Executioner Caspase-3, -6, and -7 Perform Distinct, Non-redundant Roles during the Demolition Phase of Apoptosis*

Elizabeth A. Slee, Colin Adrain, and Seamus J. Martin‡

From the Molecular Cell Biology Laboratory, Department of Genetics, The Smurfit Institute, Trinity College, Dublin 2, Ireland

Abstract

Apoptosis is orchestrated by a family of cysteine proteases known as the caspases. Fourteen mammalian caspases have been identified, three of which (caspase-3, -6, and -7) are thought to coordinate the execution phase of apoptosis by cleaving multiple structural and repair proteins. However, the relative contributions that the “executioner” caspases make to the demolition of the cell remains speculative. Here we have used cell-free extracts immuno-depleted of either caspase-3, -6, or -7 to examine the caspase requirements for apoptosis-associated proteolysis of 14 caspase substrates as well as nuclear condensation, chromatin margination, and DNA fragmentation. We show that caspase-3 is the primary executioner caspase in this system, necessary for cytochrome c-mediated cleavage of fodrin, gelsolin, U1 small nuclear ribonucleoprotein, DNA fragmentation factor 45 (DFF45), inhibitor of caspase-activated DNase (ICAD), receptor-interacting protein (RIP), X-linked inhibitor of apoptosis protein (X-IAP), signal transducer and activator of transcription-1 (STAT1), topoisomerase I, vimentin, Rb, and lamin B but not for cleavage of poly(ADP-ribose) polymerase (PARP) or lamin A. In addition, caspase-3 was also essential for apoptosis-associated chromatin margination, DNA fragmentation, and nuclear collapse in this system. Surprisingly, although caspase-6 and -7 are considered to be important downstream effector caspases, depletion of either caspase had minimal impact on any of the parameters investigated, calling into question their precise role during the execution phase of apoptosis.

Apoptosis is coordinated by a family of cysteine proteases, the caspases, which are activated upon receipt of divergent pro-apoptotic stimuli (1–6). Fourteen caspases have been identified in mammals, a subset of which are thought to be directly involved in the maturation of pro-inflammatory cytokines (3, 4, 6, 7). Caspases are synthesized as relatively inactive pro-caspases (zymogens) that require proteolytic processing for activation (3, 4, 6, 8). Caspase zymogens are typically activated at the point of recognition (CARDs), death effector domains (DEDs)) that are also present in molecules such as Fas-associated protein with death domain (FADD) or Apaf-1,1 which promote caspase autoactivation, whereas those further downstream are directly activated by apical caspases (6, 7, 9–11).

Active caspases promote apoptosis in several ways: by activating other destructive enzymes such as DNases, by promoting mitochondrial cytochrome c release via Bcl-2 family proteins such as BID, and by degrading key structural and regulatory proteins within the cell (3, 4, 7, 12). Thus far, numerous caspase substrates have been identified, and work is still ongoing to link the caspase-mediated proteolysis of particular substrates with phenotypical changes that take place during apoptosis.

Caspases implicated in apoptosis have been divided into two functional sub-groups based upon their perceived roles in this process. Upstream or apical caspases are those that are responsible for initiating the caspase cascade by becoming aggregated upon receipt of a pro-apoptotic stimulus. The latter caspases (caspase-2, -8, -9, -10) tend to have long N-terminal regions (prodomains) with motifs (caspase recruitment domains (CARDs), death effector domains (DEDs)) that are also present in molecules such as Fas-associated protein with death domain (FADD), Apaf-1, and RIP-associated Ichi-1/CED homologous protein with death domain (RAIDD), which promote their aggregation (6, 7, 9–11). The second group are downstream, or executioner, caspases (caspase-3, -6, -7) that are thought to be responsible for the actual destruction of the cell and tend to have short or absent prodomains. Much progress has been made in dissecting the routes to activation of the apical caspases, particularly in the context of apoptosis triggered by ligands of the tumor necrosis factor/nerve growth factor receptor superfamily (13). However, in sharp contrast, the contribution that each executioner caspase makes to the execution phase of apoptosis remains speculative. In particular, although caspase-6 and -7 are widely regarded to act as executioner caspases, their role during the execution phase of apoptosis is obscure.

