An APAF-1-Cytochrome c Multimeric Complex Is a Functional Apoptosome That Activates Procaspase-9

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We report here the reconstitution of the de novo procaspase-9 activation pathway using highly purified cytochrome c, recombinant APAF-1, and recombinant procaspase-9. APAF-1 binds and hydrolyzes ATP or dATP to ADP or dADP, respectively. The hydrolysis of ATP/dATP and the binding of cytochrome c promote APAF-1 oligomerization, forming a large multimeric APAF-1-cytochrome c complex. Such a complex can be isolated using gel filtration chromatography and is by itself sufficient to recruit and activate procaspase-9. The stoichiometric ratio of procaspase-9 to APAF-1 is approximately 1 to 1 in the complex. Once activated, caspase-9 disassociates from the complex and becomes available to cleave and activate downstream caspases such as caspase-3.

Several well characterized morphological features of apoptosis are caused by caspase activity. These include nuclear membrane breakdown, chromatin condensation and fragmentation, cell membrane blebbing, and formation of apoptotic bodies (1, 2). Caspases cleave a variety of cellular substrates after aspartic acid residues such as nuclear lamins, fodrin, DFF45/ICAD, gelsolin, and PAR2, leading to apoptosis (3–8).

Caspases that are involved in the execution of apoptosis are present in living cells as inactive zymogens that become activated through intracellular caspase cascades. There are two relatively well characterized caspase cascades as follows: one is initiated by the activation of cell-surface death receptors, such as Fas and tissue necrosis factor, leading to caspase-8 activation, which in turn cleaves and activates downstream caspases such as caspase-3, -6, and -7 (9–11); and the other is triggered by cytochrome c released from mitochondria, which promotes the activation of caspase-9 through APAF-1 (12).

APAF-1 is a 130-kDa protein consisting of a CED-4 homologous domain flanked by a caspase recruitment domain (CARD) and 12 WD-40 repeats (13). CED-4 is a Caenorhabditis elegans protein that plays a central role in the apoptotic program in that organism (14). The function of APAF-1 as an important apoptosis activator has been recently confirmed by knock-out experiments in mice (15–16). Animals lacking the Apaf-1 gene show excessive number of neurons in their brain, defects in facial features, and delayed recession of interdigital webbing, owing to a defect in apoptosis (15–16). Cells derived from these animals show resistance to a variety of apoptotic stimuli such as chemotherapeutic agents, UV and γ-irradiation, ceramide, and dexamethasone (15–16).

Details of the biochemical mechanism of procaspase-9 activation by APAF-1 is sketchy. Previous studies have shown that APAF-1 forms a complex with caspase-9 in the presence of dATP and cytochrome c, two co-factors for APAF-1 function (12). APAF-1 protein truncated at the COOH-terminal WD-40 repeats is constitutively active in vitro, independent of cytochrome c and dATP (17–18). In addition, truncated APAF-1 interacts with itself as detected by a co-immunoprecipitation and a yeast two-hybrid experiment, suggesting that oligomerization might be an important step for its function (17–18). Similar experiments with CED-4 protein suggest that the oligomerization of CED-4 is also important for activating CED-3, the C. elegans caspase (19). However, there has been no direct evidence either for APAF-1 oligomerization or for the roles of dATP/ATP and cytochrome c in such a process.

In the current report, we reconstituted the procaspase-9 activation pathway using highly purified cytochrome c, recombinant APAF-1, and recombinant procaspase-9. By using such a system, we are able to analyze the role of dATP/ATP hydrolysis, cytochrome c binding to APAF-1, and APAF-1 oligomerization in the de novo activation of procaspase-9. The results demonstrate a multi-stage reaction with the formation of a multi-subunit APAF-1-cytochrome c complex as the key commitment step.

