MCF-7 cells lack caspase-3 but undergo mitochondrial-dependent apoptosis via caspase-7 activation. It is assumed that the Apaf-1-caspase-9 apoptosome processes caspase-7 in an analogous manner to that described for caspase-3. However, this has not been validated experimentally, and we have now characterized the caspase-7 activating apoptosome complex in MCF-7 cell lysates activated with dATP/cytochrome c. Apaf-1 oligomerizes to produce ~1.4-MDa and ~700-kDa apoptosome complexes, and the latter complex directly cleaves/activates procaspase-7. This ~700-kDa apoptosome complex, which is also formed in apoptotic MCF-7 cells, is assembled by rapid oligomerization of Apaf-1 and followed by a slower process of procaspase-9 recruitment and cleavage to form the p35/34 forms. However, procaspase-9 recruitment and processing are accelerated in lysates supplemented with caspase-3. In lysates containing very low levels of Smac and Omi/HtrA2, XIAP (X-linked inhibitor of apoptosis) binds tightly to caspase-9 in the apoptosome complex, and as a result caspase-7 processing is abrogated. In contrast, in MCF-7 lysates containing Smac and Omi/HtrA2, active caspase-7 is released from the apoptosome and forms a stable ~200-kDa XIAP-caspase-7 complex, which apparently does not contain cIAP1 or cIAP2. Thus, in comparison to caspase-3-containing cells, XIAP appears to have a more significant anti-apoptotic role in MCF-7 cells because it directly inhibits caspase-7 activation by the apoptosome and also forms a stable ~200-kDa complex with active caspase-7.

The MCF-7 cell line was derived from a patient with metastatic breast cancer and is an often-used model system for studying estrogen receptor-positive breast cancer (for review, see Ref. 1). Because many of the problems associated with breast cancer treatment involve the development of chemo-resistance to apoptosis-inducing anti-cancer agents, there is extensive interest in using MCF-7 cells as a model for investigating the mechanisms of apoptosis in breast epithelial cells. Caspase activation is a key event in triggering the morphological and biochemical changes associated with cell death (2–4). There are two primary caspase activation pathways involving either stimulation of cell surface death receptors (the extrinsic pathway) or perturbation of mitochondria (the intrinsic pathway) (5). Many anti-cancer drugs induce apoptosis by activating the intrinsic cell death pathway, which involves the release of cytochrome c and the activation of the apoptosis-catalyzed caspase cascade (6–8). In apoptotic cells inactive procaspases are activated via this cascade mechanism in which an initiator caspase is activated and subsequently cleaves/activates an effector caspase, which then cleaves and activates the next caspase and so on.

Caspase-3 is the most active effector caspase in both the intrinsic and extrinsic pathways, where it is processed and activated by caspase-9 and caspase-8, respectively. However, MCF-7 cells do not possess caspase-3 due to a 47-base pair deletion in the caspase-3 gene (9, 10). Consequently, apoptotic cell death in MCF-7 cells must be independent of caspase-3 activation, although several studies have shown that apoptotic cell death in MCF-7 cells is accompanied by caspase activation. For example, in staurosporine-treated MCF-7 cells, recognized caspase death substrates including poly(ADP-ribose) polymerase, Rb, PAK2, gelsolin, and DFF-45 are cleaved (11). In TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis, caspase-8 is activated, which cleaves Bid to release tBid, which in turn induces cytochrome c release and caspase-9 and caspase-7 processing (12). Other studies have also shown that caspases 9, 6, 2, and 7 are cleaved/processed to their active forms (13). Thus, in the absence of caspase-3, MCF-7 cells can still activate a caspase cascade irrespective of whether the apoptosis is initiated via the intrinsic or extrinsic pathway. The intrinsic pathway, which is activated by many chemicals, including anticancer drugs, involves formation of the Apaf-1 apoptosome, a large caspase-processing complex (for review, see Refs. 6 and 7) that typically activates caspase-3. Apoptosome formation can be modeled in vitro in cell-free lysates by the addition of dATP or ATP and requires at least three apoptotic protease-activating factors (Apaf-1–3) (14–16). The CARD (caspase recruitment) domain of Apaf-1 binds to a similar domain on procaspase-9, whereas the central CED-4 domain (98–412) is involved in Apaf-1 oligomerization. In the presence of cytochrome c and dATP, Apaf-1 undergoes conformational changes, allowing it to oligomerize to form a very large apoptosome complex. Using gel filtration chromatography, we have isolated from both dATP-activated THP-1 and B chronic lymphocytic leukemia cell lysates two apoptosome complexes with apparent molecular masses of ~700 kDa and ~1.4 MDa (17–19). Furthermore, we have shown in apoptotic cells that the ~700-kDa complex predominates and is the most active complex in processing exogenous procaspase-3 (18, 19). More recently, we have used a proteomic approach to characterize the composition of this ~700-kDa apoptosome complex and have shown that it contains solely Apaf-1 and caspase-9 as its core functional proteins (20).

Several studies have reported that caspase-7 is cleaved and activated in dATP stimulated lysates (17, 21, 22), and immuno-depletion studies indicate that caspase-9 is required for the processing of both caspase-3 and -7 (21). By implication, it has been assumed that the Apaf-1-caspase-9 apoptosome complex directly processes procaspase-7 in an analogous manner to that described for procaspase-3. However, there is
no direct evidence for this, and to address this, we have characterized the role of the apoptosome in caspase-7 activation in MCF-7 cells. Our studies show that the ~700-kDa apoptosome complex is also formed in dATP-activated MCF-7 cell lysates and apoptotic MCF-7 cells and directly cleaves and activates procaspase-7. However, although the kinetics of Apaf-1 oligomerization is normal, the recruitment and processing of caspase-9 in the holoenzyme complex is much slower than in caspase-3-containing cell lysates. We also show that caspase-7, after activation by the apoptosome complex, forms a XIAP-caspase-7 complex that is not disrupted even in the presence of Smac and Omi/HtrA2. These data suggest that the absence of caspase-3 enables XIAP to have an enhanced inhibitory effect on effector caspase activation and activity and, hence, a more significant anti-apoptotic role in MCF-7 cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Apoptosis Assays, and Preparation of Control and Caspase-activated Cell Lysates—MCF-7- and MCF-7-transfected cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM Glutamax® as described previously (12). For apoptosis assays, cells were harvested by mild trypsin digestion, and the percentage of apoptotic cells with exposed phosphatidylserine was determined by annexin V-fluorescein isothiocyanate binding (Bender Medsystems, Vienna, Austria) and fluorescence-activated cell sorter essentially as previously described (12). Loss of mitochondrial membrane potential (∆Ψm) was measured with the lipophilic cationic fluorescent probe tetramethylrhodamine ethyl ester and a fluorescence-activated cell sorter scan analysis (23).

