Apoptotic cell death is characterized by a commitment and an execution phase, the latter being distinguished by a series of stereotypic morphological and biochemical changes, which require the activation of a family of caspases (1, 2). Caspases are constitutively expressed in cells as inactive proenzymes and require proteolytic cleavage to be active. Presently, fourteen caspases have been identified and these can be divided into two classes. “Initiator” caspases, such as caspases-8 and -9, possess long pro-domains and either directly or indirectly activate “effector” caspases, such as caspase-3, -6, and -7, which contain short pro-domains (3–5). Activation of initiator caspases involves interaction of their long pro-domains with either caspase recruitment domains (CARD) (6) or the death effector domains (DEDs) of adapter molecules and results in the formation of cellular complexes. Thus with receptor-mediated cell death, ligation of the CD95 (Fas/APO-1) receptor results in the formation of a death-inducing signaling complex (DISC), in which the death effector domains of the adapter molecule FADD (MORT1) interact with the N-terminal death effector domains of caspase-8 (7, 8). Many apoptotic stimuli cause cell death by inducing the release of cytochrome c (9), which together with Apaf-1 and ATP/dATP facilitates the processing of caspase-9 and initiates a caspase cascade (10, 11). Apaf-1, the first identified mammalian homologue of CED-4, is a 130-kDa protein. It contains an N-terminal CARD domain, followed by a region homologous to CED-4 and a C-terminal domain containing multiple WD-40 repeats, which are involved in protein-protein interactions (11). Both CED-4 and Apaf-1 undergo self-oligomerization, which is required for the activation of CED-3 and caspase-9, respectively (12, 13). In the presence of dATP and cytochrome c, the CARD domains of caspase-9 and Apaf-1 interact to produce an apoptosome complex in which caspase-9 is processed to its active form, which in turn activates caspase-3 (10, 14, 24).

The apoptosome has been defined as an Apaf-1-containing complex that catalyzes the activation of caspases (15, 16). Recently, using purified recombinant Apaf-1 in the presence of cytochrome c and dATP, the formation of an ~1.4-MDa apoptosome complex has been described (17, 18). This large complex recruits and activates procaspase-9, which dissociates from the apoptosome before activating caspase-3 (17, 18). In contrast, we have isolated an ~700-kDa Apaf-1 apoptosome (aposomé) complex containing activated caspase-9, -3, and -7 from dATP-activated cell lysates (19). The difference in size of the recombinant (~1.4 MDa) and native (~700 kDa) apoptosome complexes led us to question the relative significance of these two large complexes in processing caspases during dATP-dependent activation and apoptotic cell death. In the present study using Superose 6 gel filtration, we demonstrate that both the ~700-kDa and ~1.4-MDa apoptosome complexes are formed, following in vitro dATP activation of cell lysates. However, only the ~700-kDa complex is capable of processing and activating effector caspases. Furthermore we also show that, during apoptotic cell death, the ~700-kDa apoptosome complex predominates, supporting the hypothesis that this is the correctly assembled and biologically active form of the apoptosome.

**EXPERIMENTAL PROCEDURES**

Reagents and Western Blot Analysis—Most reagents, including antibodies to caspase-3, -7 and -9, were obtained from indicated sources (15, 21). Protein samples (~20 μg) were resolved on 10 or 12.5% SDS-polyacrylamide gels (PAGE) and transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia Biotech), and antibody binding was detected as described previously (22). To assess the kinetics of apoptosome assembly, Western blots of Apaf-1 from each time point were probed with identical antibody and enhanced chemiluminescence solutions and simultaneously exposed to the same photographic film. The exposed films were scanned with a Molecular Dynamics

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**Accelerated Publication**

Apaf-1 Oligomerizes into Biologically Active ~700-kDa and Inactive ~1.4-MDa Apoptosome Complexes*

(Received for publication, December 13, 1999)
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† Supported by a Glaxo Wellcome Post Graduate studentship award.
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1 The abbreviations used are: AFC, 7-amino-4-trifluoromethylcoumarin; TPCK, N-tosyl-l-phenylalanyl chloromethyl ketone; PAGE, polyacrylamide gel electrophoresis.
2 The apoptosome term has also been used to describe cytoplasmic protrusions released from cells (20). Therefore to avoid confusion we now refer to the ~700 kDa complex as the apoptosome.
Densitometer, and the images were analyzed with a Multi-Analyst Image analysis program (Bio-Rad Laboratories, CA).