**EXPERIMENTAL PROCEDURES**

*General Methods and Materials—We obtained dATP and other nucleotides and radioactive materials from Amersham Pharmacia Biotech, and molecular weight standards for SDS-PAGE and gel filtration chromatography were from Bio-Rad. General molecular biology methods were used as described in Sambrook et al. (20).*

*Construction of Alternatively Spliced Transcripts of APAF-1 in a Baculovirus Expression Vector and Site-directed Mutagenesis of APAF-1—The [α-32P]dCTP-labeled 285-bp PCR product was used to screen the HeLa λExlox cDNA library as described (13). A 1.4-kilobase pair clone was characterized by DNA sequencing and assembled with APAF-1 cDNA using DNAStar program. An additional 33 nucleotides encoding 11 more amino acids between the CARD and CED-4 homologous domain of APAF-1 was found. To confirm the existence of this alternatively spliced transcript, HeLa poly(A)+ mRNA was purified using a rapid mRNA purification kit (Amersham Pharmacia Biotech), and the first strand cDNA was carried out using the first strand cDNA synthesis kit (Amersham Pharmacia Biotech) with the specific primer 5′-AA-CACCCTCAGTACCTGCTC3′, designed from WD-40 region of APAF-1 cDNA. An aliquot of 400 ng of this first strand cDNA mixture was amplified by two primers PUP2 (5′ TAATGATCTACGTAC CTACAGTACGCTAAGTG3′ and 5′ GAAGATGATCTCAGCTACGCTACAGTC3′) (Fig. 1). The resulting 316- and 283-bp PCR products were subcloned into the PCR II vector using the TA cloning kit (Invitrogen) and sequenced. A 3.63-kilobase pair cDNA encoding the full-length APAF-1 fused
with a 9-histidine tag at the COOH terminus was subcloned into NdeI/KpnI sites of the baculovirus expressing vector pFastBac1 (Life Technologies, Inc.). To insert APAF-1 into the pFastBac1 vector, a 425-bp 5' region of APAF-1 cDNA was removed by enzymatic digestion with NotI and EcoRI from APAF-1-pFastBacI construct and replaced with 485-bp APAF-1 cDNA. An aliquot of this fusion protein mixture was then PCR amplified by two primers P3/P4 (5ꞔ-ATGGCAGCATCACGAAATAAGG 3ꞔ and 5ꞔ-ACCTTGACAGCTGAGCTGCTG 3ꞔ). Both PCR products (187 and 316 bp) were subcloned into the PCRII vector using the TA cloning kit (Invitrogen) and the sequences confirmed by DNA sequencing. This additional WD-40 motif was confirmed in frame into the full-length APAF-1 by using PCR-SOEing method (21). The full-length APAF-1-L/WD13 with 9-His tag was subcloned into pFastBac I vector at NotI and KpnI sites. The nucleotide-binding site mutations (G159E and K160T in Walker's A Box, D243A and D244A in Walker's I vector) were amplified to 100 ml and used to infect 1 liter of Sf21 cells at a density of 2x 10^6/ml. The virus stocks were amplified to 1 ml (0.2 ml) of recombinant protein was analyzed by Western blot. The virus stocks were amplified to 100 ml and used to infect 1 liter of Sf21 cells at a density of 2x 10^6/ml. The infected cells were harvested after 40 h for APAF-1 and 22 h for procaspase-9 by centrifugation and resuspended in 5 volumes of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, for APAF-1 and 22 h for procaspase-9 by centrifugation and resus- pended in the same buffer. Fractions of the fractions were directly loaded onto a TLC plate, and ATP/dATP hydrolysis was analysis as described in the legend of Fig. 2. Analysis of APAF-1/Caspase-9 Complexes—HeLa cells were set at 2x 10^6 per 150-mm dish in medium A (Dulbecco’s modiﬁed Eagle’s medium containing 100 units/ml penicillin and 100 mg/ml streptomycin (100 µg/ml), and fungizone (0.25 µg/ml). The expression of each recombinant protein has an additional 11 amino acids in between the CARD and the CED-4 homologous domain of APAF-1 (Fig. 1A). This spliced form was designated as APAF-1-L. Another alternative splicing event produces an additional exon between exon 17 and 18 encoding 43 amino acids, creating one more WD-40 repeat after the fifth WD-40 repeat. We designated this spliced form as APAF-1-L/WD13. To confirm that these alternatively spliced APAF-1s were indeed expressed in cells, we performed RT-PCR using mRNA isolated from HeLa cells as templates. After first strand cDNA synthesis, the templates were amplified by two sets of primers ﬂanking these two alternative spliced regions (Fig. 1A). As shown in Fig. 1B, each set of primers yielded two PCR products with the predicted sizes corresponding to the two alternatively spliced messages. The identity of all four PCR products was subsequently conﬁrmed by direct DNA sequencing. The WD13 spliced form of APAF-1 has been reported (GenBank™ accession number AB007875) and exists in the mouse version of APAF-1 cDNA (15). All three spliced forms of APAF-1 were expressed in SF21 insect cells using a baculovirus expression vector that fused APAF-1 to a 9-histidine tag at the COOH terminus. Each recom- binant protein was puriﬁed through a nickel affinity column and subjected to SDS-PAGE followed by Western blotting analysis using a rabbit antibody against APAF-1.
by the three alternatively spliced mRNAs, APAF-1L-WD13 has the most stable cytochrome c and dATP-dependent caspase-3 activating activity (data not shown). Thus, we used this recombinant protein in all of the following experiments and refer to it as APAF-1.