For assaying cytochrome c release, cells were resuspended in cold phosphate-buffered saline and permeabilized with 0.025% digitonin, the cell cytosol and membrane fractions were prepared by centrifugation, and the cytochrome c content of the various fractions was then determined by SDS-PAGE and Western blotting (24). Cell lysates (100,000 x g supernatants) from cells were prepared by freeze/thawing (F/T)2 (7, 18). In some experiments, to minimize mitochondrial breakage and release of pro-apoptotic proteins, MCF-7 cell lysates (digitonin and homogenization (Dig/Hom)) were prepared in an isotonic buffer (MSH), containing 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml Pefabloc SCD, one protease inhibitor mixture tablet/10 ml (Roche Diagnostics), pH 7.4, using a modified digitonin/homogenization technique (20). Caspase activation in lysates (10–15 mg/ml) was induced by incubation at 37 °C for various times with 2 mM dATP/MgCl2 plus or minus 2.0 μM cytochrome c.

Preparation of Recombinant Procapases 3 and 7—BL21 (DE3) cells were transformed with a pET-21b (Novagen) plasmid expression vector containing procaspase-3 or -7 cDNA with a C-terminal His6 purification tag and grown at 37 °C in terrific broth, 1% glucose, and ampicillin (100 μg/ml) essentially as described previously (20).

Fluorometric Assays of Caspase and Caspase-activating Activity—DEVDase activity (i.e. primarily caspase-3 and -7) of lysates or column fractions was measured fluorimetrically with 200 μM of assay buffer (20 μM Ac-DEVD-AFC, 0.1% CHAPS, 10 mM dithiothreitol, 100 mM HEPES, and 10% sucrose, pH 7.0) using a Wallac Victor2 1420 Multilabel counter (18). In some experiments the Ac-DEVD-AFC substrate concentration was increased to 100 μM. The caspase processing/activating activity of soluble apoptosome complexes was assayed using purified recombinant procaspase-3 or procaspase-7 (20). Briefly, the method assays the DEVDase activity of the sample, which is a direct measure of how much procaspase-3/-7 has been processed and activated by the apoptosome. In addition, aliquots of the DEVD assay mixtures were diluted 1:1 with 2X SDS loading buffer and analyzed for caspase-3/-7 processing by SDS-PAGE and Western blotting.

Fractionation of Cell Lysates by Gel Filtration—Lysates were fractionated by size-exclusion chromatography on either Superose-6 or Sephacryl S300 columns using a fast protein liquid chromatography (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 dithiothreitol, and 50 mM NaCl, pH 7.0, calibrated with protein standards as previously described (18). Column fractions (0.5–2 ml) were analyzed for DEVDase activity and caspase-3 or -7 processing activity as described above. Aliquots of the column fractions were also analyzed by SDS-PAGE and Western blotting for Apaf-1, caspases 9, 7, and 3 and XIAP. For the Western blot analysis, column fractions 5–17 and 18–30 were run on separate gels, and immunoblotting procedures were carried out in parallel. To ensure uniformity in signal response, the two blots from each column run were exposed simultaneously to Kodak X-Omat film. Appropriate fractions were pooled and concentrated with Vivaspin concentrators (Vivascience AG, Hannover, Germany) before immunoprecipitation experiments with the indicated antibodies.

Affinity Purification of Caspase-7 Complexes—Immunopurification of caspase-7 complexes was carried out using either an anti-active caspase-7 (Cell Signaling, New England Biolabs UK Ltd., Hertfordshire, UK) or anti-XIAP (clone 48, BD Biosciences Pharmingen) antibody covalently bound, respectively, to protein A and G Dyna Beads® (20). Immunopurification of caspase complexes was carried out from MCF-7 cell lysates (~30 mg/ml) that had first been precleared by incubating (1 h at 4 °C) with 400 μl of Sepharose protein A-coated beads/ml of lysate. The precleared lysates were then diluted to 15 mg/ml with assay buffer and dATP-activated for 2 h at 37 °C before fractionation by gel filtration. Appropriate fractions containing active caspase-7 complexes were pooled and concentrated before adding antibody-tagged beads and roller mixing at 4 °C for 4 h. Affinity purified proteins were eluted from the beads with SDS-PAGE sample loading buffer and separated by one-dimensional SDS-PAGE. In some experiments apoptosome complexes were affinity-purified using an anti-caspase-9 antibody covalently attached to protein G Dyna Beads® (20). Apoptosome complexes were captured by adding 20 μl of cross-linked beads to 90–100 μl of dATP-activated MCF-7 cell lysate and roller mixing overnight at 4 °C. Proteins were eluted from the beads as described above.

Reagents and Western Blot Analysis—Cell culture media and materials were as previously described (12, 25). Most other reagents and cells, unless indicated otherwise, were obtained from published sources (20). MCF-7 cells stably transfected with caspase-3 (MCF-7/casp-3) or pcDNA3 vector only (MCF-7/vector) cells were a gift from Dr. Alan Porter, (National University of Singapore) and originally characterized elsewhere (9). Column, cell lysates, and immunopurified samples were analyzed for various proteins by SDS-PAGE and Western blotting as previously described (20). An antibody to procaspase-7 was obtained from BD Biosciences Pharmingen. Antibodies to active caspase-3 and -7 were sourced from Cell Signaling.

