Pro-apoptotic Proteins Released from the Mitochondria Regulate the Protein Composition and Caspase-processing Activity of the Native Apaf-1/Caspase-9 Apoptosome Complex

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The apoptosome is a large caspase-activating (~700–1400 kDa) complex, which is assembled from Apaf-1 and caspase-9 when cytochrome c is released during mitochondrial-dependent apoptotic cell death. Apaf-1 is the core scaffold protein and contains CARD (caspase recruitment domain), CED-4, and multiple (15) WD40 repeat domains, which can potentially interact with a variety of unknown regulatory proteins. To identify such proteins we activated THP-1 lysates with dATP/cytochrome c and used sucrose density centrifugation and affinity-based methods to purify the apoptosome for analysis by MALDI-TOF mass spectrometry. First, we used a glutathione S-transferase (GST) fusion protein (GST-casp91–130) containing the CARD domain of caspase-9-(1–130), which binds to the CARD domain of Apaf-1 when it is in the apoptosome and blocks recruitment/activation of caspase-9. This affinity-purified apoptosome complex contained only Apaf-1XL and GST-casp91–130, demonstrating that the WD40 and CED-4 domains of Apaf-1 do not stably bind other cytosolic proteins. Next we used a monoclonal antibody to caspase-9 to immunopurify the native active apoptosome complex from cell lysates, containing negligible levels of cytochrome c, second mitochondria-derived activator of caspase (Smac), or Omi/HtrA2. This apoptosome complex exhibited low caspase-processing activity and contained four stably associated proteins, namely Apaf-1, pro-p35/p34 forms of caspase-9, pro-p20 forms of caspase-3, X-linked inhibitor of apoptosis (XIAP), and cytochrome c, which was only bound transiently to the complex. However, in lysates containing Smac and Omi/HtrA2, the caspase-processing activity of the purified apoptosome complex increased 6–8-fold and contained only Apaf-1 and the p35/p34 processed subunits of caspase-9. During apoptosis, Smac, Omi/HtrA2, and cytochrome c are released simultaneously from mitochondria, and thus it is likely that the functional apoptosome complex in apoptotic cells consists primarily of Apaf-1 and processed caspase-9.

The morphological and biochemical changes of apoptotic cell death largely result from the activation of a group of cysteine aspartic acid-specific proteases known as caspases (for review see Refs. 1–3). The activation of caspases is a central feature of apoptosis, and key components of this mechanism are highly conserved throughout evolution from Caenorhabditis elegans to Drosophila melanogaster and ultimately to mammals. In C. elegans there are at least four genes, ced-3, ced-4, ced-9, and egl-1, which are critical for the execution of apoptotic cell death. In C. elegans, CED-4 is normally bound to mitochondria and cell membranes by CED-9 (4). Up-regulation of EGL-1 inactivates CED-9, releasing CED-4, which oligomerizes/recruits and induces autolytic processing and activation of CED-3 (5). ced-3 encodes for a cysteine protease, which is homologous to interleukin-1β-converting enzyme or ICE, now known as caspase-1. Thirteen other caspses have now been identified in mammals, some of which are involved in cell death (CED-3 subfamily), whereas others are involved in inflammation (ICE subfamily).

Caspases are synthesized as inactive pro-caspases or zymogens, which in mammals are activated in a cascade mechanism, involving an initiator or activating caspase that cleaves/activates a downstream caspase, which in turn activates the next caspase and so on. There are at least two primary (extrinsic and intrinsic) caspase activation pathways, involving either stimulation of cell surface death receptors or perturbation of mitochondria (for review see Ref. 6). The intrinsic pathway is essentially homologous to C. elegans in that it involves the formation of the apoptosome, a large caspase-processing complex (for review see Refs. 7, 8). Mammalian homologues of CED-3, CED-9, and EGL-1 have been identified by conventional cloning strategies, but Apaf-1 is a mammalian homologue of CED-4 was only identified by a classical biochemical approach. In these cell-free studies, caspase activation was induced in cell lysates by dATP, and three apoptotic protease-activating factors (Apaf-1–3) were isolated (9). Apaf-1 was identified as a CED-4 homologue (10), Apaf-2 as cysteine c (9) and Apaf-3 as caspase-9 (11). Apaf-1 is a large ~135 kDa protein, which consists of an N-terminal caspase recruitment domain (CARD),1 a region homologous to CED-4 followed by a large C-terminal domain containing 12–13 WD40 repeats. The CARD domain interacts with a similar domain on procaspase-9 and the central CED-4 (98–412) region contains a putative

1 The abbreviations used are: CARD, caspase recruitment domain; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Smac, second mitochondria-derived activator of caspase; PBS, phosphate-buffered saline; DTT, dithiothreitol; Z, benzoyloxycarbonyl; FMK, fluoromethylketone; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; XIAP, X-linked inhibitor of apoptosis; IEF, isoelectrofocusing; F/T, freeze-thaw; ProT, prothymosin α; PHAP, putative HLA-DR-associated proteins.
ATPase domain with conserved Walker’s A (P-loop) and B boxes forming nucleotide binding sites (11, 12). The function of the WD40 regions is largely unknown although a critical number of 13 WD40 repeats is required for cytochrome c binding as Apaf-1 splice forms containing only 12 WD40 repeats do not bind cytochrome c and do not activate caspases (13). WD40 repeats usually form β-propeller structures arranged in a rigid circular structure and in the case of Apaf-1 it is likely that these would be arranged as two asymmetric (7- and 6-bladed) structures (8).

Apaf-1 has been shown to oligomerize in the presence of cytochrome c and dATP to form a very large apoptosome complex with a molecular weight between ~700 kDa and ~1.4 MDa (14–17). The size of the apoptosome complex varies from ~1.4 MDa when reconstituted from recombinant proteins (16) to >1.3 MDa (17) and ~1.0 MDa (18) when isolated from HeLa cell lysates, however, in dATP-activated THP-1 and B chronic lymphocytic leukemia cell lysates we have identified both an ~700-kDa and an ~1.4-MDa apoptosome complex (15, 19). The ~700-kDa complex predominates in apoptotic cells and is the most active complex in processing exogenous procaspase-9 and -3 (15, 19).

These studies suggest that the size of the apoptosome complex may vary according to cell type and/or the conditions in which the apoptosome is formed, and it is possible that other cellular proteins can bind to the Apaf-1/caspase-9 holo-enzyme complex. We therefore decided to purify the native ~700-kDa apoptosome complex to homogeneity in order to identify the additional components of the apoptosome. However, our attempts to purify the apoptosome by classical biochemical methodology using multistep chromatography were unsuccessful because of the co-purification of other large ~700-kDa protein assemblies such as the IKK (20) and rabaptin-5/rabex 5 complexes (21). We have therefore developed two affinity-based techniques to purify the apoptosome from dATP-activated lysates. First, we used a GST-caspase-9 (1–130) (GST-casp95–130) construct, which does not contain the large and small subunits of caspase-9, and acts as a dominant negative inhibitor of caspase activation by occupying the Apaf-1 CARD domain in the apoptosome complex. We then sequentially purified the ~700-kDa complex tagged with GST-casp95–130 by sucrose density gradient purification and glutathione beads. Second, we have used a monoclonal anti-caspase-9 antibody to purify the intact fully functional ~700-kDa apoptosome complex, which contains Apaf-1 and processed caspase-9. The purified apoptosome complexes were analyzed by Western blotting and MALDI-TOF mass spectrometry (M@LDI R, Micromass, Manchester, UK), and surprisingly the respective apoptosome complexes contained only Apaf-1 and GST-casp95–130 or Apaf-1 and the p35 and p34 processed forms of caspase-9. Caspase-3 was not detected in the complex and appears to only interact transiently with the apoptosome complex in conditions of low (non-physiological) ionic strength. X-linked inhibitor of apoptosis (XIAP) was also not detected in the purified complex due to the presence of endogenous Smac (second mitochondria-derived activator of caspase) and Omi/HtrA2, which sequester free XIAP and thereby prevent it from interacting with the apoptosome complex. However, when the apoptosome was formed in lysates, which were essentially free of Smac and Omi/HtrA2, we demonstrated recruitment of XIAP and caspase-3 to the apoptosome complex. These studies indicate that when the apoptosome is formed in the apoptotic cell it initially can be inhibited by XIAP, but increasing cytosolic concentrations of Smac and Omi/HtrA2 remove XIAP from the apoptosome complex, which then achieves maximum caspase-activating activity.
Separation of Apoptosome Complexes—Lysates were fractionated by size-exclusion chromatography on Superdex 6 column using an FPLC (HR 10/30 column) protein purification system (Amersham Biosciences). Columns were eluted at 4°C with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM DTT, and 50 mM NaCl, pH 7.0 calibrated with protein standards as previously described (15).

