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Apaf-1, a Human Protein Homologous to C. elegans CED-4, Participates in Cytochrome c–Dependent Activation of Caspase-3

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Summary

We report here the purification and cDNA cloning of Apaf-1, a novel 130 kd protein from HeLa cell cytosol that participates in the cytochrome c–dependent activation of caspase-3. The NH_2-terminal 85 amino acids of Apaf-1 show 21% identity and 53% similarity to the NH_2-terminal prodomain of the Caenorhabditis elegans caspase, CED-3. This is followed by 320 amino acids that show 22% identity and 48% similarity to CED-4, a protein that is believed to initiate apoptosis in C. elegans. The COOH-terminal region of Apaf-1 comprises multiple WD repeats, which are proposed to mediate protein–protein interactions. Cytochrome c binds to Apaf-1, an event that may trigger the activation of caspase-3, leading to apoptosis.

Introduction

Apoptosis is a form of cell death resulting from the activation of a genetically determined cell suicide program (reviewed by Horvitz et al., 1994; Jacobson et al., 1997). Cells undergoing apoptosis show characteristic morphological features such as condensation of cytoplasmic and nuclear contents, blebbing of plasma membranes, fragmentation of nuclei, and ultimately breakdown into membrane-bound apoptotic bodies that are rapidly phagocytosed (Kerr et al., 1972).

Genetic analysis of the nematode Caenorhabditis elegans has identified three genes that control the general apoptotic program. Two genes, *ced-3* and *ced-4*, are required for the apoptotic program (Yuan and Horvitz, 1990). *Ced-9*, which functions upstream of *ced-3* and *ced-4*, negatively regulates the apoptotic program by preventing activation of *ced-3* and *ced-4* (Hengartner et al., 1992).

The apoptotic program delineated in C. elegans is conserved in mammalian cells, which contain homologs of *ced-9* and *ced-3.* One of these homologs, *Bcl-2*, can partially substitute for *ced-9* in preventing apoptosis in C. elegans (Hengartner and Horvitz, 1994). The other homologs, including *caspase-3*, encode cysteine proteases that are closely related to *ced-3* (Yuan et al., 1993; Fernandes-Alnemri et al., 1994; Xue et al., 1996; Alnemri et al., 1996). In mammalian cells, caspase-3 normally exists as a 32 kd inactive precursor that is converted proteolytically to a 20 kd and a 10 kd active heterodimer when cells are signaled to die (Nicholson et al., 1995; Schlegel et al., 1996; Wang et al., 1996). Bcl-2, located on the outer membrane of mitochondria, prevents the activation of caspase-3 (Boulakia et al., 1996; Chinnaiyan et al., 1996; Armstrong et al., 1996; Erhardt and Cooper, 1996; Ibrado et al., 1996; Monney et al., 1996). One possible way for Bcl-2 to do this is to block mitochondria from releasing cytochrome c, a necessary cofactor for caspase-3 activation (Liu et al., 1996; Yang et al., 1997; Kluck et al., 1997).

Ced-4 is the only remaining C. elegans general apoptosis gene for which the mammalian counterpart has not been found. This gene is believed to function downstream of *ced-9* but upstream of *ced-3* in the C. elegans apoptosis pathway (Shaham and Horvitz, 1996a, 1996b). The biochemical role of the CED-4 protein is not established.

We recently established an in vitro system to study apoptosis using cytosolic fractions from normally growing HeLa cells. This system has allowed us to identify two new protein factors involved in the apoptotic pathway in mammalian cells. One factor is the previously known electron transfer protein, cytochrome c (Liu et al., 1996). The other is DNA fragmentation factor (DFF), a novel heterodimer of 45 and 40 kd subunits that functions downstream of caspase-3 to trigger fragmentation of genomic DNA into nucleosomal segments (Liu et al., 1997), a hallmark of apoptosis (Wyllie, 1980).

In addition to cytochrome c, the activation of caspase-3 requires another cytosolic factor that we initially designated as apoptotic protease activating factor-1 (Apaf-1; Liu et al., 1996). Cytochrome c has been designated as Apaf-2. Subsequent work has resolved Apaf-1 into two factors. One of these is designated as Apaf-3 and the other retains the name Apaf-1 (see Figure 1A). In the current study, we report the purification, cDNA cloning, and characterization of Apaf-1. This 130 kd protein contains a CED-3 homologous domain at the NH_2-terminus, followed by a CED-4 homologous domain, and multiple WD-40 repeats at the COOH-terminus.

Results

Fractionation of Cytosol and Reconstitution of Caspase-3 Activation

We previously established an in vitro system in which the apoptotic program is initiated by the addition of dATP (Liu et al., 1996). The initiation of apoptosis leads to activation of caspase-3 and ultimately to fragmentation of DNA in nuclei which are coincubated with the 100,000 × g supernatant (S-100) cytosolic fraction. Caspase-3 activation was monitored by cleavage of in vitro translated, ^{35}S-labeled, affinity-purified caspase-3 precursor. The proteolytic fragments are visualized by SDS-polyacrylamide gel electrophoresis (PAGE) followed by phosphorimaging (Figure 1B). Fractionation of the cytosol revealed that one of the required components was

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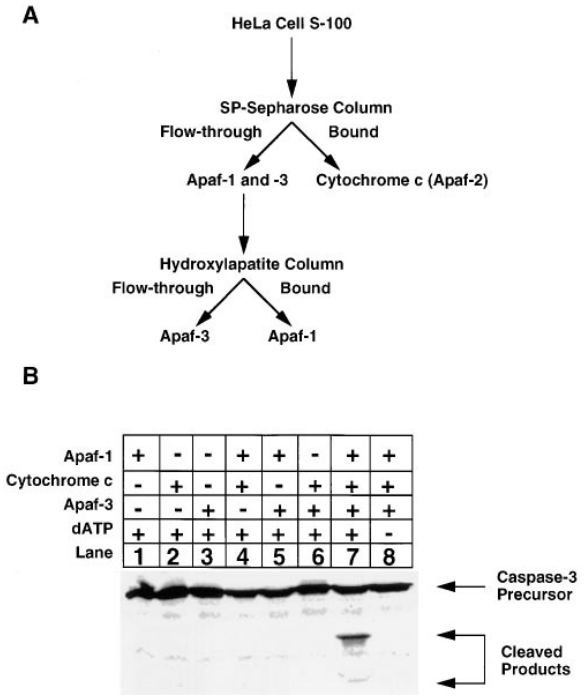

Figure 1. Fractionation and Reconstitution of dATP-Dependent Activation of Caspase-3

HeLa cell S-100 was prepared and fractionated by a Sp-Sepharose and an hydroxylapatite column chromatograph as described in Experimental Procedures.

