Caspase Activation Involves the Formation of the Aposome, a Large (~700 kDa) Caspase-Activating Complex*

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In mammals, apoptotic protease-activating factor 1 (Apaf-1), cytochrome c, and dATP activate caspase-9, which initiates the postmitochondrial-mediated caspase cascade by proteolytic cleavage/activation of effector caspases to form active ~60-kDa heterotetramers. We now demonstrate that activation of caspases either in apoptotic cells or following dATP activation of cell lysates results in the formation of two large but different sized protein complexes, the “aposome” and the “microaposome”. Surprisingly, most of the DEVDase activity in the lysate was present in the apopson and microaposome complexes with only small amounts of active caspase-3 present as its free ~60-kDa heterotetramer. The larger apomosome complex (M₅ = ~700,000) contained Apaf-1 and processed caspase-9, -3, and -7. The smaller microaposome complex (M₅ = ~200,000–300,000) contained active caspase-3 and -7 but little if any Apaf-1 or active caspase-9. Lysates isolated from control THP-1 cells, prior to caspase activation, showed striking differences in the distribution of key apoptotic proteins. Apaf-1 and procaspase-7 may be functionally complexed as they eluted as an ~200–300-kDa complex, which did not have caspase cleavage (DEVDase) activity. Pro-caspase-3 and -9 were present as separate and smaller 60–90-kDa (dimer) complexes. During caspase activation, Apaf-1, caspase-9, and the effector caspases redistributed and formed the aposome. This resulted in the processing of the effector caspases, which were then released, possibly bound to other proteins, to form the microaposome complex.

It is now widely recognized that the execution phase of apoptosis requires the activation of aspartate-specific cysteine proteases, caspases, which are related to interleukin-1β converting enzyme, or caspase-1 (1–5). So far, 11 caspases have been identified in human cells (5, 6), which all have a strict substrate requirement for cleavage of Asp-X bonds at the P₁ position. The substrate specificity of the individual caspase is then further defined by the three amino acids in the P₃, P₄, and P₂ positions of the tetrapeptide cleavage motif (5). A positional scanning combinatorial substrate approach (7) has been used to characterize the caspases into three functional subgroups. Group I caspases (caspase-1, -4, and -5) cleave at (W/L)EHD tetrapeptide motifs and are probably involved in inflammation. The effector caspases (II), which include caspase-2, -3, and -7 (and also CED-3), cleave the DEDX tetrapeptide motif, which is found in many proteins associated with apoptosis, e.g. poly-(ADP-ribose) polymerase, DNA-dependent protein kinase, 70-kDa U1 small ribonucleoprotein, and sterol regulatory element-binding proteins (5). Group III (activator) caspases include caspase-6, -8, and -9 as well as granzyme B and cleave at (I/V/L)EXD tetrapeptide sequences.

Caspases are synthesized as inactive proenzymes and activated, following cleavage at specific tetrapeptide residues (e.g. for caspase-3 this is IETD), to yield large (~20 kDa) and small (~10 kDa) subunits. The active enzyme is believed to be a heterotetramer composed of two large and two small subunits with a M₅ of ~60,000 (8). Although all of the caspases conform to this general scheme, there are considerable and significant differences in caspase structure and function (5). Thus, all have prodomains of varying length; some have linker regions, which need to be cleaved to give the small and large fragments, whereas others require only a single cleavage between the p20/p10 junction. The prodomains of the caspases may have a functional role, as “activator” caspases have long prodomains and are believed to activate the “effector” caspases, which have short prodomains (7). The cleavage/activation sites contain the P₁ Asp-X bond, and removal of the prodomains and generation of the large and small subunits are most likely carried out by caspases.

The proteolytic activity of both activator and effector caspases can be assayed with synthetic fluorogenic substrates, which contain the appropriate cleavage motif (i.e. fluorogenic I/V/L/XXD and DEXD analogues). As activation of the effector caspases represents a point of no return in the apoptotic process, it is important to understand the mechanisms underlying this process and how this influences and regulates the caspase cascade. Recent studies by Zou et al. (9) have provided new insights into these mechanisms and have indicated that for caspase-3 activation there is a requirement for cytochrome c, dATP, and two proteins designated apoptotic protease-activating factors 1 and 3 (i.e. Apaf-1 and Apaf-3). Apaf-3 has been identified as caspase-9 (10), and Apaf-1 is the first identified mammalian homologue of CED-4 (9). Apaf-1 is a large (M₅ = 130,000) soluble protein with a short N-terminal caspase recruitment domain, a central CED-4 homology domain followed by 12 WD-40 repeats, which are probably involved in protein-protein interactions (9). This and other studies provide evidence for a mechanism of caspase activation in which cytochrome c, the mammalian homologues of CED-3, and CED-4 interact as a caspase-activating complex (9–11). However, the mechanisms by which Apaf-1 activates caspase-9 and the subsequent activation of the caspase cascade are still unknown.