Reconstitution of Caspase-3 Activation with Recombinant APAF-1 and procaspase-9 have dATP and cytochrome c-dependent caspase-3 cleavage activity. Recombinant APAF-1, APAF-1L, APAF-1L-WD13, and procaspase-9 were prepared as described under “Experimental Procedures.” A, aliquots (2.5 μg) of APAF-1, APAF-1L, and APAF-1L-WD13 were subjected to 10% SDS-PAGE, and the gel was subsequently stained with Coomassie Blue. B, aliquots (2 μg) of wild type procaspase-9, C287A active site mutant, and D315A cleavage site mutant were subjected to 15% SDS-PAGE followed by staining with Coomassie Blue. C, aliquots of recombinant procaspase-9 (2 μg) with wild type sequence (lanes 1–3), or cleavage site mutant (D315A, lanes 5 and 6), or active site mutant (C287A, lanes 4 and 6), and recombinant APAF-1L-WD13 (0.8 μg) were incubated individually (lane 1) or together (lanes 2–6), in the presence (lanes 1 and 2) or absence (lanes 3–6) or presence of 0.2 μg of cytochrome c plus 100 μM dATP at 30 °C for 1 h in a final volume of 20 μl of buffer A. The samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was probed with a rabbit antibody against caspase-9. The antigen-antibody complexes were visualized by an ECL method as described under “Experimental Procedures.” The filter was then exposed to a x-ray film for 10 s. D, aliquots of recombinant procaspase-9 (2 μg) and recombinant APAF-1L-WD13 (0.8 μg) were incubated individually (lanes 1 and 2) or together (lanes 3–7), in the presence (lanes 1 and 2, 4, 6, and 7) or absence (lanes 3 and 5) of 0.2 μg of cytochrome c plus 100 μM dATP (lanes 1 and 2 and 5 and 6) or 100 μM dADP (lanes 7) with 1 μl of 35S-labeled affinity purified procaspase-3 at 30 °C for 1 h in a final volume of 20 μl of buffer A. The samples were then subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter, followed by exposing to a phosphorimaging plate for 12 h at room temperature.
APAF-1 and Procaspase-9—To study the biochemical mechanism of caspase activation, we established the caspase-3 activation reaction using purified, recombinant APAF-1 and procaspase-9. It has been a technical challenge to generate recombinant procaspases in large enough quantities for biochemical analysis since overexpression of procaspases often results in their auto-activation. In order to overcome this difficulty, we infected SF21 cells with a baculoviral vector containing the cDNA of human caspase-9 or human caspase-9 fused with a 9-histidine vector. The cells were grown in the presence of 10% bovine calf serum and were harvested 24 h after infection before auto-processing occurs. The procaspase-9 was purified either by a nickel affinity column followed by a Mono Q column or by conventional chromatography described previously (12). As shown in Fig. 2B, purified wild type procaspase-9 (1st lane), or procaspase-9 with a cysteine to alanine substitution at its active site (2nd lane), or aspartic acid to alanine substitution at its cleavage site (3rd lane) migrated as a ~50-kDa polypeptide band on an SDS gel.

The recombinant APAF-1 and procaspase-9 were then tested for their respective functions by incubating them with or without dATP and cytochrome c. As shown in Fig. 2C, wild type procaspase-9 was processed to the 35- and 10-kDa active form only when incubated with APAF-1, dATP, and cytochrome c (lane 3). No processing was observed when cytochrome c or APAF-1 was omitted (lanes 1 and 2). In contrast, the cleavage site mutant D315A, or the active site mutant C287A, could not be processed even in the presence of APAF-1, cytochrome c, and dATP (lanes 4 and 5). This experiment indicated that the processing of procaspase-9 by APAF-1, dATP, and cytochrome c occurs after Asp-315 as previously demonstrated (17), and the activation is through auto-catalysis since the active site mutant failed to be processed. Interestingly, that mixing D315A and C287A mutants did not result in any cleavage, suggesting inter-molecular cleavage does not happen (lane 6).