(A) Schematic illustration of the separation of Apaf-1, -2, and -3.

(B) Hydroxylapatite column flow-through (Apaf-3) and bound fractions (Apaf-1) were collected and dialyzed against buffer A. Aliquots of the two fractions (4 μl each) and an aliquot of cytochrome c (0.2 μg in 1 μl) were incubated individually (lanes 1–3) or in combinations of two (lanes 4–6) or all three (lanes 7 and 8), in the presence (lanes 1–7) or absence (lane 8) of 1 mM dATP with a 3 μl aliquot of in vitro translated, ^{35}S-labeled caspase-3 at 30°C for 1 hr 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. The filter was exposed to a Phosphorimaging plate for 16 hr at room temperature.

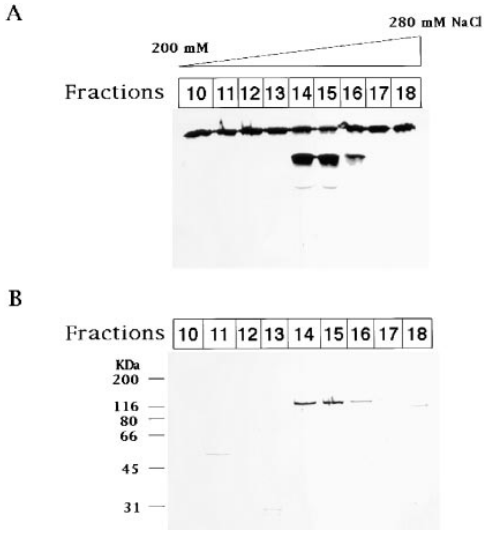

Figure 2. Mono Q Column Purification of Apaf-1

The Apaf-1 activity was purified through the Mono Q column as described in Experimental Procedures.

(A) Aliquots of 1 μl of Mono Q column fractions were incubated with aliquots of 4 μl of Apaf-3 (see Figure 1), 1 μl (0.2 μg) of cytochrome c, 3 μl of in vitro translated, ^{35}S-labeled 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 Mono Q column fractions were subjected to 8% SDS-PAGE, and the gel was subsequently stained with silver using a Silver Stain Plus kit from Bio-Rad.

cytochrome c, which was released from mitochondria during homogenization (Liu et al., 1996).

Figure 1A shows the fractionation scheme that we used for the separation of cytochrome c (Apaf-2) from two other required factors, Apaf-1 and Apaf-3. The initial step was SP-Sepharose chromatography, which separated cytochrome c (bound fraction) from Apaf-1 and Apaf-3 (flow-through). To separate Apaf-1 and -3, the flow-through fraction was loaded onto a hydroxylapatite column. The flow-through and bound fractions were collected. Neither fraction alone was competent to activate caspase-3 when incubated with cytochrome c and dATP (Figure 1B, lanes 1–6). However, when the bound and flow-through fractions were mixed in the presence of cytochrome c and dATP, caspase-3 activation was restored (Figure 1B, lane 7). No activity was detected when dATP and cytochrome c were omitted from the reaction (Figure 1B, lanes 5 and 8).

Purification of Apaf-1

During further purification, we assayed Apaf-1 by incubating various fractions with cytochrome c, dATP, the crude Apaf-3 fraction, and the substrate caspase-3. Complete purification of Apaf-1 was achieved through a six-step procedure (see Experimental Procedures). The results of the last step of purification, Mono Q column chromatography, are shown in Figure 2. Apaf-1 activity was eluted from the Mono Q column at 250 mM NaCl (Figure 2A, fractions 14–16). The same fractions were subjected to SDS-PAGE followed by silver staining and a single polypeptide band of ~130 kd coeluted with the Apaf-1 activity (Figure 2B, fractions 14–16). No other proteins were detected by silver staining in the peak fractions. Apaf-1 activity eluted from gel-filtration columns at about 130 kd, indicating that Apaf-1 exists as a monomer in solution (data not shown). About 10 μg of pure Apaf-1 was obtained from the cytosol from 100 liters of HeLa cells.

Sequencing and cDNA Cloning of Apaf-1

The 130 kd Apaf-1 protein excised from an SDS gel was subjected to trypsin and Lys-C digestion, and the resulting peptides were separated by capillary reverse-phase high pressure liquid chromatography (HPLC). The sequences of 17 peptides were determined by mass spectrometry and Edman degradation (Table 1). Protein data base searches revealed no proteins that are identical to Apaf-1. Degenerate oligonucleotides encoding peptides 2 and 4 (see Table 1) were used to prime polymerase chain reactions (PCRs) with a HeLa cDNA library as template. This yielded a 285 bp DNA fragment that also encoded peptide 3. Probing of a HeLa cDNA li-

Table 1. Peptide Sequences of Human Apaf-1 Purified from Hela Cells

Peptide #	Fraction #	MH+ Measured	MH+ Calculated	Residues in cDNA
1	K33a	1780.8	1780.0	5–18
2	K6	1255.7	1255.6	43–52
3	K43	ND	4103.82	82–100
4	T9	913.5	913.5	121–128
5	K46	3153.6[ab]	3153.6	150–178
6	K42	3160.8[a]	3161.6	188–213
7	K31	1177.8	1177.7	267–277
8	K29	1450.0	1449.8	292–304
9	K53	2747.1[b]	2745.4	503–525
10	T24	817.6	817.5	590–596
11	K33b	735.5	735.9	627–632
12	K31	615.3	615.4	639–643
13	K32	1278	1277.7	746–757
14	T17	650.5	650.4	941–945
15	T4a	559.3	558.7	946–949
16	T4b	798.4	797.9	988–994
17	K28	942.7	942.5	1169–1176

Peptides were analyzed by MALDI mass spectrometry and Edman sequencing. The tryptic and Lys-C peptides are designated by the letters T and K, respectively. The cysteine residues were alkylated with isopropylacetamide.
[a] Masses represent the average isotopic mass.
[b] The peptide contained an oxidized tryptophan resulting in an additional 32 daltons.

brary with this PCR fragment identified two overlapping cDNAs with an open reading frame that encodes 1194 amino acids with a calculated molecular mass of 136,088 daltons (Figure 3). Multiple in-frame stop codons were identified in the 5′ untranslated region of the cDNA, indicating that these cDNA clones encode full-length Apaf-1 (data not shown).