Cell Culture and Preparation of Control and Caspase-activated Cell Lysates—Human monocyte tumor cells (THP.1) were grown in RPMI 1640 media with 10% heat-inactivated fetal bovine serum in 5% CO₂ at 37 °C. Apoptosis was induced with etoposide (10 μM), a DNA topoisomerase II inhibitor, or TPCK (75 μM), a chymotrypsin-like serine protease inhibitor, as described previously (22, 23). Lysates (100,000 × g supernatants) from THP.1 cells were prepared as described previously and activated by incubation (10 mg/ml) for 1 h at 37 °C with 2 mM dATP and 2 mM MgCl₂ (19). These cell lysates contained low but detectable levels of cytochrome c and did not require the addition of exogenous cytochrome c for full caspase activation.

Fluorimetric Assay of Caspase Activity—DEVDase activity (i.e. primarily caspase-3 and -7) of lysates or column fractions was measured (λex/em = 405/510 nm) at 37 °C in 96-well plates in 200 μl of assay buffer (20 μM Z-DEVD-APC, 0.1% CHAPS, 10 mM dithiothreitol, 100 μM HEPES, and 10% sucrose, pH 7.0) using a Wallac Victor® 1420 Multilabel counter. The 96-well plates were assayed for 10 cycles, and cleavage rates were determined by linear regression. The protease activities were expressed as either pmol/min/mg protein or pmol/min/fraction.

Chromatographic Methods—Lysates were fractionated by size-exclusion chromatography, using a fast protein liquid chromatography protein purification system on analytical (16/60), preparative (26/60) Hi-Prep S300 Sephacryl high-resolution (19) and Superose 6 HR 10/30 columns (Amersham Pharmacia Biotech, Herts, UK). The Sephacryl columns were eluted at 4 °C with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM HEPES/NaOH, 5 mM dithiothreitol, pH 7.0, and fractions collected. The Superose 6 column was eluted with the same buffer supplemented with 50 mM NaCl, at 0.4 ml/min, and 0.5- or 1.0-ml fractions were collected. The columns were calibrated with protein standards (Amersham Pharmacia Biotech), including blue dextran, thyroglobulin, ferritin, catalase, bovine serum albumin, ovalbumin, and bovine heart cytochrome c (Sigma) with some of their sizes (kDa) indicated in Fig. 1. Some differences (see Results) were noted in the elution profiles of the effector caspases on S300 and Superose 6 columns. This was most probably because of addition of 50 mM NaCl in the buffer, which ensured good separation of large protein complexes on Superose 6 columns.

Assay of Apoptosome Effector Caspase Activating Activity—To assess the biological activities of the ~1.4-MDa and ~700-kDa apoptosome complexes, we developed a simple in vitro assay. Briefly, apoptosome complexes were isolated from apoptotic or dATP-treated lysates by Superose 6 gel filtration as described above. The appropriate column fractions (100 μl aliquots) were then incubated with pro-caspases (25 μg of protein) for 30 min before assaying for DEVDDase activity. The procaspases were obtained following fractionation of control lysates (fractions 18–20) by gel filtration on a preparative (26/60) Hi-Prep S300 Sephacryl high-resolution column. These fractions, which contained procaspase-3, -7, and -9, were concentrated in Vivaspin 4 (Vivascience, UK). Procaspase-3, -7, and -9, were obtained following fractionation of control lysates (fractions 100–120) by gel filtration on a preparative (26/60) Hi-Prep S300 Sephacryl high-resolution column. These fractions, which contained procaspase-3, -7, and -9, were concentrated in Vivaspin 4 (Vivascience, UK). The columns were calibrated with protein standards (Amersham Pharmacia Biotech), including blue dextran, thyroglobulin, ferritin, catalase, bovine serum albumin, ovalbumin, and bovine heart cytochrome c (Sigma) with some of their sizes (kDa) indicated in Fig. 1. Some differences (see Results) were noted in the elution profiles of the effector caspases on S300 and Superose 6 columns. This was most probably because of addition of 50 mM NaCl in the buffer, which ensured good separation of large protein complexes on Superose 6 columns.

