

Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis

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Apaf-1 plays a critical role in apoptosis by binding to and activating procaspase-9. We have identified a novel Apaf-1 cDNA encoding a protein of 1248 amino acids containing an insertion of 11 residues between the CARD and ATPase domains, and another 43 amino acid insertion creating an additional WD-40 repeat. The product of this Apaf-1 cDNA activated procaspase-9 in a cytochrome *c* and dATP/ATP-dependent manner. We used this Apaf-1 to show that Apaf-1 requires dATP/ATP hydrolysis to interact with cytochrome *c*, self-associate and bind to procaspase-9. A P-loop mutant (Apaf-1K160R) was unable to associate with Apaf-1 or bind to procaspase-9. Mutation of Met368 to Leu enabled Apaf-1 to self-associate and bind procaspase-9 independent of cytochrome *c*, though still requiring dATP/ATP for these activities. The Apaf-1M368L mutant exhibited greater ability to induce apoptosis compared with the wild-type Apaf-1. We also show that procaspase-9 can recruit procaspase-3 to the Apaf-1–procaspase-9 complex. Apaf-1(1–570), a mutant lacking the WD-40 repeats, associated with and activated procaspase-9, but failed to recruit procaspase-3 and induce apoptosis. These results suggest that the WD-40 repeats may be involved in procaspase-9-mediated procaspase-3 recruitment. These studies elucidate biochemical steps required for Apaf-1 to activate procaspase-9 and induce apoptosis.
Keywords: Apaf-1/apoptosis/caspase-3/caspase-9

Introduction

Programmed cell death, or apoptosis, an evolutionarily conserved and genetically regulated biological process, plays a critical role in the development and the regulation of tissue homeostasis of multicellular organisms (Thompson, 1995; Yuan, 1996). Genetic analyses of the nematode *Caenorhabditis elegans* have identified two genes, *ced-3* and *ced-4*, required for the execution of cell death in the worm (Hengartner and Horvitz, 1994). The product of *ced-3* is homologous to mammalian interleukin-1 β -converting enzyme, a finding that provided the first indication that cysteine proteases are critical components of the cell death machinery (Miura *et al.*, 1993). These observations led to the identification of a growing family of cysteine proteases (designated as

caspases) related to CED-3 that represent the executionary arm of the apoptotic program in mammals, flies and nematodes (Núñez *et al.*, 1998; Thornberry and Labzenik, 1998). Caspases are synthesized in cells as inactive precursors which upon stimulation with apoptotic signals are processed into mature forms composed of a tetramer of two large and two small subunits (Thornberry *et al.*, 1992; Walker *et al.*, 1994; Rotonda *et al.*, 1996). The apoptotic process is characterized by the proteolytic cascade in which upstream (initiator) caspases mediate the activation of downstream (effector) caspases (Alnemri, 1997; Núñez *et al.*, 1998). Initiator caspases contain death effector domains (DEDs) or caspase recruitment domains (CARDs) that physically link these proteases to regulatory molecules via homophilic interactions (Li *et al.*, 1997; Pan *et al.*, 1998). Caspase-9 is an upstream caspase that contains a CARD in its N-terminus; a protein module that is also present in the prodomain of several death proteases, including CED-3 and its regulator CED-4 (Hofmann *et al.*, 1997). Activation of caspase-9 initiates a protease cascade that subsequently activates downstream caspase-3 and caspase-7, leading to the cleavage of target proteins and the orderly demise of the cell (Li *et al.*, 1997; Sun *et al.*, 1999).

Our understanding of how caspases are activated in mammalian cells has been greatly enhanced by biochemical studies of the nematode proteins CED-3, CED-4 and CED-9. CED-4 physically interacts with both CED-3 and CED-9, forming a multimeric protein complex (Chinnaiyan *et al.*, 1997; Irmiler *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a,b). CED-4 promotes the activation of CED-3 and this activation process is inhibited by CED-9 (Seshagiri and Miller, 1997; Wu *et al.*, 1997b). Biochemical experiments have suggested that a mechanism exists whereby CED-3 is activated through oligomerization of CED-4 (Yang *et al.*, 1998). Activation of CED-3 is induced through aggregation of precursor CED-3 molecules, an event leading to CED-3 autoprocessing and enzymatic activation (Yang *et al.*, 1998).

Apaf-1, a mammalian homologue of CED-4, has been identified recently (Zou *et al.*, 1997; Fearnhead *et al.*, 1998). The N-terminal half of Apaf-1 shares extensive homology with CED-4, containing a CARD followed by an ATPase domain with conserved Walker's A and B motifs (Zou *et al.*, 1997). The C-terminal region of Apaf-1 lacks homology with CED-4 and consists of 12 WD-40 repeats. An important role for Apaf-1 in the regulation of apoptosis has been revealed by analysis of mutant mice deficient in Apaf-1. Mice lacking Apaf-1 showed abnormalities in several tissues characterized by the lack of developmental cell death, particularly in brain tissue (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998). Furthermore, cells derived from Apaf-1-deficient mice showed resist-

ance to a wide variety of apoptotic stimuli, including chemotherapy drugs, dexamethasone and γ -irradiation (Yoshida *et al.*, 1998). In the presence of cytochrome *c* and dATP, Apaf-1 adopts a conformation that can bind to procaspase-9, an event that leads to its proteolytic activation (Li *et al.*, 1997). A conserved mechanism similar to that proposed for CED-3 has been suggested for caspase-9 activation (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). In this model, two or more caspase-9 precursors are brought together via Apaf-1 oligomerization, which is mediated by the ATPase-like domain of Apaf-1 (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). Mutant analyses of Apaf-1 have suggested that the WD-40 repeat region (WDR) plays an inhibitory role in Apaf-1 function, since mutants lacking the WDR are constitutively active (Hu *et al.*, 1998a,b; Srinivasula *et al.*, 1998). Furthermore, the WDR has been shown to interact with the ATPase-like domain of Apaf-1, inhibiting Apaf-1 self-association. This finding suggests the existence of a mechanism whereby the WDR inhibits Apaf-1 activity (Hu *et al.*, 1998b).

In these studies, we sought to understand further the biochemical mechanism by which Apaf-1 promotes the maturation of procaspase-9 into an active protease. Analysis of wild-type (wt) and mutant Apaf-1 proteins revealed that dATP/ATP hydrolysis is required for cytochrome *c* binding to Apaf-1, Apaf-1 self-association, interaction of Apaf-1 with procaspase-9, and ultimately for caspase-9 activation and caspase-9-mediated apoptosis. We also provide evidence that the Apaf-1–caspase-9 complex recruits procaspase-3, a process that may be regulated by the WDR of Apaf-1.