Studies using cells from CASP-3 null mice as well as transformed cell lines devoid of caspase-3 have shown that caspase-3 is important for events such as DNA degradation, nuclear condensation, plasma membrane blebbing, and proteolysis of certain caspase substrates (14–17). However, some or all of the observed defects in caspase-3-deficient cells could be due to a failure to activate downstream caspases in the absence of caspase-3. In general, studies that have linked cleavage of a 1 The abbreviations used are: Apaf-1, apoptotic protease-activating factor-1; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); PARP, poly(ADP-ribose) polymerase; U1snRNP, U1 small nuclear ribonucleoprotein; RIP, receptor-interacting protein; STAT1, signal transducer and activator of transcription-1; X-IAP, X-linked inhibitor of apoptosis protein; DFF40/45, DNA fragmentation factor 40/45; PAGE, polyacrylamide gel electrophoresis.

* This work was supported by Wellcome Trust Senior Fellowship Award 047580 (to S. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Wellcome Trust Senior Fellow. To whom correspondence should be addressed. Tel.: 353-1-608 1289; Fax: 353-1-679 8558; E-mail: martinj@tcd.ie.
completed cell-free extracts. A. Jurkat cell-free extracts were prepared and immunodepleted of specific caspases as described under “Experimental Procedures.” Equal volumes (5 μl) of either mock-, caspase-3-, caspase-6-, or caspase-7-depleted extracts were separated by SDS-PAGE followed by immunoblotting for the indicated proteins. B, cell-free extracts, prepared from either Jurkat or MCF-7 cells, were incubated for 2 h at 37 °C in the presence or absence of 50 μg/ml cytochrome c and 1 mM dATP, as indicated. Reactions were then analyzed for caspase-3 by Western blot. Caspase-3 expression and cytochrome c/dATP-inducible processing is readily detected in Jurkat cells but not MCF-7 cells. C, Jurkat cell-free extracts, either mock-depleted or depleted of the indicated caspases, were incubated for 2 h at 37 °C with or without cytochrome c (50 μg/ml) and dATP (1 mM). Reactions were then analyzed by SDS-PAGE and immunoblotted for caspase-2, -3, -6, and -7 or actin as a loading control. Active forms of caspase-3, -6, and -7 were difficult to detect reproducibly with the antibodies (Ab) used, but disappearance of their proforms was readily detected. D, hierarchy of cytochrome c-inducible caspase activation events in Jurkat cell-free extracts.

As a step toward dissecting the execution phase of apoptosis, we have generated cell-free extracts devoid of individual executioner caspases to explore the impact of their removal on events that take place during the demolition phase of apoptosis. We used a cell-free system based on post-nuclear extracts, prepared from viable Jurkat cells, in which apoptosis can be induced by the addition of cytochrome c and dATP. It is well established that cytochrome c/dATP promote caspase activation via the CED-4 homologue Apaf-1 (18–22). Upon activation by Apaf-1, caspase-9 then propagates a caspase cascade, which results in the stepwise activation of caspase-3, -7, -2, -6, -8, and -10 (Refs. 7 and 22; see Fig. 1). Using Jurkat extracts immunodepleted of either caspase-3, -6, or -7, we have assessed the impact of depletion of the latter caspases on events that typically occur during the terminal phase of apoptosis. We show that caspase-3 is the primary executioner caspase, necessary for cleavage of the majority of the substrates examined (with the exception of PARP and lamin A), as well as DNA fragmentation and nuclear collapse. Surprisingly, depletion of caspase-6 or -7 from cell extracts had minimal impact on any of the parameters investigated, calling into question their role during the execution phase of apoptosis.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-caspase-3, caspase-7, RIP, X-IAP, STAT1, calcium/calmodulin-dependent protein kinase IV, and gelsolin monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Anti-caspase-6 and anti-DFF45 antibodies were from Upstate Biotechnologies (Lake Placid, NY); anti-α-fodrin monoclonal antibodies were from Chemicon International (Temecula, CA); anti-α-intermediate filament antibody was purchased from Roche Molecular Biochemicals and Pharmingen, anti-lamin B, anti-PARP, anti-topoisomerase I, and anti-U1snRNP human polyclonal autoantibodies were kindly provided by Dr. Eng Tan (Scripps Clinic, La Jolla, CA).