Large scale preparation of ~700-kDa apoptosome complexes were prepared by centrifuging TPH.1 lysates (30 ~ 80 mg) at 25,000 rpm for 17 h at 4°C on a continuous sucrose gradients (10 ~ 40%), 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM DTT, pH 7.0 at 4°C (14). The gradients were collected in 2-ml fractions, which were analyzed for the LLVYase activity of the 20 S proteasome, which co-sediments with the ~700-kDa apoptosome complex (14). Aliquots of the gradient fractions were also analyzed by SDS-PAGE and Western blotting for Apaf-1 (~165 kDa). Mass spectrometry data were analyzed automatically or manually by ProteinLynx and MAScOT software, respectively. Peptide identity was determined by submission to SWISS-PROT and TrEMBL data bases.

Affinity Purification of Apoptosome Complexes—GST-casp9~130, tagged apoptosome complexes were purified from lysates by incubating for 1 h at 4°C with an equal volume of GSH-Sepharose beads (50% slurry in PBS). Column and sucrose density fractions were extracted in the same way except that a 1:5 bead/sample volume ratio was used. The beads were washed four times in PBS by centrifugation/resuspension and then eluted with 20 mM reduced glutathione. Immunofluorophore purification of ~700-kDa apoptosome complexes was carried out by first preclearing TPH.1 cell lysates (~25 mg/ml) for 1 h at 4°C with 400 μl of lysate of protein G-Sepharose beads, which had been prewashed three times in wash buffer (assay buffer supplemented with 0.5% bovine serum albumin and 0.05% Tween 20). Precleared lysates were diluted to 15 mg/ml with assay buffer, caspase was activated and treated as indicated under “Results,” except that cytochrome c (~7 μM) was included in the activation buffer. Apoptosome capture was then carried out by adding 1 μg of cross-linking buffer containing with the separated heavy and light chains of the caspase-9 antibody, and then eluted with 20 mM reduced glutathione. Immunoaffinity purification methods potentially provide a more selective purification strategy; however, the affinity method needs to be specific for the ~700-kDa apoptosome complex by virtue of the fact that the proforms are substrates for the apoptosome and form E

Proteomic Analysis—Gels plugs (0.8 mm × 1 mm) were excised from stained SDS-PAGE or two-dimensional gels using an automated spot picker (ProPic, PerkinElmer Life Sciences). An automated digestion robot (ProCert, PerkinElmer Life Sciences) was used to perform in situ digestion on the plugs, which were sequentially reduced with 10 μM dithiothreitol, alkylated with 100 μM iodoacetamide, and digested with modified sequencing-grade porcine trypsin (Promega). Peptides were extracted from the digested gel plugs with 21 μl of 0.1% formic acid, and aliquots (5 μl) of the extracted peptides desalted with C18 Zip tips (Millipore). Protein mass analysis was then performed on a MALDI-TOF mass spectrometer using a mass range of 1,000–35,000 Da. Mass spectrometric data were analyzed automatically or manually by ProteinLynx and MAScOT software, respectively. Peptide identity was determined by submission to SWISS-PROT and TrEMBL data bases.

Reagents and Western Blot Analysis—The GST-casp9~130 construct was generated using conventional cloning strategies. Most other reagents, including antibodies to caspases-3, and -7 were obtained unless otherwise indicated from previously described sources (6, 15, 22–24). A mouse monoclonal antibody (clone 5B4) to caspase-9 was obtained from MBL laboratories (Nagaoy, Japan) and used at 0.1 μg/ml concentration. Protein samples (~20 μg) were resolved on 10 or 12.5% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Hybond C extra, Amersham Biosciences), and antibody binding was detected as described previously (15). Western blotting for XIAP was carried out, using a 0.125 μg/ml solution of a mouse monoclonal antibody (clone 48, BD Biosciences, PharMingen, San Diego, CA), which was raised to the C terminus (amino acids 286 ~ 426) of XIAP. The heavy chain of the IgG, monoclonal antibody to caspase-9 and XIAP have molecular weights of 57 and ~50 kDa, respectively and can be difficult to resolve with certainty using SDS gels and immunoblotting. Therefore in some experiments a triple sandwich (tertiary) detection method was used to identify XIAP. Briefly, the nitrocellulose blot was incubated for 1 h with a 1:2000 dilution of the primary XIAP antibody, and then blocked for 30 min with 4% bovine serum albumin in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween 20, pH 7.0). The blot was then incubated at 4°C overnight with 2 μg/ml recombinant protein G-biotin conjugate (Sigma) in TBST buffer. The protein G-biotin conjugate does not cross-react with the separated heavy and light chains of the caspase-9 antibody, and only binds to the primary XIAP antibody. The protein G-biotin conjugate was detected with ECL using a 1:1000 dilution of streptavadin-horseradish peroxidase (Amersham Biosciences).

RESULTS

In previous studies we have shown that dATP activation of TPH.1 cell lysates induces de novo formation of ~1.4-MDa and ~700-kDa Apaf-1/caspase-9-containing apoptosome complexes (15). The ~700-kDa apoptosome complex, which predominates in apoptotic cells, is the most active apoptosome when assessed by its ability to process and activate effector caspases (15). Other proteins such as caspase-3 and caspase-7 are transiently bound to the ~700-kDa apoptosome by virtue of the fact that the proforms are substrates for the apoptosome and form E/S complexes, which are easily disrupted by moderate ionic strength conditions (14, 15, 25). We initially attempted to purify the ~700-kDa apoptosome complex by conventional sequential multistep chromatographic techniques. Although the 7-step procedure we subsequently developed substantially purified the apoptosome complex (~30-fold increase in caspase-processing activity), it was apparent from two-dimensional gel analysis and mass spectrometry that the purified preparation contained other similar sized assemblies such as the I KK (20) and the ~700-kDa (21) complexes.

Proteomic Analysis—Gels plugs (0.8 mm × 1 mm) were excised from stained SDS-PAGE or two-dimensional gels using an automated spot picker (ProPic, PerkinElmer Life Sciences). An automated digestion robot (ProCert, PerkinElmer Life Sciences) was used to perform in situ digestion on the plugs, which were sequentially reduced with 10 μM dithiothreitol, alkylated with 100 μM iodoacetamide, and digested with modified sequencing-grade porcine trypsin (Promega). Peptides were extracted from the digested gel plugs with 21 μl of 0.1% formic acid, and aliquots (5 μl) of the extracted peptides desalted with C18 Zip tips (Millipore). Protein mass analysis was then performed on a MALDI-TOF mass spectrometer using a mass range of 1,000–35,000 Da. Mass spectrometric data were analyzed automatically or manually by ProteinLynx and MAScOT software, respectively. Peptide identity was determined by submission to SWISS-PROT and TrEMBL data bases.
fore we have instead developed two affinity-based approaches using caspase-9 to capture and purify the H11011 700-kDa apoptosome complex.

GST-casp91–130 Blocks dATP-dependent Caspase Activation in THP-1 Cell Lysates—The first approach was based on previous observations that the prodomain (residues 1–130) of caspase-9 inhibits caspase activation (26) by binding tightly to Apaf-1 via CARD-CARD interactions. Suitably tagged, the prodomain of caspase-9 should only bind to the CARD domain of Apaf-1 when it is accessible, i.e. after dATP activation, as in the apoptosome. We therefore reasoned that it should be possible to tag and purify the apoptosome complex with this construct, and this would allow us to identify any proteins that were interacting with the CED-4 and WD40 repeat domains. We therefore generated a recombinant GST-casp91–130 fusion protein (Fig. 1A) and showed that it inhibited dATP-activated effector caspase activation in THP-1 lysates with an IC50 = 0.8 μM (Fig. 1B). GST-casp91–130 had no effect on DEVDase activity when it was added after dATP activation (data not shown), demonstrating that it does not inhibit either caspase-3 or -7 enzymic activity. However, the inhibition of DEVDase activation by GST-casp91–130 was accompanied by blocking of caspase-9, and -3 processing (Fig. 1C, lane 6). In addition, GST-casp91–130 also completely inhibited the cleavage and processing of caspase-2 (Fig. 1C, lane 6), demonstrating that this caspase is only activated via the apoptosome-dependent activation of caspase-9 and caspase-3. Recombinant GST had no effect on dATP-dependent caspase activation (Fig. 1C, lane 4).