Results

Identification of Apaf-1XL, an alternatively spliced Apaf-1 cDNA that activates procaspase-9

In our initial studies of Apaf-1-mediated caspase-9 activation, we determined that procaspase-9 could be efficiently activated by endogenous Apaf-1 from several cell lines including HeLa and 293T cells (Hu *et al.*, 1998a; data not shown). We subsequently identified Apaf-1 cDNAs from these two cell lines. An Apaf-1 cDNA cloned from HeLa cells was identical to that previously reported (Zou *et al.*, 1997). However, the Apaf-1 cDNA cloned from 293T cells appeared to represent an alternatively spliced form of the Apaf-1 gene. This form, termed Apaf-1XL, contains an 11 amino acid (aa) insertion, GKDSVSGITSY, after the CARD (inserted between amino acids 98 and 99), and another 43 aa insertion between amino acids 811 and 812 (Figure 1A). This 43 aa region contains the last three amino acids of a WD-40 repeat followed by a large portion of another WD-40 repeat, thus creating an additional WD-40 repeat (Figure 1A). The sequence of these extra 43 aa is identical to that present in a truncated human Apaf-1 cDNA submitted to databases (DDBJ/EMBL/GenBank accession No. AB007873). Moreover, a mouse Apaf-1 cDNA isolated from brain tissue also contained this additional WD-40 repeat (Ceconi *et al.*, 1998). In addition, a recent study has identified an Apaf-1 form in HeLa cells that is identical to Apaf-1XL (Zou *et al.*, 1999). Using reverse transcriptase (RT)–PCR and Western blotting analysis, we have identified both Apaf-1XL mRNA and protein in 16 cell lines tested so far (data

not shown). Under our experimental conditions, Apaf-1XL protein expressed in 293T cells activated procaspase-9 in a cytochrome *c*- and dATP-dependent manner (Figure 1D). Under the same conditions, we were unable to demonstrate cytochrome *c*- and dATP-dependent procaspase-9 activation by the product of the original Apaf-1 cDNA (data not shown). Therefore, in the present studies we used the Apaf-1XL cDNA to examine the mechanism by which Apaf-1 activates procaspase-9 and refer to it as Apaf-1 for simplicity.

Mutation of Met368 to Leu enables full-length Apaf-1 to activate procaspase-9 independently of cytochrome *c* and dATP

We demonstrated previously that the WDR interacts with the N-terminal CED-4 homologous region of Apaf-1 and inhibits procaspase-9 activation when co-expressed with the constitutively active N-terminal Apaf-1 mutants (Hu *et al.*, 1998b). We performed experiments to determine whether the WDR with an extra WD-40 repeat is also capable of binding to N-terminal Apaf-1 with an 11 aa insertion [Apaf-1(1–570)] and inhibiting procaspase-9 activation. The analyses showed that the WDR containing the extra WD-40 repeat associated with Apaf-1(1–570) (Figure 1B) and inhibited procaspase-9 activation promoted by Apaf-1(1–570) (Figure 1C). To understand further how Apaf-1 promotes procaspase-9 activation, we tested several Apaf-1 N-terminal mutants accumulated during PCR mutagenesis for their ability to interact with the WDR. A mutant substituting amino acid Met368 for Leu, Apaf-1(1–570M368L), showed a reduced ability to bind the WDR (Figure 1B), but it retained its ability to promote procaspase-9 activation independent of cytochrome *c* and dATP (Figure 1C). In contrast to Apaf-1(1–570), however, the ability of Apaf-1(1–570M368L) to induce procaspase-9 activation was not inhibited by the WDR (Figure 1C). We next incorporated the M368L point mutation into full-length Apaf-1 and determined the ability of wt and mutant Apaf-1 to activate procaspase-9. In these experiments, we prepared cell extracts from 293T cells transiently transfected with Apaf-1 constructs and diluted the extracts so that endogenous Apaf-1 activity could not be detected in the procaspase-9 activation assay. Full-length Apaf-1 induced activation of procaspase-9 in a dATP- and cytochrome *c*-dependent manner (Figure 1D). In contrast, the point mutant Apaf-1M368L activated procaspase-9 independent of cytochrome *c* and dATP (Figure 1D). Apaf-1K160R, a mutant substituting Lys160 [an essential amino acid residue in the phosphate-binding loop (P-loop)] for Arg, failed to activate procaspase-9 in the presence or absence of dATP and cytochrome *c* (Figure 1D). This result confirmed previous observations that dATP/ATP hydrolysis is required for Apaf-1 to induce procaspase-9 activation (Li *et al.*, 1997).

The cytochrome *c*–Apaf-1 interaction requires ATP hydrolysis

To determine the role for cytochrome *c* in procaspase-9 activation, we immunoprecipitated wt and mutant Apaf-1 from cytosolic extracts in the presence or absence of dATP and assessed the binding of cytochrome *c* to Apaf-1 proteins by immunoblotting. Cytochrome *c* bound to

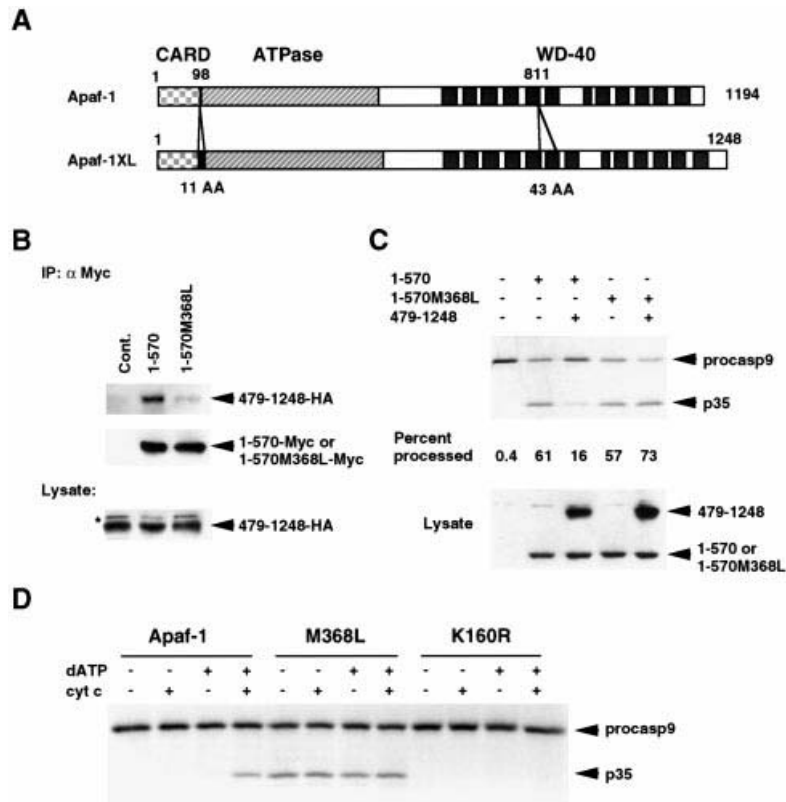


Fig. 1. Mutation of Met368 to Leu renders Apaf-1 capable of activating procaspase-9 independent of cytochrome *c* and dATP. (A) Schematic representation of the original Apaf-1 and Apaf-1XL. The 11 amino acids inserted after aa 98 are GKDSVSGITSY. The sequence of the extra 43 aa was identical to that identified in an Apaf-1 cDNA that is deposited in the DDBJ/EMBL/GenBank database (accession No. AB007873). (B) Mutation of Met368 to Leu decreases binding of Apaf-1(1–570) to WDR (WD-40 repeat region). pcDNA3 or plasmids encoding Myc-tagged Apaf-1(1–570) or Apaf-1(1–570M368L) were transfected into 293T cells with a construct producing HA-tagged WDR (aa 479–1248). Total cell lysates were immunoprecipitated with rabbit anti-c-Myc antibody and immune complexes were immunoblotted with anti-HA to detect the WDR. The asterisk indicates a non-specific band. (C) WDR fails to inhibit Apaf-1(1–570M368L)-mediated procaspase-9 activation. Twenty micrograms of cytosolic extracts containing indicated Apaf-1 mutants were incubated with *in vitro* translated [³⁵S]methionine-labeled procaspase-9 at 30°C for 30 min and separated by SDS–PAGE. Immature and cleaved caspase-9 fragments were scanned and quantitated with a phosphorimager and normalized according to the numbers of methionine residues in each form. Percentage of procaspase-9 converted into p35 is shown below each lane. Lower panel shows equal amounts of Apaf-1 mutant proteins in extracts. (D) Mutation of Met368 to Leu renders full-length Apaf-1 capable of activating procaspase-9 independent of cytochrome *c* and dATP. Two micrograms of extracts containing Apaf-1, Apaf-1M368L, or Apaf-1K160R were incubated with or without 1 mM dATP and 0.2 μg of cytochrome *c* at 30°C for 30 min.