RESULTS

Caspase Activation in dATP/Cytochrome c-treated MCF-7 Cell-free Lysates Does Not Require Caspase-3—In this study we wished to investigate apoptosome formation and function in MCF-7 cells. It was, therefore, important to characterize cytochrome c and dATP-dependent caspase activation in the cell lysates. This in vitro model has been used
FIGURE 1. dATP and cytochrome c-dependent caspase activation in MCF-7 cell lysates. In A, MCF-7 cell lysates (10 mg/ml) were activated with 2 mM dATP/MgCl₂ with or without cytochrome c (Cyto c; 2 μM) and incubated at 37 °C for 30 min. The DEVDase activity of cell lysate aliquots (100 μg) was then assayed with Ac-DEVD-AFC as described under “Experimental Procedures” (open bars). The caspase cleaving activity of the lysates was assayed by incubating activated cell lysates with recombinant procaspase-3 (200 nM) for 1 h and followed by assaying for caspase-3 (DEVDase) activity (hatched bars). Data are shown as the mean ± S.E. (n = 3). Aliquots were also taken at the end of the incubations, diluted with 2× SDS-PAGE loading buffer, and analyzed by SDS-PAGE/Western blotting (W.B.) for caspase-9 and -7 (20 μg/lane) and caspase-3 (7.5 μg/lane). In B, the time course of caspase-9, -3, and -7 processing was studied in cell lysates (10 mg/ml) prepared from MCF-7/pcDNA3 vector and MCF-7/casp-3-transfected cells. The cell lysates were incubated with dATP/MgCl₂ and cytochrome c as described above, and at the indicated times, 10-μl aliquots were removed, mixed with 90 μl of SDS-PAGE loading buffer, and analyzed by SDS-PAGE/Western blotting. Note in panel B that a longer exposure (15 min) was used to highlight the appearance of the p22 subunit of caspase-7. **, *, and † indicate protein bands non-specifically reacting with the antibody.
with a number of cell lines, and caspase-3-containing THP.1 cell lysates undergo a marked increase (−30–50-fold) in DEVDase activity (largely due to caspase-3 processing) when activated with dATP/cytochrome c (17). However, dATP/cytochrome c treatment of MCF-7 cell lysates produced only a very small increase in DEVDase activity (Fig. 1A) even though SDS-PAGE/Western blot analysis showed that caspase-9 was processed to its p35/p34 forms and caspase-7 was cleaved to its p19 active form (lane 3, Fig. 1A). In contrast, MCF-7 cell lysates, activated with dATP and then incubated with recombinant procaspase-3, exhibited a marked increase in DEVDase activity that was accompanied by procaspase-3 processing to its p19 and p17 forms (lane 6, Fig. 1A). Caspase-3 markedly influenced the processing of procaspase-9, which was initially cleaved to the p37 (caspase-3-dependent) and p35 forms when the cell lysate was activated by dATP alone (lane 5, Fig. 1A) and then fully processed primarily to the p37 and p35 forms in the presence of dATP/cytochrome c (lane 6, Fig. 1A). Also, caspase-3 accelerated the processing of procaspase-7 to its fully cleaved p19 active form (lane 6, Fig. 1A). Interestingly, in the presence of recombinant procaspase-3, the p32 form of caspase-7 was detected when the cell lysate was activated with dATP alone (lane 5, Fig. 1A). This agrees with previous studies showing that the p32 subunit is formed by caspase-3 cleavage of procaspase-7, which removes the prodomain at Asp-23 (26). The modest increase in DEVDase activity observed in the dATP/cytochrome c-treated MCF-7 cell lysates could possibly be explained by the fact that caspase-7 has a higher $K_m$ and a lower $k_{cat}$ value for the DEVD synthetic peptide substrate (26–28). The results also suggested that caspase activation in MCF-7 cell lysates was slower than in caspase-3-containing lysates. We investigated this by comparing dATP-dependent caspase activation in MCF-7 cells stably transfected with either pcDNA3 (vector) or pcDNA-casp-3. Although in MCF-7/vector cell lysates the p35 subunit was detected 5 min after dATP/cytochrome c activation, processing of the procaspase-9 was not complete until 120 min (lane 7, Fig. 1B). The p34 subunit was not detected until 60 min after dATP/cytochrome c activation, whereas in MCF-7/casp-3 cell lysates, procaspase-9 cleavage was accelerated and almost complete after 10 min (lane 12, Fig. 1B). Thus, the p35 subunit was detected at 5 min, reached a maximum at 30 min, and remained constant until 120 min before declining to barely detectable levels by 360 min. The p37 subunit was detected after the appearance of the p35 subunit at 10 min and was maximal after 30 min before disappearing by 60–120 min.

The time course also showed that caspase-7 activation in MCF-7/vector cell lysates was subsequent to caspase-9 processing, as the p19 subunit of caspase-7 was not detected until 30 min after treating with dATP/cytochrome c (lane 5, Fig. 1B). By this time there was already significant processing of procaspase-9 to its p35 form. The p22 subunit of caspase-7 (see 15-min exposure, Fig. 1B) was also detected, indicating that removal of the prodomain to produce the fully processed p19 form does not require caspase-3. In contrast, in caspase-3-containing cell lysates, caspase-7 processing was faster and more extensive than in the MCF-7/vector cell lysates and was paralleled by rapid and extensive processing of caspase-3 to the p20, p19, and p17 forms.

In MCF-7 Cell Lysates, Apaf-1 Oligomerizes to Form Two Apoptosome Complexes—We next established that Apaf-1 could form a fully functional apoptosome complex in MCF-7 cell lysates. Gel-filtration chromatography of control lysates showed that Apaf-1 eluted as an ~130-kDa monomer (fraction 18–21, Fig. 2A). After dATP/cytochrome c activation, most of the Apaf-1 eluted either as ~700-kDa (fraction 9–14, Fig. 2A) or ~1.4-MDa (fraction 5–8, Fig. 2A) apoptosome complexes. In control cell lysates procaspase-9 (~46-kDa) eluted in fractions 20–24 (Fig. 2A). After dATP activation, both the proform and processed (p35) form eluted in fractions 20–24 (Fig. 2A). However, processed caspase-9 was not associated with either the ~700-kDa or ~1.4-MDa apoptosome complexes. This contrasts with dATP-activated THP.1 cell lysates, where processed caspase-9 was detected in both apoptosome complexes and also as the free form (18).

