mutant procaspase-3 the rate of procaspase-9 cleavage was slower, such that no cleavage was observed at 2 min and the reaction had not reached completion by 20 min.

To further establish that caspase-9 cleaved at Asp330 to generate a p10 subunit is critical for full activation, we added increasing amounts of caspase-9 p35/p12 apoptosomes (at a constant 1:1 molar ratio of Apaf-1:caspase-9) to either wild-type procaspase-3 or the C163A mutant. If caspase-3-mediated feedback can enhance the ability of caspase-9 to cleave procaspase-3, then we would expect procaspase-3 cleavage to be detected at lower apoptosome concentrations in reactions containing catalytically competent caspase-3. As shown in Fig. 3C, cleavage of wild-type procaspase-3 is observed when the apoptosome concentration reaches 3.1 nM (Fig. 3C, upper panel), with substantial cleavage occurring at 50 nM. In reactions with the C163A procaspase-3 mutant, cleavage is not observed until the apoptosome concentration reaches 25 nM with only marginal cleavage at 50 nM (Fig. 3C, lower panel). The data presented in Fig. 3 therefore demonstrate that cleavage of caspase-9 at Asp330 by caspase-3 can significantly enhance the activity of the apoptosome. The magnitude of the enhancement was calculated to be up to 8-fold.

**Activities of Different Forms of Caspase-9 in the Apoptosome**—To directly demonstrate that apoptosomes containing fully processed caspase-9 are more active than those containing partially processed caspase-9, we produced the three distinct processed forms of caspase-9. Each of the three purified proteins, p35/p10, p37/p10, and p35/p12, migrated as predicted in SDS-PAGE (Fig. 4A), and the molecular masses of each individual subunit were confirmed using mass spectrometry (not shown). We first compared the activity of apoptosomes containing either p35/p12 or p37/p10 to process recombinant procaspase-3 (C163A) by titration of the apoptosome added to the reaction. Caspase-9 p37/p10 apoptosomes were able to cleave procaspase-3 at concentrations of 0.4 to 0.8 nM, whereas caspase-9 p35/p12 apoptosome required 3.1 to 6.3 nM to cleave procaspase-3 during this period (Fig. 4B). To assess the differences between apoptosomes, we used the caspase-9 tetrapeptide substrate, LEHD-AMC. After incubation of Apaf-1 with p35/p12, p37/p10, or p35/p10 to allow the formation of Apaf-1/casp-9 apoptosomes, LEHD-AMC was added to the reactions and apoptosome activity was assayed by following the liberation of AMC. The activities of p35/p10- and p37/p10-containing apoptosomes were significantly greater than that of p35/p12 over the entire range of apoptosome concentration (Fig. 4C). Together these results suggest that removal of the NH2-terminal linker region from the p12 small subunit of caspase-9 yields an apoptosome with greater specific activity.

To establish that the observed effects on LEHD-AMC cleavage were because of changes in the intrinsic catalytic properties of the apoptosomes, we determine the $K_m$ and $k_{cat}$ for each of the apoptosome species. We found that processing of caspase-9 at Asp330, to form the p10 subunit, was associated with a modest decrease in $K_m$ for LEHD-AMC (Table I). This effect was most pronounced with the p37/p10 species yielding a $K_m$ 2-fold lower than that for p35/p12. The first-order rate
The form of caspase-9 within the apoptosome can clearly impact cleavage of the synthetic substrate LEHD-AMC. To ask whether this was also true of full-length endogenous substrates, we assessed the ability of each of the distinctly processed caspase-9 species, in association with Apaf-1, to cleave and activate recombinitant procaspase-3. Apaf-1 was preincubated with p35/p12, p37/p10, or p35/p10 in the presence of dATP and cytochrome c to allow the formation of apoptosomes. Following preincubation, wild-type procaspase-3 and DEVD-AMC were added simultaneously and the time-dependent activation of caspase-3 was monitored. Apoptosomes containing either p35/p10 or p37/p10 demonstrated similar activity in this experiment, reaching a steady state level of caspase-3 activity within 10–15 min, with a rate of DEVD-AMC cleavage during this phase of ~1500 fluorescence units/h (Fig. 4D). In contrast, the activity of the reaction containing p35/p12 during this time period was ~10-fold lower at 150 fluorescence units/h (Fig. 4D). None of these caspase-9 species demonstrated appreciable activity in the absence of Apaf-1 under these conditions (Fig. 4D).

At later time points in the p35/p12 reaction the rate of DEVD-AMC cleavage begins to approach that of the other two reactions. This change in the rate of DEVD-AMC cleavage in the Apaf-1/p35/p12 containing reaction (between 30 and 50 min) is a result of feedback cleavage of p12 by active caspase-3 to yield a p10 subunit, and thus p35/p10-containing apoptosomes (Fig. 4D). The lag time required for this change in rate to occur is inversely proportional to the amount of input procaspase-3 (not shown).