To confirm that recombinant APAF-1-catalyzed procaspase-9 processing is functional, we incubated the procaspase-9 activation reaction with 35S-labeled, affinity purified procaspase-9, a caspase downstream of caspase-9 (12, 22–23). As shown in Fig. 2D, caspase-3 became activated only when APAF-1, procaspase-9, dATP, and cytochrome c were all present (lane 6). Omitting either APAF-1 (lane 1), procaspase-9 (lane 2), cytochrome c (lane 5), or dATP (lane 4) resulted in loss of procaspase-3 cleavage. dATP, which is functional in the crude system (24), does not have any activity in the purified system (lane 7), indicating the high energy bond of dATP is critical for its function.

dATP/ATP Binding and Hydrolysis by APAF-1—APAF-1 contains in its Ced-4 homologous domain consensus Walker A and B boxes for nucleotide binding (13). The nucleotide-binding sites are conserved between Ced-4 and APAF-1, suggesting the importance of nucleotide binding in their function (13, 25–26). To confirm that APAF-1 indeed binds ATP or dATP, purified APAF-1 protein was incubated with [γ-32P]ATP or dATP, and the nucleotide bound to APAF-1 was pelleted by nickel affinity resin and analyzed by thin layer chromatography (TLC). As shown in Fig. 3A, radiolabeled nucleotides were co-pelleted with APAF-1, indicating that APAF-1 indeed bound nucleotides. Interestingly, the majority of nucleotide bound to APAF-1 was ATP or dATP, rather than ATP, or dATP. This finding suggests that the nucleotides bound to APAF-1 are hydrolyzed. Incubation with cytochrome c did not affect dATP binding or hydrolysis (lanes 6 and 7). A mutant APAF-1 protein with four amino acids substitution in the conserved Walkers A and B boxes did not bind any nucleotide (lanes 4 and 6) even though the same amount of protein was precipitated (Fig. 3B).

The above data suggest that the binding and hydrolysis of ATP/dATP to ADP/dADP are important for APAF-1 function. To confirm this hypothesis, increasing amounts of a non-hydrolyzable ATP analog, ATPγS, was incubated with APAF-1 and procaspase-9, in the presence of dATP (Fig. 3C, lanes 1–4) or ATP (Fig. 3C, lanes 5–8) in a procaspase-3 activation reaction. Consistent with the previous observation, 100 μM dATP, or 1 mM ATP, is the optimal concentration in such a reaction. The presence of ATPγS inhibited the caspase-3 activation reaction in a concentration-dependent fashion with 1 mM ATPγS completely inhibiting the reaction. It is interesting to note that even at concentrations much lower than ATP or dATP present, ATPγS shows noticeable inhibitory effects (Fig. 3C, lanes 2, 3, 6, and 7).

