Cytochrome c and dATP-mediated Oligomerization of Apaf-1 Is a Prerequisite for Procaspase-9 Activation*

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To elucidate the mechanism of activation of procaspase-9 by Apaf-1, we produced recombinant full-length Apaf-1 and purified it to complete homogeneity. Here we show using gel filtration that full-length Apaf-1 exists as a monomer that can be transformed to an oligomeric complex made of at least eight subunits after binding to cytochrome c and dATP. Apaf-1 binds to cytochrome c in the absence of dATP but does not form the oligomeric complex. However, when dATP is added to the cytochrome c-bound Apaf-1 complex, complete oligomerization occurs, suggesting that oligomerization is driven by hydrolysis of dATP. This was supported by the observation that, but not the nonhydrolyzable adenosine 5'-O-(thiotriphosphate), can induce oligomerization of the Apaf-1-cytochrome c complex. Like the spontaneously oligomerizing Apaf-530, which lacks its WD-40 domain, the oligomeric full-length Apaf-1-cytochrome c complex can bind and process procaspase-9 in the absence of additional dATP or cytochrome c. However, unlike the truncated Apaf-530 complex, the full-length Apaf-1 complex can release the mature caspase-9 after processing. Once released, mature caspase-9 can process procaspase-3, setting into motion the caspase cascade. These observations indicate that cytochrome c and dATP are required for oligomerization of Apaf-1 and suggest that the WD-40 domain plays an important role in oligomerization of full-length Apaf-1 and the release of mature caspase-9 from the Apaf-1 oligomeric complex.

Caspases, a highly conserved family of cysteine proteases that cleave their substrates after an aspartate residue, play fundamental roles in the initiation and execution of apoptosis (reviewed in Refs. 1–4). Caspases are constitutively expressed in cells as single chain proenzymes that can be activated by proteolytic cleavage at specific internal aspartate residues within the procaspase polypeptide chain. Mature caspases can cleave their own proenzyme and other procaspases, suggesting that they operate in a protease cascade. Caspases have been divided into initiators and effectors, based on their place in the caspase cascade (1–4). The effectors (caspase-3, -6, and -7) are activated via the action of other caspases (i.e. initiators) and are responsible for the characteristic morphological changes of apoptosis. The initiators (caspase-8, -9, and -10) are activated by their own intrinsic autocatalytic activity with the help of other proteins with which they form complexes known as “apoptosomes” (5).

Two apoptosomes that function to activate the initiator procaspases have been identified. The death receptor apoptosis is an oligomer that is formed upon ligation of death receptors such as Fas or tumor necrosis factor receptor 1 by their ligands (6). This oligomer recruits procaspase-8 or -10 via the adaptor molecule FADD through homotypic protein-protein interactions, resulting in activation of these caspases by aggregation (7, 8). Another unrelated apoptosome is formed by Apaf-1 upon binding to cytochrome c, which is released from the mitochondria by various forms of apoptosis triggers (5, 9). The Apaf-1-cytochrome c complex then recruits procaspase-9 in a dATP/ATP-dependent manner through a CARD-CARD1 interaction, resulting in its activation and presumably the release of mature caspase-9 from the apoptosome (5, 9).

A recent study from our laboratory demonstrated that a truncated Apaf-1 variant lacking the WD-40 repeat domain (Apaf-530) can activate procaspase-9 independent of cytochrome c and dATP through spontaneous oligomerization (10). Interestingly, the truncated Apaf-1 was unable to release the mature caspase-9 from the complex, raising the possibility that the WD-40 repeats play a role in the release of mature caspase-9 from the Apaf-1 apoptosome (10).

To determine the role of cytochrome c and dATP and the function of the WD-40 repeats in the process of activation and release of caspase-9, we reconstructed an in vitro Apaf-1-caspase-9 activation system with purified recombinant full-length Apaf-1. We provide evidence that cytochrome c and dATP are required to promote oligomerization of Apaf-1 and that mature caspase-9 is released from the full-length Apaf-1 apoptosome but not from the truncated Apaf-530 complex.