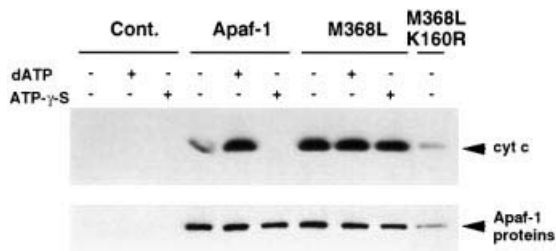


Fig. 2. Binding of cytochrome *c* to Apaf-1 requires dATP hydrolysis. Cytosolic extracts containing Myc-tagged Apaf-1, Apaf-1M368L or Apaf-1M368L/K160R were incubated with 10 μg/ml cytochrome *c* with or without 1 mM dATP or ATP-γS in the presence of monoclonal anti-Myc and protein A/G–agarose beads. After incubation at 4°C for 2 h, immunoprecipitation was performed as described in Materials and methods and cytochrome *c* associated with Apaf-1 proteins detected by immunoblotting. Wt and mutant Apaf-1 proteins precipitated from cytosolic extracts are shown in the lower panel.

Apaf-1, and this binding was greatly enhanced by the presence of dATP in the extract. Incubation of the extracts with the non-hydrolyzable ATP-γS completely abolished cytochrome *c* binding to wt Apaf-1, indicating that dATP/ATP hydrolysis is essential for Apaf-1 to interact with cytochrome *c* (Figure 2). The weak binding of

cytochrome *c* to Apaf-1 detected in the absence of dATP is presumably due to low levels of endogenous dATP/ATP in the cell extract. In contrast, cytochrome *c* interacted with Apaf-1M368L in the presence or absence of dATP (Figure 2). Addition of ATP-γS failed to inhibit cytochrome *c* binding to mutant Apaf-1M368L (Figure 2). Mutation of Lys160 (the critical P-loop residue required for ATP binding/hydrolysis) to Arg did not abolish the binding of Apaf-1M368L to cytochrome *c* (Figure 2), providing further evidence that cytochrome *c* can bind to Apaf-1M368L independent of dATP binding and hydrolysis. The diminished binding of cytochrome *c* to the double mutant Apaf-1M368L/K160R, when compared with wt Apaf-1 or Apaf-1M368L, could be explained by reduced expression of the double mutant Apaf-1 protein in the extract (Figure 2).

Apaf-1 self-association requires dATP/ATP hydrolysis

It was previously reported that Apaf-1 requires dATP/ATP to activate procaspase-9 (Li *et al.*, 1997). dATP/ATP hydrolysis could be required for Apaf-1 self-association and/or the Apaf-1–procaspase-9 interaction, since a disrupt-

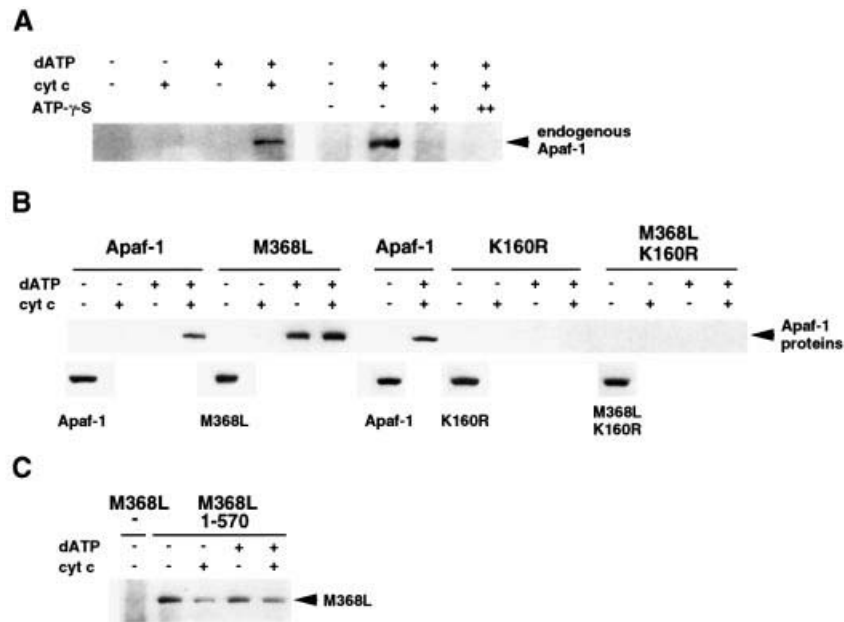


Fig. 3. Apaf-1 self-association requires cytochrome *c* and dATP hydrolysis. (A) Endogenous Apaf-1 binds Apaf-1(1–570) in the presence of cytochrome *c* and dATP. Equal amounts of 293T cytosolic extracts containing Myc-tagged Apaf-1(1–570) were incubated with rabbit anti-Myc antibody and protein A/G-agarose beads (1:1) under the indicated conditions at 4°C for 2 h. Then the beads were pelleted and washed four times with 1 ml NP-40 buffer. ‘++’ indicates that 5 mM ATP- γ S was included in the extract. (B) Interaction of wt Apaf-1, Apaf-1M368L, Apaf-1K160R, or Apaf-1M368L/K160R with Apaf-1(1–570). The experiments were performed as in (A) except that Apaf-1(1–570)-FLAG-containing extracts were mixed with lysates containing Apaf-1, Apaf-1M368L, Apaf-1K160R, or Apaf-1M368L/K160R. Expression of wt and mutant Apaf-1 proteins in total lysates is shown in lower panels. (C) Apaf-1M368L can associate with Apaf-1(1–570) independent of cytochrome *c* and dATP. Constructs producing Apaf-1(1–570) and Apaf-1M368L were cotransfected into 293T cells and prepared total lysates were used for immunoprecipitation as described in (A).

tion of either step might be sufficient to inhibit procaspase-9 activation (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). We first determined whether dATP/ATP hydrolysis is required for Apaf-1 self-association in cytosolic extracts. We transiently transfected 293T cells with a Myc-tagged Apaf-1(1–570) construct and assessed its interaction with endogenous Apaf-1 in the absence of detergent. Immunoprecipitation experiments showed that endogenous Apaf-1 associated with Apaf-1(1–570) in the presence of cytochrome *c* and dATP, but not in the absence of these factors (Figure 3A). The non-hydrolyzable ATP analogue, ATP- γ S, failed to substitute for dATP (Figure 3A), indicating that ATP hydrolysis rather than simple ATP binding is required for Apaf-1 to self-associate. We next prepared extracts from cells transfected with wt and mutant Apaf-1 constructs to analyze further the requirement of dATP and/or cytochrome *c* for Apaf-1 self-association. Immunoprecipitation analysis showed that Apaf-1 self-association required both cytochrome *c* and dATP (Figure 3B), in agreement with the results obtained with endogenous Apaf-1. The constitutively active Apaf-1M368L mutant associated with Apaf-1 in the absence of cytochrome *c*, but required dATP for association with Apaf-1(1–570) (Figure 3B). Mutation of the critical P-loop Lys160 to Arg (M368L/K160R) abolished the ability of Apaf-1M368L to associate with Apaf-1 (Figure 3B). When co-expressed with Apaf-1(1–570), Apaf-1M368L interacted with Apaf-1(1–570) without the addition of exogenous dATP (Figure 3C), suggesting that intracellular dATP/ATP can promote their association.