Figure 3 shows the predicted amino acid sequence encoded by human Apaf-1. The open reading frame of the cDNA encodes all 17 sequenced peptides that are distributed throughout the protein (indicated by underlines). The measured molecular masses of these peptides by mass spectrometry were consistent with the calculated molecular masses, indicating that there was no post-translational modification of these peptides.

Domain Structure of Apaf-1

A search of the protein data base (GenBank and Prosite) revealed that the COOH-terminal segment of Apaf-1 contains 12 WD-40 repeats (Figure 3, bold). This loosely conserved set of sequences is found in many regulatory proteins, including β-subunits of heterotrimeric G proteins (Neer et al., 1994; Wall et al., 1995; Sondek et al., 1996), the *LIS-1* gene for Miller-Dieker Lissencephaly (Reiner et al., 1993), and the SREBP cleavage-activating protein (SCAP; Hua et al., 1996).

The searches of the protein data base also revealed significant similarities between Apaf-1 and CED-3 and CED-4, two proteins that are required for the apoptotic program in C. elegans (Yuan and Horvitz, 1990). The NH_2-terminal 85 amino acids of Apaf-1 shows 21% identity and 53% similarity with the NH_2-terminal prodomain of CED-3 (Figures 4A and 4B). This domain is followed by a stretch of 320 amino acids that shows 22% identity and 48% similarity with CED-4 (Figures 4A and 4C). The two longest stretches of amino acids conserved with CED-4 lie at positions 141–157 and 227–234 of Apaf-1. These two regions correspond, respectively, to Walker's A- and B-box consensus sequences (underlined in Figure 4C) for nucleotide binding sites (Walker et al., 1982). Several amino acids that are required for CED-4 activity, including two aspartic acid residues at positions 250–251 and the isoleucine at position 258 (Yuan and Horvitz,

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MDAKARNCLLQHREALEKDIKTSYIMDHMISDGFLTISEE  40
EKVRNEPTQQQRAAMLIKMILKKDNDSYVSFYNALLHEGY  80
KDLAALLHDGIPVVSSSSVRTVLCEGGVPQRPVVFVTRKK 120
LVNAIQQKLSKLKGEPGWVTIHGMAGCGKSVLAAEAVRDH 160
SLLEGCFPGGVHWVSVGKQDKSGLLMKLQNLCTRLDQDES 200
FSQRLPLNIEEAKDRLRILMLRKHPRSLLILDDVWDSWVL 240
KAFDSQCQILLTTRDKSVTDSVMGPKYVVPVESSLGKEKG 280
LEILSLFVNMKKADLPEQAHSIIKECKGSPLVVSLIGALL 320
RDFPNRWEYYLKQLQNKQFKRIRKSSSYDYEALDEAMSIS 360
VEMLREDIKDYYTDLSILQKDVKVPTKVLCILWDMETEEV 400
EDILQEFVNKSLLFCDRNGKSFRYYLHDLQVDFLTEKNCS 440
QLQDLHKKIITQFQRYHQPHTLSPDQEDCMYWYNFLAYHM 480
ASAKMHKELCALMFSLDWIKAKTELVGPAHLIHEFVEYRH 520
ILDEKDCAVSENFQEFLSLNGHLLGRQPFPNIVQLGLCEP 560
ETSEVYQQAKLQAKQEVDNGMLYLEWINKKNITNLSRLVV 600
RPHTDAVYHACFSEDGQRIASCGADKTLQVFKAETGEKLL 640
EIKAHEDEVLCCAFSTDDRFIATCSVDKKVKIWNSMTGEL 680
VHTYDEHSEQVNCCHFTNSSHHLLLATGSSDCFLKLWDLN 720
QKECRNTMFGHTNSVNHCRFSPDDKLLASCSADGTLKLWD 760
ATSANERKSINVKQFFLNLEDPQEDMEVIVKCCSWSADGA 800
RIMVAAKNKIFLWNTDSRSKVADCRGHLSWVHGVMFSPDG 840
SSFLTSSDDQTIRLWETKKVCKNSAVMLKQEVDVVFQENE 880
VMVLAVDHIRRLQLINGRTGQIDYLTEAQVSCCCLSPHLQ 920
YIAFGDENGAIEILELVNNRIFQSRFQHKKTVWHIQFTAD 960
EKTLISSSDDAEIQVWNWQLDKCIFLRGHQETVKDFRLLK 1000
NSRLLSWSFDGTVKVWNIITGNKEKDFVCHQGTVLSCDIS 1040
HDATKFSSTSADKTAKIWSFDLLLPLHELRGHNGCVRCSA 1080
FSVDSTLLATGDDNGEIRIWNVSNGELLHLCAPLSEEGAA 1120
THGGWVTDLCFSPDGKMLISAGGYIKWWNVVTGESSQTFY 1160
TNGTNLKKIHVSPDFKTYVTVDNLGILYILQTLE        1194
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Figure 3. Amino Acid Sequence of Human Apaf-1 as Predicted from the cDNA Sequence

Amino acid residues are numbered on the right. Seventeen tryptic and Lys-C peptides that are found in purified Apaf-1 (Table 1) are underlined. Twelve putative WD-repeats are highlighted in bold (Neer et al., 1994).

