Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade

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Summary

We report here the purification of the third protein factor, Apaf-3, that participates in caspase-3 activation in vitro. Apaf-3 was identified as a member of the caspase family, caspase-9. Caspase-9 and Apaf-1 bind to each other via their respective NH$_2$-terminal CED-3 homologous domains in the presence of cytochrome c and dATP, an event that leads to caspase-9 activation. Activated caspase-9 in turn cleaves and activates caspase-3. Depletion of caspase-9 from S-100 extracts diminished caspase-3 activation. Mutation of the active site of caspase-9 attenuated the activation of caspase-3 and cellular apoptotic response in vivo, indicating that caspase-9 is the most upstream member of the apoptotic protease cascade that is triggered by cytochrome c and dATP.

Introduction

Apoptosis is a morphologically and biochemically distinct form of cell death carried out by a genetically determined cell suicide program (Kerr et al., 1972; reviewed by Horvitz et al., 1994; Jacobson et al., 1997). Several apoptosis-regulating genes identified in Caenorhabditis elegans have counterparts in humans, demonstrating that the basic mechanism of apoptosis is evolutionarily conserved (Vaux, 1997).

Three genes, *ced-3*, *ced-4*, and *ced-9*, encode the general apoptotic program in C. elegans (Horvitz et al., 1994). *ced-9* negatively regulates apoptosis while *ced-4* and *ced-3* are required to execute the apoptotic program (Yuan and Horvitz, 1990; Hengartner et al., 1992). The bcl-2 family of proteins are mammalian relatives of CED-9 (Vaux et al., 1992; Hengartner and Horvitz, 1994). Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria (Monaghan et al., 1992; Krajewski et al., 1993; de Jong et al., 1994). CED-4 is homologous to the recently identified human protein, Apaf-1, which participates in the activation of the mammalian CED-3 homolog, caspase-3 (Yuan and

Horvitz, 1992; Yuan et al., 1993; Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Tewari et al., 1995; Xue et al., 1996; Zou et al., 1997).

ced-4 has been determined genetically to function downstream of *ced-9* but upstream of *ced-3* (Shaham and Horvitz, 1996a, 1996b). Likewise, Apaf-1 functions downstream of bcl-2 but upstream of caspase-3 (Zou et al., 1997). Bcl-2 may function upstream of Apaf-1 by regulating the release of cytochrome c from mitochondria. Cytochrome c is a required cofactor for Apaf-1 function (Liu et al., 1996b; Zou et al., 1997). Consistent with this hypothesis, overexpression of bcl-2, or its close family member bcl-Xl, blocks the release of cytochrome c from mitochondria, which otherwise occurs when cells are signaled to undergo apoptosis (Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997). In addition, the bcl-2 family of proteins might also directly interact with Apaf-1 in a fashion similar to the interaction between CED-4 and CED-9 (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Spector et al., 1997; Wu et al., 1997).

Various stimuli of apoptosis lead to the activation of a family of cysteine proteases with specificity for aspartic acid residues, referred to as caspases (Alnemri et al., 1996). The activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and breaking down structural components of the cell (Nicholson and Thornberry, 1997). The targets of such cleavage events include poly(ADP-ribose) polymerase (Nicholson et al., 1995; Tewari et al., 1995), sterol regulatory element binding proteins (Wang et al., 1996), retinoblastoma (RB) protein (An et al., 1996; Janicke et al., 1996), nuclear lamins (Lazebnik et al., 1995; Orth et al., 1996; Takahashi et al., 1996), DNA-dependent protein kinase (Casciola-Rosen et al., 1996; Song et al., 1996), U1 70-K protein (Caciola-Rosen et al. 1996), and the large subunit of the DNA replication complex C (Ubeda and Habener, 1997). In addition, activated caspases lead to cleavage of the 45 kDa subunit of DNA fragmentation factor (DFF-45). This cleavage activates a pathway leading to fragmentation of genomic DNA into nucleosomal fragments, a hallmark of apoptosis (Liu et al., 1997).

Caspase-3 is activated by two sequential proteolytic events that cleave the 32 kDa precursor at aspartic acid residues to generate an active heterodimer of 20 kDa and 12 kDa subunits (Nicholson et al., 1995). The activation might be autocatalytic, or it might occur via a caspase cascade, similar to the serine protease cascade in the blood clotting process. Such a cascade might amplify apoptotic signals leading to fast and irreversible apoptosis. The active caspases are promiscuous in cleaving other caspases, making it difficult to identify the initial triggering event in caspase activation (Tewari et al., 1995; Liu et al., 1996a; Muzio et al., 1996; Srinivasula et al., 1996a; Wang et al., 1996).

We have recently established an in vitro system in which caspase-3 is activated by the addition of dATP to cytosol from normally growing cells (Liu et al., 1996b). This system enabled us to study the triggering events in caspase-3 activation. Fractionation of HeLa cytosol

A

B

C

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MDEADRRLLRRCRLRLVEELQVDQLWDALLSSELFRPHMI  40
EDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISCLEDT  80
GQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPEIRKPEV  120
LRPETPRPVDIGSGGFGDVGALESLRGNADLAYILSMEPC  160
GHCLIINNVNFCRESGLRTRTGSNIDCEKLRRRFSSPHFM  200
VEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQ  240
ASHLQFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGKPK  280
LFFIQACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQ  320
EGLRTFDQLDISSLPTPSDIFVSYSTFPGFVSWRDPKSGS  360
WYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYKQMP  400
GCFNFLRKKLFFKTS  416
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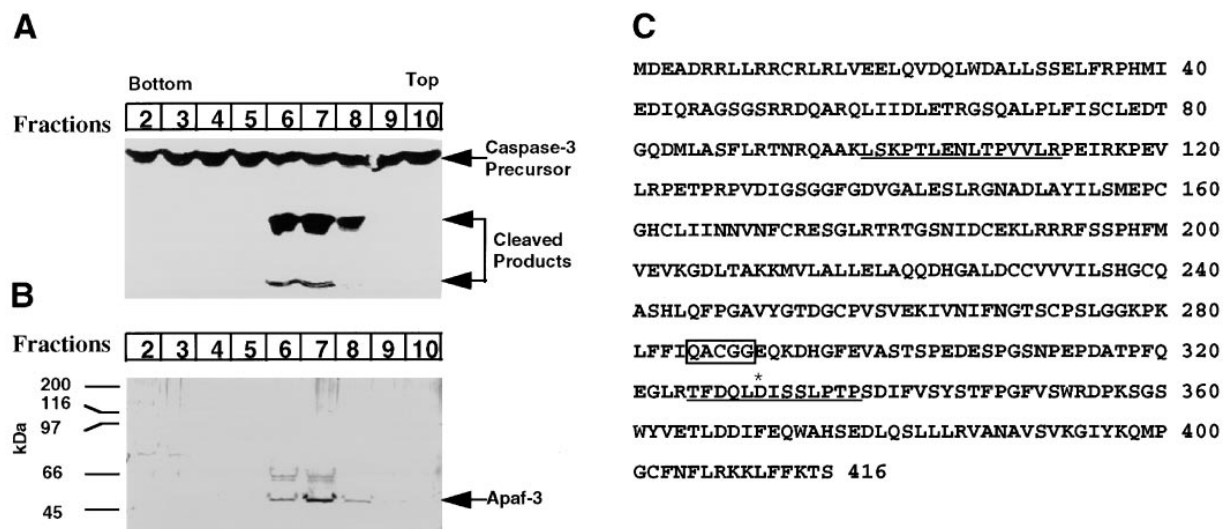
Figure 1. Glycerol Gradient Purification of Apaf-3 and Its Identification as Caspase-9/Mch6/ICE-LAP6

The Apaf-3 activity was purified through the glycerol gradient step as described in Experimental Procedures.