Next, we immunoblotted the column fractions for caspase-7, and in MCF-7/WT control lysates procaspase-7 eluted predominantly in fractions 20–24 (Fig. 2A), corresponding to a molecular mass of ~60–80 kDa. However, after dATP activation, the active p19 form of caspase-7 eluted in fractions 18–21, which corresponds to a molecular mass of ~200–300 kDa (Fig. 2A). The ~200–300-kDa caspase-7 complex we detected in MCF-7/WT cell lysates did not contain Apaf-1 or caspase-9 (Fig. 2A). To determine whether or not the elution pattern of caspase-7 was different from that of caspase-3, we also fractionated MCF-7/casp-3 lysates by Superose-6 gel filtration chromatography. In these lysates Apaf-1 and caspase-9 exhibited similar profiles of oligomerization and cleavage, respectively, as did the MCF-7/WT lysates (Fig. 2B). Procaspase-3 eluted in fractions 21–24 (Fig. 2B) and is slightly smaller than procaspase-7. However, in dATP/cytochrome c-treated lysates, the active p19/17 forms of caspase-3 eluted in fractions 22–26 and not in the caspase-7-containing fractions (Fig. 2B). This is consistent with the known quaternary structure of active caspase-3, which is a 58–60-kDa tetramer and indicated that active caspase-7 forms a larger ~200-kDa complex with one or more proteins.

The ~700-kDa Apoptosome Complex Directly Processes Procaspase-7—We next investigated the ability of the ~700-kDa and ~1.4-MDa apoptosome complexes to directly activate effector caspases. Column fractions were incubated with procaspase-3, which is cleaved/activated by the Apaf-1-caspase-9 apoptosome. Caspase activation was determined by measuring DEVDase activity, which was predominant in fractions 9–14 (Fig. 3A). These fractions corresponded to the ~700-kDa apoptosome complex, as determined by their Apaf-1 content (Fig. 2A). Those fractions corresponding to the ~1.4-MDa apoptosome complex did not activate procaspase-3, and column fractions taken from fractionated non-activated cell lysates also did not activate this procaspase (Fig. 3A). From this we concluded that the ~700-kDa complex was the only active apoptosome complex. The fact that it processed and activated procaspase-3 indicated that it should also contain caspase-9. However, we were unable to detect caspase-9 in the column fractions (Fig. 2A), as possibly the concentration of apoptosis was too low. To show that this was an Apaf-1-caspase-9 holoenzyme complex, we used an antibody to caspase-9 that only immunoprecipitates Apaf-1 when caspase-9 is a constituent part of an active apoptosome holoenzyme complex (20). The anti-caspase-9 antibody immunoprecipitated the p35 form of caspase-9 and Apaf-1 from the ~700-kDa complex (Fig. 3B) but did not capture Apaf-1 from the ~1.4-MDa complex (Fig. 3B). We then incubated the respective apoptosome complexes with procaspase-7 (Fig. 3C) or procaspase-3 (data not shown) and assayed for DEVase activity using 100 μM Ac.DEVD.AFC (optimal concentration for assaying caspase-7 activity) and immunoblotted for active caspase-7 subunits. The ~700-kDa apoptosome complex directly processed and activated procaspase-3 (Fig. 3A) and procaspase-7 (Fig. 3C), as shown by the stimulation of DEVase activity and the generation of the p19 subunit (Fig. 3C). The ~1.4-MDa complex had little or no caspase-processing ability with either procaspase-3 or -7. Thus, the ~700-kDa apoptosome complex directly processes and activates procaspase-7. Interestingly, without caspase-3 it was still possible to generate the p19 subunit of caspase-7 (lane 4, Fig. 3C). Cleavage of the pro-domain or N-peptide of procaspase-7 at Asp-23 is believed to catalyze by caspase-3. However, in caspase-3 null MCF-7/WT cells the ~700-kDa
FIGURE 2. Apoptosome formation in MCF-7 cell lysates is accompanied by the formation of an ~200-kDa active caspase-7 complex. MCF-7/WT and MCF-7/casp-3 cell lysates (15 mg/ml) were activated with 2 mM dATP/MgCl₂ and cytochrome c (Cyt c, 2 μM) for 30 min at 37 °C. The lysates were then separated by Superose-6 gel-filtration chromatography, and the column fractions were assayed for DEVDase activity and for Apaf-1 and caspase-9, -3, and -7 by SDS-PAGE and Western blotting (W.B.) as described under "Experimental Procedures." Panel A shows in MCF-7/WT cell lysates oligomerization of Apaf-1 (un-oligomerized) to form ~1.4-MDa and ~700-kDa apoptosome complexes, with the attendant caspase-9 and -7 processing. The same analysis was carried out for cell lysates prepared from MCF-7 cells, stably transfected with caspase-3. Essentially similar elution profiles to the MCF-7/lysate were obtained for Apaf-1, caspase-9, and caspase-7. However, in MCF-7/casp-3 lysates, active caspase-3 was detected and eluted later than active caspase-7, demonstrating that active caspase-3 has a lower molecular mass than active caspase-7, which appears to be complexed to one or more other proteins (active caspase-7 complex). As in Fig. 1, **, *, and † indicate protein bands non-specifically reacting with antibody.
FIGURE 3. In MCF-7/WT cell lysates procaspase-7 is directly cleaved and activated by the ~700-kDa apoptosome complex. In A, MCF-7/WT cell lysates (15 mg/ml) were activated with 2 mM dATP/MgCl₂ and cytochrome c (Cyto c, 2 μM for 30 min), and 3 mg of lysate was separated on a Superose-6 gel filtration column. Aliquots (50 μl) of each fraction were incubated with 200 nM procaspase-3 at 37°C, then analyzed for DEVDase activity. In B, fractions 5–7 and 10–15, corresponding, respectively, to the ~1.4-MDa and ~700-kDa apoptosome complexes, were pooled and concentrated to 100 μl. The concentrated complexes were then incubated overnight with an anti-caspase-9 antibody cross-linked to Dyna beads as described under “Experimental Procedures.” The beads were washed 4 times and then eluted in 50 μl of 2× SDS-PAGE loading buffer before being analyzed by SDS-PAGE/Western blotting (W.B.). I.P., immunoprecipitate. The input, supernatant, and elution volumes loaded onto the gel were equivalent to 6.2, 7.5, and 36%, respectively, of the original concentrated fractions. In C, the pooled 1.4-MDa and ~700-kDa fractions were concentrated to 250 μl, and 50 μl of each fraction was incubated with procaspase-7 for 1 h at 37°C. Ac-DEVD.AFC was then added to a final volume of 200 μl to give a final substrate concentration of 100 μM for caspase-7. At the end of the assay, 50 μl of the reaction mixture was diluted 1:1 with 2× SDS-PAGE loading buffer and analyzed by SDS-PAGE/Western blotting.
apoptosome complex clearly catalyzes the formation of the p22 subunit of caspase-7, which then autocatalytically cleaves off the prodomain to produce the p19 subunit.