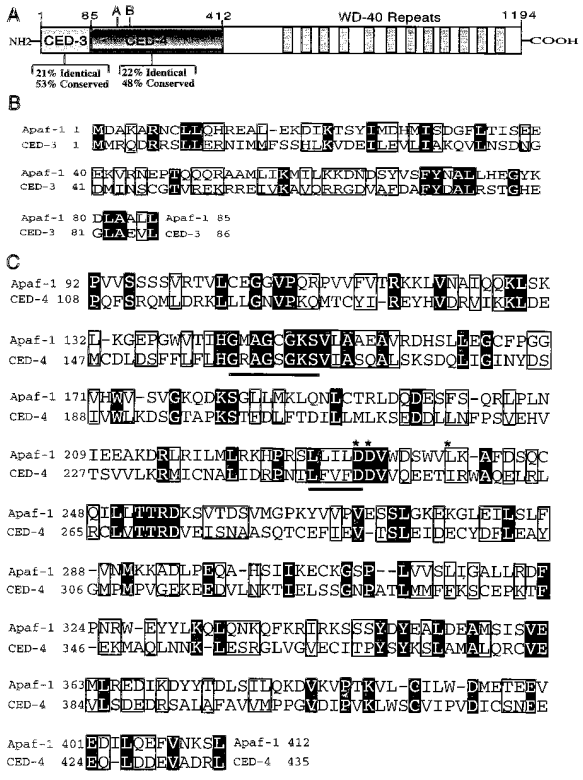

Figure 4. Domain Structure of Apaf-1

(A) A schematic representation of Apaf-1 is shown by the horizontal bar, with the numbers corresponding to the amino acid residues in Figure 3. The CED-3 and CED-4 homologous regions, the Walker's A- and B-box consensus sequences for nucleotide binding sites (Walker et al., 1982), and 12 WD repeats are indicated.

(B) The amino acid sequence of the CED-3 homologous region of Apaf-1 and the NH_2-terminal prodomain of CED-3 (Yuan et al., 1993) were aligned by the Lipman-Pearson method of the DNASTAR program. Identical amino acids and conserved amino acids are shown by closed and open boxes, respectively.

(C) The amino acid sequence of the CED-4 homologous region of Apaf-1 and the amino acids of CED-4 (Yuan and Horvitz, 1992) were aligned as above. Identical amino acids and conserved amino acids are shown by closed and open boxes, respectively. The Walker's A- and B-box consensus sequences for nucleotide binding sites at amino acid residues 143–150 and 228–232 of Apaf-1 are underlined. Asterisks denote the amino acid residues that are known to be important for CED-4 function (Yuan and Horvitz, 1990; Chinnaiyan et al., 1997).

1992; Chinnaiyan et al., 1997), are conserved in Apaf-1 (Figure 4C, asterisks).

Tissue Distribution of Apaf-1

Figure 5 shows the tissue distribution of Apaf-1 mRNA analyzed by Northern blotting of human adult and fetal tissues. In all tissues examined, including adult brain, heart, liver, spleen, skeletal muscle, lung, pancreas, thymus, small intestine, colon, peripheral blood leukocytes, kidney, testis, ovary and fetal brain, liver, kidney, and lung, a predominant mRNA of ~8 kb nucleotides was detected, indicating ubiquitous expression of Apaf-1. Expression was highest in adult spleen and peripheral blood leukocytes and in fetal brain, kidney, and lung,

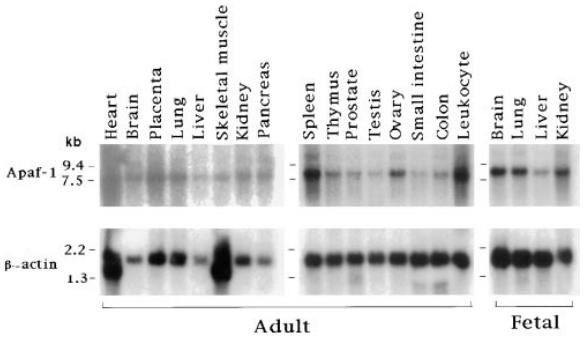

Figure 5. Distribution of Apaf-1 mRNA in Human Tissues

Apaf-1 was hybridized to poly(A)$^+$ RNA from the indicated tissue (2 μg/lane) as described in Experimental Procedures. The left filter was exposed to a X-ray film with an intensifying screen at −80°C for 8 days, and the right two filters were exposed to a X-ray film with an intensifying screen at −80°C for 4 days. The same filters were subsequently hybridized with human β-actin and exposed to film for 2 hr (left filter) or 30 min (right two filters) at −80°C with an intensifying screen. The samples from human adult or fetal tissue are indicated.

correlating with the high level of apopotosis in these tissues.

Production of Recombinant Apaf-1

A polyclonal antibody raised against a recombinant protein containing amino acids 10–254 of Apaf-1 reacted with the purified protein (Figure 6A, lane 5). The same antibody also reacted with the Apaf-1 produced in human embryonic kidney 293 cells after transfection with an expression vector containing human Apaf-1 (Figure 6A, lanes 2 and 4). Control cells transfected with the vector alone show less Apaf-1, representing the endogenous Apaf-1 in 293 cells (Figure 6A, lanes 1 and 3). The recombinant Apaf-1 migrated to approximately the same position as purified Apaf-1, confirming that the cDNA encoded full-length Apaf-1.

To test the function of recombinant Apaf-1, we cotransfected a cDNA encoding the hamster caspase-3 (Wang et al., 1996) with Apaf-1 in 293 cells. The 32 kd hamster caspase-3 overexpressed in 293 cells was detected by immunoblot analysis with a polyclonal antiserum against the NH_2-terminal region (amino acids 12–20) of hamster caspase-3 (Wang et al., 1996; Figure 6A, lanes 3 and 4). This antibody does not recognize human caspase-3 (Figure 6A, lanes 1 and 2; Wang et al., 1996). Interestingly, a higher level of caspase-3 was consistently observed when cotransfected with Apaf-1 (Figures 6A and B). One possible explanation for this observation is that Apaf-1 may bind caspase-3 directly and slow down its normal turnover rate. No activation of caspase-3 was observed in vivo by overexpression of recombinant Apaf-1, confirming that Apaf-1 alone was not sufficient to trigger caspase-3 activation (Figure 6A, lanes 3 and 4). However, when the cell extracts from Apaf-1 overexpressing cells were incubated with dATP, caspase-3 was rapidly activated (Figure 6B, lane 8). In contrast, caspase-3 in the extracts from cells transfected with vector alone was only slightly activated during the same period of incubation. These cell extracts