MATERIALS AND METHODS

Purification of Apaf-1L Protein from Sf-9 Cells—All the purification steps were carried out at 4 °C. Apaf-1L was expressed in Sf-9 cells by infecting the cells with recombinant Apaf-1 baculovirus. An S-100 extract was prepared from a 1-liter suspension culture of the infected cells in 25 mM HEPES buffer (pH 7.5) containing 300 mM NaCl, 10 mM KCl, 1.5 mM MgCl2, 10% glycerol, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. 280 mg of total proteins were loaded onto a 2-ml bed volume column of Ni2+-nitrilotriacetic acid agarose (Novagen) at a flow rate of 0.05 ml/min in the presence of 10 mM imidazole. After washing the column with 2 × 20 ml of each fraction containing Apaf-1L (~90 mM-150 mM imidazole) were pooled and con-

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1 The abbreviations used are: CARD, Caspase recruitment domain; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; γ-S-ATP, adenosine 5'-O-(thiotriphosphate); AMC, 7-amino-4-methylcoumarin.
centrated (Centricon-30; Amicon), and the final concentration of NaCl was adjusted to 20 mM in a final volume of 2.0 ml. Subsequently, the concentrated Apaf-1L sample (600 µg) was applied to an FPLC Mono Q column (1.0 ml; Amersham Pharmacia Biotech) at a flow rate of 0.05 ml/min. After washing the column with 10 ml of the HEPES buffer containing 50 mM NaCl, the protein was eluted with a 20-ml gradient of 20–300 mM NaCl at a flow rate of 0.2 ml/min. The peak fractions containing Apaf-1L were pooled and concentrated, and the concentration of NaCl was adjusted to 50 mM in a final volume of 0.5 ml (110 µg).

Finally, 2 × 250 µl of the Mono Q purified Apaf-1L was loaded separately onto a Superose 12 FPLC column (Amersham Pharmacia Biotech) at a flow rate of 0.2 ml/min. 15-µl aliquots from each 250-µl fraction were separated by SDS-PAGE and analyzed by Western blotting with anti-Apaf-1 antibody. The peak fractions of Apaf-1L protein were pooled and concentrated (1 ml; 60 µg of protein), and the purity of the protein was verified by SDS gel electrophoresis and Coomassie staining. By comparison with gel filtration protein standards (Amersham Pharmacia Biotech), the peak fraction of Apaf-1L from the Superose 12 column corresponded to apparent molecular size of 125 kDa (this value was calculated by linear extrapolation from the calibration protein standards; data not shown).

Oligomerization of Apaf-1 Protein—All oligomerization reactions of Apaf-1L were carried out by incubating Apaf-1 (3 µg) with or without cytochrome c (7 µg) or dATP (1 mM) or both at 4 °C for 70 min in a final volume of 100 µl of 25 mM HEPES buffer (pH 7.5) containing 50 mM NaCl, 1.5 mM MgCl₂, 10% glycerol, and 0.1 mM DTT (oligomerization buffer). In some experiments dATP was substituted with ATP (1 mM), or y-S-ATP (1 mM). After oligomerization an additional 150 µl of the oligomerization buffer was added to each sample, and the reaction mixture was directly applied to a Superose 6 FPLC column at a flow rate of 0.2 ml/min. 45-µl aliquots of the 500-µl fractions were fractionated by SDS-PAGE and assayed for the presence of Apaf-1 protein by immunoblotting with anti-Apaf-1 antibody. Approximate molecular masses of the different forms of Apaf-1L protein were obtained by linear extrapolation from the calibration protein standards.

Fractionation of Apaf-1 and Caspase-9 on Sephacryl S-400 HR Column—Initially, 100 µl of in vitro translated ³⁵S-labeled pro-caspase-9 was fractionated on a 15-ml open column of Sephacryl S-400 HR (Amersham Pharmacia Biotech) at a flow rate of 0.05 ml/min. 15-µl aliquots of 200-µl fractions were separated on 10% polyacrylamide gels, and the elution peak of pro-caspase-9 was determined by autoradiography. 1 × 10⁵ trichloroacetic acid counts of the partially purified pro-caspase-9 were incubated with 3.0 µg of pure Apaf-1L and 7.0 µg of cytochrome c in a final volume of 100 µl of the oligomerization buffer containing 1.0 mM dATP. The mixture was incubated at 30 °C for 45 min to allow processing of pro-caspase-9 followed by loading onto the Sephacryl S-400 column. The elution of caspase-9 forms and Apaf-1L protein were assessed by autoradiography and Western blotting with anti-Apaf-1 antibody, respectively. Similarly, 25 µg of affinity purified Apaf-530 protein (10) were incubated with the partially purified pro-caspase-9 and fractionated on the same gel filtration column.