Detection of the ~700-kDa Apoptosome Complex in Apoptotic MCF-7 Cells—To determine whether the ~700-kDa apoptosome complex is also formed in apoptotic MCF-7 cells, we treated MCF-7 cells with staurosporine, which is a well characterized inducer of cell death. Staurosporine induced apoptosis, as assessed by time-dependent poly(ADP-ribose) polymerase cleavage, and a decrease in mitochondrial membrane potential (ΔΨm) to increased phosphatidylserine exposure as described under “Experimental Procedures.” The Western blots (W.B.) show caspase-dependent cleavage of the apoptotic specific poly(ADP-ribose) polymerase protein substrate to the cleaved p85 form and release of cytochrome c (Cyto c) from the mitochondria (mitos) to the cytosol. In 6B cells were seeded in large flasks (3 per treatment) at 5 × 10^6 cells per flask and cultured for 18 h. The cells were then incubated for 1 h plus or minus Z-VAD.fmk (100 μM) before inducing apoptosis with staurosporine (1 μM). After 6 h the cells were harvested and resuspended in 150 μl of cell lysis buffer, and cell-free lysates were produced by freeze/thawing as described under “Experimental Procedures.” The lysates were then separated by Superose-6 gel filtration as described in Fig. 3, and the fractions were analyzed for Apaf-1 and caspase-9 (data not shown) and caspase-7 (see also Fig. 6D) by SDS-PAGE and Western blotting. The fractions corresponding to the 1.4-MDa and ~700-kDa apoptosome fractions were pooled and concentrated to 110 μl and then incubated overnight with anti-caspase-9 antibody cross-linked to Dyna beads as described in Fig. 3. The washed beads were then eluted with SDS-PAGE loading buffer and analyzed for Apaf-1 and caspase-9 by SDS-PAGE and Western blotting. The input, supernatant, and elution volumes loaded onto the gel were equivalent to 6.0, 6.0, and 66.0%, respectively, of the pooled and concentrated fractions. IP, immunoprecipitate.

Using this technique, we were able to detect the ~700-kDa apoptosome complex in lysates isolated from staurosporine-treated cells (lane 8, Fig. 4B). Furthermore, when we inhibited caspase activity with Z-VAD.fmk, increased amounts of Apaf-1 were immunoprecipitated from those fractions corresponding to the ~700-kDa complex (lane 9, Fig. 4B). Interestingly, in the presence of Z-VAD.fmk we detected both the pro and p35 forms of caspase-9 bound to the ~700-kDa apoptosome complex. We were unable to detect the 1.4-MDa apoptosome complex in apoptotic MCF-7 cell lysates (Fig. 4B).