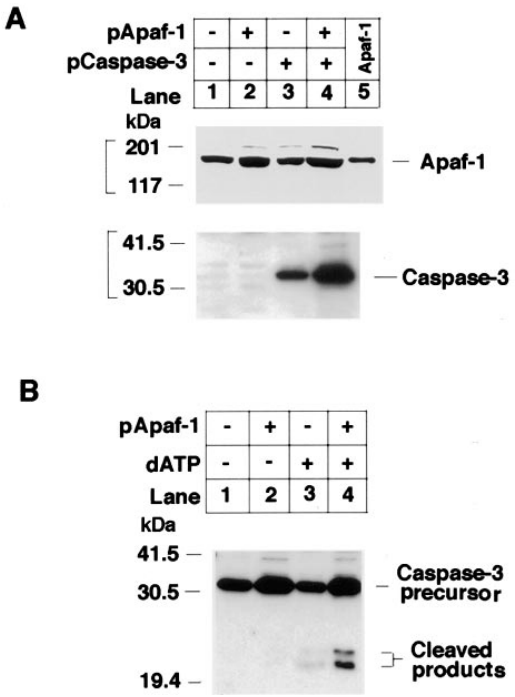

Figure 6. Expression of Apaf-1 in 293 Cells

Human embryonic kidney 293 cells were set up and transfected as described in Experimental Procedures.

(A) 293 cells were transfected with vector alone (lane 1), Apaf-1 (lane 2), hamster caspase-3 (lane 3), or Apaf-1 plus hamster caspase-3 (lane 4). The cells were harvested 36 hr after transfection and the cytosol was prepared as described in Liu et al. (1996). Aliquots of 30 μg of cytosol were subjected to 8% SDS-PAGE (upper panel) or 15% SDS-PAGE (lower panel) and blotted onto nitrocellulose filters. The filters were probed with either a rabbit anti-Apaf-1 (1:2000) (upper panel) or a rabbit anti-hamster caspase-3 (1:2000) (lower panel) antibody, respectively, and the antigen-antibody complexes were visualized by an ECL method as described in Experimental Procedures. The films were exposed for 30 s (upper panel) or 40 s (lower panel) respectively. Lane 5 is 3 μl of Apaf-1 protein purified to Mono Q column step as described in Experimental Procedures.

(B) Aliquots of 40 μg of cytosol prepared from 293 cells transfected with indicated plasmids were incubated either with buffer A (lanes 1 and 2) or in the presence of 1 mM dATP (lanes 3 and 4) at 30°C for 30 min in a final volume of 20 μl adjusted with buffer A. The samples were subsequently subjected to 15% SDS-PAGE followed by Western blotting analysis using a rabbit antibody against hamster caspase-3 (1:2000), and the antigen-antibody complexes were visualized by an ECL method as described in Experimental Procedures. The film was exposed for 30 s.

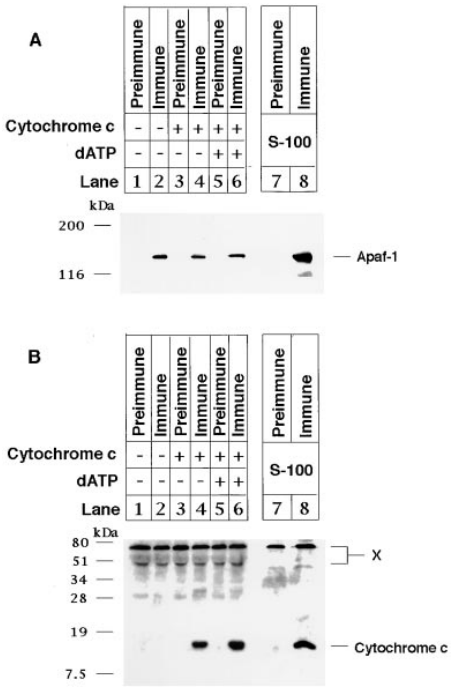

Figure 7. Cytochrome c Binds to Apaf-1

Rabbit polyclonal antiserum against Apaf-1 was generated as described in Experimental Procedures. For immunoprecipitation, an aliquot of 300 μl of protein A–agarose (Santa Cruz) was incubated with an aliquot of 500 μl of preimmune or immune serum at 4°C overnight. The protein A–agarose beads were then pelleted by centrifugation and washed three times with buffer A. The beads were resuspended in 300 ml buffer A and incubated with same volume of BSA (1 mg/ml) at 30°C for 20 min before use. Aliquots of 100 μl of partially purified Apaf-1 (same as used in Figure 1) were incubated alone or with 2 μg of cytochrome c (lanes 3–6) in the absence (lanes 1–4) or presence of 1 mM dATP (lanes 5 and 6) in a final volume of 200 μl of buffer A at 30°C for 20 min followed by the addition of 30 μl of antibody–protein A agarose beads prepared as above. In lanes 7 and 8, aliquots of 30 μl of antibody–protein A-agarose beads were added to aliquots of 1 ml HeLa cell S-100 fraction directly. After incubation at room temperature for 3 hr in a rotator, the mixtures were pelleted by centrifugation and the beads were washed four times with buffer A. The beads were then resuspended in 200 μl of 1× SDS loading buffer. After boiling for 3 min, the beads were pelleted by centrifugation and the supernatants were collected.

(A) Aliquots of 30 μl of resulting superanatants were subjected to 8% SDS-PAGE followed by electroblotting to a nitrocellulose filter. The filter was probed with the serum against Apaf-1 (1:5000 dilution). The antigen–antibody complexes were visualized by an ECL method as described in Experimental Procedures. The filter was exposed to a Kodak X-OMAT X-ray film for 5 s.

(B) Aliquots of 30 μl of resulting superanatants were subjected to 15% SDS-PAGE followed by electroblotting to a nitrocellulose filter. The filter was probed with a monoclonal antibody against cytochrome c as described in Liu et al. (1996). X denotes a cross-reactive band to this antibody. The antigen–antibody complexes were visualized by an ECL method as described in Experimental Procedures. The filter was exposed to a Kodak X-OMAT X-ray film for 3 min.

contain similar amounts of cytochrome c released during homogenization (data not shown).