In THP-1 cells, we previously demonstrated the activation of caspase-2, -3, -6, -7 and -8 during apoptosis (12). In the current study, we have used gel filtration and sucrose density centrifugation to examine the cytosolic distribution and molecular speciation/composition of caspases and Apaf-1. Our data show
that in THP.1 cells the process of caspase activation involved the formation of two large soluble protein complexes hereafter referred to as the aposome and microaposome. Surprisingly most of the effector caspase activity (DEVVDase) was present primarily as these two large complexes rather than as the free ~60-kDa heterotetramer. In lysates isolated from control cells, Apaf-1 was present in its native state as a dimer and eluted from a gel filtration column as a complex of 200–300 kDa. Procaspase-9 was also present in its native state as a dimer (~70–90 kDa) and, after activation of caspases with dATP, redistributed with Apaf-1 to form the large aposome ($M_r = 700,000$) complex. The aposome recruited and processed the effector caspases, which were then released in the microaposome complex.

**MATERIALS AND METHODS**

**Cell Culture and Preparation of Cell Lysates—**Human monocytic tumor cells (THP.1) were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 100 units of penicillin, and 100 µg/ml streptomycin in 5% CO$_2$ at 37 °C. Apoptosis was induced with cycloheximide (25 µM) plus N$^o$-p-tosyl-L-lysine chloromethyl ketone (TLCK)$^1$ (100 µM) as described previously (12). THP.1 cell lysates (100,000 g of supernatants) were prepared by resuspending cells in a buffer containing 50 mM PIPES/ROH, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol, 20 µg/ml leupeptin, 10 µg/ml pepstatin A, 20 µg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 6.5, and freeze/thawing in liquid nitrogen (12). Activation of lysates (10 mg/ml) was assayed by measuring DEVVDase (see “Results”) activity following *in vitro* incubation for 1 h at 37 °C with 2 mM dATP and 2 mM Mg$^{2+}$. These cell lysates did not require the addition of exogenous cytochrome c for full activation. Recombinant His-tagged caspase-3 was expressed in BL21 (DE30) bacteria and purified on a Ni$^{2+}$ affinity column (13).

**Fluorometric Assay of Caspase Activity—**Spectrofluorometric assays of proteolytic activity were carried out as described previously (12) using synthetic fluorogenic substrates, i.e. benzoylxy carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVVD-AMC) to measure DEVVDase or effector caspase activity (i.e. primarily caspase-3 and -7) and benzoylxy carbonyl-Ileu-Glu-Thr-Asp-7-amino-4-methylcoumarin (Z-IETD-AMC) to measure caspase activator activity. In some experiments benzoylxy carbonyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin (Z-VEID-AMC) was used to assay not only caspase activator activity but also the activity of caspase-6, which is believed to be the protease responsible for lamin cleavage (14). In other experiments, the chymotrypsin-like (LLVYase) activity of the 20 S proteasome was measured using Val-Tyr-AMC (Pepro Tech Inc., Rocky Hill, NJ, USA). Liberation of AMC and AMC from the substrates was measured continuously using excitation/emission wavelength pairs (λ$_{exc}$/λ$_{em}$) of 400/505 nm and 380/460 nm, respectively. Lysates or column fractions (10 µl/25–50 µg of protein) were assayed in 1.25 ml of 0.1% CHAPS, 10 mM dithiothreitol, 100 mM HEPES, and 10% sucrose, pH 7.0. The reaction was started with 20 µM substrate, and the reaction followed for 2–4 min. In some experiments with recombinant enzymes and dATP activation experiments where the enzyme activity was much higher (e.g. recombinant or partially purified enzymes), the protein concentration was adjusted accordingly. The protease activity was calculated using FL WinLab software (Perkin-Elmer Corp.) and expressed as either pmol/mg of protein/min or pmol/fraction/min.