XIAP Regulates the MCF-7 ~700-kDa Apoptosome Complex—Previously, in THP.1 cell lysates we have shown that the proapoptotic proteins Smac and Omi/HtrA2 regulate XIAP binding to the apoptosome (20). Thus, in Smac- and Omi/HtrA2-free lysates the apoptosome contains Apaf-1, caspase-9, caspase-3, and XIAP, whereas in the presence of Smac and Omi the apoptosome complex contains only caspase-9 and Apaf-1. MCF-7 cell lysates prepared by F/T contained substantial amounts of Smac and Omi/HtrA2 (Fig. 5A). In contrast, a combination of digitonin permeabilization and homogenization (Dig/Hom) produces cell lysates with very low or negligible levels of cytochrome c, Smac, and Omi/HtrA2 (Fig. 5A). These lysates still respond to dATP/cytochrome c and process/activate recombinant procaspase-3 as shown by the increase in DEVDase activity (lane 6, Fig. 5B). However, F/T lysates exhibited a ~2-fold greater increase in procaspase-3 activating activity (lane 3, Fig. 5B), indicating that the caspase activating activity of the Dig/Hom lysates was inhibited. We, therefore, used the anti-caspase-9 immunoprecipitation method to assess apoptosome composition in the two types of lysate. In the F/T lysates, apoptosome formation as determined by the ability of the anti-caspase-9 antibody to capture Apaf-1 was essentially maximal at 15 min and was accompanied
FIGURE 5. In Smac- and Omi-free lysates, XIAP binds to the MCF-7 apoptosome. Lysates were prepared by either F/T or Dig/Hom as described under “Experimental Procedures.” In A, the respective lysates and cellular fractions were analyzed for cytochrome c (Cyto c), Smac, and Omi/HtrA2. In B, F/T and Dig/Hom lysates (10 mg/ml) were activated with 2 mM dATP/MgCl₂ and cytochrome c (2 μM) for 30 min at 37 °C. An aliquot was taken for the DEVDase activity assay (open boxes), and the remaining lysate was incubated with procaspase-3 (200 nM) for 1 h and before analyzing again for the DEVDase activity. In C and D, lysates (15 mg/ml) were precleared with Sepharose protein G beads and then activated with 2 mM dATP/MgCl₂ and cytochrome c (2 μM) from 10 to 30 min at 37 °C. At the indicated time caspase activation was terminated with Z-VAD.fmk (20 μM) and an aliquot (20 μg) was taken for the input sample; the remaining lysate (100 μl) was incubated overnight at 4 °C with 20 μl of anti-caspase-9 complexed to Dyna beads. The supernatants were removed, and the beads were washed 4 times before eluting in 30 μl of SDS-PAGE loading buffer, which was analyzed by immunoblotting for Apaf-1, caspase-9, and XIAP. The input, supernatant, and elution volumes loaded onto the SDS-PAGE gels were 1, 2, and 66%, respectively, of the pooled and concentrated fractions. In C, an additional immunoblot is shown for caspase-7, which was only detected in the eluate fractions from the Dig/Hom lysates and only when the blot (W.B.) was overexposed. The asterisk refers to a nonspecific band, which is mitochondrial Hsp60 (D. Twiddy, G. M. Cohen, M. MacFarlane, and K. Cain, unpublished data) and is detected in the F/T but not the Dig/Hom lysates. In E, F/T and Dig/Hom lysates were fractionated as described in Fig. 2, and the fractions (15–22), which should contain the active caspase-7 complex, were analyzed for the p19 form of caspase-7.
FIGURE 6. Active caspase-7 forms an ~200–300-kDa complex that is distinct from active caspase-3. As described in Fig. 2, MCF-7/WT and MCF-7/casp-3 cell lysates (15 mg/ml) were activated with 2 mM dATP/MgCl₂ and cytochrome c (2 μM) for 30 min at 37 °C. The lysates were separated by Superose-6 gel-filtration chromatography, and column fractions were assayed for DEVDase activity. In A, the DEVDase activity for the MCF-7/WT and MCF-7/casp-3 fractions is shown as open and solid symbols, respectively. Unactivated (Control) and cytochrome c/dATP-activated (Cyt c/dATP) lysates are shown as □ and ○ and as ◐ and ●, respectively. Note the different y axis scales, respectively, for the MCF-7/WT and MCF-7/casp-3 fractions.
by processing of caspase-9 to form the p35 subunit (Fig. 5D). A similar
time course for apoptosis formation was seen in the Dig/Hom lysates
(Fig. 5C). Thus, the decreased caspase-processing activity of the Dig/
Hom lysates was not due to reduced apoptosis formation or caspase-9
processing.

We next immunoblotted for XIAP, which interacts with the ATPF
motif of the p12 subunit of caspase-9, generated by autocatalytic cleav-
age of procaspase-9 at Asp-315 (30). In Dig/Hom lysates, co-prefrac-
tion with XIAP with Apaf-1 and caspase-9 was detected within 1 min of
initiating caspase activation with dATP/cytochrome c (Fig. 5C). Fur-
thermore, the XIAP-Apaf-1-caspase-9 holoenzyme complex was stable
for at least 30 min. In marked contrast, in F/T lysates, XIAP did not
co-precipitate with Apaf-1 and caspase-9 (Fig. 5D).

We also immunoblotted for caspase-7 in both F/T and Dig/Hom
lysates and were unable to detect significant amounts of caspase-7 in the
apoptosome complex (results not shown). However, using heavily
exposed immunoblots, we detected very small amounts of active
caspase-7 associated with the apoptosome complex as isolated from
Dig/Hom lysates (see the 30-min time point, Fig. 5C). Furthermore, the
majority of the processed caspase-7 (p19 subunit) was detected in the
supernatant fractions (Fig. 5C). Thus, although caspase-7 processing is
abrogated in Dig/Hom lysates, it is clear that the cleaved form of
caspase-7 is still not bound to the apoptosome. Therefore, we analyzed
the Dig/Hom lysate by gel filtration (Fig. 5E) and found that small
amounts of processed caspase-7 were instead associated with the
~200–300 kDa complex (see Fig. 2). This is very different from
caspase-3, which in the absence of Smac and Omi/HtrA2 binds to the
apoptosome (20) by simultaneous binding to caspase-9 and XIAP (22).
However, in the absence of Smac and Omi/HtrA2, XIAP binds to and is
an integral component of the MCF-7 Apaf-1-caspase-9 apoptosis
complex. Thus, our results show that caspase-7 is not stably associated
with the apoptosome, irrespective of the presence of XIAP.

Processed Caspase-7 Eluates as an ~200–300-kDa Complex—The predicted molecular mass of tetrameric active caspase-7 in dATP-acti-
vated and apoptotic cell lysates is ~60 kDa. However, the elution behav-
ior of active caspase-7 in MCF-7 cell lysates indicated that it was com-
plexed with other proteins (Fig. 2). In this respect previous studies have
suggested that caspase-7 after activation can bind to other proteins and
subcellular organelles (31, 32). Our gel filtration results indicated that
caspase-7 is cleaved/activated by the ~700-kDa apoptosome and then
redistributes and binds with a protein(s) to form a ~200–300-kDa com-
plex (Fig. 2). In this respect caspase-7 appeared to be quite different
from caspase-3, and to confirm this conclusion we analyzed column
fractions from fractionated MCF-7/WT and MCF-7/casp-3 lysates for
DEVDase activity. In dATP-activated MCF-7/WT lysates a small peak
of DEVDase activity eluted in fractions 18–21 (Fig. 6A). In contrast, the
DEVDase activity of dATP-activated MCF-7/caspase-3 lysates was
much higher (~10–15-fold) than the MCF-7/WT lysates and eluted in
fractions 22–26 (Fig. 6A).