Cytochrome c Binds to Apaf-1

As noted above, the activation of caspase-3 by Apaf-1 and Apaf-3 requires cytochrome c as well as dATP. To determine whether Apaf-1 interacts directly with cytochrome c, we incubated cytochrome c with crude Apaf-1 (same as in Figure 1) and then immunoprecipitated the mixture with a polyclonal antiserum against Apaf-1. The immunoprecipitates were then subjected to SDS-PAGE and immunoblot analysis with antibodies against Apaf-1 or cytochrome c. As shown in Figure 7A, antiserum against Apaf-1 precipitated Apaf-1 while the preimmune serum from the same animal did not. The antiserum against Apaf-1 also precipitated cytochrome c (Figure 7B), indicating that cytochrome c forms a complex with Apaf-1. Preimmune serum caused no such

precipitation. The binding of cytochrome c to Apaf-1 was not influenced by the presence or absence of dATP (Figure 7B, lanes 3–6). The precipitation of cytochrome c by this antiserum requires the presence of Apaf-1 in the mixture since cytochrome c alone cannot be precipitated by this antibody (data not shown).

To confirm that cytochrome c interacts with Apaf-1 in the crude cytosolic extracts, the immunoprecipitation experiment was performed using unfractionated HeLa cell S-100. Apaf-1 in the S-100 fraction was precipitated by this antiserum (Figure 7A, lanes 7 and 8). Cytochrome c, which is released to the cytosol during homogenization (Liu et al., 1996), coprecipitated with Apaf-1 (Figure 7B, lane 8). The preimmune serum caused neither protein to precipitate (Figures 7A and 7B, lane 7).

Discussion

Activation of Caspase-3

In the present study, we identified, purified, and cloned a 130 kd protein that participated in caspase-3 activation reaction in vitro. Caspase-3 normally exists in the cytosol of cells as a 32 kd precursor that is activated proteolytically into a 20 kd and a 10 kd heterodimer when cells are signaled to undergo apoptosis in response to serum withdrawal, activation of Fas, treatment with ionization, and a variety of pharmacological agents (Chinnaiyan et al., 1996; Datta et al., 1996, 1997; Erhardt and Cooper, 1996; Hasegawa et al., 1996; Jacobson et al., 1996; Schlegel et al., 1996). The activated caspase-3 is capable of autocatalysis as well as cleaving and activating other members of the caspase family, leading to rapid and irreversible apoptosis (Wang et al., 1996; Srinivasula et al., 1996). Activated caspase-3 will cleave and activate the 45 kd subunit of DNA fragmentation factor, DFF, which in turn leads to the degradation of DNA into nucleosomal fragments (Liu et al., 1997), a hallmark of apoptosis (Wyllie, 1980).

The ability of activated caspase-3 to trigger downstream apoptotic events implies that the activation process has to be highly regulated to prevent unwanted cell death. Indeed, Bcl-2 prevents the activation of caspase-3 in response to a variety of apoptotic signals (Boulakia et al., 1996; Chinnaiyan et al., 1996; Armstrong et al., 1996; Erhardt and Cooper, 1996; Ibrado et al., 1996; Monney et al., 1996). One possible mechanism by which Bcl-2 prevents the activation of caspase-3 is to prevent cytochrome c release from mitochondria. Consistent with this possibility, the release of cytochrome c from mitochondria in response to apoptotic stimuli is blocked in cells overexpressing Bcl-2 (Kluck et al., 1997; Yang et al., 1997).

Once released from mitochondria, cytochrome c binds to Apaf-1, which may trigger the activation of caspase-3 in the presence of dATP. Consistent with this model, cytochrome c was shown to bind to Apaf-1 in a coimmunoprecipitation assay (Figure 7). The levels of Apaf-1 and Apaf-3 do not seem to be regulated by Bcl-2 since similar amounts of Apaf-1 and Apaf-3 are found in the cytosol of control and Bcl-2 overexpressing cells (X. Liu and X. Wang, unpublished observation).

Apaf-1 Is a Mammalian Homolog of CED-4

Several lines of evidence suggest that Apaf-1 might be the mammalian homolog of CED-4. First, Apaf-1 shows significant sequence homology with CED-4 over 320 amino acids; second, several important regions of CED-4, including conserved nucleotide binding regions and an isoleucine at position 258, are conserved in Apaf-1 (Figure 4C); third, the biochemical function of Apaf-1, which is to mediate the activation of caspase-3, is consistent with the function of CED-4 as delineated by genetic studies. The genetic studies indicate that *ced-4* functions downstream of *ced-9* but upstream of *ced-3* since the killing of the C. elegans ALM neurons by overexpression of CED-4 is greatly reduced in a *ced-3* mutant, whereas killing of these neurons by overexpression of *ced-3* is unaffected by mutations in *ced-4* (Hengartner et al., 1992; Shaham and Horvitz, 1996a, 1996b). Similarly, Bcl-2 functions upstream of Apaf-1 by controlling the release of cytochrome c, a cofactor for Apaf-1 activity (Kluck et al., 1997; Yang et al., 1997). In addition, the Bcl-2 family of proteins might directly interact with Apaf-1 in a fashion similar to the interaction between CED-9 and CED-4 (Chinnaiyan et al., 1997; Wu et al., 1997). Such an interaction could negatively or positively regulate caspase-3 activation mediated by Apaf-1, depending on the anti- or proapoptosis nature of the proteins.

Mechanism for Triggering Caspase-3 Activation

Apaf-1 itself does not seem to be a caspase. The conserved active site pentapeptide QACR(or Q/G)G that is present in all identified caspases (Alnemri et al., 1996) is not present in Apaf-1. An interesting observation made by Neer et al. (1994) is that most of the WD repeat-containing proteins are regulatory and none is an enzyme. Therefore, the biochemical mechanism for cytochrome c–triggered caspase-3 activation remains to be worked out. The eventual understanding will require the purification of Apaf-3 and reconstitution of the caspase-3 activation reaction with purified factors. The identification of two regions in the *ced-4* homologous domain of Apaf-1 that correspond to the Walker's nucleotide binding sites (see Figures 4A and 4C) may explain the requirement of dATP in the caspase-3 activation reaction. The homology of the NH_2-terminal region of Apaf-1 with CED-3 prodomain raises an interesting question about the evolutionary origin of CED-3 and CED-4 compared with that of caspase-3 and Apaf-1. The function of this ced-3 homologous region should also be an interesting focus of future studies.