Procaspase-9 binding to Apaf-1 requires dATP/ATP hydrolysis

To examine the role of dATP/ATP hydrolysis in procaspase-9 binding, we first determined whether dATP/

ATP binding/hydrolysis is required for endogenous Apaf-1 to bind procaspase-9 in cytosolic extracts. We began by transiently transfecting 293T cells with hemagglutinin (HA)-tagged procaspase-9 (C287S) and assessing the interaction of procaspase-9 with endogenous Apaf-1 in the absence of detergent. Immunoprecipitation of procaspase-9 showed that endogenous Apaf-1 binds to procaspase-9 in the presence of cytochrome *c* and dATP, but not in the absence of these factors (Figure 4A). In addition, the non-hydrolyzable ATP analogue, ATP- γ S, inhibited procaspase-9 binding to endogenous Apaf-1 (Figure 4A), indicating that ATP hydrolysis is required for Apaf-1 to bind procaspase-9. We next performed immunoprecipitations with cytosolic extracts from cells transfected with wt and mutant Apaf-1 constructs to further dissect the requirement of dATP and/or cytochrome *c* for the binding of procaspase-9 to Apaf-1. To avoid the formation of Apaf-1 protein complexes in living cells, we prepared cellular extracts expressing wt or mutant Apaf-1 and incubated them with separately prepared extracts containing procaspase-9 (C287S). As we showed with endogenous Apaf-1, exogenous Apaf-1 required both cytochrome *c* and dATP to interact with procaspase-9 (Figure 4B). Mutation of Lys160 to Arg abolished procaspase-9 binding to Apaf-1, confirming that dATP/ATP hydrolysis is needed for the Apaf-1–procaspase-9 interaction. The constitutively active Apaf-1M368L mutant interacted with procaspase-9 in the absence of dATP and cytochrome *c* (Figure 4B), which is consistent with the observation that this mutant does not require these factors for activation of procaspase-9. Furthermore, in contrast to endogenous Apaf-1, incubation with ATP- γ S did not inhibit the binding of mutant Apaf-1M368L to procaspase-9 (data not shown). Surprisingly, mutation of

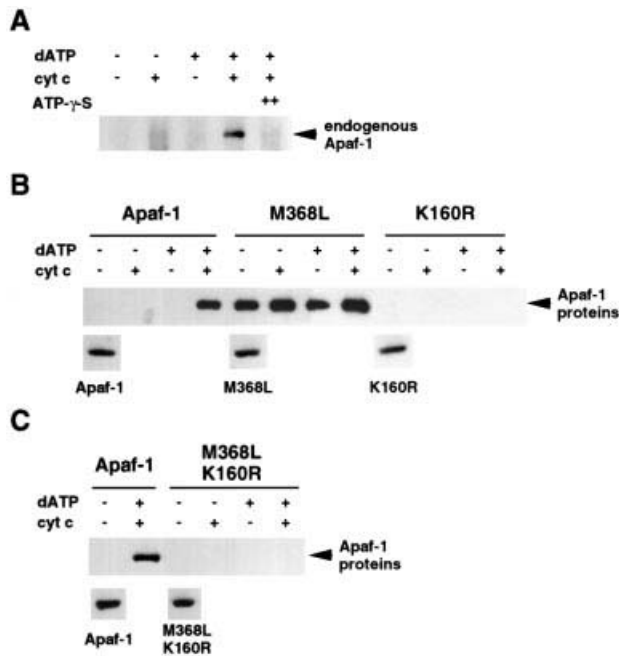


Fig. 4. Cytochrome *c* and ATP hydrolysis are required for Apaf-1 to interact with procaspase-9. (A) Procaspase-9 interacts with endogenous Apaf-1 in a cytochrome *c* and dATP-dependent manner. Equal amounts of 293T cytosolic extracts containing procaspase-9 (C287S)-HA were incubated with rabbit anti-HA antibody and protein A/G-agarose beads (1:1) under the indicated conditions at 4°C for 2 h. Then the beads were pelleted and washed four times with 1 ml NP-40 buffer. ‘++’ indicates that 5 mM ATP- γ S was included in the extract. (B) Procaspase-9 interacts with Apaf-1M368L in the absence of cytochrome *c* and dATP. The experiments were performed as in (A) except that procaspase-9(C287S)-HA-containing extracts were mixed with lysates containing Apaf-1, Apaf-1M368L or Apaf-1K160R. (C) A double-point mutant, Apaf-1M368L/K160R fails to interact with procaspase-9. Expression of wt and mutant Apaf-1 proteins in total lysates is shown in the lower panels in (B) and (C).

Lys160 to Arg abolished the ability of Apaf-1M368L to interact with procaspase-9 (Figure 4C), suggesting that dATP/ATP binding/hydrolysis is indeed required for this point mutant to associate with procaspase-9. These apparently conflicting data for the dATP/ATP requirement are probably explained by the pre-formation of Apaf-1M368L oligomeric complex within the cell after exposure to endogenous dATP/ATP.

ATP hydrolysis is only required for a brief period of time for Apaf-1 to activate procaspase-9

Next we performed experiments to determine whether dATP/ATP hydrolysis is continuously required for Apaf-1-mediated activation of procaspase-9. To do this, we added the non-hydrolyzable ATP analog, ATP- γ S, to the procaspase-9 activation assay at various times, incubating the reaction for a total of 30 min. As depicted in Figure 5, procaspase-9 activation was greatly inhibited by ATP- γ S when added at the beginning of the reaction. ATP- γ S inhibited procaspase-9 activation by ~70% when added after 10 s and by ~30% after 20 s (Figure 5). ATP- γ S minimally inhibited or could no longer inhibit procaspase-9 activation when it was added after 40 s (Figures 5). These experiments suggest that dATP/ATP hydrolysis is required for a brief period of time to activate procaspase-9.

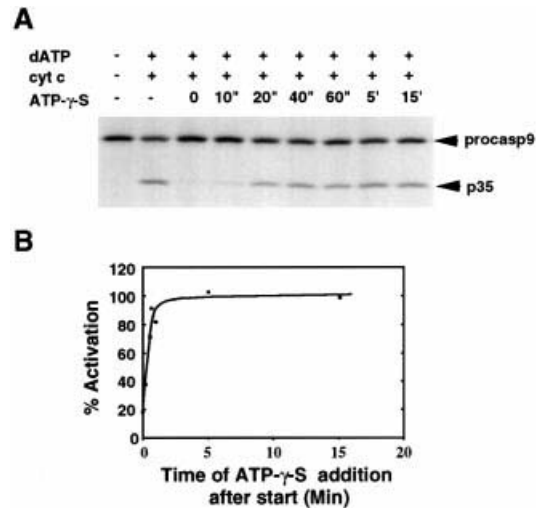


Fig. 5. dATP hydrolysis is only required for a short time in Apaf-1-mediated procaspase-9 activation. Two micrograms of Apaf-1-containing extract was mixed with 1 μ l [³⁵S]methionine-labeled procaspase-9 on ice in the presence of 0.1 mM dATP and 0.2 μ g of cytochrome *c*. One millimolar ATP- γ S was added to the reaction mixture at different time intervals after the tubes containing reaction mixtures were transferred into 30°C waterbath. The reaction was incubated for a total of 30 min at 30°C. (A) Procaspase-9 activation profile in the presence of ATP- γ S when added at different times after the beginning of the reactions. (B) Time course of inhibition of procaspase-9 processing by ATP- γ S. The amount of unprocessed and processed caspase-9 (p35) forms were scanned and quantitated using a phosphoimager. First, the amount of p35 processed forms was normalized to full-length procaspase-9 and added to the corresponding unprocessed procaspase-9 to obtain the total amount of procaspase-9 used in each lane. Then, p35 bands were normalized relative to total procaspase-9 in each lane. Finally, normalized p35 fragments were divided by the p35 fragment without ATP- γ S (positive control).