**Fully Active Caspase-9 Is a Target for XIAP-mediated Inhibition through the BIR3 Domain—**Srinivasula et al. (19) have reported that the Arg169–Thr185 motif (Arg169–Thr185 motif) at the NH2-terminus of the p12 domain, a motif similar to the AVPI motif responsible for SMAC-mediated BIR3 binding, is critical for inhibition of caspase-9 by the BIR3 domain of XIAP (19). Because feedback cleavage of caspase-9 by caspase-3 removes the ATPase motif as part of the linker, we were interested in determining whether p35/p10 or p37/p10 caspase-9 were less sensitive to inhibition by XIAP. Accordingly, we reconstituted the apoptosome with various forms of caspase-9 and tested the ability of BIR3 to inhibit caspase-9-mediated activation of procaspase-3. As expected, BIR3 inhibited caspase-3 activation by apoptosomes containing procaspase-9 (not shown) or caspase-9 p35/p12 (Table II, procaspase-3 as substrate, Fig. 5A). Surprisingly, apoptosomes containing the p35/p10 form of caspase-9 were inhibited with nearly the same IC50 values as procaspase-9- and p35/p12-containing apoptosomes (Table II and Fig. 5A). These data implied that other regions within the caspase-9 protein are able to mediate inhibition by BIR3. The NH2-terminus of the fully cleaved p10 small subunit of caspase-9 also contains a tetrapeptide motif, Arg169–Thr185, with similarity to the SMAC and caspase-9 p12 motifs (Fig. 5B). Although Srinivasula et al. (19) found little binding of BIR3 to this motif, we expressed point mutants targeting both of the potential BIR3 binding motifs within caspase-9 (Fig. 5, C and D) to test whether the p10 NH2-terminal motif is responsible for BIR3-mediated inhibition of fully processed caspase-9. Apoptosomes containing caspase-9 p35/p10 A331G/I332G (M4 in Table II and Fig. 5C), or caspase-9 p35/p12 A316G/T317G (M3) were no longer sensitive to BIR3-mediated inhibition in the fully reconstituted system (Fig. 5A). This result is consistent with a model where the AISS motif present at the NH2-terminus of p10 is responsible for the inhibition by BIR3 of caspase-9 p35/p10 observed in Fig. 5A. Interestingly, caspase-9 p35/p12 A331G/I332G (M2) was also insensitive to inhibition by BIR3, even though the input caspase-9 had a p12 subunit with the

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**Table I**

| Apoptosome          | $K_m$ (µM LEHD-AMC) | $k_{cat}$ (1/s) | Catalytic rate ($k_{cat}/K_m$, 1/s·µM) |
|---------------------|---------------------|----------------|---------------------------------------|
| Apaf-1/p35/p12      | 792                 | 0.12           | 151.5                                 |
| Apaf-1/p35/p10      | 550                 | 0.34           | 618.2                                 |
| Apaf-1/p37/p10      | 382                 | 0.43           | 1125.7                                |

**Kinetic parameters of different forms of caspase-9 in the apoptosome**

The $K_m$ and $k_{cat}$ for LEHD-AMC was determined for each form of Apaf-1/caspase-9 apoptosome, and each value was calculated using a non-linear regression method to fit Michaelis-Menten equation as described under "Experimental Procedures."
ATPF BIR3 recognition motif. These data can be accommodated by at least two models, either ATPF-mediated inhibition by BIR3 is reversible by removal of the linker region by caspase-3 cleavage of Asp330, or caspase-3 cleavage of Asp330 occurs more rapidly than binding of BIR3 to the NH2 terminus of p12. Finally, caspase-9 p35/p12 A316G/T317G (M1) was inhibited by BIR3 with nearly the same IC50 as caspase-9 p35/p10, suggesting this the M1 mutant was inhibited following cleavage at Asp330 and exposure of the AISS motif.

To demonstrate more directly that each of the NH2 termini of the small subunits of caspase-9 can mediate BIR3 inhibition, we tested the various apoptosomes in the absence of caspase-3 (and thus without feedback cleavage at Asp330) using the LEHD-AMC substrate. We observed potent BIR3 inhibition of p35/p12 WT and the p35/p12 M2 mutant, whereas the p35/p12 M1 mutant was not sensitive to BIR3 (Table II, LEHD as substrate, and Fig. 5E). As expected from Fig. 5A, we also observed inhibition by BIR3 of caspase-9 p35/p10 WT but no inhibition of the p35/p10 M4 mutant or the p35/p12 M3 mutant. Finally, to demonstrate that the above observations are relevant to BIR3-mediated inhibition in the context of full-length XIAP, we repeated the experiment using purified full-length XIAP. As shown in Fig. 5F, the ability of full-length XIAP to inhibit the apoptosome is dependent on the presence of