Unlike CED-4 of C. elegans, Apaf-1 contains multiple WD repeats in the COOH-terminal region. WD repeats are involved in protein–protein interactions (Neer et al., 1994; Sondek et al., 1996; Wall et al., 1995). The seven WD repeats in the β-subunit of heterotrimeric G protein form a circularized 7-fold β propeller. In $G_{i\alpha}\beta_1\gamma_2$, blades of the WD propeller interact with $G_{i\alpha_1}$ subunit and the γ subunit, thereby bridging the heterotrimeric complex (Wall et al., 1995). It is possible that the activation of caspase-3 is through a similar scenario, with the WD repeats of Apaf-1 bridging cytochrome c and Apaf-3, to induce the de novo activation of caspase-3, leading to apoptosis.

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. Protein concentrations were determined by the Bradford method; general molecular biology methods were used as described in Sambrook et al. (1989).

Assay for Caspase-3 Activation

Caspase-3 was translated and purified as described (Liu et al., 1996). 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-1 from HeLa S-100

All purification steps were carried out at 4°C. All chromatography steps except the SP-Sepharose column (Pharmacia), and first hydroxylapatite column (Bio-Rad) were carried out using an automatic fast protein liquid chromatography (FPLC) station (Pharmacia).

Seven hundred milliliters (4.9 g of protein) of HeLa S-100 from 100 liters of suspension cultured HeLa cells were prepared as described in Liu et al. (1996) and applied to a SP-Sepharose column (200 ml bed volume) equilibrated with buffer A. The 800 ml flow-through fraction (3,648 mg of protein) was collected and loaded directly onto a hydroxylapaptite column (50-ml bed volume) equilibrated with buffer A. The column was washed with three column volumes of buffer A containing 1 M NaCl followed by two column volumes of buffer A. The bound material was eluted with 200 ml of 0.3 M KPO_4, pH 7.5. The protein peak from the column (115 ml, 287 mg of protein) was dialyzed against buffer A and then loaded onto a second hydroxylapaptite column (10 ml bed volume) equlibrated with buffer A. The column was eluted with a 200 ml buffer A to 250 mM KPO_4, pH 7.5 linear gradient followed by a 100 ml, 250 mM to 500 mM KPO_4, pH 7.5 linear gradient. Fractions of 10 ml were collected and assayed for Apaf-1 activity. Active fractions (50 ml, 11 mg of protein) were pooled and dialyzed against buffer A and then loaded onto a 5-ml heparin-Sepharose column (Pharmacia) equlibrated 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 mM NaCl to 400 mM NaCl, both in buffer A. Fractions of 4 ml were collected and assayed for Apaf-1 activity. The active fractions (8 ml, 0.86 mg of protein) were pooled and loaded directly onto a Superdex 200 16/60 gel-filtration column equlibrated with buffer A containing 100 mM NaCl. The column was eluted with the same buffer (four runs), and fractions of 4 ml were collected starting from 30 ml of elution. The fractions were assayed for Apaf-1 activity, and the active fractions were pooled (16 ml, 32 μg of protein) and loaded directly onto a Mono Q 5/5 column (Pharmacia) equlibrated with buffer A containing 100 mM NaCl. The column was eluted with a 20 ml linear gradient from 100 mM NaCl to 300 mM NaCl, both in buffer A. Fractions of 1 ml were collected and assayed for Apaf-1 activity.

Protein Sequencing of Apaf-1

The 130 kd band (4–8 pmol) from the Mono Q column was electroblotted onto and excised from a PVDF membrane (ProBlott, Applied Biosystem). The band was reduced and alkylated with isopropylacetamide followed by digestion in 20 μl of 0.05 M ammonium bicarbonate, 20% acetonitrile with 0.2 μg of trypsin (Promega) or Lysine-C (Wako) at 37°C for 17 hr as described previously (Henzel et al., 1993). The solution was then directly injected onto a 0.32 × 150 mm C18 capillary column (LC Packing, Inc.). Solvent A is 0.1% aqueous trifluoroacetic acid (TFA) and solvent B is acetonitrile containing 0.07% TFA. The peptides were eluted with a linear gradient of 0–80% B in 120 min. Peptide peaks were detected at 195 nm and hand collected into 0.5 ml Eppendorf tubes.

An aliquot (0.2 μl) of each of the isolated HPLC fractions was applied to a premade spot of matrix (0.5 μl of 20 mg/ml a-cyano-4-hydroxycinammic acid plus 5 mg/ml nitrocellulose in 50% acetone/50% 2-propanol) on the target plate (Shevchenko et al., 1996). Ions were formed by matrix-assisted laser desorption/ionization with a 337 nm nitrogen laser. Spectra were acquired with a Perseptive Biosystems Voyager Elite time-of-flight mass spectrometer, operated in linear delayed extraction mode. Subsequently, fragment ions for selected precursor masses were obtained from postsource decay (PSD) experiments (Kaufmann et al., 1994). To enhance the ion abundances at low mass, collision gas (air) was introduced to the collision cell during the acquisition of the lower portion (<200 Da) of the fragment ion spectrum. Each peptide mass and its associated fragment ion masses were used to search an in house sequence data base with an enhanced version of the FRAGFIT program (Henzel et al., 1993).