Transfection, Immunoprecipitation, and Western Analyses—These were performed as described previously (9, 11).

RESULTS AND DISCUSSION

Expression and Purification of Functional Recombinant Apaf-1—To obtain sufficient quantities of human Apaf-1 for functional and biochemical characterization, we engineered baculoviruses encoding two C-terminally His₆-tagged Apaf-1 isoforms. One isoform (Apaf-1S) is identical in sequence to the published Apaf-1 (GenBank™ accession number AF013263) and has 13 WD-40 repeats (residues 413–1194). Apaf-1L (1237 residues) has 14 WD-40 repeats (residues 413–1237) and was cloned from a human Jurkat cDNA library. The CARD domain (residues 1–97) and the CED-4 homology domain (residues 98–412) are indicated.

The two isoforms have C-terminal His₆ tags to facilitate their purification. B, Western blot analysis of partially purified recombinant Apaf-1S and Apaf-1L. Apaf-1S (lane S) and Apaf-1L (lane L) were expressed in SF-9 cells by infecting the cells with their respective baculoviruses. 48 h after transfection the cells were harvested and lysed, and the lysates were bound to Ni²⁺ affinity resin. The bound Apaf-1 proteins were then eluted with imidazole and analyzed by 10% SDS-PAGE and immunoblotting. C, activity of Apaf-1S and Apaf-1L toward pro-caspase-9. Pro-caspase-9 was in vitro translated in the presence of [³⁵S]methionine. Following translation pro-caspase-9 was desalted by gel filtration through a biospin column (Bio-Rad) to remove unincorporated methionine and free nucleotides and incubated with Apaf-1S (second through fifth lanes) or Apaf-1L (sixth through ninth lanes) in the presence or absence of cytochrome c or dATP or both for 2 h at 30 °C. Samples were then analyzed by SDS-PAGE and autoradiography. D, Coomassie-stained recombinant Apaf-1L (lane L) was purified to complete homogeneity as described under "Materials and Methods," fractionated on 10% SDS gel, and then stained with Coomassie. Lane M, molecular mass markers.

Apaf-1 stability and its overall tertiary structure. We observed that Apaf-1S is less soluble than Apaf-1L and that the majority of the expressed protein accumulates as insoluble occlusion bodies in SF-9 cells. However, we did not see any precipitation of the soluble Apaf-1S during the incubation period with pro-caspase-9 (data not shown). Based on these data and on the published sequence of mouse Apaf-1, we believe that the human Apaf-1L isoform is the functional form of Apaf-1 in human cells.

Cytochrome c and dATP Are Required for Oligomerization of Apaf-1L—Cytochrome c and dATP are necessary for Apaf-1-mediated activation of pro-caspase-9 (9). However, their exact role in this process remains to be determined. Our recent studies demonstrated that deletion of the entire WD-40 domain of Apaf-1 produced a constitutively active Apaf-1 variant (Apaf-530) that can spontaneously oligomerize and induce activation of pro-caspase-9 independent of cytochrome c and dATP (10). Because the process of oligomerization appears to be critical for activation of pro-caspase-9, we hypothesize that cytochrome c and dATP regulate oligomerization of full-length Apaf-1, possibly by changing the conformation of the WD-40 domain, making it less favorable for oligomerization.

To test this possibility we purified recombinant Apaf-1L to complete homogeneity (Fig. 1D), incubated it with or without cytochrome c or dATP or both, and then analyzed its elution profile by gel filtration on an FPLC Superose-6 column. We reasoned that if cytochrome c or dATP both induce oligomerization of Apaf-1, we should be able to separate the oligomeric form of Apaf-1 from its monomeric form on the basis of molec-
Procaspase-9 Activation by Apaf-1