GST-casp91–130 Binding to Apaf-1 Requires dATP—We next determined under what conditions GST-casp91–130 would bind to Apaf-1. GSH-Sepharose beads were used to affinity capture GST-casp91–130 and any attendant binding proteins, which were then eluted from the beads with 20 mM GSH. Lysates were incubated for 30 min with and without 0.5 μM GST-casp91–130 (lanes 5 and 6). Each lysate sample was assayed for DEVDase activity (expressed as fold-increase over the nonactivated THP-1 lysate). The cell lysates (20 μg/lane) were also analyzed for caspase-3, caspase-9, and caspase-2 by SDS-PAGE/Western blotting as described under “Experimental Procedures.” The proform and processed p19/p17 forms of caspase-3 are indicated, as are the proforms of caspase-9 and caspase-2.

Fig. 1. GST-casp91–130 acts as a dominant negative inhibitor of dATP activation in THP-1 cell lysates. A, schematic representation of the fusion protein GST-casp91–130. GST-casp91–130 was added to THP-1 lysates (10 mg/ml) with 2 mM dATP/MgCl2 for 30 min at 37 °C in the presence of increasing concentrations of GST-casp91–130. Caspase activation was measured using DEVD-AFC as a substrate as described under “Experimental Procedures” and expressed as a percentage of the dATP-activated control. C, THP-1 lysates (10 mg/ml) were incubated for 30 min at 37 °C with either 2 μM GST (lanes 3 and 4) or 2 μM GST-casp91–130 (lanes 5 and 6). Each lysate sample was assayed for DEVDase activity (expressed as fold-increase over the nonactivated THP-1 lysate). The cell lysates (20 μg/lane) were also analyzed for caspase-3, caspase-9, and caspase-2 by SDS-PAGE/Western blotting as described under “Experimental Procedures.” The proform and processed p19/p17 forms of caspase-3 are indicated, as are the proforms of caspase-9 and caspase-2.
Although Apaf-1 was detected in the lysates (Input, Fig. 2A, lanes 1–4) and flow-through fractions (Fig. 2A, lanes 5–8), it was only captured in the presence of GST-casp91–130 and dATP/Mg2+ (Fig. 2A, lane 12). Thus, GST-casp91–130 only binds to Apaf-1 under conditions that promote apoptosome formation. Furthermore, when GST-casp91–130 was added to the lysates post-caspase activation, it did not inhibit DEVDase activity (results not shown) and was unable to displace the endogenous caspase-9, judged by its inability to bind Apaf-1 to the GSH-Sepharose beads (Fig. 2B, lanes 3 and 4). Thus, without dATP activation, GST-casp91–130 does not bind to Apaf-1, presumably because the conformation of monomeric Apaf-1 occludes the CARD binding site.

We then used Western blotting to determine whether XIAP and caspase-3 were associated with the GST-casp91–130-tagged apoptosome complex. Although, both proteins were detected in the lysates and flow-through fractions (Fig. 2A, lanes 1–8), they were not detected in the glutathione eluates (Fig. 2A, lanes 9–12). Thus, XIAP and caspase-3 do not bind directly to the GST-casp91–130/Apaf-1 complex or alternatively may be bind-
ing to complexes in the flow-through fractions that have not been captured by the GSH-Sepharose beads. Previously, XIAP and caspase-3 have been shown to bind to the native apoptosome complex via interactions with XIAP, which simultaneously binds caspase-9 and caspase-3 (27). The binding of the BIR3 domain of XIAP to caspase-9 requires the cleavage of caspase-9 at Asp315 to yield an N-terminal ATPF motif, which binds to a conserved surface groove on the XIAP-BIR3 domain (28). Although this interaction is important for inhibition, a second protein-protein interface between XIAP-BIR3 and the small subunit of caspase-9 has also been identified, which produces an inactive BIR3-caspase-9 dimeric complex instead of an active caspase-9 homodimer (29). In contrast the inhibition of caspase-3 and -7 by XIAP involves an 18-peptide sequence immediately preceding the BIR2 domain, which interacts with the catalytic groove and occupies the substrate binding pockets of caspase-3/7 (30, 31). Furthermore, catalytically active caspase-9 is required for recruitment of caspase-3 to the apoptosome (27). Thus, in the GST-casp91–130 apoptosome complex where the ATPF and caspase-9 active site binding motifs are absent it would be predicted that XIAP and caspase-3 would not be recruited to the apoptosome complex. Thus, these results show conclusively that without a fully functional and intact caspase-9 there is no direct interaction/binding of XIAP and caspase-3 to the apoptosome complex.

Interestingly, caspase-2 also did not bind to the GST-casp91–130-tagged apoptosome complex (Fig. 2A), demonstrating that there is no interaction between caspase-2 and the apoptosome.

**Fig. 3.** GST-casp91–130 binds to Apaf-1 only when oligomerized into apoptosome complexes. THP-1 lysates (15 mg/ml) were activated with 2 mM dATP for 1 h at 37 °C plus or minus 2 μM GST-casp91–130. The lysates were separated by Superose-6 gel filtration chromatography. Fractions were then analyzed for their ability to activate procaspase-3 (A) as described under "Experimental Procedures" and analyzed by SDS-PAGE/Western blotting for Apaf-1 and GST-casp91–130 (B). Fractions corresponding to the ~700-kDa (fractions 10–14) and ~1.4-MDa apoptosome (fractions 5–7) complexes were pooled, concentrated, and incubated with GSH-Sepharose beads for 1 h at 4 °C. After centrifugation, the flow-through was removed and the beads washed thoroughly (5×) in cold PBS. The beads were eluted in 20 mM reduced glutathione, and the eluates (beads) were analyzed by immunoblotting for Apaf-1 and GST-casp91–130 (C).
casp9\textsuperscript{1–130} predominantly eluted as a non-oligomerized protein and did not affect the elution behavior of Apaf-1, demonstrating that it does not bind to Apaf-1 in the absence of dATP. Furthermore, GST-casp9\textsuperscript{1–130} did not affect the oligomerization of Apaf-1 to form the \(-1.4\)-MDa and \(-700\)-kDa complexes. However, in the presence of GST-casp9\textsuperscript{91–130} the caspase-activating activity of the \(-700\)-kDa complex was completely inhibited (Fig. 3A). After dATP activation, a significant proportion of the GST-casp9\textsuperscript{91–130} now co-migrated with the \(-1.4\)-MDa complex. Smaller amounts of GST-casp9\textsuperscript{91–130} were also detected in the fractions corresponding to the \(-700\)-kDa complex (see below). The fractions corresponding to the \(-1.4\)-MDa (Fig. 3B, lanes 5–8) and the \(-700\)-kDa (Fig. 3B, lanes 10–14) complexes were pooled and concentrated, and the apoptosome complexes affinity-purified with GSH-Sepharose, and subsequently analyzed for Apaf-1 and GST-casp9\textsuperscript{91–130} by SDS-PAGE and Western blotting. In the concentrated pooled samples Apaf-1 and GST-casp9\textsuperscript{91–130} were detected in both the \(-1.4\)-MDa and \(-700\)-kDa complexes (Fig. 3C, lane 9), albeit with apparently different Apaf-1/GST-casp9\textsuperscript{91–130} ratios. The beads were washed repeatedly to remove all loosely bound Apaf-1 and GST-casp9\textsuperscript{91–130} and then eluted with 20 mM glutathione. The \(-1.4\)-MDa complex contained more GST-casp9\textsuperscript{91–130} per mol of Apaf-1 than the \(-700\)-kDa complex (Fig. 3C, lane 9). The data also showed that the use of GST-casp9\textsuperscript{91–130} to capture apoptosome complexes directly from lysates could co-purify both the \(-1.4\)-MDa and \(-700\)-kDa complexes.

**Large Scale Purification of GST-casp9\textsuperscript{1–230}-tagged \(-700\)-kDa Apoptosome Complexes**—As we wished to specifically identify only those proteins that were interacting with the \(-700\)-kDa apoptosome complex, it was necessary to purify sufficient quantities of the GST-casp9\textsuperscript{1–130}-tagged \(-700\)-kDa apoptosome complex. Therefore, we prepared 30 mg of GST-casp9\textsuperscript{91–130}-tagged lysate that was fractionated on a 10–40% sucrose density gradient (25,000 rpm for 17 h). Under these conditions the \(-700\)-kDa apoptosome complex co-sediments with the 20 S proteasome (14), which was detected by analyzing the gradient fractions for chymotrypsin-like (LLVYase) activity, using the synthetic substrate, suc-LLVY-A MC (data not shown). The fractions were also analyzed by SDS-PAGE and Western blotting for Apaf-1 and GST-casp9\textsuperscript{1–130} (Fig. 4A). In agreement with our previous studies, most of the monomeric Apaf-1 in the nonactivated lysates was detected at the top of the gradient mainly in fractions 11–16 (Fig. 4A), whereas GST-casp9\textsuperscript{91–130} that is smaller than Apaf-1, sedimented in fractions 13–17. However, in dATP-activated lysates most of the Apaf-1 was oligomerized and sedimented in fractions 8–11, which also contained LLVYase activity (data not shown). Residual amounts of nonoligomerized Apaf-1 were detected at the top of the gradient (fractions 13–16), whereas the \(-1.4\)-MDa complexes were detected in the higher density fractions (1–7). Although small amounts of GST-casp9\textsuperscript{91–130} were detected in the fractions corresponding to the \(-700\)-kDa and \(-1.4\)-MDa apoptosome complexes, the majority of the GST-casp9\textsuperscript{91–130} was present as the uncomplexed form in fractions 13–16. In the nonactivated lysate, GST-casp9\textsuperscript{91–130} was only detected in fractions 13–16, confirming that the construct only binds to Apaf-1 when it is incorporated into the apoptosome complex.