(A) Aliquots of 1 μl of glycerol gradient fractions were incubated with aliquots of 4 μl of Apaf-1 fraction bound to hydroxylapatide column (Zou et al., 1997), 1 μl (0.2 μg) of cytochrome c, 3 μl of in vitro–translated, ^{35}S-labeled, and affinity-purified Caspase-3, and 1 mM dATP at 30°C for 1 hr in a final volume of 20 μl of buffer A. After 1 hr at 30°C, the samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a phosphorimaging plate for 16 hr at room temperature.

(B) Aliquots (30 μl) of the indicated glycerol gradient fractions were subjected to 8% SDS-PAGE, and the gel was subsequently stained with silver using a Silver Stain Plus kit from Bio-Rad.

(C) The Apaf-3 peak fractions from the Mono Q column as described in the Experimental Procedures were subjected to 8% SDS-PAGE and electroblotted onto a PVDF membrane. The 46 kDa polypeptide band correlated with Apaf-3 activity was excised from the membrane and subjected to trypsin digestion. The resulting peptides were separated by a reverse-phase HPLC column, and the amino acids sequences of two individual peptides (underlined) were determined by Edman degradation in an Applied Biosystems sequencer. The amino acid sequence of caspase-9 was as reported in Srinivasula et al. (1996a). The conserved active site pentapeptide QACGG is boxed. The asterisk denotes the amino acid residue after which the cleavage occurs during caspase-9 activation (Srinivasula et al., 1996a).

revealed that three protein factors, designated apoptotic protease activating factors (Apafs), are necessary and sufficient to reconstitute dATP-dependent caspase-3 activation (Zou et al., 1997). The NH$_2$-terminal region of Apaf-1 shares sequence similarity with the prodomain of CED-3 and other caspases with long prodomains, including caspase-1/ICE (Thornberry et al., 1992), caspase-2/ICH-1 (Wang et al., 1994), and caspase-9/Mch6/ICE-LAP6 (Duan et al., 1996; Srinivasula et al., 1996a). This domain may serve as a caspase recruitment domain (CARD) by binding to caspases that have similar CARDs at their NH$_2$ termini (Hofmann et al., 1997; Zou et al., 1997). The CARD of Apaf-1 is followed by a stretch of 320 amino acids that are homologous to CED-4. The C-terminal part of Apaf-1 is composed of 12 putative WD40 repeats, a protein motif believed to mediate protein–protein interactions (Neer et al., 1994; Wall et al., 1995; Sondek et al., 1997). Apaf-2 has been identified as cytochrome c, which was released to the cytosol during homogenization (Liu et al., 1996b).

In the current studies, we have purified the third apoptotic protease activating factor, Apaf-3, and we have used it to reconstitute the caspase-3 activation reaction using purified components. Protein sequencing and immunoblot analysis revealed that Apaf-3 is identical to caspase-9/Mch6/ICE-LAP6 (Alnemri et al., 1996; Duan et al., 1996; Srinivasula et al., 1996a). Caspase-9 binds to Apaf-1 in a cytochrome c– and dATP-dependent fashion and becomes activated under such condition. Activated

caspase-9 in turn cleaves and activates caspase-3. An active site mutant of caspase-9 blocks caspase-3 activation and cellular apoptosis in vivo. These data demonstrate an orderly activation of the caspase cascade during apoptosis.

Results

Purification of Apaf-3 and Its Identification as Caspase-9

Caspase-3 activation was assayed by incubating in vitro–translated, ^{35}S-labeled, and affinity-purified caspase-3 with HeLa S-100 cytosolic fraction. Caspase-3 activation was initiated by the addition of dATP. The activated 20 kDa and 12 kDa fragments were separated from the 32 kDa precursor by SDS-PAGE (polyacrylamide gel electrophoresis) and visualized by phosphorimaging (Liu et al., 1996b; Zou et al., 1997).

The separation of Apaf-1, -2, and −3 was initiated by applying HeLa cell S-100 to an SP-Sepharose column. Cytochrome c (Apaf-2) bound to the column while Apaf-1 and Apaf-3 flowed through it. Apaf-1 and Apaf-3 were further separated on a hydroxylapatite column, which bound Apaf-1 but not Apaf-3. Apaf-3 was further purified by the following six additional steps: Q-Sepharose ion-exchange chromatography, phenyl-sepharose hydrophobic-interaction chromatography, heparin-agarose ion-exchange chromatography, Superdex 200 gel filtration chromatography, Mono Q ion-exchange chromatography,
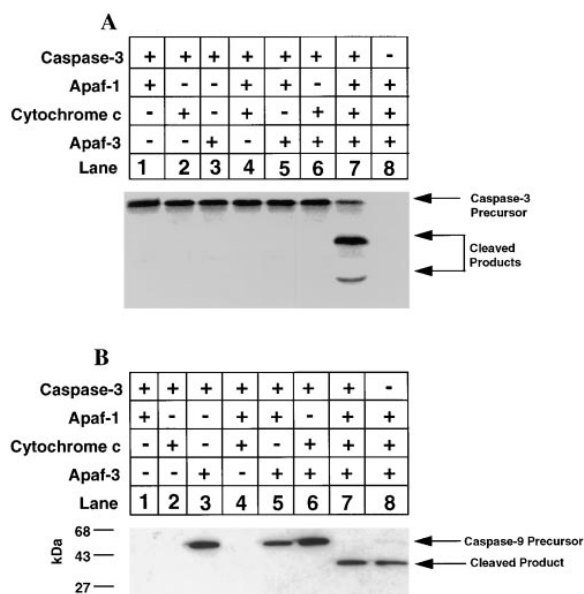
Figure 2. Reconstitution of Caspase-3 Activation with Purified Components

Aliquots (2 μl) of in vitro–translated, ^{35}S-labeled, and affinity–purified caspase-3 were incubated at 30°C for 1 hr with aliquots of 2 μl Apaf-1 purified through the Mono Q column step as described in Zou et al. (1997) (lanes 1, 4, 5, and 7), or 1 μl (0.3 μg) cytochrome c (lanes 2, 4, 6, and 7), or 2 μl of purified Apaf-3 (12.5 ng) (lanes 3 and 5–7) in a final volume of 20 μl buffer A supplemented with 1 mM dATP and 1 mM MgCl$_2$. In lane 8, Apaf-1, cytochrome c and Apaf-3 were incubated as described above in the absence of in vitro-translated ^{35}S-labeled caspase-3. After incubation, samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter.
(A) The filter was exposed to a phosphorimaging plate for 2 hr at room temperature.
(B) The same filter was probed with a polyclonal anti-caspase-9 antibody generated as described in the Experimental Procedures. The antigen/antibody complex was visualized by an ECL method as described in the Experimental Procedure. The filter was exposed to a Kodak X-Omat AR X-ray film for 10 s.