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RESULTS
dATP Activation of Cell Lysates Produces Both ~700-kDa and ~1.4-MDa Apoptosome Complexes—In our previous study, a Sephacryl S300 column was used to characterize the apoptosome as an ~700-kDa complex (19). Because this column cannot accurately resolve complexes of 800–1500 kDa, we used a Superose 6 high-resolution analytical gel filtration column, which can resolve complexes up to 4 MDa. In control lysates, both procaspase-9 and -3 eluted as their free unprocessed zymogens (Fig. 1A, fractions 22–28) and Apaf-1 eluted as a single peak (Fig. 1A, fractions 18–24), with an estimated Mₐ ≤ 158,000, indicating that it was most likely a monomer. After dATP activation of the lysate for 1 h, a dramatic change in the elution behavior of Apaf-1 and the caspases was observed. Apaf-1 was now associated predominantly with ~1.4-MDa and ~700-kDa apoptosome complexes (Fig. 1B, fractions 6–8 and fractions 10–15, respectively) with a small amount remaining as monomeric Apaf-1 (Fig. 1B, fractions 19–22). After dATP activation, most of the procaspase-9 was processed to its catalytically active large subunits (p35/p37 subunits), which eluted with the ~1.4-MDa and ~700-kDa apoptosome complexes, and also as the free processed caspase (Fig. 1B). Procaspase-3 was completely processed to its active large subunit (C, the ~700 kDa but not the ~1.4-MDa apoptosome complex possessed the ability to process and activate most of the effector caspases. The Apaf-1-containing fractions (fractions 5–21, Fig. 1B) were assayed for caspase processing activity by measuring DEVDDase activity only produced when the fractions were incubated with "free caspases" as described under "Experimental Procedures." Thus, the ~700-kDa apoptosome complex (fractions 10–15) initially had very little or no DEVDDase activity but had the inherent ability to activate exogenous effector caspases. All the fractions from panel B were also initially assayed for DEVDDase activity (C—C). The NaCl in the elution buffer resulted in the DEVDDase (i.e. free active effector caspase-3 and -7) activity eluting in fractions 22–28. The elution positions of M markers are indicated with arrows.

Fig. 1. Two large ~1.4-MDa and ~700-kDa apoptosome complexes are formed following dATP activation of cell lysates. Lysates (10 mg/ml) from THP.1 cells were incubated with or without dATP (2 mM) for 1 h, and the lysate (2 mg) was separated on a Superose 6 column and immunoblotted. A, Apaf-1 from control lysates eluted as a monomer and caspase-3 and -9 as unprocessed zymogens. B, after dATP activation, Apaf-1 eluted both as an ~1.4-MDa and an ~700-kDa apoptosome complex. Almost all of the caspase-9 was processed to its p35 and p37 forms, which eluted with the ~1.4-MDa and the ~700-kDa apoptosome complexes, and as free heterotetramers. Caspase-3 was processed to its active large subunit. C, the ~700-kDa but not the ~1.4-MDa apoptosome complexes were formed, but only the ~700-kDa complex actively processed the effector caspases as assayed by the induced DEVDDase activity. Thus, following dATP activation, two large apoptosome complexes (~700 kDa and ~1.4 MDa) were formed, but only the ~700-kDa complex
Biologically Active andInactive Apaf-1 Complexes

Kinetics of Formation of the ~700-kDa and ~1.4-MDa Apoptosome Complexes—To understand the relationship between the ~700-kDa and ~1.4-MDa apoptosome complexes, we studied their time-dependent formation and activities following dATP activation. The ~700-kDa complex was more rapidly formed than the ~1.4-MDa complex (Fig. 2A). Production of the ~700-kDa complex was maximal within 5 min of initiating dATP activation, whereas assembly of the ~1.4-MDa complex did not reach a maximum until after 20 min (Fig. 2A). However, only the ~700-kDa apoptosome complex exhibited significant biological activity as assessed by its ability to process caspases and yield active effector caspase activity (DEVDase, Fig. 2B). The biological activity of the assembled ~700-kDa complex was almost maximal 5 min after activation was initiated, whereas the ~1.4-MDa complex was virtually inactive at all time points measured (Fig. 2B).