Fractions 8–10 were selected so as to exclude contamination from free Apaf-1 and the \(-1.4\) MDa apoptosome. We then combined and concentrated these fractions both from the control and dATP-activated sucrose density gradients and then affinity-purified the tagged complexes with GSH-Sepharose beads. In the pooled control fractions only trace amounts of Apaf-1 and GST-casp9\textsuperscript{91–130} could be detected by SDS-PAGE/Western blotting (Fig. 4B, lane 1). Furthermore, Apaf-1 was also detected in the flow-through fraction (lane 2) but was not bound to the GSH-Sepharose beads. In contrast, in the dATP-activated pooled fractions, high concentrations of Apaf-1 and GST-casp9\textsuperscript{91–130} were detected (Fig. 4B, lane 5), a significant proportion of which was affinity-purified by the GSH-Sepharose beads (Fig. 4B, lane 8). We further analyzed the pooled fractions by SDS-PAGE and silver staining, which showed that the flow-through fractions from both control and dATP-activated pooled fractions contained numerous proteins (Fig. 4C, lanes 1 and 2). By contrast, the GSH-Sepharose beads contained very few proteins (Fig. 4C, lanes 3 and 4). Both the control and dATP-activated pooled samples contained two non-specific bands with molecular weights of around 25–30 kDa (Fig. 4C, lanes 3 and 4). Furthermore, these bands were most likely contaminants as they were also detected in blank lanes (not shown). Two prominent silver-stained bands were detected in the dATP-activated samples corresponding to the Apaf-1 and GST-casp9\textsuperscript{91–130} bands that were detected by Western blotting (Fig. 4B). Duplicate gels were stained with SYPRO Ruby, and the band corresponding to Apaf-1 was analyzed by MALDI-TOF (Fig. 5). The peptide analysis confirmed that the band detected was indeed Apaf-1 and interestingly one of the identified peptides corresponded to residues 101–111 of Apaf-1, which includes 9 amino acids from the 11-amino acid insert, which is only seen in Apaf-1XL and Apaf-1LN (13). Furthermore, peptide 803–818 also includes amino acids that are derived from the extra WD40 repeat, which is inserted at residue 811, and thus characterizing this protein as the Apaf-1XL splice isoform (\(M_r = 1,417,333\); HSSP accession number O14727-00-08).

We repeated this affinity purification another two times and in each case identified Apaf-1 and GST-casp9\textsuperscript{91–130} by SDS-PAGE/Western blotting. Protein staining of the gels with Sypro Ruby detected Apaf-1 and GST-casp9\textsuperscript{91–130} but did not detect any other proteins. However, we also ran SDS-PAGE gels, which we stained with a more sensitive silver stain, and in one of the experiments several other proteins were detected although these were present in substoichiometric (as compared with the Apaf-1) amounts and could not be identified by MALDI-TOF mass spectrometry. Thus, the GST-casp9\textsuperscript{91–130}/Apaf-1 apoptosome complex does not appear to bind significant amounts of any other proteins.

**Time-dependent Formation of the Apoptosome as Measured by Immunoprecipitation of Caspase-9**—The results with GST-casp9\textsuperscript{1–230}-tagged apoptosome complex indicated that if there were any other proteins binding to the apoptosome complex, then they could only do so via caspase-9 and associated interactions. It was therefore necessary to immunopurify the apoptosome complex with an intact caspase-9. Initially, we screened a panel of Apaf-1 and caspase-9 antibodies and finally selected an anti-caspase-9 antibody, which was raised to the CARD domain of caspase-9 and would only could capture Apaf-1 when it was bound to the apoptosome complex. Initial experiments confirmed that the antibody only captured Apaf-1 when the lysate was activated with dATP (data not shown). We have previously established using Superose 6 chromatography that formation of the \(-700\)-kDa apoptosome complex in THP-1 lysates is maximal 10–15 min after dATP activation (15) and that after 30 min it decreases, presumably because caspase-3 cleavage of Apaf-1 degrades the complex (32). As we wished to establish the optimal time for analyzing the complex by two-dimensional gels we carried out a time course experiment in which we took samples and captured caspase-9 and associated proteins with the anti-caspase-9 monoclonal antibody. The lysates (inputs), unbound proteins (supernatants), and captured proteins (beads) were then analyzed by Western blotting for Apaf-1, caspase-9, XIAP, and caspase-3.
FIG. 4. Only GST-casp9\(^{1-130}\) and Apaf-1 can be detected in the purified GST-casp9\(^{1-130}\), inhibited ~700-kDa apoptosome. THP-1 lysates (15 mg/ml) were activated with 2 mM dATP in the presence of 2 \(\mu\)M GST-casp9\(^{1-130}\) for 1 h at 37 °C. Lysates were layered onto 10–40% sucrose gradients and centrifuged at 25,000 rpm for 17 h. The fractionated gradients were analyzed for LLVYase activity (data not shown) and immunoblotted for Apaf-1, GST, and GST-casp9\(^{1-130}\) (A). Fractions corresponding to the ~700-kDa apoptosome were pooled, concentrated, and incubated with GSH-Sepharose for 4 h at 4 °C. After centrifugation, the flow-through was removed, and the beads were washed in cold PBS (4×). The beads were then resuspended in 2× SDS loading buffer, heated at 50 °C for 30 min, and centrifuged to pellet the beads. The eluate from the beads was then analyzed by SDS-PAGE/Western blotting for Apaf-1 and GST-casp9\(^{1-130}\) (B), and other proteins by silver staining (C). Duplicate samples were also run and stained with SYPRO Ruby and used for tryptic digestion and MALDI-TOF analysis.
of the apoptosome complex as determined by the appearance of Apaf-1 in the bead fraction, was very rapid and even at 0 min, significant amounts of Apaf-1 was bound to the beads (Fig. 6A, lane 15). It should be stressed that in the absence of dATP, Apaf-1 did not bind to caspase-9 and consequently was not captured by the beads. The binding of the Apaf-1 to the beads at 0 min probably reflects the fact that the apoptosome assembly is so rapid, that even cooling the lysate to 4 °C slows but does not completely stop apoptosome assembly. Binding of Apaf-1 to the beads was maximal at 5/10 min and remained relatively constant for up to 30 min (Fig. 6A, lanes 18–20), before decreasing significantly at 60 min (data not shown).

The cleavage of procaspase-9 occurs at the Asp315 (autocatalytic, apoptosome-dependent) and the Asp330 (caspase-3-dependent) sites to yield the p35 and p37 large subunits, respectively (28). A third autocatalytic site at Glu306 has also been identified, which produces a p34 large subunit (28). Our analysis of caspase-9 processing and binding to the beads showed that even at zero time in the presence of dATP, captured caspase-9 was partially processed to the p35 subunit (Fig. 6A, lane 15). At 5–10 min (lanes 18 and 19), caspase-9 was fully processed as only the p35 and p34 subunits were bound to the beads. From 10 to 30 min, there was little change in the levels of processed caspase-9 that bound to the beads, but thereafter there was a significant decrease in the amount of caspase-9 bound to the beads (data not shown). We did not detect the p37 form of caspase-9 in these experiments, which is to be expected of the apoptosome complex as determined by the appearance of Apaf-1 in the bead fraction, was very rapid and even at 0 min, significant amounts of Apaf-1 was bound to the beads (Fig. 6A, lane 15). It should be stressed that in the absence of dATP, Apaf-1 did not bind to caspase-9 and consequently was not captured by the beads. The binding of the Apaf-1 to the beads at 0 min probably reflects the fact that the apoptosome assembly is so rapid, that even cooling the lysate to 4 °C slows but does not completely stop apoptosome assembly. Binding of Apaf-1 to the beads was maximal at 5/10 min and remained relatively constant for up to 30 min (Fig. 6A, lanes 18–20), before decreasing significantly at 60 min (data not shown). The binding of Apaf-1 to the beads was accompanied by a corresponding decrease in the level of Apaf-1 in the supernatants (Fig. 6A, lanes 9–14).
as caspase-9 processing appears to be so rapid that by the time the caspase-3 is activated (see below) there is very little pro-caspase-9 left to be cleaved at the Asp330 site.