and finally, glycerol gradient centrifugation. The results of the glycerol gradient are shown in Figure 1. After centrifugation, the fractions were collected from bottom to top and assayed for Apaf-3 activity by incubating each fraction with partially purified Apaf-1 (hydroxylapatite bound fraction), cytochrome c, and ^{35}S-labeled caspase-3 in the presence of dATP. Apaf-3 activity appeared in fractions 6–8 with a peak at fraction 7 (Figure 1A). The same fractions were subjected to SDS-PAGE followed by silver stain (Figure 1B). A single polypeptide band migrating just above the 45 kDa molecular weight marker coeluted with the Apaf-3 activity. The NH$_2$ terminus of this protein band was blocked to Edman degradation. To obtain protein sequence, the polypeptide band was blotted onto a PVDF membrane and subjected to tryptic digestion. The resulting tryptic peptides were separated by a reverse-phase HPLC column, and the sequences of two peptides were determined by Edman degradation. A database search revealed that the two peptide sequences, LSKPTLENLTPVVLR and TFDQLD ISSLPTP, matched perfectly with amino acid residues

98–112 and 325–337, respectively, of human caspase-9/MCH6/ICE-LAP6 (Figure 1C, underlined).

Reconsititution of Caspase-3 Activation with Purified Components
Figure 2A shows the reconstitution of the caspase-3 activation reaction using three purified proteins: Apaf-1, cytochrome c, and Apaf-3. No single component nor any combination of two was sufficient to activate caspase-3 (lanes 1–6). All three proteins plus dATP were required to cleave caspase-3 (lane 7).

Using a polyclonal antibody generated against a recombinant caspase-9 fusion protein, we examined the cleavage of caspase-9 during the caspase-3 activation reaction by immunoblot analysis (Figure 2B). Apaf-3 was recognized by the anti-caspase-9 antibody, confirming that Apaf-3 is caspase-9 (lanes 3 and 5–8). Caspase-9 was activated in the presence of Apaf-1, cytochrome c, and dATP regardless of whether caspase-3 was present (lanes 7 and 8), indicating that caspase-9 activation is independent of caspase-3 activity.

Caspase-9 Is Required for Caspase-3 Activation
To further demonstrate that caspase-9 is upstream of caspase-3, we immunodepleted caspase-9 from the HeLa cell S-100 fraction using the antibody against caspase-9. As shown in Figure 3, S-100 depleted of either caspase-9 or cytochrome c failed to activate caspase-3 (lanes 3, 4, 7, and 8). Caspase-3 was not depleted by the antibodies against caspase-9 or cytochrome c since the caspase-3 precursor was present at approximately the same level in all reactions (Figure 3B). Addition of purified Apaf-3 or cytochrome c to extracts depleted of the respective proteins restored the cleavage of caspase-3 (lanes 5, 6, 9, and 10). The endogenous caspase-3 precursor present in these extracts was cleaved in the same fashion as the ^{35}S labeled caspase-3 (Figure 3B).

Activated Caspase-9 Cleaves Caspase-3
The above data indicate that caspase-9 is the upstream caspase in a protease cascade that activates caspase-3. To demonstrate that active caspase-9 cleaves caspase-3, we generated recombinant active caspase-9 enzyme by overexpressing it in bacteria.

As shown in Figure 4, activated caspase-9 cleaves and activates caspase-3 in as early as 15 min. This implies that, once caspase-9 becomes activated, it will initiate a protease cascade leading to the rapid activation of caspsase-3, a major caspase activity in cells undergoing apoptosis (Faleiro et al., 1997; Martins et al., 1997; Takahashi et al., 1997). The enzymatic activity of caspase-9 can be inhibited by zVAD-fmk, a general caspase inhibitor that blocks cellular apoptotic response (data not shown).

Evidence that Hydrolysis of dATP or ATP Is Required for Caspase-9 Activation
Using only purified components, we reconstituted the caspase-3 activation reaction in the presence of varying concentrations of either dATP or ATP (Figure 5). Caspase-9 and caspase-3 were both activated in the presence of 1 μM dATP (lane 3, panels A and B). Similar

A

	S-100		S-100 --Casp.9				S-100 --Cyt.c			
Cytochrome c	-	-	-	-	-	-	-	-	+	+
Caspase-9	-	-	-	-	+	+	-	-	-	-
dATP	-	+	-	+	-	+	-	+	-	+
Lane	1	2	3	4	5	6	7	8	9	10

← Caspase-3 Precursor

← Cleaved Products

B

	S-100		S-100 --Casp.9				S-100 --Cyt.c			
Cytochrome c	-	-	-	-	-	-	-	-	+	+
Caspase-9	-	-	-	-	+	+	-	-	-	-
dATP	-	+	-	+	-	+	-	+	-	+
Lane	1	2	3	4	5	6	7	8	9	10

← Caspase-3 Precursor
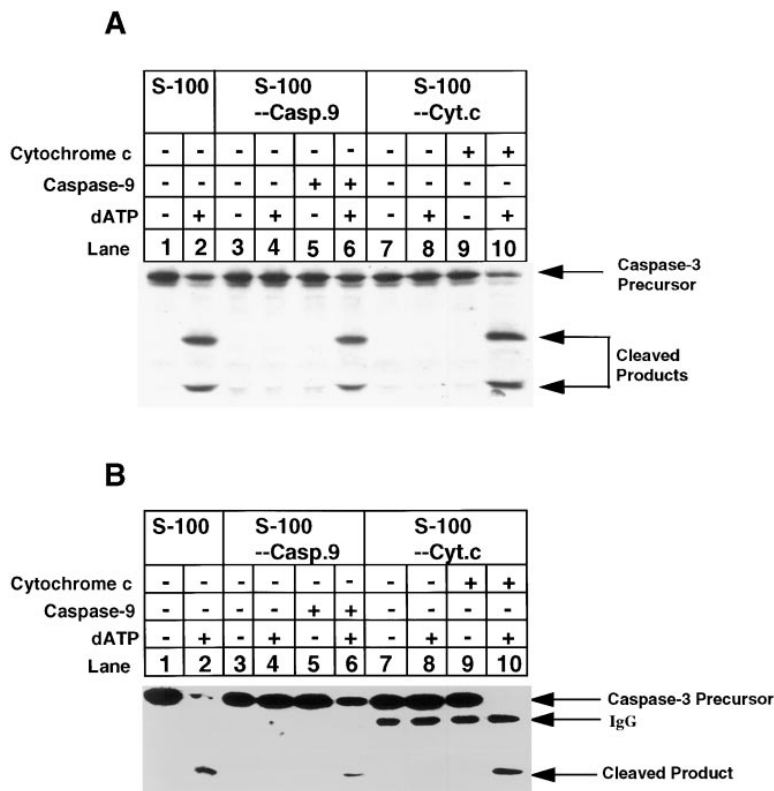
← IgG

← Cleaved Product

Figure 3. Caspase-9 and Cytochrome c Are Required for Caspase-3 Activation

Depletion of caspase-9 from HeLa cell S-100 was performed as described in the Experimental Procedures. Depletion of cytochrome c was as described in Liu et al. (1996b).