**Chromatographic Methods—**The aposome and microaposome complexes were isolated by size-exclusion chromatography with a HiPrep 16/60 S-300 Sephacryl high-resolution column (Amersham Pharmacia Biotech) and a Waters 650E Advanced Protein Purification system. The column was equilibrated with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM dithiothreitol, pH 7.0, and all separations were carried out at 4 °C. Lysates (20–30 mg of protein) were applied to and eluted from the column at a flow rate of 0.4 ml/min, and 2 ml fractions were collected and assayed for DEVVDase activity. Appropriate fractions were concentrated in Vivaspin 4 (Vivascience, Lincol, United Kingdom) concentrators (10-kDa cut-off) and stored at −70 °C. The column was calibrated with an Amersham Pharmacia Biotech HMW gel filtration protein standards kit containing, thyroglobulin ($M_r = 669,000$), ferritin ($M_r = 418,000$), catalase ($M_r = 206,000$), bovine serum albumin ($M_r = 67,000$) protein standards, and Ni$^{2+}$-column-purified recombinant His-tagged caspase-3 ($M_r = 60,000$, assuming a tetrameric configuration).

**Sucrose Density Gradient Centrifugation and Fractionation—**Aposome and microaposome complexes prepared as described above were carefully layered onto continuous sucrose gradients (10–40%), 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM dithiothreitol, pH 7.0, at 5 °C. The loaded gradients were centrifuged in a Beckman SW-28 rotor at 25,000 rpm for 17 h at 5 °C. The gradients were then fractionated into 17 × 2-ml fractions, which were analyzed for protein and caspase activity before being concentrated and stored at −70 °C. Sucrose densities were determined by refractometry.

**Western Blot Analysis—**Protein samples (20 µg) were resolved on 10, 13, or 15% SDS-polyacrylamide gel electrophoresis depending on the protein of interest and blotted onto nitrocellulose (Hybond-C extra, Amersham Pharmacia Biotech) as described previously (12). Samples were normally prepared for SDS-polyacrylamide gel electrophoresis by diluting in concentrated SDS sample buffer and then loading the gels with the appropriate amount. Membranes were blocked to prevent nonspecific binding before probing with the appropriate dilution of antibody. Anti-caspase-3 antibody was a kind gift from Dr. D. W. Nicholson, Merck Frosst, Canada. Rabbit anti-Apaf-1 (9) was a generous gift from Dr. X. Wang, University of Texas. A polyclonal antibody that recognizes the pro- and p19 forms of caspase-7 was prepared as described previously (12). Anti-caspase-9 antibody that recognized both the inactive proform (46 kDa) and the activated ~37- and 35-kDa processed forms was a kind gift from Dr. D. Green, La Jolla Institute for Allergy and Immunology, San Diego, California. A mouse monoclonal antibody (MCP-20) to the α subunit HC2 of the 20 S subunit and a rabbit polyclonal antibody to the ATPase subunit p45 of the human 19 S proteasome were obtained from Affiniti Research Products Ltd., Exeter, United Kingdom. Antibody binding was detected by incubation with an appropriate secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG) conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham Pharmacia Biotech).

**RESULTS**

**IETDase Is Distinct from DEVVDase Activity—**The aim of this study was to characterize the process of caspase activation in THP.1 cells. Initially we tried to identify the IETDase enzyme(s) capable of cleaving the IETD motif necessary to activate caspase-3. Cell lysates isolated from nonapoptotic (control) THP.1 cells have low DEVVDase activity, which increases to a maximum approximately 2 h after apoptosis is induced with cycloheximide and TLCK (12). Similarly in the present study, DEVVDase activity increased 10-fold from 83 to 837 pmol/mg/min 2 h after the induction of apoptosis (Table I). Furthermore, in the current study, we also showed that the cell lysates exhibited a constitutive IETDase activity of 160 pmol/mg/min. However, 2 h after the induction of apoptosis, the IETDase activity only increased by 40–50% to 232 pmol/mg/min (Table I). Slightly higher increases (2–3-fold) were observed when VEID-AMC cleavage (VEIDase) was used to measure activator caspase activity (Table I), probably due to activation of caspase-6 (14), the lamin cleaving enzyme, which is processed during apoptosis in THP.1 cells (12). Caspases can also be