FIG. 3. ATP binding and hydrolysis by APAF-1. Recombinant wild type (W) and nucleotide-binding sites mutant (M) APAF-1 were purified as described under “Experimental Procedures.” A, aliquots of 2 μg of wild type or mutant APAF-1 were incubated with 100 nM ATP plus 2 μCi of [α-32P]ATP (lanes 3 and 4), or 100 nM dATP plus 2 μCi of [α-32P]dATP (lanes 5 and 6) in the presence of 0.4 μg of cytochrome c (lane 7) at 30 °C for 1 h at a final MgCl2 concentration of 2 mM and final volumes of 50 μl of buffer A. After 1 h incubation, the samples were mixed with 400 μl of buffer A and an aliquot of 50 μl of nickel beads. After incubation at 4 °C for 2 h in a rotator, the mixtures were pelleted by centrifugation, and the beads were washed five times with buffer A. The beads were then resuspended in 50 μl of buffer A containing 250 mM imidazole, and the beads were pelleted by centrifugation. Aliquots of 10 μl of the supernatants were collected and loaded on a TLC plate and developed in 1 M formic acid plus 0.5 M LiCl. For the control (lanes 1 and 2), aliquots of [α-32P]ATP or [α-32P]dATP in 50 μl of buffer A were incubated at 30 °C for 1 h, followed by directly loading the TLC plate together with the above samples. B, aliquots of 5 μl of resulting supernatants as in A were subjected to 10% SDS-PAGE followed by Western blotting analysis using a rabbit antibody against the Ced-4 homologous domain of APAF-1 (1:2000). The antibody-antigen complexes were visualized by an ECL method as described under “Experimental Procedures.” The film was exposed for 10 s. C, aliquots of 0.8 μg of recombinant APAF-1L-WD13 were incubated with aliquots of 0.2 μg of recombinant procaspase-9 purified as described under “Experimental Procedures,” 1 μl of in vitro translated 35S-labeled caspase-3, 0.2 μg of cytochrome c, 100 μM dATP (lanes 1–4) or 1 mM ATP (lanes 5–8), and increasing amounts of ATPγS in a final volume of 20 μl in buffer A. After 1 h incubation at 30 °C, the samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter followed by exposure to a phosphorimaging plate for 12 h at room temperature. The film was exposed for 10 s. The film was exposed for 10 s.
Interactions Between Caspase-9, APAF-1, and Cytochrome c—Previous studies have demonstrated that APAF-1 interacts with caspase-9 only in the presence of dATP/ATP and cytochrome c (12). To study further the interaction between these three proteins, we incubated purified APAF-1 (His-tagged), procaspase-9 (without tag), and cytochrome c in different combinations, and we precipitated APAF-1 with nickel resin. The supernatants and pellets were probed with antibodies against these three proteins. Shown in Fig. 4C, the same amounts of APAF-1 were precipitated by the nickel resin independent of cytochrome c, dATP, or procaspase-9. No APAF-1 was detected in the supernatant (data not shown). On the other hand, caspase-9 was pelleted only in the presence of APAF-1, dATP, and cytochrome c (Fig. 4B, lane 6). Interestingly, the majority of pelleted caspase-9 was already processed into the 35-kDa active form, indicating that procaspase-9 was rapidly auto-activated once bound to APAF-1. When dATP, APAF-1, or cytochrome c was omitted in the reaction, procaspase-9 stayed in the supernatant (Fig. 4A, lanes 5 and 7–9). Noticeably, some of the processed caspase-9 was also in the supernatant (Fig. 4A, lane 6), suggesting that once processed, caspase-9 can be released from APAF-1. Consistent with the previous finding, cytochrome c was co-precipitated with APAF-1 in the absence and presence of dATP (13) (Fig. 4D). However, the amount of cytochrome c that was co-precipitated with APAF-1 was consistently increased in the presence of dATP, suggesting that dATP may stabilize the binding of cytochrome c to APAF-1.

Formation of APAF-1-Cytochrome c-Caspase-9 Complex in the Presence of dATP—The co-precipitation experiments with either nickel resin (Fig. 4) or antibody against caspase-9 (12) indicate that caspase-9 interacts with APAF-1 in the presence of cytochrome c and dATP. To characterize further the biochemical nature of this APAF-1-caspase-9 complex formed in the presence of cytochrome c and dATP, we separated these proteins and the complex on a gel filtration column. In the absence of dATP, the mixture of APAF-1, procaspase-9, and cytochrome c were separated by the sizing column (Fig. 5A). The protein peaks of APAF-1, procaspase-9, and cytochrome c were found at fractions 14, 16, and 21, respectively. Each protein migrates at the position corresponding to its mono-
Cytochrome c Multimeric Complex Activates Procaspase-9

Both dATP and Cytochrome c Are Required to Form a Caspase-9-activating APAF-1-Cytochrome c Complex—To dissect further the individual steps leading to the formation of this multimeric protein complex, recombinant APAF-1 protein was analyzed on the same gel filtration column either alone (Fig. 6A), or preincubated with cytochrome c plus ATPγS (Fig. 6B), or dATP (Fig. 6C), or dATP plus cytochrome c (Fig. 6D). Preincubation of APAF-1 with cytochrome c plus nonhydrolyzable ATP or dATP alone did not cause significant change in their column behavior. In addition, after preincubating APAF-1 with dATP, the reaction mixture was loaded on the same gel filtration column, and the resulting column fractions were assayed for APAF-1 activity in the absence or presence of dATP. The APAF-1 activity was only observed in the presence of dATP and the activity peak was observed at fraction 14, correlating with the monomeric form of APAF-1 (Fig. 6C, lower panel). The APAF-1 protein that migrated larger than fraction 14 was not active and therefore most likely represents nonspecific protein aggregates. Indeed, recombinant APAF-1 alone was found to have a tendency to self-aggregate into non-functional complexes (data not shown). These data suggest that incubation of APAF-1 with dATP, or cytochrome c, was not sufficient to drive APAF-1 into the functional multimeric complex nor forgo the requirement for dATP in activating procaspase-9 (Fig. 6C, lower panel).