Preparation of Cell Extracts—Jurkat extracts were prepared as described previously (22, 23). Briefly, cells were lysed by homogenization with ~40 strokes of a B-type pestle after incubation in ice-cold cell extract buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Crude extracts were then centrifuged for 30 min at 15,000 × g to remove nuclei, unbroken cells, and other debris. Post-nuclear extracts were then used for immuno-depletions as described below. MCF-7 extract was prepared in the same manner except that the MCF-7 cells were lysed by three rounds of freeze-thaw on liquid nitrogen before centrifugation at 15,000 × g for 15 min.

Immuno-depletion of Extracts—Jurkat extracts were immunodepleted of specific caspases as described previously (22, 23). Briefly, 40 μl of a 50% slurry of protein A/G-agarose beads (Santa Cruz Biotechnology) were coated with 5 μg of anti-caspase antibody by continual rotation for 3 h at 4 °C in phosphate-buffered saline, pH 7.2 (final volume, 200 μl). Purified rabbit or mouse IgG (Sigma) was used as a control. Antibody-coated beads were then washed twice with phosphate-buffered saline, pH 7.2, before adding to cell extracts. Cell extracts (100 μl) were immuno-depleted overnight at 4 °C under constant rotation, followed by removal of antibody-coated beads by centrifugation. Depleted extracts were used immediately and were not refrozen.

Induction of Apoptosis in Cell-free Extracts—Reactions were set up in a final volume of 10 μl or multiples thereof. A typical 10-μl reaction contained 5 μl of cell extract (~10 μg of protein) and 2 μl of purified CEM nuclei (~4 × 106) and were brought up to their final volume with cell extract buffer or cytochrome c and dATP dissolved in the same buffer. To initiate apoptosis, extracts were incubated for 2 h in the presence of bovine heart cytochrome c (50 μg/ml final concentration; Sigma) and dATP (1 mM; Roche Molecular Biochemicals). CEM and 293T nuclei were prepared as described previously (24). Cell-free reac-
tions were analyzed for substrate proteolysis by Western blotting, as described previously (22, 25). Reactions were analyzed for DNA fragmentation using agarose gel electrophoresis, as described previously (24). In some experiments, nuclei within the extracts were visually scored for apoptotic features by staining with ethidium bromide (2.5 μg/ml final concentration) in 15 mM PIPES-NaOH, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 5 mM MgCl₂, 200 mM sucrose, 3.7% formaldehyde followed by examination under UV microscopy.

Coupled in Vitro Transcription/Translation—[35S]Methionine-labeled enhanced green fluorescent protein lamin A was prepared by coupled in vitro transcription/translation of pcDNA3. Enhanced green fluorescent protein (GFP)-lamin A (a gift from Dr. Bill Earnshaw, University of Edinburgh) using the TNT kit (Promega) and translation grade [35S]methionine (PerkinElmer Life Sciences) as described previously (22). [35S]Methionine-labeled enhanced green fluorescent protein lamin A was then included in the cell-free reactions as described above. [35S]Methionine-labeled enhanced green fluorescent protein lamin A breakdown was analyzed by SDS-PAGE followed by autoradiography.