The supernatant fractions contained only residual traces of caspase-9 (Fig. 6A, lanes 9–14), demonstrating that under the conditions employed there was almost complete binding of caspase-9 to the beads. We then looked at the recruitment and processing of caspase-3, and at zero time we only detected the

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**Fig. 6. Time-dependent apoptosome formation as measured by immunoprecipitation of caspase-9.** Precleared THP-1 lysates were activated with dATP as described under “Experimental Procedures.” At the indicated times, the lysates were treated with Z-VAD.fmk (200 μM) and transferred onto ice. Samples of the lysates (Input) were taken for the SDS-PAGE/Western blotting, and the remaining lysates then incubated with the anti-caspase-9 monoclonal antibody for 2 h at 4°C. The lysates were then incubated overnight at 4°C with Protein G Dyna beads. The beads were removed from the supernatants and washed before eluting with SDS-sample buffer (bead). The input (lanes 3–8), supernatants (lanes 9–14), and bead samples (lanes 15–20) were then separated by SDS-PAGE and immunoblotted for Apaf-1, caspase-9, caspase-3, and XIAP as described under “Experimental Procedures.” A, a time course from 0 to 30 min is shown with a control and dATP (30 min)-activated lysate added as marker lanes (1 and 2) for the indicated proteins. The position of the heavy and light chains of the IgG1 and protein G are as indicated. B, a longer time course for XIAP detection is shown in which the film has been overexposed so as to resolve the XIAP degradation products (1–3). Band 2 corresponds to the ~29 kDa (BIR 3 ring containing) fragment, which is generated by caspase-3 cleavage at Asp242. C, the time course samples shown in B were also probed with an antibody raised to Smac as described previously (22).
proform of caspase-3 in the input and supernatant fractions (Fig. 6A, lanes 3 and 9). At the later time points, in both the inputs and supernatants (2 min and onward), we detected increasing amounts of the p20 subunit, followed by the later appearance of the p19/p17 forms (Fig. 6A, lanes 5–8 and 11–14). Complete processing of caspase-3 to the p19/p17 forms was a slower process than observed for caspase-9 as even after 30 min, small amounts of the p20 form could still be detected (Fig. 6A, lanes 8 and 14). Surprisingly, we did not detect any caspase-3 binding to the beads, although previously, we have shown that the ~700-kDa apoptosome complex isolated by S300 Sephacryl chromatography in low salt buffers contains caspase-3 (14). In this context, however, in the presence of 50 mM NaCl, caspase-3 is stripped off the apoptosome complex during Superose 6 gel filtration chromatography (15). Therefore, we conclude that the binding of caspase-3 to the apoptosome complex is a weak, transient phenomenon, which reflects the fact that caspase-3 is essentially a substrate for the Apaf-1/caspase-9 holoenzyme complex.

XIAP is believed to interact with the ATPF motif of the p12 subunit of caspase-9, which is generated when the procaspase is cleaved at Asp\(^{135}\) (14, 28). Previous studies have also suggested that in dATP-activated lysates XIAP associates with oligomerized Apaf-1 and/or processed caspase-9 (27). However, in this time-course study we were unable to detect any XIAP (~57 kDa) binding to the beads, which instead remained in the supernatants (Fig. 6A, lanes 9–14). The IgG\(_1\) heavy chain (M\(_r\) ~50) and light chain (~25) of anti-caspase-9 monoclonal antibody were clearly detected on the beads (Fig. 6A, lanes 15–20). In a separate time course experiment we achieved essentially similar results (Fig. 6B, lanes 14–19) although a very faint band was detected migrating at a slightly higher position than the IgG\(_1\) heavy chain. In this Western blot we deliberately used longer exposures to try and detect XIAP, which was blotted against an XIAP antibody raised to the C terminus (268–426) of the protein. This antibody will recognize the full-length protein or truncated proteins that contain the BIR3 domain. XIAP is cleaved by caspase-3 at Asp\(^{242}\) generating ~27 and ~29 kDa (BIR 3-containing) fragments (33) which were detected in the inputs and supernatants at 10–60 min post dATP activation (Fig. 6B, lanes 4–8). The generation of this fragment appears to occur at or around the time that caspase-3 is extensively processed to the p19/p17 subunits (Fig. 6A, lanes 7 and 13). Interestingly, we also detected two other immuno reactive/cleavage products of XIAP that were produced in a time-dependent manner and were detected as early as 5 min after dATP activation (Fig. 6B, lanes 3 and 9). The absence of XIAP in the immunoaffinity-purified apoptosome complex was surprising as there is a considerable amount of evidence to show that XIAP will bind to the ATPF motif of the p35 subunit. One possible explanation is the presence of Smac, which is released into the cytosol during apoptotic cell death (28, 34, 35). Smac binds to XIAP and relieves the inhibition of caspase-9, and in naïve 293 cell lysates, exogenous GST-XIAP immunoprecipitates Smac (24). We therefore probed the time course samples, for the presence of Smac, which was detected in the lysate (Fig. 6B, lane 1), inputs (Fig. 6B, lanes 2–7), and supernatant fractions (Fig. 6C, lanes 8–13). However, Smac was not detected in the bead eluates demonstrating that Smac was not binding to the apoptosome complex. The presence of Smac in the lysates strongly suggested that XIAP was sequestered by Smac. Therefore, we used Smac (22) and XIAP antibodies to immunoprecipitate Smac and XIAP, respectively, from THP-1 lysates and found that XIAP coprecipitated with Smac (data not shown). This result confirmed that the lack of XIAP in the complexes precipitated by the anti-caspase-9 antibody was due to the presence of endogenous Smac in the lysates.

**Large Scale Immunoaffinity Purification of the ~700-kDa Apoptosome Complex**—In order to identify any other binding proteins which were recruited to the apoptosome complex, we devised a large scale immunoaffinity purification scheme to purify the apoptosome complex from 75 mg of THP.1 lysate (Fig. 7A). This involved a 5-min activation with dATP and cytochrome c, after which the reaction mixture was cooled to 4 °C in the presence of Z-VAD.fmk (200 μM), which was added to inhibit caspase activity. The lysate was then subjected to sucrose density gradient centrifugation in order to isolate the ~700-kDa apoptosome complex, which on the basis of its Apaf-1 content sedimented in fractions 8–10 (Fig. 7B). The same fractions contained the processed p35/34 subunits of caspase-9 as identified by Western blotting. Duplicate gradients contained nonactivated THP.1 lysate, and in these gradients Apaf-1 and the proform of caspase-9 sedimented predominantly in fractions 12–16 and 15–18, respectively. The indicated fractions containing the ~700-kDa apoptosome complex and equivalent fractions from the control gradients were concentrated and incubated with the anti-caspase-9 monoclonal antibody. However, in preliminary experiments we failed to capture the apoptosome complex on the magnetic beads, and subsequent experiments showed that the sucrose gradient solutions had to be supplemented with 25 mM NaCl to obtain efficient binding to the protein G Dyna beads. The affinity-purified complexes were analyzed by SDS-PAGE and Western blotted for Apaf-1, caspase-9, XIAP, and caspase-3. The apoptosome complexes were found to contain Apaf-1 and the p35/34 subunits of caspase-9 (Fig. 7C). We immunoblotted for XIAP using conventional antibody techniques, which again showed that XIAP was only found in the supernatant (Fig. 7C). Two bands corresponding to the heavy and light chains of the IgG\(_1\) anti-caspase-9 monoclonal antibody were also bound to the beads. As the heavy chain and XIAP are not easily separated on SDS-PAGE, we used a triple sandwich detection technique to probe for XIAP. This secondary antibody did not cross-react with the heavy or light chain of the anti-caspase-9 monoclonal antibody and only detected XIAP in the lysates or supernatant fractions (Fig. 7C). XIAP was not detected in the bead sample, and we conclude that in the presence of endogenous Smac it is not bound to the ~700-kDa apoptosome complex (Fig. 7C). We next probed for caspase-3, which on the sucrose gradient eluted at the top of the gradient (results not shown), and consequently was not detected in the immunofinity-purified apoptosome complex.