Strikingly, when APAF-1 was preincubated with dATP and cytochrome c, the peak of APAF-1 was shifted around fraction 11 and associated with cytochrome c (Fig. 6D). This APAF-

fractions were incubated with 5 μl (0.17 μg) of recombinant pro- caspase-9 and 1 μl of in vitro translated 35S-labeled procaspase-3 in the presence or absence of 100 μM dATP and 0.2 μg of cytochrome c at 30 °C for 1 h in a final volume of 35 μl of buffer A. The samples were then subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a phosphorimaging plate for 14 h at room temperature. E, recombinant cleavage site mutant (D315A) pro- caspase-9 was purified as described under “Experimental Procedures.” The APAF-1 and procaspase-9 (Asp-315) standards were quantified by amino acid analysis. An aliquot of 150 μl (25 μg) of recombinant APAF-1 was incubated with aliquots of 180 μl (45 μg) of recombinant procaspase-9 (D315A), 4.8 μg of cytochrome c, and 100 μl dATP in the final volume of 360 μl in buffer A. After 1 h incubation in 30 °C, the sample was then fractioned in the gel filtration column as in A. The fraction 10 and fraction 11 were concentrated to a final volume of 30 μl and subjected to 10% SDS-PAGE. The gel was subsequently stained with Coomassie Blue and scanned in a densitometer.
1cytochrome c complex was now fully functional in activating procaspase-3 when purified procaspase-9 was added (Fig. 6D, lower panel). dATP and cytochrome c were no longer required for such a reaction.

APAF-1 and Procaspe-9 Are At a 1:1 Ratio in the Complex—To estimate the molar ratio of APAF-1 and procaspase-9 in the complex, we incubated APAF-1 with an excessive amount of procaspase-9 with the D315A mutation (~5 to 1 molar ratio). This mutant cannot be processed within this complex and therefore cannot be released. The APAF-1:procaspase-9 D315A complex was isolated from the fraction 10–11 of the gel filtration column after preincubating with dATP and cytochrome c and subjected to SDS-PAGE together with the known amounts of APAF-1 and procaspase-9 followed by Coomassie Blue staining (Fig. 6E). No cytochrome c was detected by this staining method even though it can be detected by Western blot analysis (data not shown). The bands corresponding to APAF-1 and procaspase-9 were scanned by a densitometer. The results suggest that APAF-1 and procaspase-9 are present at an approximately 1:1 ratio in this complex.

DISCUSSION

The reconstitution of the procaspase-9 activation pathway with highly purified, recombinant APAF-1 and procaspase-9 described above revealed that caspase-9 activation was achieved by a three-step reaction as illustrated in Fig. 7. First, dATP/ATP binds to APAF-1 through its consensus nucleotide-binding domain and is hydrolyzed to dADP or ADP, respectively; second, cytochrome c binds to APAF-1 and promotes the multimerization of APAF-1:cytochrome c complex when the dATP/ATP bound to APAF-1 is being hydrolyzed; third, once the multimeric complex is formed, procaspase-9 is recruited to the complex in a 1:1 molar ratio to APAF-1, and it becomes activated through auto-catalysis. The activated caspase-9 is then released from the complex, allowing it to cleave downstream caspases and new procaspase-9 to come into the complex to be processed.

Role of ATP/dATP Hydrolysis—The direct demonstration that APAF-1 binds to and hydrolyzes dATP/ATP confirms the notion that the evolutionarily conserved nucleotide-binding sites are important for its function. Mutations at the conserved nucleotide-binding site diminished the nucleotide binding capacity of APAF-1 and rendered the protein inactive (Fig. 3A, and data not shown). The inhibitory effect of a non-hydrolyzable ATP analog for both caspase activation (Fig. 3C) and ATP binding to APAF-1 (data not shown) indicates that both the binding of ATP/dATP to APAF-1 and their hydrolysis to ADP/dADP are important for its function. Interestingly, ATPγS showed an inhibitory effect even at concentrations much lower than the ATP present (Fig. 3C). One interpretation for this observation is that each subunit of APAF-1 must hydrolyze ATP/dATP to form the functional complex. The subunit that binds ATPγS not only inactivates itself but also effectively inhibits other ATP-bound APAF-1 molecules that were going to form a complex with it. This observation supports the importance of APAF-1 multimerization.