Fig. 2. Gel filtration and immunoprecipitation analysis of Apaf-1L. A purified Apaf-1L was incubated with or without cytochrome c or dATP, or both. The major peaks in each run were labeled as follows: peak I, buffer; peak II, dATP; peak III, cytochrome c, peak IV, cytochrome c plus dATP. The insets in D and E represent immunoprecipitates of selected fractions. F, 350 μg of S100 extracts of FLAG- and T7-tagged Apaf-1L were mixed and incubated with or without cytochrome c, dATP, or both and then immunoprecipitated with a FLAG antibody. The immunoprecipitates were fractionated by SDS gel electrophoresis and immunoblotted with a T7 antibody (upper panel). The corresponding resin-bound FLAG-Apaf-1L protein in each sample is shown underneath (lower panel).

ular size differences. Preincubation of Apaf-1L with dATP alone did not change its elution profile from that of the buffer control (Fig. 2, A-C). Both the buffer control and dATP-Apaf-1L eluted as single peaks around fraction 20 (Fig. 2, B and C, respectively). The approximate size of Apaf-1L in the peak fraction was ~125 kDa, suggesting that it is a monomer and that dATP alone is not sufficient to induce its oligomerization. Preincubation of Apaf-1L with cytochrome c alone resulted in a small shift in the Apaf-1L elution profile (Fig. 2, A and D). The majority of Apaf-1L eluted in a large peak around fraction 30, which corresponds to a size of ~170 kDa. In this fraction, cytochrome c co-eluted with Apaf-1L, indicating that this fraction contains a complex of cytochrome c-bound monomeric Apaf-1L. In addition to this peak, a smaller peak of free monomeric Apaf-1L eluted around fraction 33. Unbound cytochrome c eluted around fraction 40 (not shown). These results suggest that cytochrome c alone does not induce oligomerization of Apaf-1.

Interestingly, preincubation of Apaf-1L with cytochrome c and dATP resulted in a dramatic shift in the Apaf-1L elution profile (Fig. 2, A and E). The majority of Apaf-1L eluted around fraction 20. The remaining Apaf-1L eluted in two minor peaks around fractions 30 and 34, which correspond to cytochrome c-bound Apaf-1L and free monomeric Apaf-1L, respectively. The size of Apaf-1 in the major peak fraction is ~1.4 MDa. This and the presence of cytochrome c in this fraction suggest that the major peak contains a large oligomeric complex of Apaf-1L and cytochrome c. Based on the observed sizes of this oligomer (peak IV, ~1.4 MDa) and the cytochrome c-bound Apaf-1L monomer (peak III, ~170 kDa) and assuming that the oligomer is globular, we calculated that this oligomer contains at least eight molecules of Apaf-1L. These data demonstrate that Apaf-1 exists as a monomer and that binding of cytochrome c and dATP to Apaf-1 induces formation of an octamer of cytochrome c-bound Apaf-1.

To confirm the gel filtration results, we performed immunoprecipitation experiments using FLAG- and T7-tagged Apaf-1L. S100 extracts from 293 cells transfected with FLAG- or T7-tagged Apaf-1L were mixed and incubated with or without cytochrome c, dATP, or both and immunoprecipitated with a FLAG antibody. The immunoprecipitates were then fractionated by SDS gel electrophoresis and immunoblotted with a T7 antibody. As expected, only in the presence of both cytochrome c and dATP was there a significant association of the two tagged Apaf-1L proteins with each other (Fig. 2F). A small amount of association that was observed with cytochrome c alone could be because of the presence of residual amounts of ATP or dATP in the S100 extract. No association was observed in the buffer or dATP controls.