Although, the immunoblotting data showed that we had affinity-purified Apaf-1 and processed caspase-9, it was important to demonstrate that both of these proteins were bound to the beads as components of a functional apoptosome. We confirmed this by carrying out a parallel purification run as described in Fig. 7A, except that we omitted the ZVAD.FMK treatment. The apoptosome bead complexes were then incubated with recombinant procaspase-3 for 1 h at 37 °C, before magnetically separating the beads and the captured apoptosome complexes from the supernatants. The latter were assayed for DEVDase activity and caspase-3 processing. Significantly, a high DEVDase activity coupled with extensive processing of procaspase-3 to the p19 and p17 subunits was only detected in the supernatant, which was obtained from incubating the apoptosome-bead complexes with procaspase-3 (Fig. 7D, lane 2). In the absence of recombinant procaspase-3 there was little or no DEVDase activity and caspase-3 was not by detected by SDS-PAGE and immunoblotting (Fig. 7D, lane 1). Procaspase-3 in the absence of the apoptosome bead complex had a very low DEVDase activity, and only one band with
FIG. 7. Large scale purification of the ~700-kDa apoptosome complex. A, scheme summarizes the strategy used for preparing the purified apoptosome complex. Precleared THP.1 lysate (75 mg) was activated at a concentration of 15 mg/ml with dATP and cytochrome c for 5 min at 37 °C as described under “Experimental Procedures.” The reaction was terminated by cooling to 4 °C and adding 200 μM Z-VAD.fmk, before layering the lysate onto two 10–40% sucrose gradients, which were centrifuged at 25,000 rpm for 17 h. Equivalent amounts of control nonactivated precleared lysates were also separated in the same manner. The fractionated gradients were analyzed for LLVYase activity (data not shown) and immunoblotted for Apaf-1, and caspase-9. A representative gradient for each treatment is shown which illustrates the distribution of Apaf-1 and caspase-9 (B). Fractions 8–10 of each gradient were pooled and concentrated before immunopurifying with anti-caspase-9 antibody and protein G Dyna beads. The concentrated ~700-kDa complexes before and after immunoaffinity purification were analyzed by SDS-PAGE/Western blotting for Apaf-1, caspase-9, caspase-3, and XIAP (C). Lanes 1 and 2 show the inputs from control and dATP-activated lysates. The concentrated ~700-kDa complexes isolated from the control and dATP-activated lysates are shown in lanes 3 and 4, respectively. The results of the immunopurification (i.e. supernatants and beads) for the control and dATP-activated complexes are shown in 5 and 6, and 8 and 9, respectively.
a molecular weight of \(-33\) kDa (procaspase-3) was detected by SDS-PAGE and immunoblotting (Fig. 1D, lane 3).

Thus, the captured apoptosome complexes were functionally competent and in order to identify other potential apoptosome-binding proteins, we ran eluates obtained from apoptosome bead complexes on two-dimensional gels and stained the proteins with SYPRO Ruby (Fig. 5A), which were then analyzed by MALDI-TOF mass spectrometry. In the dATP-activated \(-700\) kDa apoptosome complex, we detected various glycosylated forms of the heavy chain of the antibody (Fig. 8A), but these were also detected in the control non-dATP-activated complex (results not shown). At the top of the two-dimensional gels a number of spots were identified and found to contain peptide masses from IgG, and probably represent poorly solubilized antibody protein. An additional spot (Apaf-1?) was detected with a molecular weight and pI similar to Apaf-1, but we failed to get an identity by mass spectrometry. We have found that during two-dimensional gel electrophoresis, Apaf-1, like many other high molecular weight proteins (>125 kDa) is only poorly transferred from the first (IEF) to the second dimension (SDS-PAGE). However, in a separate experiment we ran the purified apoptosome complex on SDS-PAGE and identified a \(-130\) kDa protein, which we confirmed as Apaf-1 by MALDI-TOF and LC-MS/MS. In the two-dimensional gel of the purified apoptosome two other major spots were detected that were not present in the control sample. These spots on the basis of their molecular weight, pI difference and MALDI-TOF spectra were unequivocally identified as the p35 and p34 processed subunits of caspase-9 (Fig. 8, A and B).

In the Absence of Mitochondrial Pro-apoptotic Proteins, XIAP and Caspase-3 Are Rapidly Recruited to the Apoptosome—The apoptosome complex in the apoptotic cell is assembled in the presence of pro-apoptotic proteins released from the mitochondrion, such as Smac and Omi/HtrA2 (36). Thus, the analysis we have carried out in effect characterizes the proteome of the apoptosome complex in an apoptotic cell. However, in the absence of Smac and Omi/HtrA2, XIAP should bind to and modulate the activity of the apoptosome complex. Previous experiments in our laboratory have shown that GST-XIAP constructs can be added to dATP-activated lysates and bind to the apoptosome via interactions with caspase-9 and caspase-3 (27). However, in our current experiments, the lysates were prepared by F/T, and contain relatively high levels of Smac and Omi/HtrA2 (Fig. 9A), which have the potential to sequester the endogenous XIAP. We therefore devised an alternative method for breaking open THP-1 cells without significantly damaging the mitochondria (see “Experimental Procedures”). This method, in contrast to the F/T method produced a lysate (Dig/Hom), which was essentially free of cytochrome c and contained only very low or negligible amounts of Smac and Omi/HtrA2 (Fig. 9A). Significantly, in comparison to the F/T lysates the Dig/Hom lysates exhibited a much diminished response to dATP and did not activate with dATP unless exogenous cytochrome c (7 \(\mu\)M) was added (data not shown). Furthermore, in the presence of cytochrome c the level and kinetics of the dATP-dependent increase in DEVDase activity were much lower and significantly slower than that observed in F/T lysates (Fig. 9B). This data indicated that caspase-3 activation/activity was inhibited probably by the presence of XIAP and this suggestion was supported by the observation that this effect was antagonized by excess Smac. Thus, there was a marked increase in the dATP-dependent DEVDase activity of the Dig/Hom lysates, when preincubated with recombinant Smac (1 \(\mu\)M, Fig. 9C). Significantly, the same concentration of Smac had no effect on dATP activation in the F/T lysate (Fig. 9C).

We then immunoprecipitated the apoptosome complexes using the anti-caspase-9 antibody covalently cross-linked to magnetic beads. Using this method we avoided any possible confusion in detecting XIAP, as we did not get any contamination from the heavy chain of the caspase-9 antibody (data not shown). In the Dig/Hom lysates, Apaf-1 rapidly oligomerized to form the apoptosome, which was captured on the magnetic beads (lanes 15–20, Fig. 9D). The kinetics of Apaf-1 oligomerization in the Dig/Hom lysates were very similar to those observed in the F/T lysates (compare Fig. 9D, lanes 15–20 with Fig. 6A), demonstrating that the lower DEVDase activity in these lysates was not due to impaired apoptosome formation. However, in the Dig/Hom lysates, although caspase-9 was rapidly recruited to the apoptosome, its processing to the active p35/p34 forms was much slower, such that at 30 min, significant amounts of procaspase-9 were detected in the apoptosome (Fig. 9D, lane 20). Furthermore, the amount and extent of processing of caspase-3 was retarded in the Dig/Hom lysates with only limited processing of the proform to the cleaved p20 form (Fig. 9D, lanes 3–8), thus explaining the reduced DEVDase activity (Fig. 9B). Also, in contrast to the F/T lysates, we were able to detect small amounts of procaspase-3 and its cleaved p20 subunit in the apoptosome complexes (compare, Fig. 9D, lanes 17–20 with Fig. 6A). Furthermore, unlike the F/T lysates we observed rapid recruitment of XIAP to the apoptosome complex when the Dig/Hom lysates were dATP-activated. These results show that in the absence of Smac and Omi/HtrA2, XIAP will bind to the apoptosome and inhibit its caspase-9 processing activity, in turn abrogating caspase-3-dependent processing of caspase-9 (to the p37 subunit) and caspase-3 autocatalytic processing to its p19 and p17 subunits.

We also detected small amounts of cytochrome c in the apoptosome complexes obtained from the Dig/Hom lysates (Fig. 9D, lanes 15–17). However, cytochrome c was only detected transiently at these early time points, and by 5 min it was virtually undetectable by Western blotting (Fig. 9D, lane 18). This may explain why we were not able to detect cytochrome c in large scale purification experiments, because the apoptosome complexes were prepared with a 5-min dATP activation protocol.