(A) Aliquots (50 μg) of HeLa cell S-100 (lanes 1 and 2), or Hela S-100 immunodepleted of caspase-9 (lanes 3–6), or Hela S-100 immunodepleted of cytochrome c (lanes 7–10) were incubated with aliquots (3 μl) of in vitro–translated, ^{35}S-labeled caspase-3 in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of 1 mM dATP in a final volume of 20 μl of buffer A. Aliquots of 6 μl of Apaf-3 (37.5 ng) purified to the Mono Q step (lanes 5 and 6), or 0.2 μg purified cytochrome c (lanes 9 and 10) were supplemented to the indicated reactions. After incubating at 30°C for 1 hr, the samples were subjected to 15% SDS-PAGE, and the gel was subsequently transferred to a nitrocellulose filter. The filter was exposed to film for 3 days at −80°C.

(B) Same reactions were performed as in (A) except the in vitro–translated, ^{35}S-labeled caspase-3 was omitted. The samples were then subjected to 15% SDS-PAGE followed by electroblotting to a nitrocellulose filter. The filter was probed with 25 μg of a monoclonal antibody to caspase-3 (Transduction Laboratories). The antigen/antibody complexes were visualized by an ECL method as described in Experimental Procedures. The filter was exposed to Kodak X-OMAT X-ray film for 10 min.

activation occurred in the presence of ATP, but a much higher concentration of ATP (1 mM) was required (lane 7). This result is consistent with our previous observation that dATP is the preferred nucleotide for activation (Liu et al., 1996b). Neither caspase-9 nor caspase-3 was cleaved in the presence of the nonhydrolyzable ATP analogs, AMP-PNP or ATP-γ-S, suggesting that hydrolysis is required for the reaction (lanes 8–13). dADP, a nucleotide that substitutes for dATP in the crude system (Liu et al., 1996b), did not function in the purified system (data not shown). We suggest that dADP was converted to dATP in the crude system.

Apaf-1 and Caspase-9 Form a Complex in the Presence of dATP and Cytochrome c
To further explore the mechanism of caspase-9 activation, we studied the interaction of caspase-9 with Apaf-1

Time (min)   0   15   30   45   60   120
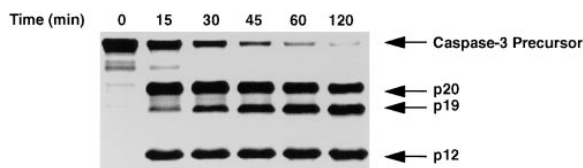
← Caspase-3 Precursor
← p20
← p19
← p12

Figure 4. Caspase-9-Mediated Cleavage of Caspase-3

Active caspase-9 was prepared as described in the Experimental Procedures. Aliquots (10 ng) of active caspase-9 were incubated with aliquots (0.5 μl) of in vitro–translated, ^{35}S-labeled, and affinity-purified caspase-3 precursor for the indicated times at 37°C. The samples were subsequently subjected to 15% SDS-PAGE and exposed to X-ray film for 12 hr at room temperature.

by coimmunoprecipitation using the antibody against caspase-9. The precipitates were analyzed by immunoblotting with an antibody against Apaf-1 (Zou et al., 1997). As shown in Figure 6A, the anti-caspase-9 antibody precipitated Apaf-1 in the presence of cytochrome c, dATP and Apaf-3 (lane 10). Preimmune serum from the same animal did not precipitate Apaf-1 (lane 9). Omission of dATP (lanes 7–8), cytochrome c (lanes 3–6), Apaf-3 (lanes 11–12), or Apaf-1 (lanes 1–2) obliterated coimmunoprecipitation of Apaf-1.

To further demonstrate that the interaction of caspase-9 with Apaf-1 requires cytochrome c and dATP, we performed the same coimmunoprecipitation experiment with HeLa S-100 immunodepleted of cytochrome c (Figure 6B). This extract possessed no caspase-3 activating activity unless purified cytochrome c was added to the reaction (Figure 3, lanes 7–10). Apaf-1 was coimmunoprecipitated with caspase-9 in this extract only when both cytochrome c and dATP were added (lane 8). Preimmune serum did not precipitate Apaf-1 (lane 7).

Caspase-9 Binds to Apaf-1 through Interaction of CARDs
Several caspases, including caspase-9, have long prodomains at their NH$_2$ termini. This domain has been proposed to function as a CARD, allowing proteins with such domains to interact with each other (Hofmann et al., 1997). Although Apaf-1 is not a caspase, its NH$_2$-terminal region contains a CARD, suggesting that Apaf-1 may recruit caspase-9 through their respective CARDs (Zou et al., 1997). To test this possibility directly, we
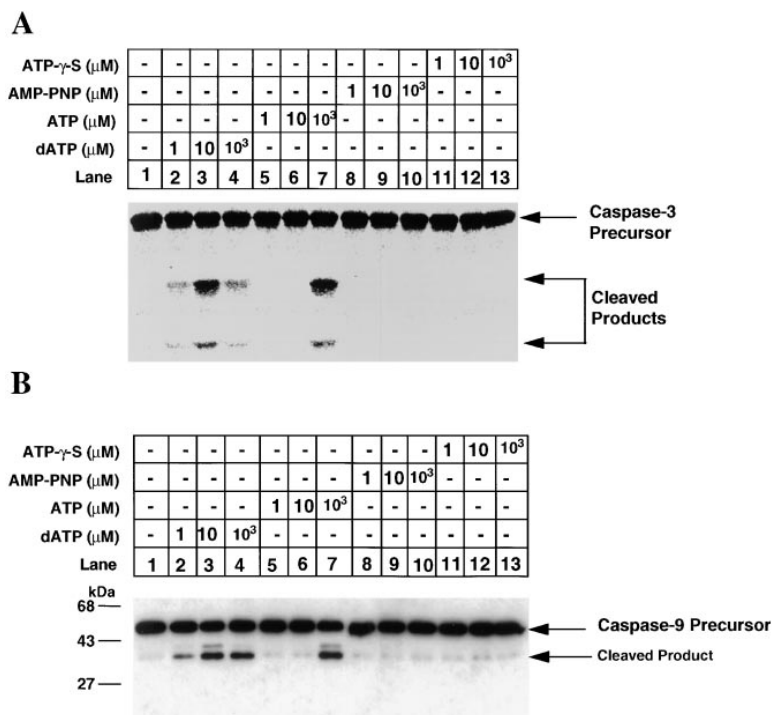
A

ATP-γ-S (μM)	-	-	-	-	-	-	-	-	-	-	1	10	10^3
AMP-PNP (μM)	-	-	-	-	-	-	-	1	10	10^3	-	-	-
ATP (μM)	-	-	-	-	1	10	10^3	-	-	-	-	-	-
dATP (μM)	-	1	10	10^3	-	-	-	-	-	-	-	-	-
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13