During Apoptosis, the ~700-kDa Apoptosome Complex Predominates—To determine whether the ~700-kDa and/or ~1.4-MDa apoptosome complexes were formed during apoptosis, lysis were prepared from control and apoptotic cells and fractionated using a Superose 6 column. Apoptosis was induced in THP.1 cells by two different stimuli, i.e. etoposide and TPCK (22, 23), which gave 30 and 38% apoptosis, respectively, as determined by Annexin V binding. All of the Apaf-1 in lysates isolated from control cells eluted as the free ~130-kDa monomeric form (Fig. 3A). In marked contrast, Apaf-1, in lysates from etoposide or TPCK-treated THP.1 cells, eluted mainly as the free monomeric form or as the ~700-kDa complex, and little if any Apaf-1 eluted as an ~1.4-MDa complex (Fig. 3, B and C). Furthermore, only the ~700-kDa apoptosome complex processed and activated effector caspases (Fig. 3, B and C), suggesting that this was the biologically active complex which was formed during etoposide- or TPCK-induced apoptosis. Thus, in THP.1 cells, induction of apoptosis by either etoposide or TPCK resulted in the formation of an ~700-kDa caspase processing complex.

DISCUSSION

Reconstitution studies with recombinant proteins have shown that Apaf-1 and caspase-9 in the presence of cytochrome c and dATP forms a very large (~1.4 MDa) apoptosome complex (17, 18). This complex was active as judged by its ability to process 35S-labeled procaspase-9 and -3. However, in the current study we have demonstrated that, during dATP-dependent activation of cell lysates, two large apoptosome complexes with M, values of ~700 kDa and ~1.4 MDa were formed (Figs. 1 and 2). The rate of formation of the ~700-kDa complex was very rapid (within 5–10 min) compared with the formation of the ~1.4-MDa complex (~20 min). Moreover, the ~700-kDa complex efficiently processed exogenous caspases, whereas the ~1.4-MDa complex was relatively inactive (Figs. 1 and 2). Why the ~1.4-MDa apoptosome complex was incapable of caspase processing is unclear because both complexes contained oli-
gomerized Apaf-1 and processed caspase-9 (Fig. 1). We cannot rule out the possibility that an inhibitor is associated with the larger complex or alternatively that the ~1.4-MDa complex may be inappropriately oligomerized and structurally different from the ~1.4-MDa complex previously described (17, 18). Nevertheless, the ~700-kDa complex does not appear to be formed when reconstitution of the apoptosome is carried out with recombiant Apaf-1, procaspase-9, cytochrome c, and dATP (17, 18).

The biological significance of two large Apaf-1 apoptosome complexes is as yet unclear. However, our experiments with apoptotic lysates provide important evidence which indicates that the ~700-kDa complex is the biologically significant complex that is formed during apoptosis. Thus, in THP-1 cells using two unrelated stimuli, i.e. a DNA topoisomerase II inhibitor (etoposide) and a serine chymotrypsin-like protease inhibitor (TPCK), we have demonstrated that the predominant complex formed is an ~700-kDa apoptosome complex (Fig. 3). Taken together our results suggest that the ~700-kDa complex is the major form of the apoptosome formed in vivo and that the correct assembly of the apoptosome may require other factors present in cell lysates, in addition to Apaf-1, cytochrome c, dATP, and caspase-9. Thus, clear differences exist in the apoptosome complexes formed in vitro from recombinant proteins when compared with those assembled in cell lysates and during apoptosis.

In summary, following dATP activation of lysates, two Apaf-1-containing complexes are formed, a biologically inactive ~1.4-MDa and a biologically active ~700-kDa apoptosome. Kinetic analysis demonstrates a more rapid formation of the smaller ~700-kDa complex, which exhibits significant caspase-activating activity. In apoptotic cells, the ~700-kDa complex predominates, and we therefore propose that the ~700-kDa complex is the functionally active form of the apoptosome in apoptosis.

Acknowledgments—Anti-caspase-3 and -caspase-9 antibodies were kind gifts from Dr. D. W. Nicholson, Merck Frosst, Canada, and Dr. D. Green, La Jolla Institute for Allergy and Immunology, San Diego, CA, respectively. Rabbit anti-Apaf-1 was kindly provided by Dr. X. Wang, University of Texas Southwestern Medical Center, Dallas, TX.

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J. Biol. Chem. 2000, 275:6067-6070.
doi: 10.1074/jbc.275.9.6067

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