To verify that cleaved caspase-3 and -7 eluted differently on the gel
filtration column, we probed the fractions with alternative antibodies
that were specific to active caspase-7 and -3 and also to procaspase-7.
The immunoblots showed that the p19 form of caspase-7 eluted in
MCF-7/WT and MCF-7/caspase-3 lysates in fractions 17–22 (peaking
in fraction 19, Fig. 6, B and C). In contrast, in the caspase-3-containing
lysates, the p19/p17 active caspase-3 subunits eluted later, in fractions
22–26 (Fig. 6C). Significantly, procaspase-7 in control MCF-7/WT and
MCF-7/casp-3 lysates eluted predominantly in fractions 20–24 (peak-
ing in fraction 21). Together with our previous data (Fig. 2) these results
demonstrated unequivocally that processed caspase-3 and -7 eluted as
different sized protein complexes.

The ~200–300-kDa complex was also detected in lysates obtained
from apoptotic MCF-7/WT cells treated for 6 h with staurosporine (Fig.
6D). Control lysates did not contain this complex and, significantly,
Z-VAD.fmk abolished caspase-7 processing and formation of the
~200–300-kDa complex. Because the active caspase-7 complex was
also formed in apoptotic cells, we wanted to carry out a proteomic
analysis of this complex. Therefore, we used gel filtration chromato-
graphy to partially purify the complex, which was then immunoprecipi-
tated with the active anti-caspase-7 antibody. The p19 form of caspase-7
was identified by liquid chromatography-tandem mass spectroscopy
data (not shown) and immunoblotting (lane 6, Fig. 7A) in the complexes
prepared from dATP-activated but not heat-activated lysates. However,
mass spectrometry analysis of other captured proteins revealed that
there were no significant differences between the heat- and dATP-acti-
vated samples (data not shown).

Identification of the ~200–300-kDa Complex as a XIAP-Caspase-7
Complex—IAP proteins are potential candidates for binding to active
caspase-7, and we therefore probed the ~200–300-kDa caspase-7 com-
plex for XIAP, cIAP1, and cIAP2. XIAP was not detected in the immu-
noprecipitated complex (lane 6, Fig. 7A), and cIAP1 and cIAP2, which
were detected in the input fractions of both control and dATP-activated
samples, were also not present in the eluate fractions (lane 6, Fig. 7A).
However, some faint nonspecific bands (asterisks) were detected with
the cIAP1 and cIAP2 antibodies in both the control and dATP-activated
samples (lanes 5 and 6, Fig. 7A). Significantly, the intensity of these bands
did not vary or correlate with the intensity of the p19 subunit of
caspase-7. The cIAP1-nonspecific band runs very close to the expected
position of cIAP1, so we performed a second pull-down using cell
lysates. In this experiment (see Supplemental Fig. 1) we extended the
running time on the SDS-PAGE and achieved a clear separation
between the nonspecific band in the eluate and the cIAP1 band in the
input and supernatant fractions.

It was possible that the anti-caspase-7 antibody that binds to a neo-
epitope in the active site could displace proteins (e.g. XIAP) which were
binding to the active site of caspase-7. So we again purified the ~200–
300-kDa complex by gel filtration and then immunoaffinity-purified
the complex with an antibody to XIAP. We then immunoblotted the pull-
downs for caspase-7, XIAP, cIAP1, and cIAP2, and this showed that
XIAP was complexed with caspase-7 but not with cIAP1 or cIAP2 (lane
6, Fig. 7B). Furthermore, Smac was not detected in the eluted fractions,
and in the absence of dATP and cytochrome c, the XIAP antibody still
pulled XIAP (Fig. 7B, compare lanes 5 with 6) but did not co-precipitate
the p19 large subunit of caspase-7. Thus, after processing by the apo-
ptosome, caspase-7 forms a stable complex with XIAP but not with
cIAP1 or cIAP2.

DISCUSSION

In this study we have characterized the caspase-7-activating apopto-
some complex in apoptotic MCF-7 cells and in dATP/cytochrome c-ac-
activated MCF-7 cell lysates. The rate of Apaf-1 oligomerization to form the ~700-kDa apoptosome complex is identical to that observed in caspase-3 containing THP-1 and Jurkat cell lysates (18, 20, 33). To form a fully active caspase-processing holoenzyme complex, caspase-9 must bind to the apoptosome. However, in MCF-7 cell lysates we had to immunoprecipitate caspase-9 from column fractions before we were able to detect caspase-9 in the apoptosome fractions (Fig. 3B and 4B). This apparent absence of caspase-9 in the ~700-kDa apoptosome complex may simply be a detection problem due to relatively low levels of apoptosome complex in the MCF-7 cells. Alternatively it may imply that the apoptosome complexes are heterogeneous, containing varying amounts of caspase-9. In this respect the structure of caspase-9 in the apoptosome complex is still poorly understood (for review, see Refs. 7, 8, and 34). Procasapase-9, unlike effector caspases, has an unusually long flexible linker peptide which allows active site formation that gives thezymogen low but significant cleavage activity. This is markedly enhanced when caspase-9 is bound to the apoptosome in dATP/cytochrome c-activated cell lysates (18, 35, 36). Interestingly, non-cleavable caspase-9 mutants will also process procaspase-3 when incubated with dATP/cytochrome c-activated cytosol (22, 35). This observation can be explained by the “induced-proximity model,” which proposes that monomeric procaspase-9 dimerizes at high concentrations, forcing anallosteric rearrangement of one of the monomers in the dimer to produce a catalytically active site (37, 38). It is suggested that Apaf-1 recruitment of caspase-9 facilitates caspase-9 dimerization/activation and that caspase-9 cleavage stabilizes the dimer (39). However, caspase-9 recruitment to the apoptosome is accompanied by simultaneous processing of the proform, as the p35 subunit was detected within 1–5 min of dATP/cytochrome c activation of MCF-7 cell lysates (Fig. 1B and Fig 5, C and D)). Significantly, caspase-7 processing was not detected until 30 min post-dATP activation and always lagged behind that of caspase-9 irrespective of the presence of caspase-3. Interestingly, in lysates isolated from apoptotic cells, Z-VAD.fmk seemed to freeze or stabilize procaspase-9 in the apoptosome. This suggests that caspase-9 processing destabilizes its binding to the apoptosome, resulting in the release of free caspase-9.