← Caspase-3 Precursor

← Cleaved Products

B

ATP-γ-S (μM)	-	-	-	-	-	-	-	-	-	-	1	10	10^3
AMP-PNP (μM)	-	-	-	-	-	-	-	1	10	10^3	-	-	-
ATP (μM)	-	-	-	-	1	10	10^3	-	-	-	-	-	-
dATP (μM)	-	1	10	10^3	-	-	-	-	-	-	-	-	-
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13

kDa
68 —
43 — ← Caspase-9 Precursor
← Cleaved Product
27 —

Figure 5. Titration of dATP, ATP, and Nonhydrolyzable ATP Analogs in the Activation of Caspase-3 and Caspase-9

Aliquots (2 μl) of Apaf-1 purified through the Mono Q column step as described in Zou et al. (1997) were incubated at 30°C for 1 hr with aliquots of 2 μl of purified Apaf-3 (12.5 ng), 1 μl (0.3 μg) cytochrome c, and 2 μl of in vitro–translated, ^{35}S-labeled, and affinity-purified caspase-3 in a final volume of 20 μl of buffer A in the absence of nucleotides (lane 1), or in the presence of increasing concentrations of dATP (lanes 2, 3, and 4), ATP (lanes 5, 6, and 7), AMP-PNP (lanes 8, 9, and 10), or ATP-γ-S (lanes 11, 12, and 13). Samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter.
(A) The filter was exposed to a phosphorimaging plate for 2 hr at room temperature.
(B) The same filter was probed with a polyclonal anti-caspase-9 antibody generated as described in the Experimental Procedures. The antigen/antibody complex was visualized by an ECL method as described in the Experimental Procedure. The filter was exposed to a Kodak X-Omat AR X-ray film for 10 s.

expressed the first 97 amino acids of Apaf-1 fused to a GST protein and studied its interaction with caspase-9. As shown in Figure 7, wild-type caspase-9 translated in vitro bound to the CARD of Apaf-1. The active site mutation, cysteine 287 to alanine, also bound to this fusion protein. Caspase-9 without its prodomain no longer bound. Caspase-3, which does not have a long prodomain, did not bind to the CARD of Apaf-1 either. GST alone did not bind caspase-9. In contrast to the results with full-length Apaf-1, we found that the CARD-GST fusion protein bound caspase-9 in the absence of cytochrome c and dATP (data not shown). These data suggest that, within the context of full-length Apaf-1, the CARD is not accessible for caspase-9 binding. Cytochrome c and dATP may induce a conformational change in Apaf-1 that exposes its CARD.

Active Site Mutation of Caspase-9 Blocks Caspase-3 Activation and Apoptosis In Vivo

Inasmuch as the active site mutant version of caspase-9 binds to Apaf-1, we designed experiments to determine whether this mutant protein exerts a dominant-negative influence on activation of caspase-3 and cellular apoptosis response in vivo.

When human breast carcinoma MCF-7 cells were transiently transfected with an expression vector, about 17% of the cells showed spontaneous apoptosis (Figure 8A). When the vector encoded full-length Apaf-1 or wild-type caspase-9, there was a slight increase to 26% and 29%, respectively. When Apaf-1 and wild-type caspase-9 were coexpressed, almost 60% of the cells underwent apoptosis. When the active site mutant of caspase-9 (C287A) was coexpressed with Apaf-1, the number of cells undergoing apoptosis decreased to about 10%.

Bax is a member of the bcl-2 family that opposes the action of bcl-2 and accelerates cell death when overexpressed in mammalian cells (Oltvai et al., 1993). Overexpression of Bax in human MCF-7 cells elicited apoptosis in nearly 90% of the transfected cells (Figure 8). Cotransfection with the active site mutant of caspase-9 reduced the percentage of cells undergoing apoptosis to about 20%, which is near the background level.

To confirm that the active site mutant of caspase-9 (C287A) blocks apoptosis by inhibiting caspase-3 activation, we analyzed caspase-3 in human embryonic kidney cells (293 cells) that were transfected with Bax, or Bax plus the mutant caspase-9 (C287A). As shown in Figure 8B, activation of caspase-3 was detected in cells that were overexpressing Bax (lane 2). The activation of caspase-3 was decreased to about 50% when an equal amount of mutant caspase-9 was coexpressed with Bax, and the activation of caspase-3 was completely blocked when a 4-fold excess amount of mutant caspase-9 was used (lanes 3–4).

Discussion

In the current report, we present experimental data that suggest a mechanism for the activation of caspase-3/CPP32, a major caspase activity in cells undergoing apoptosis (Faleiro et al., 1997; Martins et al., 1997; Takahashi et al., 1997). The model, shown in Figure 9, indicates that caspase-3 activation begins when caspase-9 binds to Apaf-1 in a reaction triggered by cytochrome c and dATP. Complex formation between Apaf-1 and caspase-9 is mediated by the CARD on the two proteins. Binding leads to the cleavage of caspase-9, converting it to an active protease. Active caspase-9 then cleaves
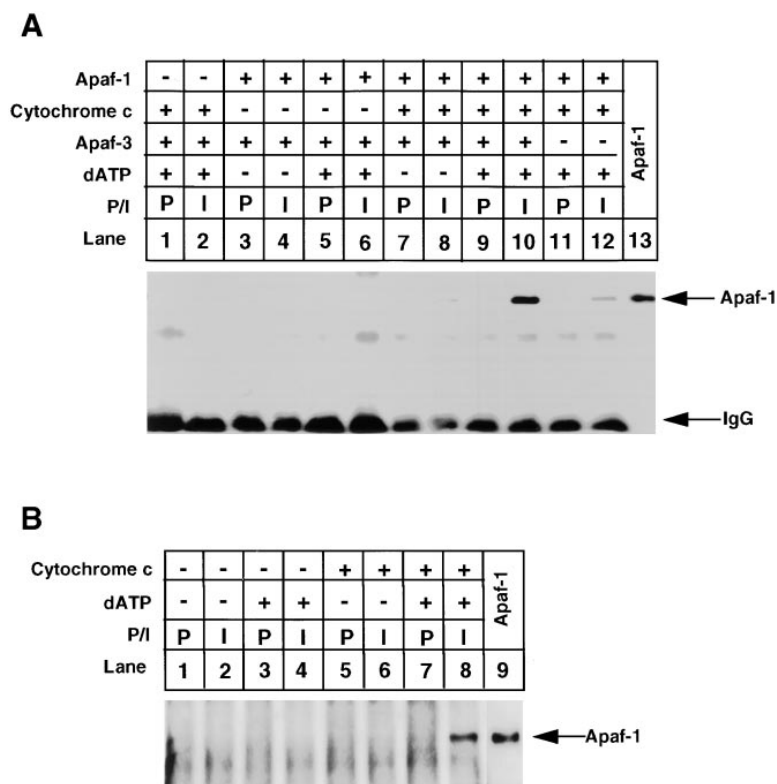
A

Apaf-1	-	-	+	+	+	+	+	+	+	+	+	+	
Cytochrome c	+	+	-	-	-	-	+	+	+	+	+	+	
Apaf-3	+	+	+	+	+	+	+	+	+	+	-	-	Apaf-1
dATP	+	+	-	-	+	+	-	-	+	+	+	+	
P/I	P	I	P	I	P	I	P	I	P	I	P	I	
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13

◄— Apaf-1

◄— IgG

B

Cytochrome c	-	-	-	-	+	+	+	+	
dATP	-	-	+	+	-	-	+	+	Apaf-1
P/I	P	I	P	I	P	I	P	I	
Lane	1	2	3	4	5	6	7	8	9