Automated protein sequencing was performed on models Procise 494A and 494CL Applied Biosystems sequencers equipped with an on-line parathyroid hormone (PTH) analyzer. Peaks were integrated with Justice innovation software using Nelson Analytical 760 interfaces. Sequence intepretation was performed on a DEC Alpha computer (Henzel et al., 1987).

cDNA Cloning of Apaf-1

A 1 μl (10^8 pfu) aliquot of λExlox HeLa cDNA library (Yokoyama et al., 1993) was heated at 99°C for 15 min to release the DNA, which were directly amplified with 300 pmol of primer-1 5′-AA(A/G)GT(A/T/C/G)(A/C)G(A/T/C/G)AA(T/C)GA(A/G)CC(A/T/C/G)AC-3′ and 20 pmol SP6 primer 5-ATTTAGGTGACACTATAGAA-3′ using the PCR reaction with 5 cycles of 94°C for 30 s; 60°C for 30 s; and 72°C for 1 min followed by 25 cycles of 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min. The PCR product was purified by passing a PCR Purification column (Qiagen). One-fiftieth of the purified product was further amplified using 300 pmol of primer-2 5′-AA(T/C)GA(A/G)CC(A/T/C/G)AC(A/T/C/G)CAACAACA-3′ and 300 pmol of primer-3 5′-TT(T/C)TG(T/C)TG(T/A/G)AT(A/T/C/G)GC(A/G)TTCAC-3′ in a PCR reaction as described above. A 285 bp PCR product was obtained and subsequently sequenced after subcloning into the PCR II vector using the TA cloning kit (Invitrogen). The 285 bp PCR product was labeled with [α-^{32}P]dCTP using *redi* prime RANDOM Primer Labeling kit (Amersham) and used to screen the HeLa λExlox cDNA library by hybridizing duplicate filter at 42°C overnight in Rapid-hyb buffer (Amersham). The filters were washed twice with 1× saline citrate (SSC)/0.1% SDS for 15 min at room temperature and once with 0.5× SSC/0.1% SDS for 10 min at 65°C. Out of 6×10^5 plaques screened, four positive clones were identified and a 1.4 kb partial clone was characterized. The 1.4 kb insert was excised and labeled with [α-^{32}P]dCTP as described above. The same library was rescreened with this 1.4-kb cDNA fragment as described above. Forty-five positive clones were identified and characterized. The longest 3 kb clone that contains the 5′ part of Apaf-1 was sequenced. An aliquot of 40 ng of this plasmid was amplified by two PCR primers, APPN 5′-ACATCACGAATCTTTCCCGC-3′ and APPC 5′-AACACTT CACTATCACTTCC-3′, designed from the 3′ end of the 3 kb insert. A 604 bp PCR fragment was generated and labeled with [α-^{32}P]dCTP as above. The same filters were rescreened with this PCR fragment, and 35 positive clones were identified and characterized. The longest one with an insert of 4.5 kb was sequenced. This clone contains the 3′ part of Apaf-1, which overlaps with the 3 kb 5′ clone by 472 bp. The full-length cDNA was obtained by ligating the two clones at an EcoRI site located within the 472 bp overlapping region.

Western Blot Analysis

Western blot analysis for cytochrome c was performed as described previously (Liu et al., 1996). Anti-Apaf-1 antiserum was generated by immunizing rabbits with a recombinant Apaf-1 fusion protein (see below). Immunoblot analysis was performed with a horseradish peroxidase conjugated goat anti-mouse (cytochrome c) or goat anti-rabbit (Apaf-1) immunoglobulin G using enhanced chemiluminescence Western blotting detection reagents (Amersham).

Production of Apaf-1 Fusion Protein

The primers 5′-GCAAAAGCTCGAAATCATATGCTTCAACATAGAG-3′ and 5′-TCGCGGCCGCCTCGAGGGCTCTGGTTGTAAG-3′ were designed to PCR-amplify the 1.4 kb plasmid Apaf-1 cDNA open reading frame, and the amplified 800 bp fragment encoding amino acids 10–254 of Apaf-1 was subcloned in-frame into the NdeI–XhoI sites of the bacterial expression vector pET-15b (Novagen). The expression plasmid was transformed into bacteria BL21(DE3). In a typical Apaf-1 preparation, a 5 ml overnight cultured of bacteria containing Apaf-1 expression vector was added into a 500 ml LB broth and 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 shaking continued for another 2 hr. The bacteria were pelleted by centrifugation, and the bacterial pellet was resuspended in 10 ml of buffer B (6 M GuHCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0). After centrifugation at 10,000 × g for 15 min, the supernatant was loaded onto a nickel affinity column (6 ml). The column was washed with 300 ml buffer B followed by 300 ml buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0). The column was eluted with Buffer C containing 250 mM imidazole. About 10 mg of Apaf-1 protein was purified from a 500-ml culture.

Northern Blotting Analysis

Poly(A)$^+$ RNA blots containing 2 μg of poly(A)$^+$ RNA per lane from multiple human adult and fetal tissues were purchased from Clontech. Blots were hybridized with 2×10^6 cpm/ml random primed 604-bp Apaf-1 PCR fragment corresponding to amino acid 590–792 (amplified with the APPN and APPC primers) in Rapid-hyb buffer (Amersham) at 65°C over night. The filters were washed twice with 1× SSC/0.5% SDS for 15 min at 65°C followed by 0.5× SSC/0.5% SDS for 20 min at 65°C. The same filters were also hybridized at 65°C for 2 hr with a 2.0 kb β-actin cDNA probe, and the filters were then washed as above.

Expression of Apaf-1 in 293 Cells

A 5.7 kb cDNA containing the entire coding region of Apaf-1 plus 577 bp of 5′ untranslated region and 1.5 kb of 3′ untranslated region was subcloned into NotI and EcoRI sites of a pcDNA 3.1(−) vector (Invitrogen), and the plasmid was designated as pApaf-1 and prepared using a Qiagen Mega plasmid kit. Human embryonic kidney 293 cells were set up at 1×10^6 per 100 mm dish in medium A (Dulbecco's modified Eagle's medium containing 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum, and grown in monolayer at 37°C in an atmosphere of 6%–7% CO_2. After incubation for 24 hr, each dish was transfected with either 15 μg of vector alone 10 μg of pApaf-1 plus 5 μg of vector, 10 μg of vector plus 5 μg of pCPP32 (Wang et al., 1996), or 10 μg of pApaf-1 plus 5 μg of pCPP32 as indicated in the legend to Figure 6 using the MBS Transfection Kit (Stratagene) as described (Hua et al., 1996). After 36 hr, the cells were harvested, and the S-100 fractions were prepared as described in Liu et al. (1996).

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GenBank Accession Number

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