The Oligomeric Apaf-1L-Cytochrome c Complex Can Induce Activation of Procaspase-9 without Additional dATP or Cytochrome c—To determine the activity of Apaf-1L in the major peak fractions of the four gel filtration experiments (Fig. 2, B-E), we incubated samples of the peak fractions with procaspase-9 in the presence or absence of cytochrome c or dATP or both. As shown in Fig. 3A, the oligomeric Apaf-1L (peak IV) was capable of processing procaspase-9 without additional cytochrome c and dATP. The cytochrome c-Apaf-1L complex (peak III), on the other hand, was capable of processing procaspase-9 only when both cytochrome c and dATP were added. Based on these data and the gel filtration data, we suggest that cytochrome c can bind to Apaf-1 in the absence of dATP but cannot induce its oligomerization. However, in the presence of dATP, the cytochrome c-Apaf-1L complex will form an oligomer that is capable of activating procaspase-9. This was further confirmed by incubating the pooled peak III (Fig. 2D) with dATP and then fractionating it on Superose 6 column. As shown in Fig. 3B, dATP induced a complete shift in the elution profile of the cytochrome c-bound Apaf-1L. All Apaf-1L in peak III eluted in a single peak around fraction 20, which corresponds to the oligomeric Apaf-1L. This observation demonstrates that dATP is required for oligomerization of the cytochrome c-bound Apaf-1 monomer. This may have certain physiological implications. For example, cytochrome c release from the mitochondria of injured cells may not be sufficient to induce oligomerization and activation of procaspase-9. Only under conditions where sufficient dATP or ATP are available can cytochrome c release from the mitochondria induce apoptosis.
Oligomerization of Apaf-1 Requires a Hydrolyzable ATP—

Studies with purified Apaf-1 demonstrated that ATP in the presence of cytochrome c could also induce activation of procaspase-9, although at a higher concentration than dATP (9). However, substitution of ATP or dATP by the nonhydrolyzable ATP analogue γ-S-ATP prevented activation, suggesting that hydrolysis of the γ-phosphate group is necessary for Apaf-1 function (9). Because dATP is required to induce oligomerization of the cytochrome c-bound Apaf-1L (Fig. 3, A and B), we reasoned that ATP, but not γ-S-ATP, should be able to induce the same effect. To test this hypothesis we incubated purified Apaf-1L with cytochrome c and dATP, ATP, or γ-S-ATP and then analyzed its elution profile by gel filtration on an FPLC Superose-6 column. As expected, dATP and ATP, but not γ-S-ATP, were able to induce the formation of the Apaf-1L oligomeric complex, which eluted around fraction 20 (Fig. 3C). However, the amount of the oligomeric Apaf-1L induced by ATP was less than that induced by dATP. This is consistent with earlier observations that dATP is more effective than ATP in inducing procaspase-9 activation in S100 lysates and by purified Apaf-1 (9, 14). This also indicates that the effectiveness of the dATP analogues in inducing activation of procaspase-9 by Apaf-1 depends on their ability to induce oligomerization of Apaf-1. Our finding that γ-S-ATP cannot induce oligomerization of Apaf-1 explains its inability to induce activation of procaspase-9 by purified Apaf-1 (9) and suggests that hydrolysis of ATP or dATP might be critical in the oligomerization process.

To determine whether purified Apaf-1L has an ATPase activity, we incubated increasing amounts of pure Apaf-1L at 37 °C for 30 min in a reaction buffer (20 mM HEPES (pH 7.5), 75 mM NaCl, 10 mM MgCl₂, 5 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.075 mM EDTA, 15% glycerol, 75 μg/ml acetylated bovine serum albumin, 500 μM unlabeled ATP, and 16.5 μM γ-[32P]ATP. Picomoles of ATP hydrolyzed were plotted against increasing amounts of Apaf-1 (nm).

FIG. 3. Procaspase-9 processing activity of Apaf-1L in peaks I-IV and the role of dATP. A, ³⁵S-labeled procaspase-9 was incubated with buffer control (lanes C) or aliquots of peaks I-IV (Fig. 2, B-E) in the presence or absence of cytochrome c or dATP or both. Samples were then analyzed by SDS-PAGE and autoradiography. Procaspase-9 (pcasp-9) and the p35 fragment of mature caspase-9 are indicated. B, elution profile of the Apaf-1-cytochrome c complex (peak III) after preincubation with dATP. Pooled peak III fractions from the cytochrome c run (Fig. 2D) were incubated with dATP for 1 h and then loaded on Superose-6 FPLC column. Equal volumes of the column fractions were analyzed for Apaf-1L by immunoblotting as described in the legend to Fig. 2. C, effect of substitution of dATP with ATP or γ-S-ATP on oligomerization of Apaf-1L. Purified Apaf-1L was incubated with cytochrome c and dATP, ATP, or γ-S-ATP and then loaded on Superose-6 FPLC column. Aliquots of the column fractions from the three runs were analyzed for Apaf-1L by immunoblotting as described in the legend to Fig. 2. D, ATPase activity of purified Apaf-1L. ATPase assay was performed similar to the method described by Gradia et al. (16). In brief, increasing amounts of pure Apaf-1L was incubated at 37 °C for 30 min in a reaction buffer (20 μl) consisting of 25 mM HEPES (pH 7.5), 75 mM NaCl, 10 mM MgCl₂, 5 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.075 mM EDTA, 15% glycerol, 75 μg/ml acetylated bovine serum albumin, 500 μM unlabeled ATP, and 16.5 μM γ-[32P]ATP. Picomoles of ATP hydrolyzed were plotted against increasing amounts of Apaf-1L (nm).