Apaf-1M368L exhibits enhanced ability to induce apoptosis

The experiments shown in Figure 1D indicate that the Apaf-1M368L mutant can activate procaspase-9 independently of cytochrome *c*. To determine the significance of these findings for induction of apoptosis, we transiently transfected wt and mutant Apaf-1 constructs into 293T cells and assessed their ability to induce apoptosis 19 h after transfection. Wt Apaf-1 failed to induce cell death at low concentrations, but induced low-level apoptosis at higher concentrations of plasmid DNA (Figure 6). In contrast, Apaf-1M368L induced significant apoptosis even at low levels of plasmid DNA and exhibited greater proapoptotic activity than wt Apaf-1 at higher levels of plasmid (Figure 6B). The P-loop mutant of Apaf-1 (K160R) deficient in Apaf-1 self-association and procaspase-9 binding failed to induce apoptosis at all concentrations of plasmid tested (Figure 6B). Apaf-1M368L-induced apoptosis was effectively inhibited by a catalytically inactive caspase-9 mutant (C287S) that acts as a dominant-negative inhibitor (Figure 6B) (Li *et al.*, 1997; Pan *et al.*, 1998), indicating that apoptosis induced by Apaf-1M368L is caspase-9 dependent. Significantly, the Apaf-1 double mutant, M368L/K160R, did not induce apoptosis consistent with a requirement of dATP/ATP hydrolysis for Apaf-1M368L to self-associate and binding to procaspase-9 (Figure 6C).

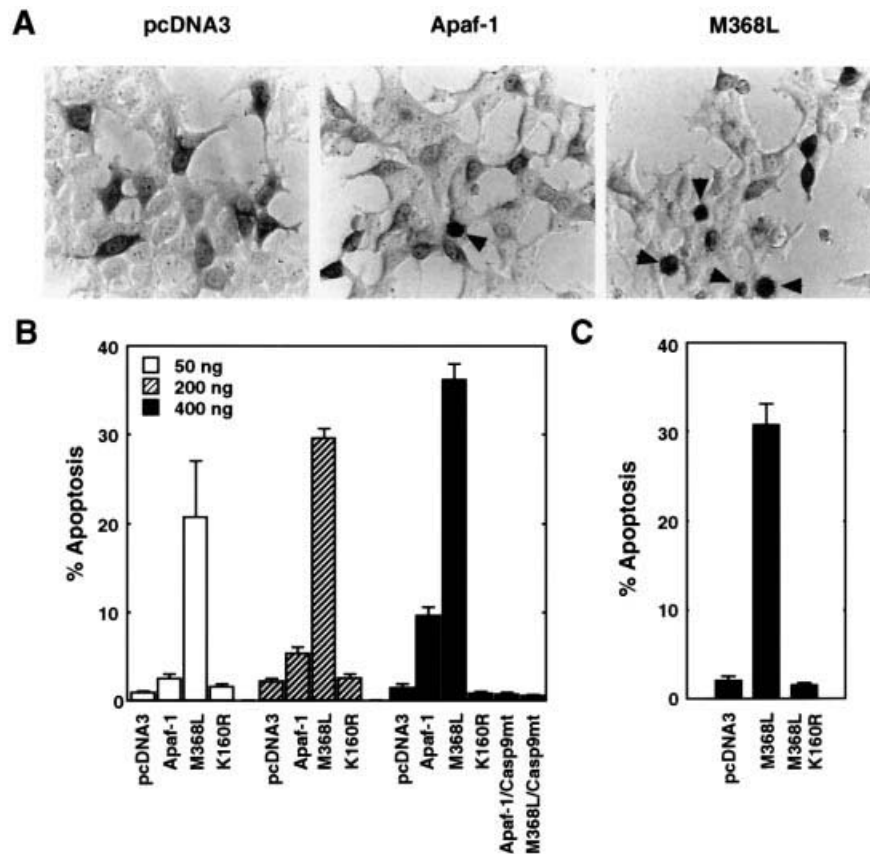


Fig. 6. Induction of apoptosis by wt and Apaf-1 mutants. (A) Representative field of 293T cells transfected with 400 ng of indicated control and Apaf-1 constructs and stained for β -galactosidase activity. Darker cells represent transfected and β -galactosidase-positive cells. The arrowheads indicate round-up apoptotic cells with membrane blebbing. (B) Apaf-1M368L induces apoptosis in 293T cells. 293T cells were transiently transfected with a reporter pcDNA3-galactosidase plus the indicated plasmids. The results represent the percentage of blue cells that exhibit morphological features of apoptosis and are given as the mean \pm SD of triplicate cultures. Casp9mt represents a catalytically inactive mutant of procaspase-9 (C287S). (C) Apaf-1M368L/K160R failed to induce apoptosis. Four-hundred nanograms of indicated plasmids were used to transiently transfect 293T cells as in (B).

Apaf-1(1–570) fails to induce apoptosis and activate procaspase-3

Like the Apaf-1M368L mutant, Apaf-1 mutants lacking the WDR can activate procaspase-9 independent of cytochrome *c* and dATP (Figure 1C) (Hu *et al.*, 1998a,b; Srinivasula *et al.*, 1998). Therefore, we compared the ability of Apaf-1M368L and Apaf-1(1–570) to induce apoptosis. Significantly, Apaf-1(1–570) did not kill 293T cells while Apaf-1M368L induced significant apoptosis in transfected cells (Figure 7A). Immunoblotting analysis with anti-caspase-9 antibody showed that both Apaf-1M368L and Apaf-1(1–570) induced processing of endogenous procaspase-9 into its signature p35 fragment (Figure 7B, upper panel), indicating that the differential activity of these Apaf-1 mutants cannot be explained by lack of procaspase-9 activation. To assess the activation of procaspase-3, a downstream caspase activated by caspase-9, we immunoblotted the same extracts with anti-caspase-3 antibody. Expression of Apaf-1M368L induced processing of procaspase-3, as determined by the appearance of the p18 subunit and reduction of procaspase-3 levels (Figure 7B, lower panel, reduced levels of procaspase-3 could be observed in a shorter exposure; data not shown). Significantly, procaspase-3 from cells transfected with Apaf-1(1–570) remained unprocessed even though procaspase-9 was cleaved (Figure 7B). Thus,

the differential proapoptotic activity exhibited by the constitutively active Apaf-1 mutants, Apaf-1(1–570) and Apaf-1M368L, might be explained by the differential processing of procaspase-3. To gain more insight into this mechanism, we further assessed the activation of procaspase-3 and procaspase-9 in cytosolic extracts prepared from Apaf-1M368L and Apaf-1(1–570)-transfected cells. In Apaf-1M368L extracts, procaspase-9 could be effectively processed into the signature p35 proteolytic fragment resulting from autoactivation of procaspase-9 and p37, a fragment resulting from cleavage of procaspase-9 by active caspase-3 (Figure 7C, upper panel) (Srinivasula *et al.*, 1998). In contrast, procaspase-9 was only cleaved into p35 but not p37 in the Apaf-1(1–570) extract (Figure 7C), providing additional evidence that caspase-3 was not activated in extracts expressing Apaf-1(1–570). To verify this result, we assessed the processing of procaspase-3 in the same extracts. The analysis confirmed that Apaf-1M368L but not Apaf-1(1–570) promoted procaspase-3 activation (Figure 7C, lower panel).