◄—Apaf-1

Figure 6. Formation of Apaf-1/Caspase-9 Complex

(A) Apaf-1 and Apaf-3 were seperated by a hydroxyapatite column as described in Zou et al. (1997) and used for the immunoprecipitation experiment. Aliquots of 50 μl of the Apaf-1 fraction, 100 μl of the Apaf-3 fraction, 0.5 μl (10 mg/ml) of purified cytochrome c, and dATP to a final concentration of 1 mM were added as indicated to a final volume of 500 μl of buffer A. 500 μg of BSA was also added to all reactions to reduce nonspecific binding to the beads. After incubating at 30°C for 20 min, aliquots (25 μl) of anti-caspase-9 antibody/protein A beads prepared as in Experimental Procedures were added to the reaction. After incubation with rotation overnight at 4°C, the beads were centrifuged and washed with 1 ml of buffer A three times. The beads were then resuspended in 60 μl of 1× SDS loading buffer. After boiling for 5 min, the beads were pelleted by centrifugation and the supernatants were collected. Aliquots of 15 μl of resulting supernatants were subjected to 8% SDS-PAGE followed by electroblotting to a nitrocellulose filter. Purified Apaf-1 (5 ng) was loaded on lane 13. The filter was probed with the serum against Apaf-1 (1:2000 dilution) as described in Zou et al. (1997). The antigen/antibody complexes were visualized by an ECL method as described in Experimental Procedures. The filter was exposed to Kodak X-OMAT X-ray film for 1 min at room temparature. P, preimmune serum, I immune serum. (B) Aliquots of 120 μl (900 μg protein) of the Hela cell S-100 extract immunodepleted of cytochrome c (see Figure 3) were incubated in the absence (lanes 1, 2, 5, and 6) or presence (lanes 3, 4, 7, and 8) of 1 mM dATP at 30°C for 20 min in a final volume of 500 μl of buffer A. Purified cytochrome c (5 μg) was added to lanes 3, 4, 7, and 8. Purified Apaf-1 (5 ng) was loaded on lane 9. After incubation, the samples were subjected to immunoprecipitation and Western blot analysis as described in (A). The filter was exposed to Kodak X-OMAT film for 1 s (lanes 1–8) or 15 s (lane 9).

and activates caspase-3, thereby setting in motion the events that lead to DNA fragmentation and cell death.

Role of Apaf-1

The current data suggest that Apaf-1 serves as a docking protein for caspase-9 and cytochrome c. Apaf-1 is a 130 kDa protein that possesses three distinct domains (Zou et al., 1997). The NH$_2$-terminal 85 amino acids are homologous to the CARD segment in the prodomain of several caspases, including CED-3, caspase-1, caspase-2, and caspase-9 (Thornberry et al., 1992; Yuan et al., 1993; Wang et al., 1994; Duan et al., 1996; Srinivasula et al., 1996a). CARDs have been proposed to mediate the recruitment of caspases (Hofmann et al., 1997). Indeed, the CARD of Apaf-1 readily binds to caspase-9 (Figure 7). These data do not exclude the possibility that caspase-9 interacts with other regions of Apaf-1 as well. Apaf-1 binds caspase-9 only in the presence of cytochrome c and dATP. This suggests that the CARD of Apaf-1 is normally not exposed when cytochrome c is absent from the cytosol.

Role of Cytochrome c

Cytochrome c is absolutely required in our in vitro system for the activation of caspase-3 and caspase-9. When cytochrome c was depleted from HeLa cell S-100 extracts by a monoclonal antibody, the ability to activate caspases was abolished. Activity was restored when purified cytochrome c was added back (Figure 3). The essential role of cytochrome c was further demonstrated in the coimmunoprecipitation experiment. When cytochrome c was depleted, caspase-9 failed to bind Apaf-1 even in the presence of dATP. The binding was restored when purified cytochrome c was added (Figure 6). These data strongly support the suggestion that cytochrome c initiates apoptosis by inducing the formation of the Apaf-1/caspase-9 complex.

Holocytochrome c is normally present exclusively in mitochondria. Apocytochrome c that is newly synthesized in the cytosol cannot activate apoptosis (Yang et al., 1997). In response to a variety of apoptosis-inducing agents, cytochrome c is released from mitochondria to the cytosol (Liu et al., 1996; Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997). In cells overexpressing bcl-2, or its close family member bcl-xl, the release of cytochrome c is blocked, aborting the apoptotic response (Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997). Cytochrome c binds to Apaf-1 in the absence of dATP (Zou et al., 1997), but this complex will not bind caspase-9 unless dATP or ATP is present (Figure 6). It is likely that the nucleotide induces a conformational change in the Apaf-1/cytochrome c complex that exposes the CARD domain of Apaf-1, allowing caspase-9 to bind. The precise region of Apaf-1 that binds cytochrome c has not yet been mapped.
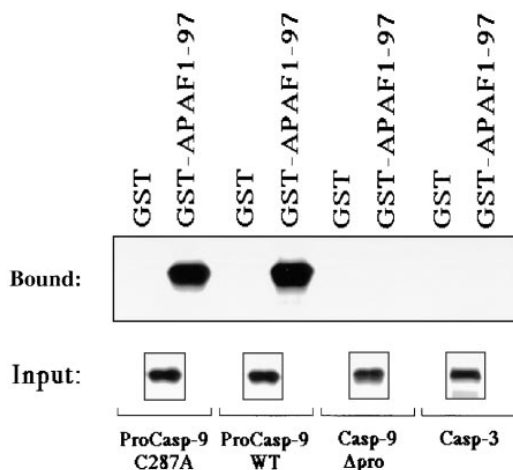
Figure 7. Apaf-1 and Caspase-9 Interact via their CARDs

The CARD domain of Apaf-1 (residues 1–97) was subcloned into the bacterial expression vector pGEX-5X-3 in-frame with an N-terminal GST tag. GST and GST-APAF-1-CARD fusion protein were expressed in E. coli DH5α bacteria and then immobilized on glutathione-Sepharose. Labeled interacting proteins were prepared as in Figure 4, and aliquots of 1/10 of the input for binding assay were subjected directly to SDS-PAGE and visualilzed by autoradiography. Equal amounts of the indicated translation reactions were diluted to 150 μl with GST binding buffer (50 mM Tris-HCl [pH 7.6], 120 mM NaCl, and 0.5% Brij supplemented with protease inhibitors) and allowed to incubate with the immobilized GST or GST-Apaf-1(1–97) at 4°C for 2 hr with rotation. The beads were pelleted by centrifugation and washed four times with the same buffer and boiled for 3 min in 1× SDS sample buffer. After a brief centrifugation, the resulting supernatants were subjected to a 10% SDS gel and visualized by autoradiography.