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1 The abbreviations used are: TLCK, N$^o$-p-tosyl-L-lysine chloromethyl ketone; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; AFC, 7-amino-4-trifluoromethylcoumarin; AMC, 7-amino-4-methylcoumarin; CHO, aldehyde 2.
Cell lysates from nonapoptotic and apoptotic cells were prepared as described in Table I and preincubated for 20 min at 37 °C with varying concentrations of the indicated inhibitors before adding the appropriate substrate to initiate the reaction. The effects of the inhibitors on the IETDase and DEVDase activity were expressed as a percentage of the activity of the uninhibited (control) lysates that had been preincubated for 20 min at 37 °C with the Me2SO solvent. The results are given where possible as the concentration of inhibitor giving 50% inhibition of activity (I50).

|     | Z-IE TD-CHO | Ac-DEVD-CHO | Z-VAD-FMK | Ac-YVAD-CMK |
|-----|-------------|-------------|-----------|-------------|
| IETDase (nonapoptotic lysate) | 100 nM | 10% inhibition at 400 nM | No inhibition at 400 μM | 25% inhibition at 400 μM |
| DEVDase (apoptotic lysate)    | 100 nM | 1–2 nM | 1–2 μM | 0.5–1 μM |

Ma r (kDa) 669 418 206 67

![Fig. 1. Activator and effector caspase activity is found in an ~700-kDa complex, the aposome. Aliquots (2 ml) of lysates (10 mg/ml) isolated from nonapoptotic cells were fractionated by size exclusion chromatography before (-dATP; ○ ○ ○) or after caspase activation with 2 mM dATP (+dATP; ● ● ●) as described under "Materials and Methods." Fractions (fr) (2 ml) were collected after discarding the first 80 ml of the column eluate (i.e. most of the void volume) and assayed for VEIDase (A), IETDase (B), and DEVDase (C) activity and protein content. The column was calibrated with blue dextran and Amersham Pharmacia Biotech HMW gel filtration protein standards (indicated in the figure) and purified His-tagged recombinant caspase-3 (Mr ~ 60,000), which was detected by measuring its DEVDase activity (arbitrary units, ~—~). The elution profile shows the activator and effector caspase activity of each fraction plotted against the respective fraction number.

**Isolation of the Aposome, an ~700-kDa Complex with Caspase Cleavage Activity**—To further characterize the IETDase activity, we used size exclusion chromatography to fractionate lysates from both control and activated lysates (i.e. lysates prepared from apoptotic cells or lysates activated in vitro with dATP). Surprisingly, when an apoptotic (results not shown) or a dATP-activated lysate was separated on a Sephacryl S-300 column, the DEVDase activity mainly eluted as two large complexes with Mr of ~700,000 and ~200,000–300,000 (Fig. 1C). This was totally unexpected as the active forms of effector caspases are believed to exist as heterotetramers with a Mr of ~60,000 (8) as shown with recombinant His-tagged caspase-3 (Fig. 1C) and from previous purification studies using ion exchange chromatography (16, 17). In our studies, only a small amount of DEVDase activity was detected in those fractions corresponding to a Mr of 60,000–80,000 (Fig. 1C). This latter peak was not always detected, and in some separations, the large ~700-kDa complex, which we have termed the aposome, had the highest DEVDase activity. The aposome also contained caspase activator activity as shown by its IETDase and VEIDase content (Fig. 1, A and B). In addition a second smaller complex with high DEVDase activity of ~200–300 kDa molecular mass (hereafter referred to as the microaposome) was generated during caspase activation (Fig. 1C). The microaposome had low levels of IETDase and VEIDase activity (Fig. 1, A and B) and consequently much higher DEVDase/

activated in vitro by incubating cell lysates with dATP (15). In the case of THP.1 cells, a 30–60-fold increase in DEVDase activity (~3000 pmol/mg/min) was achieved by incubating the lysates (10–15 mg/ml) with 2 mM dATP/2 mM Mg2+ for 1 h (results not shown). However, even with these high levels of effector caspase activity, there was only an ~2- and 3-fold increase in IETDase and VEIDase activities, respectively. These small increases were always coincident with the increase in DEVDase activity and could be attributed to the effector caspases ability to cleave the IETD and VEID substrates, albeit with much reduced efficiency. Thus, irrespective of how the effector caspases were activated there was no significant preceding induction of activator caspase activity.