**Table II**

IC50 (nM) of XIAP or BIR3 mediated inhibition of caspase-9 and caspase-9 mutants in apoptosome

| Substrate Inhibitor | Caspase-9 |
|---------------------|----------|
|                     | p35/p12 (WT) | p35/p12 (M2) | p35/p12 (M3) | p35/p10 (WT) | p35/p10 (M4) |
| p35/p12 BIR3        | 4.5       | >500         | >500          | 13.3         | >500         |
| LEHD_{AMC} BIR3     | 17.3      | >1000        | 108.3         | 10.1         | >1000        |
| LEHD_{AMC} XIAP     | 12.0      | >1000        | 7.5           | 1.5          | >1000        |

**Fig. 5.** Either of the two conserved XIAP binding motifs within caspase-9, ATPF or AISS, are sufficient for inhibition by XIAP or its BIR3 domain. A, caspase-9 or its mutant were incubated with Apaf-1, procaspase-3, dATP, cytochrome c, and different amounts of BIR3. The activity of processed caspase-3 was measured by the cleavage of DEVD-AMC. Open circles, p35/p12 (WT); open diamonds, p35/p10 (WT); open squares, p35/p12 (M1); filled diamonds, p35/p12 (M2); filled triangle, p35/p12 (M3); filled square, p35/p10 (M4). Symbols are the same for A, E, and F. B, alignment of the NH2-terminal amino acids from mouse, *Xenopus*, and human caspase-9 p10, along with the NH2-terminus of human caspase-9 p12 and human SMAC/Diablo. C, details of caspase-9 p35/p12 p35/p10 point mutants used to assess amino acid requirements for BIR mediated inhibition. D, Coomassie Blue-stained gel of caspase-9 small subunit NH2-terminal mutants. In E and F, caspase-9 or the caspase-9 mutants were incubated with Apaf-1 dATP, cytochrome c, and either BIR3 or full-length XIAP. The activity of Apaf-1/caspase-9 holoenzyme was directly measured by the cleavage of LEHD-AMC.
either ATPF or AISS exposed at the NH_{2} terminus of the small subunit. The IC_{50} values for each of these reactions is shown in Table II (XIAP inhibitor, LEHD as substrate). These data support a model where XIAP is able to inhibit caspase-9 at all levels of activation by virtue of a conserved motif at the NH_{2} terminus of the less active p12 subunit as well as the more active p10 subunit.

**DISCUSSION**

In the present study, we have addressed the potential physiological role(s) of caspase-3-mediated feedback cleavage of caspase-9 using purified, recombinant Apaf-1, caspase-9, and caspase-3 proteins in a reconstituted apoptosome in vitro. Because caspase-9 can be cleaved by itself (Asp315) or by caspase-3 activation of the apoptosome (Fig. 2), we set out to investigate this possibility and provide biochemical support for two recent studies that support this possibility. Fujita et al. (19) would predict that

Recent work by Srinivasula et al. (19) would predict that feedback cleavage of caspase-9 by caspase-3, and loss of the BIR3 binding motif, would be accompanied by a loss of sensitivity to inhibition by XIAP, representing a potential point of no return for the cell. Thus, we were surprised to observe that both full-length XIAP or the isolated BIR3 domain is equally effective at inhibiting p10-containing forms of caspase-9 (p35/p12, p37/p10) as the p12-containing caspase-9 (p35/p12, Table II and Fig. 5). Cleavage by caspase-3 at Asp330 exposes a tetrapeptide motif AISS that appears to be responsible for XIAP inhibition (Fig. 5). Two lines of evidence support this conclusion: 1) the similarity of the AISS sequence to the SMAC/reaper tetrapeptide motif (Fig. 5B), and 2) the nearly complete loss of function when the NH_{2}-terminal alanine of p10 is mutated to glycine (Fig. 5, A, E, and F). This p10 NH_{2} terminus is well conserved (Fig. 5B), indicating that it is important that caspase-3 feedback-cleaved caspase-9 still be subject to regulation by IAPs.

The catalytic activity of caspase-9 in the apoptosome is regulated at multiple levels. First, noncovalent association with Apaf-1 via CARD/CARD interaction causes an increase in protease activity (18, 28). The precise activation mechanism is unknown but may involve allosteric changes in procaspase-9 induced upon binding to Apaf-1. Second, autolytic cleavage at Asp315, perhaps because of the induced proximity (14) of caspase-9 molecules in the multivalent apoptosome, further increases caspase-9 protease activity. Third, feedback cleavage at Asp330 by caspase-3 increases caspase-9 activity still further, providing for an amplification of the cascade. How the loss of the 15-amino acid linker region leads to enhancement of the catalytic activity is not clear. In the recently published structures of caspase-9 the NH_{2} terminus of the p12 subunit was not resolved (28). However, the NH_{2} terminus of the p12 subunit was adjacent to the catalytic site, suggesting that removal of the NH_{2} terminus could affect either substrate recognition or catalysis by caspase-9. Finally, all forms of activated caspase-9 are subject to inhibition by XIAP. Thus, even after full activation of the apoptosome, it appears that XIAP may be capable of setting a threshold over which sufficient active caspase-9 must be generated before a cell can complete the apoptotic program.

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