Role of dATP/ATP

On the COOH-terminal side of the CARD domain, Apaf-1 contains a region of 320 amino acids that is homologous to CED-4. This region contains Walker's A and B boxes and the nucleotide p-loop (Walker et al., 1982; Zou et al., 1997), suggesting that it serves as a nucleotide binding site. Mutations of residues in CED-4 that are required for nucleotide binding destroy its ability to promote caspase activation (Chinnaiyan et al., 1997; Seshagiri and Miller, 1997). Similar mutations of Apaf-1 also obliterate its activity (S. M. S. and E. S. A., unpublished data). ATP will replace dATP, but the required concentration is much higher (1 μM for dATP versus 1 mM for ATP) (Figure 5). The intracellular concentrations of ATP and dATP are in the range of 0.2–10 mM and 10–20 μM, respectively (Skoog and Bjursell, 1974), raising the possibility that ATP, as well as dATP, may support apoptosis within the cell. Our observation that nonhydrolyzable analogs of ATP fail to subsitute for ATP suggests that hydrolysis of the high-energy bond is required for caspase activation.

The suggestion that ATP is required to carry out the cell suicide program is supported by recent observation that cells depleted of ATP undergo necrosis instead of apoptosis in response to apoptotic stimuli (Eguchi et al., 1997; Leist et al., 1997). Restoring ATP levels restored the apoptotic response (Eguchi et al., 1997; Leist et al., 1997).
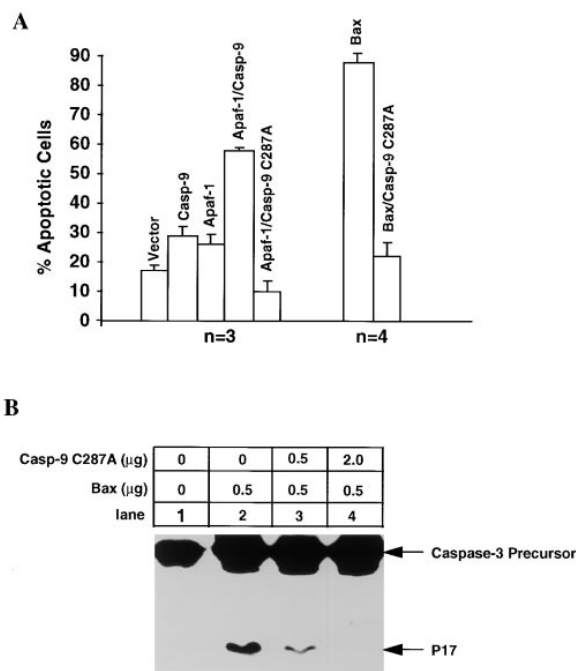

Figure 8. Inhibition of Apoptosis and Caspase-3 Activation by an Active Site Mutant of Caspase-9

Human MCF-7 and 293 cells were transiently transfected with the indicated plasmids as described in the Experimental Procedures.
(A) The graph shows the percentage of round blue apoptotic MCF-7 cells as a function of total blue cells under each condition.
(B) Human embryonic kidney cells (293 cells) were transfected with the indicated amount of Bax in pCIneo vector together with the indicated amount of plasmid containing C287A mutant caspase-9 as described in the Experimental Procedures. Cells were collected 30 hr after transfection, and the cell lysates were analyzed by Western blot analysis using a polyclonal antibody against human caspase-3.

The Role of Caspase-9

All of our data are consistent with the suggestion that caspase-9 is the caspase that is directly activated by Apaf-1 and cytochrome c, at least in the cellular extracts that we studied. When the HeLa S-100 extracts were immunodepleted of caspase-9, they lost the ability to activate caspase-3, and activity was restored by addition of purified caspase-9. The initial cleavage of caspase-9, whether it is through autocatalysis or another mechanism, is currently under investigation.

Several other caspases, including caspase-1, -2, -8, and -10, also contain long NH$_2$-terminal CARDs like that of caspase-9 (Thornberry et al., 1992; Wang et al., 1994; Boldin et al., 1996; Muzio et al., 1996; Srinivasula et al., 1996b). It is possible that one or more of these caspases might substitute for caspase-9 in tissues in which their expression is high.

Experimental Procedures

General Methods and Materials

We obtained dATP and other nucleotides from Pharmacia, radioactive materials from Amersham, and molecular weight standards for SDS-PAGE and gel filtration chromatography from Bio-Rad and GIBCO-BRL. Protein concentrations were determined by the Bradford method; general molecular biology methods were used as described in Sambrook et al. (1989).
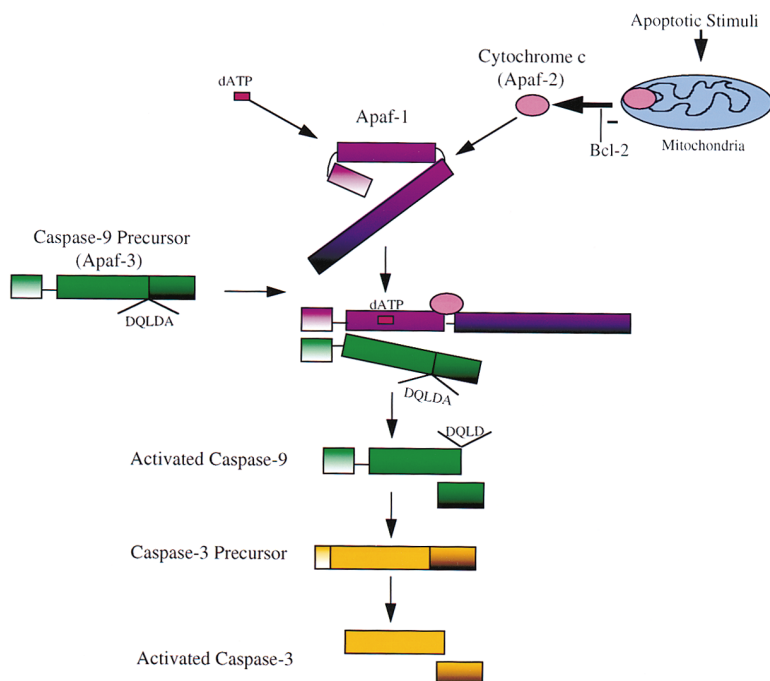
Figure 9. Model of Caspase-3 Activation
See the text for details. Mitochondria, Apaf-1, cytochrome c, caspase-9, and caspase-3 are indicated.

Assay for Caspase-3 Activation
Caspase-3 was translated and purified as described (Liu et al., 1996a). A 3 μl aliquot of the in vitro–translated caspase-3 was incubated with the indicated protein fraction in the presence of 1 mM dATP and 1 mM of additional $MgCl_2$ at 30°C for 1 hr in a final volume of 20 μl of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF). At the end of the incubation, 7 μl of 4× SDS sample buffer was added to each reaction. After boiling for 3 min, each sample was subjected to a 15% SDS-PAGE. The gel was transferred to a nitrocellulose filter, which was subsequently exposed to a phosphorimaging plate and visualized in a Fuji BAS-1000 Phosphorimager.

Purification of Apaf-3 from HeLa S-100
All purification steps were carried out at 4°C. All chromatography steps except the SP-Sepharose column (Pharmacia), hydroxylapatite column (Bio-Rad), and Q-Sepharose (Pharmacia) were carried out using an automatic fast protein liquid chromatography (FPLC) station (Pharmacia).