Fig. 4. Analysis of mature caspase-9 release from Apaf-1L and Apaf-530. A and B, purified recombinant Apaf-1L or Apaf-530 were incubated with partially purified ³⁵S-labeled procaspase-9 and then fractionated by gel filtration on Sephacryl S-400 column as described under “Materials and Methods.” The fractions were analyzed by immunoblotting (Apaf-1L) and autoradiography (caspase-9), and the intensities of the bands were plotted against the fraction number. C, peak fractions 22 and 38 or the corresponding pooled peaks (fractions 18–28 and fractions 34–42, respectively) were incubated with or without ³⁵S-labeled procaspase-3 for 1 h at 30 °C. The samples were then analyzed by SDS-PAGE and autoradiography. The p20 and p12 fragments of processed caspase-3 are indicated. The p35 fragment of the released caspase-9 (fourth and fifth lanes) and procaspase-9 (ninth lane) are similar in size and migrate in SDS gels as 35-kDa proteins. The p12 of mature caspase-9 is not detectable because of its low density. D, activity of the released caspase-9. Indicated concentrations of bacterial or released caspase-9 were incubated with DEVD-AMC (50 μM) in a buffer control or S100 extract (50 μg) from Apaf-1-deficient embryonic fibroblasts for 30 min at 30 °C. After incubation the released AMC was determined by luminescence spectrometry and represented as arbitrary units. The concentration of caspase-9 was estimated by quantitative immunoblotting. Cytochrome c (5 ng/ml) and dATP (1 mM) were added as a negative control to the S100 extract to demonstrate that these factors do not induce DEVD-AMC cleavage in this extract.

ATP or dATP might be critical in the oligomerization process.
22 and 38 or the corresponding pooled peaks with \(^{35}\text{S}\)-labeled procaspase-3. Peak fraction 38 or the corresponding pooled peak (fraction 34–42) was capable of processing procaspase-3 to the p20 and p12 fragments of active caspase-3 (Fig. 4, lanes 1–3) or on an S100 extract from 293 S100 cells (lanes 4–7) supplemented with \(^{35}\text{S}\)-labeled procaspase-9 or procaspase-3 were incubated with or without Apaf-530 in the presence or absence of cytochrome c and dATP. Samples were then analyzed by SDS-PAGE and autoradiography.

In conclusion, we have demonstrated that Apaf-1 undergoes oligomerization upon binding to cytochrome c in a dATP-dependent manner. This oligomeric complex can recruit procaspase-9 directly and activate it and then release the mature caspase-9 from the complex to initiate the caspase cascade. Thus Apaf-1 functions as a cytosolic death receptor that is activated upon binding to its ligand, cytochrome c, in the presence of dATP.

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Fig. 5. Activity of the Apaf-1L- and Apaf-530-caspase-9 complexes. A. \(^{35}\text{S}\)-labeled procaspase-3 (lanes 1–6) or \(^{35}\text{S}\)-procaspase-9 (lanes 7–9) were incubated with or without Apaf-1L or Apaf-530 and cytochrome c plus dATP in the presence or absence of nonradiolabeled procaspase-9. Samples were then analyzed by SDS-PAGE and autoradiography. B. Apaf-530 inhibits processing of procaspase-3 in 293 S100 extracts. A buffer control (lanes 1–3) or an S100 extract from 293 cells (lanes 4–7) supplemented with \(^{35}\text{S}\)-labeled procaspase-9 or procaspase-3 were incubated with or without Apaf-530 in the presence or absence of cytochrome c and dATP. Samples were then analyzed by SDS-PAGE and autoradiography.