In the current study we provide new insights to the processing and structure of caspase-7 in cell lysates. Crystallographic studies on recombinant caspases 1, 3, and 7 have shown that the catalytically active enzymes are heterotetramers (40–42) and at physiological concentrations form stable inactive homodimers (17, 18, 43, 44). Cleavage of the linker peptide loop produces the large and small subunits of the heterotetramer, inducing conformational changes that form the functional active site. Although recombinant caspase-7 and caspase-3 elute on gel
filtration columns with the predicted molecular mass of ~60 kDa (Ref. 17 and data not shown), our current study shows that a very different picture exists in cellular lysates (even if they contain Smac and Omi/HtrA2). Furthermore, there are marked differences between caspases 3 and 7. First, the elution pattern of procaspase-3 and its processed form (Fig. 2A and Fig. 6) is consistent with a molecular mass of ~60 kDa and agrees with studies on recombinant proteins. However, processing of procaspase-7 to produce the p19 form of caspase-7 is accompanied by a marked shift in the size of the cleaved protein, which elutes as an ~200-kDa complex (Fig. 2A and Fig. 6). This complex was detected in both MCF-7/WT (casp-3 null) and MCF-7/casp-3 cells using two separate antibodies to caspase-7. Furthermore, immuno-affinity purification established that the ~200-kDa complex contained XIAP bound to the active form of caspase-7 (Fig. 7). The stability of this interaction seems to be very high as it was not disrupted by the presence of Smac or Omi/HtrA2 in the lysates.

The role of XIAP in mammalian apoptotic cell death is still controversial even though there is evidence that some apoptic and effector caspases are regulated by IAPs (45). In Drosophila, caspase inhibition by DIAP1 is essential for survival (46). However, the loss of XIAP in mammalian cells does not result in a significant phenotype (47), although overexpression of XIAP suppresses apoptosis induced by various stimuli (48). XIAP inhibits caspases 9, 3, and 7 via interactions between processed caspases and the BIR2 and BIR3 domains, and some IAPs can regulate effector caspases by binding to the catallytically active site. However, recent studies show that XIAP inhibits caspase-7 activity by multiple interactions between the N-terminal linker region of the BIR2 domain and the active site of caspase-7 and by binding to Ala-Asn-Pro, an IAP binding motif (IBM) that is revealed by cleavage at Asp-206 (49). In Drosophila, DIAP1 binds to an IBM located at the N terminus of the large subunit of Drosophila-related interleukin-converting enzyme (50), whereas cIAP1 can also bind to caspase-7 via an N-terminal Ala-Lys-Pro (AKP) IBM generated by cleavage of procaspase-7 at Asp-23. Thus, XIAP can potentially bind to the active site of caspase-7 and two separate IBM motifs. Removal of the prodomain at Asp-23 to reveal the AKP motif can be catalyzed by caspase-3 before Asp-198 cleavage by an apical caspase or granzyme B (51). However, we and others have found that active caspase-7 in vitro can also remove its own prodomain in dATP-activated MCF-7 lysates after cleavage at Asp-198 (Fig. 1 (26)). Furthermore, recombinant procaspase-7 is processed by the ~700-kDa apoptosome complex to yield both the p19 and p22 forms of caspase-7 (Fig. 3C). Interestingly, the p22 form of caspase-7 was not found in the ~200–300-kDa complex and instead eluted as a smaller-sized complex, indicating that it was probably not complexed with other proteins (fractions 22–24, Fig. 6B). This suggests that removal of the prodomain is required for forming the 200–300-kDa complex. Thus, in the absence of caspase-3, apoptosome-dependent activation of caspase-7 involves cleavage at Asp-198 followed by autocatalytic cleavage at Asp-23. The exposed AKP (IBM) sequence could in theory bind cIAP1, but we could not detect this IAP in the immunoaffinity-purified ~200-kDa complex (Fig. 7A). Indeed, XIAP was the only IAP bound to caspase-7, and furthermore, an antibody that was raised to the active site of caspase-7 displaced XIAP in the immunoprecipitation experiments (Fig. 7A), demonstrating that XIAP interacts with active site of caspase-7. Although this confirms the presence of an active caspase-7/XIAP complex, it does not exclude the possibility that cIAP1/cIAP2 could also form separate complexes by binding to the active site of caspase-7.

The absence of caspase-3 in MCF-7 cells inevitably means that the response of these cells to apoptotic stimuli is markedly altered, as they now rely on caspase-7 to be the primary executioner caspase. In a recent study, 75% of breast tumors lacked caspase-3 transcript and protein expression (52). Thus, the MCF-7 cell line seems to be a good cell model for this particular disease, and significantly, reconstitution of caspase-3 in this cell line augments the apoptotic response of these cells to doxorubicin and other apoptotic stimuli (52, 53). In this context we have delineated the activation and fate of caspase-7 during apoptosis-dependent cell death in caspase-3 null MCF-7 cells. In these cells, the rapid process of Apaf-1 oligomerization to form the ~700-kDa apoptosome complex is similar to caspase-3-containing cells. After binding to the apoptosome, procaspase-9 is cleaved to yield the processed p35 form of caspase-9. This process is slower in MCF-7/WT cells when compared with MCF-7/casp-3 cells. Similarly, in the absence of caspase-3, although the ~700-kDa Apaf-1-caspase-9 apoptosome complex catalyzes the processing of procaspase-7 to its active p19 form, it does so relatively slowly. Significantly, after processing, active caspase-7 does not associate with the apoptosome because, even in Smac/Omi-containing cells, it has such a high affinity for XIAP that it forms a relatively stable ~200-kDa XIAP-caspase-7 complex. This suggests that XIAP has a more pronounced anti-apoptotic effect in MCF-7 cells than it does in caspase-3-containing cells. The combination of slow processing/activation of caspase-7 and its strong binding to XIAP may explain in part why MCF-7 cells are relatively insensitive to apoptotic stimuli.

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