Thus other factors in addition to IETDase activity were important in determining how the caspases were activated. To investigate this further, it was important to establish that IETDase activity was distinct from DEVDase activity. We examined the sensitivity of ly-rate IETDase and DEVDase activities to various caspase peptide inhibitors whose structures are based on the substrate specificity motif favored by the particular caspases (5–7). Ac-DEVD-CHO and Z-IE TD-CHO differentially inhibited the DEVDase activity of recombinant caspase-3 with I50 values of ~1–2 nM and ~100 nM, respectively. In lysates isolated from control cells, Z-IETD-CHO was a potent inhibitor of IETDase activity (Table II). However, IETDase activity was insensitive to Ac-DEVD-CHO at concentrations in excess of that needed to completely inhibit DEVDase (i.e. caspase-3 and caspase-7) activity of apoptotic lysates. Furthermore, other caspase inhibitors, i.e. Z-VAD-FMK and Ac-YVAD-CMK, which potentely inhibited the DEVDase activity of apoptotic lysates, were ineffective against IETDase activity even at very high concentrations (Table II). These data demonstrated that IETDase activity was not due to a nonspecific cleavage activity of effector caspases. Thus, the DEVDase and IETDase activities were clearly due to separate and distinct enzymes.
IETDase and DEVDase/VEIDase ratios than the aposome. In an unactivated lysate prepared from control cells, the IETDase and VEIDase activities were also found to be associated with a large complex, which was of a similar size to the aposome, but unlike the latter had a very low DEVDase activity (Fig. 1). Thus following caspase activation, most of the DEVDase activity was predominantly associated with two large complexes, the aposome and microaposome.

Formation of the Aposome Results in Recruitment of Apaf-1 and Caspase-9—The gel filtration data strongly suggested that we had identified two large multimeric complexes, which were involved in caspase processing. To further characterize these complexes, column fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The column fractions were not pre-concentrated to minimize any possible artifacts due to autocatalytic processing. In control lysates, Apaf-1 was located almost entirely in those fractions corresponding to a $M_r$ of approximately 60,000–80,000, suggesting that it existed primarily in its unactivated form as a dimer (Fig. 2A). After dATP activation, all of the proform disappeared and was accompanied by the appearance of the p17 catalytically active large subunit of caspase-3 in both the aposome and microaposome fractions (Fig. 2C). The p17 subunit was also detected in fractions 3–6 (Fig. 2C). In both control and dATP-activated lysates, an immunoreactive band with a $M_r$ of 29,000 was also detected in those fractions 3–6 (Fig. 2C), corresponding to the aposome complex. Initially we thought this was due to the processing of procaspase-3 at Asp-28. However, we did not detect this band using a monoclonal antibody to caspase-3 (Transduction Laboratories); it suggested that our normal form, which was found predominantly in the ~700-kDa aposome complex (Fig. 2B, lanes 4–6). These results raised the possibility that caspase-9 was recruited to the complex with Apaf-1 and then activated. Only small amounts of “free” active caspase-9 were detected in fractions 18 and 19, which may have been released from the aposome. Thus, in control lysates, caspase-9 exhibited a completely different size distribution from Apaf-1. However on activation with dATP, Apaf-1 and caspase-9 were both found in the ~700-kDa aposome complex, which exhibited effector caspase activity.

Procaspses-3 and -7 Are Present as Different Sized Complexes—In control lysates, procaspase-3 was detected in fractions 17–21 corresponding to a $M_r$ of approximately 60,000–80,000, suggesting that it existed primarily in its unactivated form as a dimer (Fig. 2C). After dATP-dependent caspase activation, all of the proform disappeared and was accompanied by the appearance of the p17 catalytically active large subunit of caspase-3 in both the aposome and microaposome fractions (Fig. 2C). The p17 subunit was also found in fractions 17–21, indicating that some free heterotetrameric caspase was present. The overall distribution of the p17 subunits in the column fractions closely paralleled the DEVDase activity profile (compare Figs. 1C and 2C). In both control and dATP-activated lysates, an immunoreactive band with a $M_r$ of 29,000 was also detected in those fractions 3–6 (Fig. 2C), corresponding to the aposome complex. Initially we thought this was due to the processing of procaspase-3 at Asp-28. However, we did not detect this band using a monoclonal antibody to caspase-3 (Transduction Laboratories); it suggested that our normal
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Surprisingly, procaspase-7 eluted in fractions 11–14, similar to Apaf-1 (Fig. 2D), which suggested that they might be associated in a complex in control cells. After dATP activation, procaspase-7 was fully processed and only the active p19 subunits were detected. Thus before processing, procaspase-3 probably existed as a dimer, whereas procaspase-7 was either in a higher oligomeric form or alternatively was associated in a complex possibly with Apaf-1. During caspase activation, the catalytically active forms of both effector caspases were detected in the aposome complex, suggesting that they were recruited to and processed by the aposome. These results demonstrated that the effector caspase-3 and -7 were present in control lysates as different sized protein complexes.