Apaf-1M368L but not Apaf-1(1–570) can recruit procaspase-3 to the Apaf-1–procaspase-9 complex through procaspase-9

Next we wanted to determine the mechanism by which procaspase-3 is activated in extracts from Apaf-1M368L

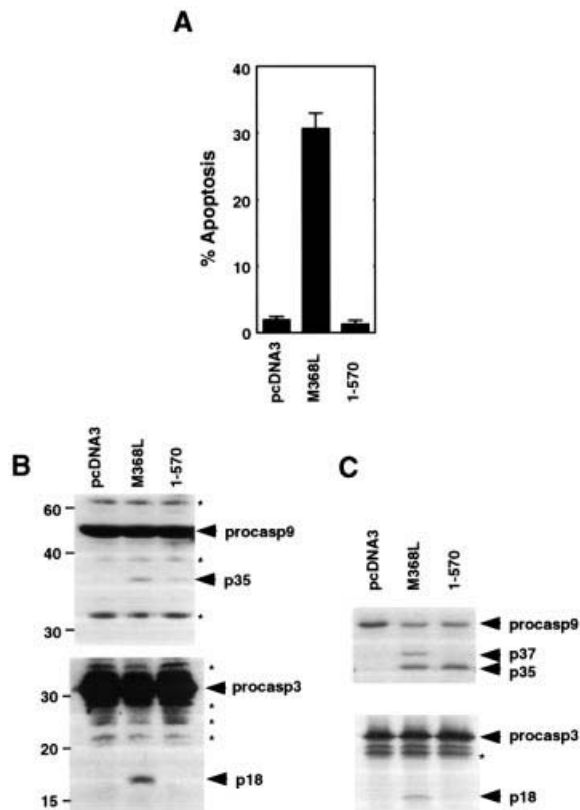


Fig. 7. Apaf-1(1–570) fails to induce apoptosis and activate procaspase-3. (A) Apaf-1(1–570) is unable to induce apoptosis of 293T cells. 293T cells were transiently transfected with a reporter pcDNA3-galactosidase plus 0.4 μ g of the indicated plasmids. The results represent the percentage of blue cells that exhibit morphological features of apoptosis and are given as the mean \pm SD of triplicate cultures. (B) Procaspase-9 but not procaspase-3 is processed in Apaf-1(1–570)-transfected 293T cells. Total cell lysates from 293T cells as indicated in (A) were separated on SDS-PAGE gel and immunoblotted for caspase-9 (upper panel) and caspase-3 (lower panel). Molecular weight markers are indicated on the left of each blot. (C) Both Apaf-1M368L and Apaf-1(1–570) can activate procaspase-9 but only Apaf-1M368L is able to activate procaspase-3. Forty micrograms of cytosolic extracts containing Apaf-1M368L or Apaf-1(1–570) were incubated with [35 S]methionine-labeled procaspase-9 (upper panel) or procaspase-3 (lower panel) in the absence of cytochrome *c* and dATP at 30°C for 30 min. Arrows indicate immature and processed forms of caspase-9 (upper panel) and caspase-3 (lower panel). p35 and p18 represent processed forms of procaspase-9 and -3 respectively. Asterisks indicate non-specific bands.

transfected cells but not from those expressing Apaf-1(1–570). One possibility we considered is that procaspase-3 could be recruited to the Apaf-1M368L–procaspase-9 complex but not to the Apaf-1(1–570)–procaspase-9 complex. To examine this, we immobilized various Apaf-1 mutants on agarose beads in the presence or absence of procaspase-9 and incubated the immobilized Apaf-1 or Apaf-1–procaspase-9 complexes with *in vitro* translated procaspase-3. In the absence of procaspase-9 (C2827S), procaspase-3 interacted weakly with Apaf-1M368L, while procaspase-3 did not associate with Apaf-1(1–570) or Apaf-1(479–1248) (Figure 8A). The presence of procaspase-9 greatly enhanced the binding of procaspase-3 to Apaf-1M368L (Figure 8A). In contrast, procaspase-3 did not bind to Apaf-1(1–570) even in the presence of procaspase-9 (Figure 8A). To determine whether procaspase-3 interacts with procaspase-9 alone

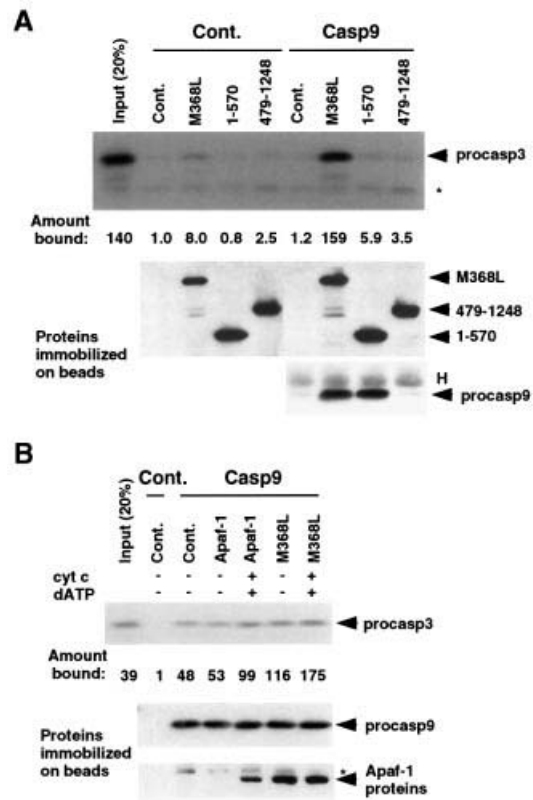


Fig. 8. Procaspase-3 is recruited into the Apaf-1–caspase-9 complex but not into the Apaf-1(1–570)–caspase-9 complex. (A) Procaspase-3 is recruited into Apaf-1M368L–caspase-9 complex. Apaf-1 proteins were immobilized on agarose beads and packed into gel filtration columns as described in Materials and methods. *In vitro*-translated [35 S]methionine-labeled procaspase-3 was loaded onto the beads. After extensive washing proteins bound to the beads were eluted, separated by SDS-PAGE and transferred to nitrocellulose membrane. (B) Procaspase-3 binds to procaspase-9 directly. Experiments were performed as in (A) except procaspase-9 (C287S) was immobilized on agarose beads. Procaspase-3 bound was detected by autoradiography and Apaf-1 proteins and procaspase-9 (C287S) were detected by immunoblotting. The upper panels show an autoradiograph of procaspase-3 bound and amounts of bound procaspase-3 as quantitated with a phosphoimager (procaspase-3 bound to control beads was arbitrarily designated as 1). Apaf-1 proteins and procaspase-9 (C287S) bound to beads were shown in lower two panels. Asterisks indicate non-specific bands. H, immunoglobulin heavy chain; Cont., control lysates transfected with pcDNA3.

or with the Apaf-1M368L–procaspase-9 complex, we performed similar experiments with immobilized procaspase-9 (Figure 8B). As shown in Figure 8B, procaspase-3 did bind to procaspase-9 in the absence of Apaf-1. Addition of Apaf-1M368L or wt Apaf-1 in the presence of cytochrome *c* and dATP enhanced the binding of procaspase-3 to immobilized procaspase-9 by 2- to 4-fold (Figure 8B). This effect is presumably due to increased recruitment of procaspase-9 to the complex by binding to Apaf-1M368L or Apaf-1 in the presence of cytochrome *c* and dATP. A direct interaction between procaspase-3 and procaspase-9 was also confirmed by co-immunoprecipitation analysis (data not shown). Thus, wt Apaf-1 and Apaf-1M368L but not Apaf-1(1–570) can recruit procaspase-3 to the Apaf-1–procaspase-9 complex through procaspase-9.