The effects of binding and hydrolysis of ATP/dATP seem to be transient since preincubation of dATP with APAF-1 did not bypass the requirement of dATP (Fig. 6C, lower panel). Since intracellular ATP and dATP concentrations are around 10 μM and 10 μM, respectively (27), it is conceivable that ATP/dATP is hydrolyzed by APAF-1 constantly. This hydrolysis, however, does not have any functional consequence since cytochrome c is usually sequestered away from APAF-1 in mitochondria.

Neer et al. (28) compared all the known WD-40 repeat-containing proteins and noted that most are regulatory, but none is an enzyme. ATP/dATP hydrolysis activity by APAF-1 proved it to be an exception.

Role of Cytochrome c in Promoting APAF-1 Multimerization—Holocytochrome c exists exclusively in the intermembrane space of mitochondria in living cells. The newly translated apocytochrome c in cytosol does not have apoptosis promoting activity (29). However, when cells undergo apoptosis in response to a variety of stimuli, cytochrome c is released from mitochondria to cytosol, where APAF-1 is located (30). No detectable amount of APAF-1 is found in the membrane fraction in both living or apoptotic cells (data not shown). Cytochrome c was found to interact with APAF-1 in the absence or presence of dATP as shown by co-immunoprecipitation experiment (13) (Fig. 4D). However, when subjected to gel filtration chromatography, the cytochrome c and APAF-1 complex was...
only detected in the presence of dATP, indicating that the interaction between APAF-1 and cytochrome c in the absence of dATP is not stable and can be easily separated. The binding and hydrolysis of dATP did not seem to be influenced by the presence of cytochrome c, suggesting that the function of cytochrome c is not to regulate the dATP binding and hydrolysis by APAF-1 (Fig. 3A). In the presence of dATP, cytochrome c promotes the multimerization of APAF-1 from a monomeric form to a large complex that consists at least 8 subunits of APAF-1 (calculated based on its size >1.3 million daltons). Such a complex is fully functional in activating procaspase-9 and neither dATP nor cytochrome c is required any longer (Fig. 6D, lower panel). These data are consistent with a model that cytochrome c bound to APAF-1 will stabilize the transient conformational change of APAF-1 resulting from dATP/ATP hydrolysis, allowing them to multimerize. The functions of both dATP and cytochrome c are probably accomplished once the multimerized APAF-1/cytochrome c is formed (Fig. 6D). However, because cytochrome c is still detected in the functional caspase-9-activating complex, it is still possible that cytochrome c plays an additional role in procaspase-9 activation such as stabilizing this complex.

Oligomerized APAF-1-Cytochrome c Complex: a Functional Apoptosome?—Several features of this stable APAF-1 and cytochrome c multimeric complex make it a candidate of apoptosis as follows: first, it is a big complex (>1.3 million daltons measured by gel filtration chromatography); second, the formation of this complex requires the hydrolysis of high energy bond of ATP, or dATP; and third, this complex is functional in terms of activating procaspase-9. The formation of this complex will likely be the commitment step for cells to activate their caspases in response to stimuli that cause cytochrome c release. This complex is able to trigger many characteristic apoptotic features of dying cells through caspase activation. Future studies are needed to determine the exact molecular compositions of this complex.

Why multimeric complex? Since the full occupancy of procaspase-9 in the apoptosome results in approximately 1:1 molar ratio of APAF-1 to procaspase-9 (Fig. 6E), one obvious reason for apoptosome is to bring multiple procaspase-9 in close proximity, allowing them to cleave each other. Similar hypothesis have been proposed for CED-4 and APAF-1 WD-40 truncation (17–19). However, there is no evidence that inter-molecular cleavage indeed occurs, and simply mixing D315A and C287A mutants did not result in the cleavage of C287A even though both of them can be recruited onto the apoptosome (Fig. 3C and data not shown). Another reason for the multimeric complex could be to increase the threshold of apoptosis to ensure nonspecific leakage of cytochrome c does not cause apoptotic response.

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