Dissociation of the Aposome into the ~200–300-kDa Microaposome Complex—The gel filtration data provided evidence that the aposome was an ~700-kDa multi-enzyme complex. However, as the aposome eluted close to the void volume of the column, it was not possible to determine whether or not there was more than one type of complex. Sucrose density gradient centrifugation has been widely used to purify and analyze large cellular complexes, such as the cytosolic chaperonin-containing T-complex polypeptide 1 and the 20 S proteasome (18, 19). Partially purified aposome complexes, which were free of the microaposome complex, when prepared by gel filtration chromatography (fractions 5 and 6, Fig. 1), were sedimented on a 10–40% (w/v) sucrose density gradient, and the fractions were assayed for activator and effector caspase activity. Most of the DEVDase activity (~70%) sedimented in fractions 7–11, i.e. very similar to the thyroglobulin marker (Fig. 3C). The remaining DEVDase activity (approximately 17%) banded at a lower sucrose concentration (i.e. fractions 12–16). All the IETD/VEIDase activity banded as a homogenous peak in fractions 7–11. Thus, further purification by sucrose density gradient centrifugation of the partially purified aposome from the gel filtration step showed that the large core complex of ~700 kDa was relatively stable as it still exhibited IETDase, VEIDase, and DEVase activities. On the basis of the caspase activator activity, the combination of gel filtration and sucrose gradient centrifugation resulted in a significant purification of the aposome. Thus, the specific activities of the IETDase and VEIDase increased from 107 and 262 to 2702 and 1300 pmol/mg/min, respectively, representing a 20–25-fold increase in purity. Although there were increased levels of DEVase in the purified aposome, it was not possible to use this as a measure of purification, as the increase was due to caspase activation as well as purification. The sucrose density gradient profile also indicated that some of the DEVase activity had dissociated from the aposome and now co-eluted with the microaposome complex (Fig. 3C). Further purification by sucrose density centrifugation of the microaposome complex, initially prepared by gel filtration chromatography, showed that it sedimented as a single homogenous band in fractions 13–16 (Fig. 3C). This suggested that the effector caspases were recruited and activated in the aposome and then released in the microaposome complex.

IETDase and VEIDase Activities Co-elute with the Proteosome in Control Lysates—The size of the aposome was remarkably similar to the 20 S proteasome, which is a cylindrical particle of an 11-nm diameter and a 15-nm length and a Mr of 740,000 (for review see Refs. 20 and 21). The 20 S proteasome forms the catalytic core of the 26 S proteasome and is composed of 14 separate subunits arranged in 4 rings as an α-β-α-β-α-β complex. Although originally defined as a complex with multiple peptidase activities, it is now generally recognized as having three main protease activities (21). One of these is the chymotrypsin-like activity, assayed using Suc-Leu-Leu-Val-Tyr-AMC as a substrate. Control and dATP-activated lysates from THP-1 cells had high levels of LLVYase activity (1100–1300 pmol/mg/min), which co-eluted with the IETDase/VEIDase activity on gel filtration (results not shown). The ~700-kDa complex from control cells, which contained the IETDase/VEIDase activity, was further purified by sucrose density gradient centrifugation. The IETDase and VEIDase activities both sedimented as symmetrical peaks of activity in fractions 7–11 (Fig. 4A). These fractions also contained LLVYase activity (Fig. 4B), the 20 S α-subunit of the 20 S proteasome (Fig. 4C), and the ATPase subunit SUG1, which is part of the 19 S regulator complex (Fig. 4D). The presence of SUG1 indicated that either the intact 26 S proteasome was present or alternatively the 19 S regulator complexes, which also have a Mr of ~700,000, had dissociated from the 26 S proteasome. Thus in control cells, IETDase and VEIDase activities both co-eluted with the proteosome (Fig. 4) but not with Apaf-1 or the pro-caspases (Fig. 2). This suggested that IETDase and VEIDase may be novel enzymic activities, possibly associated with the proteosome.