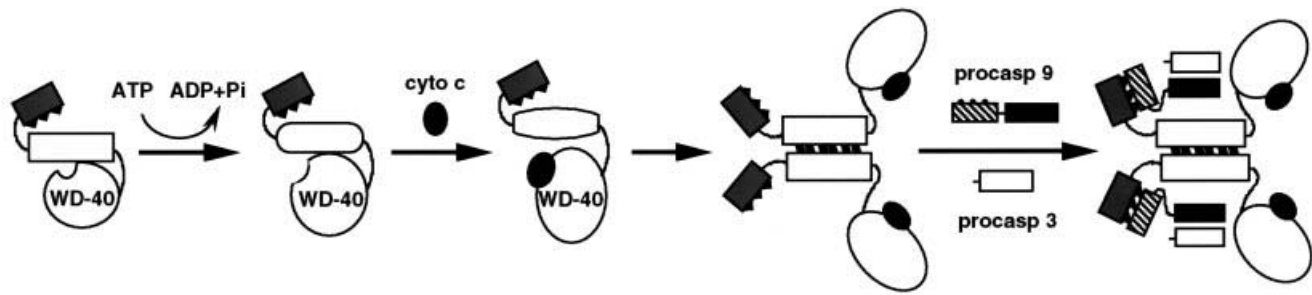


Fig. 9. A model depicting the mechanism of apoptosome formation and procaspase-9 activation. Apaf-1 undergoes sequential conformational changes induced by ATP hydrolysis and cytochrome *c* binding and acquires the ability to oligomerize. Subsequent binding of procaspase-9 to the Apaf-1 oligomer results in recruitment of procaspase-3 and possibly procaspase-7 to this complex to form a functional apoptosome. Apaf-1M368L may bypass the first two steps and adopts a conformation that is competent in oligomerizing and binding to procaspase-9 in the presence of dATP. Alternatively, Apaf-1M368L may assume a conformation that bypasses cytochrome *c* binding but still requires a single dATP/ATP hydrolysis event for oligomerization and procaspase-9 binding. This model is presented in greater detail in the Discussion.

Discussion

In this report we used Apaf-1 and various mutants to elucidate the mechanism by which Apaf-1 mediates procaspase-9 activation and induces apoptosis. Our studies revealed that the binding of cytochrome *c* to Apaf-1 requires dATP/ATP hydrolysis. We also demonstrated that two essential steps involved in procaspase-9 activation, Apaf-1 self-association and procaspase-9 recruitment to Apaf-1, require dATP/ATP hydrolysis and cytochrome *c* binding to Apaf-1. These steps are important for proapoptotic activity since Apaf-1 mutants deficient in dATP/ATP hydrolysis or cytochrome *c* binding are impaired in their ability to promote procaspase-9 activation and apoptosis. Finally, we provide evidence that procaspase-3 is recruited into the Apaf-1–procaspase-9 complex via interaction with procaspase-9. The WDR may play an important role in the recruitment of procaspase-3 to the Apaf-1–procaspase-9 complex, since mutant Apaf-1(1–570) lacking the WDR can bind to procaspase-9 but cannot recruit procaspase-3 and is deficient in apoptosis.

Our observations that the WDR with an extra WD-40 repeat can interact with Apaf-1(1–570) and inhibit activation of procaspase-9 mediated by Apaf-1(1–570) (Figure 1B and C) support conclusions of our previous paper obtained with the original Apaf-1 cDNA clone (Hu *et al.*, 1998b). Based on the results of our present study and previous studies (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998), we provide a model depicting the events that lead to the formation of a functional apoptosome (Figure 9). In this model, dATP/ATP hydrolysis induces a conformational change so that Apaf-1 acquires the ability to bind cytochrome *c*. Upon cytochrome *c* binding, Apaf-1 undergoes further conformational changes to allow Apaf-1 oligomerization and CARD exposure. Procaspase-9 is then recruited into Apaf-1 oligomers via CARD–CARD homophilic interactions, and procaspase-3 is recruited into the complex via interaction with procaspase-9. Once recruited to the apoptosome, procaspase-9 is activated via autocatalysis by Apaf-1 oligomerization-induced proximity of multiple procaspase-9 molecules (Hu *et al.*, 1998b; Srinivasula *et al.*, 1998). Procaspase-9 might be cleaved and activated by adjacent procaspase-9 molecules (intermolecular catalysis). Alternatively, procaspase-9, forming part of the apoptosome, might adopt a conformation that results in intramolecular

cleavage. Activated caspase-9 then cleaves and activates procaspase-3 recruited to the apoptosome. Once the functional apoptosome is formed, dATP/ATP hydrolysis would no longer be required for procaspase-9 recruitment, activation or release of active caspase-9, as suggested by our finding that dATP/ATP hydrolysis is only required for a brief period of time in order for Apaf-1 to activate procaspase-9. A model of Apaf-1 function that is consistent with our findings has been proposed recently based on studies with purified Apaf-1 and procaspase-9 proteins (Zou *et al.*, 1999).

In this study, we generated and characterized an Apaf-1 mutant that activates procaspase-9 independent of cytochrome *c*. Apaf-1M368L constitutively self-associates when synthesized in living cells. However, additional mutation of Lys160 to Arg abrogated its ability to self-associate and bind procaspase-9, as well as its capacity to promote procaspase-9 activation and apoptosis. Although the precise mechanism that accounts for its constitutional activation remains to be determined, these results suggest that Apaf-1M368L may bind and hydrolyze dATP/ATP *in vivo*, and induce Apaf-1 oligomerization and procaspase-binding and activation as intracellular dATP or ATP levels are available in cells at concentrations of 10 μ M and 10 mM, respectively (Skoog and Bjursell, 1974). The ability of Apaf-1 to bind and hydrolyze dATP/ATP was demonstrated by a recent paper that was published while this paper was under review (Zou *et al.*, 1999). In the absence of dATP/ATP hydrolysis, the ability of wt Apaf-1 to oligomerize and to bind procaspase-9 might be repressed by an interaction between its N-terminal region and the WDR (Figure 1B and C) (Hu *et al.*, 1998b). One role of dATP/ATP hydrolysis might be to induce a conformational change that enables cytochrome *c* to bind Apaf-1. This hypothesis is supported by our finding that cytochrome *c* binding to Apaf-1 requires dATP/ATP hydrolysis. Binding of cytochrome *c* to the WDR may disrupt or alter the negative interaction between the N-terminal region and the WDR, allowing Apaf-1 to oligomerize and to bind procaspase-9. This model is supported by the analysis of the Apaf-1M368L mutant. This mutant exhibited reduced interaction between the N-terminal region and the WDR, bypassing the requirement of cytochrome *c* to disrupt the negative interaction. The lack of requirement for cytochrome *c* suggests that Apaf-

1M368L spontaneously adopts a conformation that is competent for oligomerization and procaspase-9 binding in the presence of dATP/ATP. Mutation of Met368 to Leu, therefore, might mimic conformational changes induced by dATP hydrolysis and cytochrome *c* binding. Thus, our findings that Apaf-1M368L still requires dATP/ATP binding or hydrolysis suggest that a second dATP/ATP binding or hydrolysis event may be required. Alternatively, Apaf-1M368L may assume a conformation that bypasses cytochrome *c* binding but still requires a single dATP/ATP hydrolysis event for oligomerization and procaspase-9 binding. CED-4, the *C.elegans* homologue of Apaf-1, lacks the WDR implying that CED-4 could constitutively activate CED-3, unless repressed by its physical association with CED-9. This model is supported by genetic analysis of *C.elegans* that showed that loss-of-function mutants of *ced-9* are lethal due to increased developmental cell deaths, but can be rescued by loss-of-function mutations in *ced-4* (Hengartner *et al.*, 1992). Furthermore, biochemical experiments in mammalian cells have revealed that the ability of CED-4 to activate CED-3 is inhibited by the association of CED-9 with CED-4 (Seshagiri and Miller, 1997; Wu *et al.*, 1997b). Interestingly, the equivalent position of Met368 is conserved in mouse Apaf-1 but is a leucine residue in CED-4. These observations suggest that cytochrome *c* is probably not a factor required for CED-4-mediated CED-3 activation. However, additional studies to test this hypothesis need to be performed in *C.elegans*.