HeLa S-100 (700 ml [4.9 g of protein]) from 100 liters of suspension-cultured HeLa cells was prepared as described in Liu et al. (1996b) and applied to a SP-Sepharose column (200 ml bed vol) equilibrated with buffer A. The 800 ml flow-through fraction (3648 mg of protein) was collected and loaded directly onto a hydroxylapaptite column (50 ml bed vol) equilibrated with buffer A. Flow-through fraction (2741 mg of protein) of the hydroxylapatite column was directly loaded on to a Q-Sepharose column (100 ml bed vol). The column was washed with three colum vol of buffer A. The bound material was eluted with three 200 ml steps elution with buffer A containing 0.1 M NaCl, 0.2 M NaCl, and 0.3 M NaCl. The fractions from step elution were assayed for Apaf-3 activity. Ammonium sulfate (1 M) was added directly to the active faction (0.2 M NaCl elution, 279 mg of protein), and after rotating at 4°C for 1 hr, the precipitate was pelleted by centrifugation in a SA-600 rotor (Sorvall) at 15,000 rpm for 20 min, and the supernatant was directly loaded onto a Phenyl-Superose 10/10 column (Pharmacia) equilibrated with buffer A containing 1 M ammonium sulfate. The column was eluted with a 200 ml linear gradient of buffer A containing 1 M ammonium sulfate to buffer A. Fractions of 4 ml were collected and assayed for Apaf-3 activity after diluting 1/10 with buffer A. Active fractions (20 ml, 9.8 mg of protein) were pooled and dialyzed against buffer A and then loaded onto a 5 ml heparin-agarose column (Pharmacia) equilibrated

with buffer A. The column was washed with 20 ml of buffer A containing 100 mM NaCl and eluted with a 50 ml linear gradient from 100–300 mM NaCl, both in buffer A. Fractions of 4 ml were collected and assayed for Apaf-3 activity. The active fractions (12 ml, 3.4 mg of protein) were pooled and loaded directly onto a Superdex 200 16/60 gel-filtration column equilibrated with buffer A containing 100 mM NaCl. The column was eluted with the same buffer (6 runs), and fractions of 4 ml were collected starting from 30 ml of elution. The fractions were assayed for Apaf-3 activity and the active fractions were pooled (48 ml, 480 μg of protein). After diluting with equal volume of buffer A, the sample was loaded directly onto a Mono Q 5/5 column (Pharmacia) equilibrated with buffer A containing 50 mM NaCl. The column was eluted with a 20 ml linear gradient from 50–250 mM NaCl, both in buffer A. Fractions of 1 ml were collected and assayed for Apaf-3 activity. A 0.5 ml aliquot of the peak Apaf-3 fraction (12 μg) from the Mono Q column was directly loaded onto a 5 ml 10%–30% linear glycerol gradient in buffer A. After centrifugation at 55,000 rmp for 16 hr in a SW 60 rotor (Beckman), fractions of 0.5 ml were collected from bottom to top and assayed for Apaf-3 activity.

Production of Caspase-9 Fusion Protein
The full-length caspase-9 coding region (Srinivasula et al., 1996a) was cloned into the BamHI site of pET-15b vector (Novagen). The expression plasmid was transformed into bacteria BL21 cells (Novagen). In a typical caspase-9 preparation, a 5 ml overnight culture of bacteria containing the caspase-9 expression vector was added into a 1 liter LB broth, cultured for 3 hr by shaking at 220 rpm in 37°C. Isopropyl-1-thio-B-D-galactopyranoside (IPTG) was added to the culture in a final concentration of 1 mM and continued shaking for another 2 hr. The bacteria were pelleted by centrifugation, and the bacterial pellet was resuspended in 10 vol of buffer A. The resuspended cells were lysed in buffer A by sonication. After centrifugation at 10,000 g for 15 min, the supernatant was loaded onto a nickel affinity column (2 ml). The column was washed with 20 ml buffer A followed by 20 ml buffer A containing 1 M NaCl. After reequilibrating the column with 20 ml buffer A, the bound protein was eluted with buffer A containing 250 mM imidazole. The identity of caspase-9 was verified by SDS-PAGE followed by Coomassie blue staining and used to immunize rabbits.

The active caspase-9 was generated by transforming bacterial competent cells BL21(DE3) (Novagen) with a full-length caspase-9, an independent clone that differs from the published sequence at

amino acid 197, P197L (Srinivasula et al., 1996a), cloned into pET-21b vector (Novagen). The expression of the recombinant caspase-9 was induced with IPTG for 3 hr at room temparature. The recombinant protein was subsequently purified as described above.

Western Blot Analysis

Western blot analysis for Apaf-1 was performed as described previously (Zou et al., 1997). Anti-caspase-9 antiserum was generated by immunizing rabbits with a recombinant caspase-9 fusion protein (see above). Immunoblot analysis was performed with a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G using enhanced chemiluminescence Western blotting detection reagents (Amersham).

Immunodepletion of Caspase-9 from HeLa S-100

An aliquot of 500 μl of protein A–agarose (Santa Cruz) was incubated with an aliquot of 500 μl of preimmune or immune anti-caspase-9 serum at 4°C overnight. The antibody/protein A–agarose beads were then pelleted by centrifugation and washed five times with buffer A. The beads were resuspended in 1 ml of buffer A.

An aliquot of 50 μl of these beads was incubated with 100 μl BSA dissolved in buffer A (50 mg/ml) at room temperature for 2 hr. The beads were pelleted by centrifugation and incubated overnight at 4°C with 1 ml (8.3 mg/ml protein) HeLa cell S-100 extracts. The beads were subsequently pelleted by centrifugation, and the supernatant was used as extracts immunodepleted of caspase-9.

Transfection of MCF-7 and 293 Cells

Human MCF-7 cells were set at 1×10^5 cells per well in a 12-well plate at day 0. On day 2, the cells were transfected with 0.5 μg of pRSC-LacZ double expression vector (Invitrogen) plus 0.5 μg of pFLAG-CMV-2 vector (IBI Kodak) (well 1); or 0.5 μg of pRSC-LacZ vector plus 0.5 μg of pFlag-CMV-2 containing full-length Apaf-1 (pApaf-1/flag) (well 3); or 0.5 μg of pRSC-LacZ vector containing wild-type caspase-9 (pCasp-9) plus 0.5 μg of pFLAG-CMV-2 vector (well 2); or 0.5 μg of pRSC-LacZ vector containing the wild-type caspase-9 plus 0.5 μg of pFLAG-CMV-2 vector containing full-length Apaf-1 (well 4); or 2 μg of pCasp-9/C287A plus 0.5 μg of pApaf-1/flag and 0.5 μg of pRSC-LacZ vector (well 5); or 2 μg of pcDNA 3 vector plus 0.5 μg of full-length Bax in pRSC-LacZ (pRSC-LacZ-Bax) (well 6); or 2 μg of pCasp-9/C287A plus 0.5 μg of pRSC-LacZ-Bax (well 7) using the LipofectAMINE (Life Technologies, Inc.) method. The amount of DNA in all transfection experiments was made equal by including respective amounts of vector. Cells were stained for β-galactosidase expression and examined for morphological signs of apoptosis 30 hr after transfection.

Human embryonic kidney cells (293 cells) were set at 4×10^5 per well in a 6-well plate at day 0. On day 2, the cells were transfected with 0.5 μg of Bax in pCIneo vector together with the indicated amount of plasmid containing C287A mutant caspase-9 as described above.

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