The Aposome after Sucrose Density Purification Contains

![Figure 3](image-url)

**Fig. 3. Sucrose density gradient purification of the aposome.** Aposome and microaposome complexes were prepared by S-300 gel filtration of lysates isolated from control (nonapoptotic) cells after dATP activation as described in Fig. 1. The two most active fractions (fr) from the aposome and microaposome peaks were layered onto 10–40% sucrose gradients and centrifuged at 25,000 rpm for 17 h. The fractionated gradients were analyzed for VEIDase (A), IETDase (B), and DEVase (C) activity. Thyroglobulin was run as a high Mr (669,000) marker, and its sedimentation position is indicated with the bar and arrows. The closed circles and the open squares represent the aposome and microaposome complexes, respectively. The dashed line indicates the sucrose gradient.
and Western blotting for the proteasome subunits, 20 S fractions were also analyzed by SDS-polyacrylamide gel electrophoresis shown), the 20 S stability fractions also contained LLVYase activity (results not redistributed to the microaposome complex. The sucrose den- 
trification step either by further degradation or alternatively by 
of caspase-7 was lost during the sucrose density gradient pu-
to loading on the gradient. This suggested that the active form 
the gradient-purified aposome complex fractions (results not 
Small amounts of Apaf-1 and caspase-9 were detected in frac-
the active forms of caspase-9 and -3 (Fig. 5, A-C). These 
filtration characteristics to the proteasome (see “Discussion”). 
Further purification of the microaposome complex by sucrose 
density gradient centrifugation showed that little if any Apaf-1 
or caspase-9 was present in the microaposome complex, but it 
that only the active forms of the effector caspases were asso-
ciated with the microaposome complex, and given the size of 
this complex it is likely that other proteins were part of the 

**DISCUSSION**

The most striking finding of the present study was the ob-
ervation that effector caspase activity was found in two large 
multimeric complexes, the aposome and the microaposome. 
The larger complex, the aposome, had a \( M_r \) of \( \sim 700,000 \) and 
**FIG. 4.** IETDase and VEIDase activities co-elute with the pro-
tasome in control lysates. Control lysate was fractionated and 
separated by gel filtration chromatography as described in Fig. 1. The 
two most active IETDase/VEIDase-containing fractions (\( P \)), which also 
contained the most active proteasome (LLVYase) activity, were pooled 
and further purified by sucrose density gradient centrifugation as 
described in Fig. 3. The fractions were then analyzed for IETDase, 
VEIDase, and LLVYase activity, which are shown in A and B. All the 
fractions were also analyzed by SDS-polyacrylamide gel electrophoresis 
and Western blotting for the proteasome subunits, 20 S subunit HC2 
and 19 S regulator subunit (D), SUG1.

**FIG. 5.** After sucrose density centrifugation, the partially pu-
rified aposome contains Apaf-1, caspase-3, and caspase-9. Apos-
ome and microaposome complexes were prepared by gel filtration 
chromatography and further purified by sucrose density centrifugation 
as described in Fig. 3. Fractions 6–17 from the sucrose gradient were 
then analyzed for Apaf-1 (A and E), caspase-9 (B and F), caspase-3 (C 
and G), and the 20S \( \alpha \)-subunit (D). The positions of processed caspase 
subunits are indicated with arrowheads.

Apop-1, Caspase-3, and Caspase-9—Following further purifica-
tion of the aposome by sucrose density gradient centrifugation, 
fractions were obtained and analyzed by immunoblotting. This 
revealed that fractions 7–10 contained most of the Apaf-1 as 
well as the active forms of caspase-9 and -3 (Fig. 5, A-C). These 
fractions also contained most of the DEVDase activity and 
virtually all of the IETDase and VEIDase activities (Fig. 3). 
Small amounts of Apaf-1 and caspase-9 were detected in frac-
tions 14–17. The p19 subunit of caspase-7 was not detected in 
the gradient-purified aposome complex fractions (results not shown), 
although it was present in the S-300 preparation prior to 
loading on the gradient. This suggested that the active form 
of caspase-7 was lost during the sucrose density gradient pu-
rification step either by further degradation or alternatively by 
redistribution to the microaposome complex. The sucrose 
density fractions also contained LLVYase activity (results not 
shown), the 20 S \( \alpha \)-subunit of the proteasome (Fig. 5D), and 
SUG1, the 19 S proteasome subunit (data not shown). These 
data indicated that either Apaf-1, caspase-3, and caspase-9 
were in a complex, which was interacting with the proteasome, or 
alternatively the aposome complex had comparable size and sed-
imentation characteristics to the proteasome (see “Discussion”). 