DISCUSSION

The assembly of the apoptosome complex represents the initiating step for the activation of the caspase cascade. Reconstitution studies with recombinant Apaf-1, caspase-9, and cytochrome c show that the resultant apoptosome complex is fully functional in terms of its ability to cleave and activate procaspase-3 (16, 17, 23). The composition of the apoptosome assembled in the presence of other cellular components has not been fully characterized, and in this study we have used two alternative methods to characterize the constituent components of the native apoptosome. First, we have used a dominant negative inhibitor of the apoptosome complex, namely GST-

Lane 7 is the anti-caspase 9 antibody that was run as a control for XIAP immunoblotting, which was detected conventionally, and also with the triple sandwich (tertiary) detection method as described under “Experimental Procedures.” A nonspecific immunoreactive band (N.S.) was detected in the caspase blots. D, in a parallel experiment the apoptosome complex was purified in the absence of Z-VAD.FMK and the caspase-activating activity of the immunoprecipitated apoptosome complex assessed by incubating with recombinant procaspase-3 (200 nM) for 1 h at 37 °C as described under “Experimental Procedures.” The beads plus apoptosome complexes were then removed, and caspase-3 processing and DEVDase activity in the supernatant were analyzed by Western blotting and fluorimetry (lanes 1–3). Note that the proform of the recombinant caspase-3 (lanes 2 and 3) contain a C-terminal His\(_6\) tag and is therefore slightly larger than the endogenous procaspase-3 (lane 4) detected in the control lysate.
casp91–130, which only binds to Apaf-1 when it is oligomerized to the 700-kDa and a 1.4-MDa apoptosome complexes. This construct blocks caspase activation by binding to the exposed CARD domain of Apaf-1 when activated by dATP. We have prepared large quantities of the 700-kDa apoptosome complex, using a sucrose density gradient, followed by affinity purification on GSH-Sepharose beads. The purified apoptosome complex was analyzed by SDS-PAGE/Western blotting and MALDI-TOF mass spectrometry and found to contain only Apaf-1 and the GST-casp91–130 proteins. We therefore conclude that the WD40 domains and other regions of the Apaf-1 apoptosome complex do not bind any other cytosolic proteins.

Second, we purified the 700-kDa apoptosome complex with intact full-length caspase-9, using 75 mg of lysate and an initial sucrose density purification. The conditions we used in this experiment were designed to ensure maximum assembly of the complex and minimum degradation due to caspase-3 cleavage of Apaf-1. We therefore carried out a detailed time course of apoptosome assembly in F/T lysates and confirmed that oligomerization of Apaf-1 is a very rapid process being virtually complete within 5 min of initiating apoptosome assembly with

![Image](http://www.jbc.org/)

**Fig. 8. Identification of the p35 and p34 forms of caspase-9 in the ~700-kDa apoptosome complex.** Apoptosome complexes captured on protein G Dyna beads (as described in the legend to Fig. 7) were eluted with IEF rehydration buffer and 50 μl applied to 17-cm IPG strips (pH 5–8) and isoelectrofocused as described under “Experimental Procedures.” The strips were then run on 7.5% SDS-PAGE gels and stained with SYPRO Ruby. After imaging (A), the indicated spots were cut and analyzed by tryptic digestion and MALDI-TOF mass spectrometry. The p35 (B) and p34 (C) spots were identified by MALDI-TOF to be caspase-9. The migration in the SDS gel and the predicted pl values are compatible with them being the p35 and p34 subunits derived from cleavage of caspase-9 at Asp315 and Glu306 respectively. The two-dimensional gels after spot cutting were restained with a more sensitive silver-staining method, but no other additional proteins were detected (data not shown).
In THP.1 lysates with very low levels of Smac and Omi/HtrA2, the apoptosome rapidly recruits XIAP and caspase-3. THP.1 lysates (Dig/Hom) were prepared in isotonic buffer using a combination of digitonin permeabilization and homogenization as detailed under "Experimental Procedures." Lysates were dATP activated as described in Fig. 6 and at the indicated times, aliquots of lysates were analyzed for DEVDase activity while the remaining sample was treated with Z-VAD.fmk (200 \( \mu \)M), and aliquots taken (Input) for SDS-PAGE/Western blotting. Apoptosome complexes were then isolated from the remaining lysate by incubating overnight at 4 °C with anti-caspase-9 antibody cross-linked to protein G Dyna Beads®. The beads were removed from the supernatants and washed before eluting with SDS-sample buffer (bead). The input, supernatants, and bead samples were separated by SDS-PAGE and immunoblotted for Apaf-1, caspase-9, caspase-3, XIAP, and cytochrome c as described under "Experimental Procedures." A, SDS-PAGE/Western blotting was used to compare the cytochrome c, Smac, and Omi/HtrA2 content of naïve Dig/Hom lysates with the normal lysate (F/T) as prepared by the freeze/thawing method. The time course of dATP activation of the DEVDase activity (before the addition of 200 \( \mu \)M Z-VAD.FMK) in the Dig/Hom lysate is shown in B and compared with an equivalent F/T lysate. C, Dig/Hom and F/T lysates were preincubated at 4 °C with 1 \( \mu \)M recombinant SmacΔ55 before measuring the DEVDase activity after dATP activation (15 min). Note that the DEVDase activity of the Dig/Hom lysate (in contrast to the F/T lysate) could not be activated by dATP without the addition of exogenous cytochrome c (7 \( \mu \)M). D, the apoptosome complexes isolated from the time course experiment with the Dig/Hom lysate were analyzed by SDS-PAGE/Western blotting. A control and dATP (30 min)-activated lysate were added as reference lanes (1 and 2) for the indicated proteins. With the anti-caspase-9 antibody covalently linked to the beads only the light chain of the IgG 1 is carried over into the SDS eluate. For Apaf-1 and caspase-9, 5% of the total inputs (lanes 3–8), supernatants (lanes 9–14), and bead samples (lanes 15–20) were loaded onto the gel. In the case of caspase-3, XIAP and cytochrome c to aid visualization, the total bead sample was increased to 20% of the input. The caspase-3 antibody also detects a nonspecific band at 25 kDa and is indicated (*).
dATP. Furthermore, the processing of caspase-9 as detected by Western blotting is extremely rapid and is initially cleaved to the p35 form. Interestingly, the p34 subunit is also produced but at a rate slower rate than the p35 form, which seems to be degraded to the smaller subunit. The p34 subunit was initially reported to be generated by cleavage at Ser207 and shown to require a catalytically competent active site in caspase-9 (37). However, mutational analysis in recombinant proteins has suggested that the p34 is generated by autocatalytic cleavage at Glu306 (28). Surprisingly, the autocatalytic cleavage of caspase-9 to the p34 subunit is much more sensitive to Z-VAD-FMK inhibition than the corresponding process for p35 generation.  

The time course data also showed that caspase-3 processing is a slower process than caspase-9 processing, and only occurs after the Apaf-1/caspase-9 holoenzyme complex is formed. However, we were unable to detect caspase-3 in the immunoprecipitated apoptosome complex isolated from F/T lysates, which is in contrast to our earlier studies, using Sephacyr S300 gel chromatography (15). It is clear from these experiments that caspase-3 is only a transitory component of the apoptosome complex and that in the normal ionic milieu of the cell caspase-3 is recruited, processed, and rapidly released from the complex.

The time course experiments with F/T lysates also showed that XIAP was not associated with the complex even at early time points. Furthermore, XIAP could not be detected in the apoptosome complex, purified by sucrose density gradient centrifugation, and immunoaffinity purification. The lack of XIAP in the apoptosome complex was unexpected as the BIR3 domain of XIAP is known to bind to the p12 subunit of caspase-9 via interactions with the N-terminal ATPF motif. The absence of XIAP in the complex could be attributed to a number of reasons: First, THP.1 lysates contained detectable levels of endogenous Smac and Omi/HtrA2 (Figs. 6C and 9D, lanes 1 and 2), which can complex with XIAP. The concentration of Smac in the cell lysates and the affinity of XIAP for this protein are probably sufficient to sequester any free XIAP, thus preventing binding of XIAP to the newly generated ATPF motif. It should be noted that XIAP has been shown to bind several different proteins, including TAB 1, a protein associated with the transforming growth factor-β1 signaling pathway (38), and a process of GSPT1/eRF3, a protein involved in G2 to S phase transition (39). Thus, there are potentially several other proteins in addition to Smac that can sequester free XIAP. Second, the p12 subunit should be generated when the p35 subunit is produced, but caspase-3 cleavage at Asp330 can potentially remove the linker peptide containing the ATPF motif and any bound XIAP (24, 27). However, we were unable to detect the p12 or p10 subunits so we cannot exclude the possibility that caspase-3 had cleaved off the linker region of caspase-9 together with XIAP. The significance of this process on apoptosome formation is difficult to judge as recent studies with recombinant proteins have shown that the p10 subunit of caspase-9, which has an AISS motif can also bind XIAP, resulting in the inhibition of caspase-processing (40). Moreover, even at the very earliest time points we did not detect the p37 subunit of caspase-9, which is also generated by caspase-3 cleavage. Although, previous studies have indicated that XIAP is associated with oligomerized Apaf-1 and/or processed caspase-9 (27) it should be stressed that these experiments were done with apoptosome complexes isolated by Sephacyr S300 gel filtration using salt-free buffers. It may be that under these assay conditions it is possible to detect weak and transitory interactions between XIAP and caspase-9 in the apoptosome.