Apaf-1M368L acts as a gain-of-function mutation in that, unlike wt Apaf-1, it activates procaspase-9 independent of cytochrome *c* and dATP *in vitro* and exhibits enhanced ability to induce apoptosis *in vivo*. In contrast, another mutant, Apaf-1(1–570), activates procaspase-9 independent of cytochrome *c* and dATP *in vitro*, but fails to induce apoptosis. Our results indicate that procaspase-9 complexed with Apaf-1M368L, but not procaspase-9 complexed with Apaf-1(1–570), can recruit and activate procaspase-3, providing an explanation for the differential phenotypes. Our studies support a model whereby procaspase-3 is recruited into the apoptosome formed by the Apaf-1–procaspase-9 complex via its interaction with procaspase-9. Apaf-1(1–570) lacking the WDR binds and activates procaspase-9 suggesting that its inability to recruit procaspase-3 is independent of these activities. Several possibilities could be envisioned to explain these results. First, conformational changes in the procaspase-9 bound to wt Apaf-1 and Apaf-1M368L could be critical for the recruitment of procaspase-3. Procaspase-9 bound to Apaf-1(1–570) might assume a conformation that is unsuitable for procaspase-3 recruitment. Another possibility is that the WDR, which is lacking in Apaf-1(1–570), might be involved in the recruitment of procaspase-3 to the Apaf-1–procaspase-9 complex. In the absence of the WDR, Apaf-1(1–570) may be unable to induce the conformational changes of procaspase-9 required for recruitment of procaspase-3.

The cytochrome *c*/Apaf-1/caspase-9/caspase-3 pathway is now known to contribute to apoptosis induced by various stresses, including UV and γ -irradiation and chemotherapeutic drugs (Hakem *et al.*, 1998; Yoshida *et al.*, 1998). In addition, inactivation of either Apaf-1 or caspase-9 in this pathway contributes to cellular trans-

formation and tumorigenesis (Soengas *et al.*, 1999). Under normal conditions, cytochrome *c* is unavailable to induce procaspase-9 activation since it resides in the intermembrane space of the mitochondria. After apoptotic stimuli lead to mitochondrial damage, cytochrome *c* is released and can activate procaspase-9 in an Apaf-1-dependent manner (Zou *et al.*, 1997; Fearnhead *et al.*, 1998). Prosurvival Bcl-2 family members are overexpressed in many tumors and inhibit caspase-9-mediated apoptosis by binding and inhibiting procaspase-9 activation directly and/or preventing the release of cytochrome *c* from mitochondria (Kharbanda *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997; Yang *et al.*, 1997; Hu *et al.*, 1998a). Therefore, it will be interesting to examine whether prosurvival Bcl-2 family members like Bcl-X_L can directly inhibit Apaf-1M368L-mediated procaspase-9 activation. If Bcl-X_L can no longer exert its inhibitory effect on Apaf-1M368L-mediated activation of procaspase-9, the ability of Apaf-1M368L to activate procaspase-9 in the absence of cytochrome *c* could provide a potential method of inducing apoptosis in cancer cells in which cytochrome *c* release is blocked by overexpression of anti-apoptotic members of the Bcl-2 family.

Materials and methods

Plasmids and reagents

The Apaf-1XL cDNA was isolated by RT-PCR amplification from RNA template isolated from 293T cells and cloned into the pcDNA3 plasmid. The nucleotide sequence of the Apaf-1XL cDNA has been submitted to the DDBJ/EMBL/GenBank database (accession No. AF149794). pcDNA3-Apaf-1XL-M368L and pcDNA3-Apaf-1XL-K160R were generated by replacing the *Bst*EII–*Eco*RV fragment of pcDNA3-Apaf-1XL with the corresponding fragments from pcDNA3-Apaf-1(1–559M357L) and pcDNA3-Apaf-1(1–559K149R), respectively (Hu *et al.*, 1998b). pcDNA3-Apaf-1XL-M368L/K160R was constructed by ligation of a *Bst*EII–*Xba*I fragment from pcDNA3-Apaf-1(1–559K149R) and a *Xba*I–*Eco*RV fragment from pcDNA3-Apaf-1(1–559M357L) into *Bst*EII–*Eco*RV-digested pcDNA3-Apaf-1XL. All the constructs were verified by nucleotide sequencing. Polyclonal anti-Apaf-1 antibody was kindly provided by Xiaodong Wang, Southwestern Medical School, Dallas, TX. Polyclonal anti-caspase-9 and anti-caspase-3 antibodies were gifts from Donald Nicholson (Merck).

Transfection, immunoprecipitation and Western blot analysis

Human embryonic kidney 293T cells ($2\text{--}5 \times 10^6$) were transfected with 5 μg of the indicated plasmid DNA by the calcium phosphate method as reported (Inohara *et al.*, 1997). Cytosolic extracts were made essentially as described previously (Hu *et al.*, 1998a). Protein immunoprecipitation and Western blot analysis with relevant antibodies were performed as described previously (Inohara *et al.*, 1997). The proteins were detected by an enhanced chemiluminescence system (ECL; Amersham).

In vitro caspase-9 and caspase-3 assay

Cytosolic extracts were prepared and stored frozen at -80°C . Procaspase-9 was translated *in vitro* from a pcDNA3-caspase-9 plasmid in the presence of [^{35}S]methionine (Amersham) with a Promega TNT transcription/translation kit and purified through a desalting column free of radioactive methionine and ATP (Pharmacia). Procaspase-3 was translated *in vitro* using the same kit. To avoid contaminating dATP/ATP from cytosolic extracts in testing cytochrome *c* and dATP-dependent activation of procaspase-9 by Apaf-1 proteins, the cytosolic extracts were passed through a desalting column (Pharmacia). We incubated 2 μg (Figures 1D and 5A) or 40 μg (Figure 7C) of cellular extracts with or without dATP (1 mM) or bovine cytochrome *c* (0.2 μg , Sigma) in the presence of 1 μl *in vitro* translated procaspase-9 in a final volume of 25 μl . The mixtures were incubated for 30 min at 30°C and the reactions stopped by adding 5 \times SDS loading buffer and boiling for 5 min.

Apoptosis assay

293T cells (1×10^5) were seeded in each well of 12-well plates. After 18 h, cells were transiently transfected with 0.2 μ g of the reporter pcDNA3-galactosidase plasmid plus indicated amount of test plasmids, as reported (Hu *et al.*, 1998a). pcDNA3 was used to adjust total plasmid DNA to an equal amount. The percentage of apoptotic cells was determined 19 h after transfection in triplicate cultures as described previously (Hu *et al.*, 1998a).

Procaspase-3 recruitment assay

Cytosolic extracts containing Myc-tagged Apaf-1 proteins and control or caspase-9 extracts were incubated with either anti-c-Myc (to immobilize Apaf-1 protein) or anti-HA (to immobilize procaspase-9) antibodies and protein A/G-agarose beads (1:1) for 2 h. The beads were washed three times with 1 ml NP-40 buffer (Inohara *et al.*, 1997) and once with 1 ml buffer A (Hu *et al.*, 1998a). Apaf-1- or procaspase-9-coated beads were packed into disposable gel filtration columns. Then *in vitro* translated caspase-3 was loaded onto the beads and washed four times with 1 ml buffer A. Bound proteins were eluted with 50 μ l of hot $1 \times$ SDS loading buffer. We quantitated bound caspase-3 using a PhosphorImager from Molecular Dynamics.

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