Further purification of the microaposome complex by sucrose 
density gradient centrifugation showed that little if any Apaf-1 
or caspase-9 was present in the microaposome complex, but it 
was highly enriched in active caspase-3 subunits, which were 
found in fractions 14–16 (Fig. 5, E-G). Active caspase-7 was 
also found in these fractions, but neither of the proteasome 
subunits was detected (data not shown). These results showed 
that only the active forms of the effector caspases were asso-
ciated with the microaposome complex, and given the size of 
this complex it is likely that other proteins were part of the 
complex.
The Aposome, a Large ~700-kDa Caspase-activating Complex

procaspase-3 also appeared to exist as a dimer, whereas procaspase-7 eluted as a 200–300-kDa complex (Fig. 2). These results raise the possibility that procaspase-7 may be associated with and regulate the activity of Apaf-1 in control cells. Alternatively, procaspase-7 may be associated with other protein(s).

Following dATP-dependent activation, Apaf-1 and both the p35 and p37 active subunits of caspase-9 were associated with the aposome (Figs. 2 and 5). Whereas the initial processing of procaspase-9 to its p35 subunit is autocatalytic, the generation of the p37 subunit is mediated by caspase-3 (22). In this regard it was of interest that active caspase-3 was also associated with the aposome and was likely responsible for the formation of the p37 subunit.

The DEVDase containing the microaposome complex was most likely derived from the aposome as it was only detected when the caspases were activated and when the aposome was generated (Figs. 1 and 3). The relationship between the aposome and microaposome may be analogous to the cytosolic chaperonin-containing T-complex polypeptide 1 complex, which is a large cytosolic complex with a 

$M_t$ of ~950,000 that exists in equilibrium with smaller microcomplexes of 120–250 kDa (23). The microaposome may be a proteolytically degraded form of the aposome, but this seems unlikely given that little or no Apaf-1 was present in the microaposome (Fig. 5E). Another possibility was that the appearance of active caspases in the microaposome complex occurred after caspase activation, when they were released from the large ~700-kDa aposome complex to form the smaller ~200–300 kDa sized microaposome. In support of this, the microaposome contained active effector caspases but not Apaf-1 (Fig. 2 and 5E). The size of the microaposome complex also suggested that it might contain other proteins in addition to the active caspases. Although we have not characterized the complex, we have shown that Hsp-90 co-eluted with the microaposome complex.2 Thus other molecules, such as Hsp-90, may be involved in transporting or targeting the active caspases to key intracellular targets, e.g. the nucleus or endoplasmic reticulum. In this regard, it should be stressed that prior to caspase activation, procaspase-3 and -7 were located in different sized soluble complexes (Fig. 2). This may be of functional significance as we have recently shown that following Fas-induced apoptosis in mouse liver, activated caspase-3 and -7 are targeted to different subcellular locations (24).

The large size of the aposome was similar to that of energy-dependent self-compartmentalizing proteases, such as the proteasome and ClpAp proteases found in Escherichia coli and mitochondria (20, 21). As similar methods were used to purify the aposome as well as these complexes, it was not totally unexpected that the proteasome co-purified with the aposome complex (Fig. 5). Interestingly IETDase/VDEADase activities of the control lysate co-purified with the proteasome (Fig. 4), suggesting that they may be previously uncharacterized proteolytic activities of the proteasome. Such IETDase activity may be responsible for the activation of effector caspases. It is unclear at the moment whether or not the proteasome and the aposome are part of one larger complex or are independent complexes, which may possibly interact during caspase activation. As yet we cannot answer this question, because we have only partially purified the aposome and not separated it from the proteasome. In preliminary studies, high concentrations of lactacystin, a potent proteasome inhibitor (25), inhibited neither dATP-dependent caspase activation nor IETDase activity (data not shown), which suggested that the proteasome was not directly involved with caspase activation. However, further experiments are clearly required to resolve this issue. Many studies have implicated an important but as yet undefined role for the proteasome in apoptosis (reviewed in Ref. 26). Our present findings may help to define this role.

In conclusion, we have demonstrated for the first time that active effector (DEVDase) caspase activity is associated with two large cellular complexes, the aposome and the microaposome. The aposome is an ~700-kDa multimeric complex, which is formed during apoptosis by the recruitment of Apaf-1 together with caspase-9, -3, and -7. The microaposome appears to be released from the aposome and contains primarily active effector caspase-3 and -7. Our results suggest that these two large interacting complexes play a key role in the activation and execution of the caspase cascade.

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