However, the most likely explanation for the absence of XIAP in the apoptosome complex was the presence of Smac and/or Omi/HtrA2, which could remove XIAP from the complex and we confirmed this by establishing an alternative method of disrupting THP.1 cells, which produced cytosols with very low or negligible levels of cytochrome c, Smac, and Omi/HtrA2 (Fig. 9A). Caspase activation by dATP in these lysates had an obligatory requirement for exogenous cytochrome c and showed only limited DEVDase activity unless preincubated with Smac (Fig. 9, B and C). In the Dig/Hom lysates the time course of the Apaf-1 oligomerization and recruitment of procaspase-9 to the apoptosome were essentially the same as in the F/T lysates (Fig. 9D, lanes 15–20). However, the rate of caspase-9 processing was noticeably slower in the apoptosome complexes prepared from the Smac and Omi/HtrA2-free cytosols and furthermore, the amount and extent of procaspase-3 processing was markedly inhibited, with the p20 form predominating (Fig. 9D, lanes 3–8). Caspase-3 is initially cleaved to the p20 form by active caspase-9 and then subsequently by active caspase-3 to the p19 and p17 forms. Thus, the cleavage profile indicates that it is the latter autocatalytic step, which is particularly sensitive, and interestingly it has been reported that caspase-3 is more sensitive to XIAP inhibition than caspase-9 (36). In any event caspase-3 and caspase-9 are potently inhibited by XIAP, which in the Dig/Hom lysates was rapidly recruited to the apoptosome complex (Fig. 9D, lanes 15–20). Thus, the presence of XIAP in the complex is consistent with the cleavage profiles observed for caspase-9 and caspase-3. Furthermore, we also detected procaspase-3 and the p20 subunit in the apoptosome complex (Fig. 9D, lanes 15–20) a result which supports previous studies showing that XIAP can simultaneously bind active caspase-3 and -9, thereby preserving the association of caspase-3 within the apoptosome complex (24).

While, the studies with the Smac and Omi/HtrA2 free cytosol showed that XIAP and caspase-3 could be retained in the apoptosome complex it is clear that this is not the most likely situation that occurs in the cytosol of an apoptotic cell. Real-time single cell analysis of cells, stably expressing Smac-yellow fluorescent protein (Smac-YFP) and cytochrome c green fluorescent protein show the simultaneous release of Smac and cytochrome c during apoptosis (41). This would indicate that Smac, and probably Omi/HtrA2 would be present in the cytosol when the apoptosome is formed. Thus, the apoptosome produced in the freeze/thawed lysates is essentially being assembled in the presence of pro-apoptotic proteins, which would also be present in the cytosol of the apoptotic cell. Under these conditions the SDS-PAGE two-dimensional gel electrophoresis and proteomic analysis showed that in addition to Apaf-1 there were only two other protein spots present in the apoptosome complex and both of these were identified as caspase-9 by MALDI-TOF. The molecular weight and pI positions of the proteins are consistent with these being the p35 and p34 large subunits of caspase-9. It was possible that that these two forms represent differentially phosphorylated forms of the same cleaved (p35) subunit, and a recent study has shown that caspase-9 processing is inhibited by phosphorylation of Thr125 by ERK MAPK (42). However, the two caspase-9 subunits were separated by ~1 kDa on both SDS and two-dimensional gels. This mass difference would require phosphorylation of most of the serines and threonines of the p35 subunit, which would result in a very large acidic shift in the pI value. This is clearly not the case and we conclude that during dATP activation of THP.1 lysates, caspase-9 is cleaved to give both the p35 and p34 subunits, which are tightly bound to the apoptosome.

K. Cain and C. Langlais, unpublished results.
The two-dimensional gel analysis of the F/T lysates did not identify significant amounts of any other proteins that co-purified with the apoprtosome. However the apoprtosome complex was clearly functional because it cleaved and activated recombinant caspase-3 (Fig. 7D). The lack of other proteins associated with the apoprtosome complex was an unexpected finding, considering the size and possible structure of the apoprtosome complex. Several studies have suggested that other proteins such as Aven, NAC, Hsp70, and Hsp-90 (43–47) are associated with the apoprtosome, but we failed to detect any of these proteins in the immunoaffinity-purified apoprtosome complex. Recent studies have also identified the tumor suppressor PHAP and the oncoprotein prothymosin α (ProT) as modulators of apoprtosome formation and function (18). ProT apparently inhibits apoprtosome formation and is antagonized by high levels of dATP whereas PHAP promotes apoprtosome formation and caspase-9 activation. Interestingly, there is no evidence to suggest a direct association of ProT and PHAP with the apoprtosome and significantly the effect of these proteins could not be demonstrated in reconstitution experiments with purified proteins, suggesting the involvement of additional as yet unknown or unidentified regulatory proteins. It is also possible that initial protein-protein interactions are important to trigger conformations changes leading to apoprtosome formation, but are required once Apaf-1 has unfolded and oligomerized. But once the complex is formed these interacting proteins may bind less tightly and be lost during purification. It is significant that cytochrome c which is essential for formation appears to be only associated with the complex for a brief period of time (Fig. 9D, lanes 15–17). We have also failed to identify cytochrome c in apoprtosomes purified by Superose-6 gel filtration.4 Interestingly, other studies have also reported that the apoprtosome holoenzyme complex purified by sucrose density gradient purification does not contain cytochrome c (25). It is possible that cytochrome c induces conformational changes in Apaf-1, which then oligomerizes and in doing so destabilizes and weakens the Apaf-1-cytochrome c binding interactions. The task of identifying these transient interactions is extremely difficult and we have treated dATP-activated lysates with various cross-linking reagents5 to try and trap other proteins in the apoprtosome complex. However, these experiments produced a large array of cross-linked filamentous proteins and other large complexes, which proved impossible to analyze and separate.

While it is possible that various proteins are interacting transiently with the apoprtosome complex it should be realized that in vitro, the formation of the ~700-kDa apoprtosome complex is easily disrupted by the ionic strength of the incubating media (23). This effect can be overcome by increasing the cytochrome c concentration and appears to be due to disruption by K+ of the strong electrostatic interactions between cytochrome c and Apaf-1 (49). The formation of the ~1.4-MDa complex is not affected by the ionic strength of the media. In this respect we have recently found that commercial preparations of purified Hsp70 will abrogate formation of the ~700-kDa apoprtosome but not the ~1.4-MDa complex.6 However, commercial preparations of Hsp70 and other heat shock proteins invariably contain 100–150 mM NaCl and under our assay conditions it is difficult to ascertain the precise contribution of the ionic environment to the observed inhibitory effects of Hsp 70 and other heat shock proteins. These considerations highlight that in vitro formation and protein-protein interactions of the ~700-kDa complex apoprtosome are markedly affected by ionic strength and purported interactions between supraphysiologically concentrated proteins and the apoprtosome complex may not necessarily occur in vivo.

So far no one has succeeded in producing crystals of the apoprtosome and the only structural evidence available comes from a recent low resolution (27 Å) electron microscopy study using recombinant proteins (48). The apoprtosome complex appears to be a wheel-like structure containing 7 Apaf-1 molecules arranged radially with the CARD and part of the CED-4 domains packing together to form a central hub. The WD40 domains appear in a Y-shaped configuration of two lobes of unequal size (7- and 6-bladed propellers), which may in the nonactivated monomeric Apaf-1 bind and occlude the hub domain. It is envisaged that cytochrome c, displaces the hub domain allowing binding and conformational changes, which allow oligomerization to form the apoprtosome complex. Previous studies with recombinant Apaf-1 and caspase-9 had indicated a 1:1 stoichiometry (17) but in the electron microscopy (EM)-derived structures there does not seem to be enough room on the hub to accommodate 7 caspase-9 molecules. Furthermore when the apoprtosome is formed in the presence of a non-cleavable mutant of caspase-9 it can apparently dimerize via the hub structures to form an even larger apoprtosome structure composed of two wheels bound though their respective hubs (48). The EM structural model of the recombinant apoprtosome offers intriguing insights into its formation and function, but there is as yet no evidence as to the actual structure of the native apoprtosome when it is assembled in cell lysates or cells undergoing apoptotic cell death. In this respect, our data clearly show that in cell lysates in the presence of Smac and Omi/HtrA2, apoprtosome assembly only involves Apaf-1 and caspase-9, which constitute the core components of the apoprtosome. However, the possibility remains that in the intact cell, other proteins released from cellular organelles such as the mitochondrion could transiently bind to the apoprtosome and modulate its function.

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Pro-apoptotic Proteins Released from the Mitochondria Regulate the Protein Composition and Caspase-processing Activity of the Native Apaf-1/Caspase-9 Apoptosome Complex

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