RESULTS

To explore the contributions of individual executioner caspases (caspases-3, -6, and -7) to the execution phase of apoptosis, we used a cell-free system based on post-nuclear extracts of Jurkat cells in which apoptosis can be triggered by addition of cytochrome c and dATP. We have previously shown that these cell extracts support a whole variety of apoptosis-associated changes including caspase activation, proteolysis of multiple caspase substrates, nuclear condensation and fragmentation, and internucleosomal DNA cleavage (22–25). Using cytochrome c/dATP as a pro-apoptotic stimulus, we previously defined the order of caspase activation events that occur in the cytochrome c/Apaf-1 pathway in these extracts (22). Thus, the impact of specific caspases on downstream caspase activation events can be readily appreciated in this context (Fig. 1).

Caspase Depletion from Cell-free Extracts—Caspase-3, -6, or -7 were immuno-depleted from Jurkat cell extracts using specific antibodies coupled to protein A/G-agarose beads. As a control, we also mock-depleted the extracts with total mouse or rabbit IgG using similar procedures. Removal of specific caspases from the extracts was confirmed by Western blot analysis (Fig. 1A). In some experiments, we also used extracts generated from human MCF-7 cells that are devoid of caspase-3 (Fig. 1B) due to a frameshift in exon 3 that results in the introduction of a premature stop codon into the caspase-3 transcript (14). As previously reported (22), depletion of caspase-3 abolished cytochrome c/dATP-inducible activation of caspase-2 and -6 in the extracts and also partially attenuated activation of caspase-7 (via a feedback loop involving caspase-9; Fig. 1C). Depletion of caspase-7 failed to inhibit cytochrome c/dATP-inducible activation of any other caspase (Fig. 1C). Depletion of caspase-6 also failed to inhibit activation of any other executioner caspase but did inhibit the late activation of caspase-8 and -10 in the extracts (Ref. 22; data not shown). Thus, caspase-3 plays an important role in propagating the cytochrome c/Apaf-1 pathway in addition to its proposed role as an effector caspase (Fig. 1D).

Caspase-3 and -7 Are Redundant with Respect to Proteolysis of PARP but Not α-fodrin, Gelsolin, or U1snRNP—We then explored the impact of caspase-3, -6, or -7 deletion on proteolysis of the caspase substrate proteins α-fodrin, PARP, gelsolin, and U1snRNP in this pathway (26–29). As illustrated in Fig. 2, removal of caspase-6 and -7 from the extracts had no effect on cytochrome c/dATP-initiated breakdown of α-fodrin, PARP, gelsolin, or U1snRNP. In contrast, extracts depleted of caspase-3 were incapable of supporting proteolysis of the latter substrates, with the notable exception of PARP (Fig. 2). Proteolysis of α-fodrin to a 150-kDa breakdown product was detected in the absence of caspase-3; however, this cleavage product has previously been shown to be produced by calpain (30–31). In contrast, the appearance of the 120-kDa cleavage product of α-fodrin was completely blocked in the absence of caspase-3 but not in the absence of either caspase-6, or -7.

The observation that PARP cleavage was unaffected by the removal of either caspase-3, -6, or -7 was surprising and suggested either that there is redundancy between the caspases with respect to PARP cleavage or that the most apical caspase in the cytochrome c/Apaf-1 caspase cascade (caspase-9) is responsible for this cleavage event. Clearly, redundancy was not detected with respect to cleavage of α-fodrin, gelsolin, or U1snRNP, despite the fact that caspase-2, -3, and -7 have apparently overlapping substrate specificities (32, 33), and all are activated in these extracts (22, 23).

To explore the possibility of redundancy, we generated extracts devoid of all three caspases (-3, -6, -7) and compared these with extracts depleted of single caspases. Fig. 2B shows that whereas extracts depleted of single caspases exhibited complete proteolysis of PARP as before, extracts depleted of all three caspases (-3, -6, and -7) failed to support proteolysis of this substrate. Because caspase-6 is not activated in the absence of caspase-3 (Fig. 1C; Ref. 22), it is unlikely that caspase-6 contributes to PARP proteolysis. Thus, redundancy exists between the caspase-3 and -7 at the level of PARP proteolysis but not with respect to proteolysis of α-fodrin, gelsolin, or U1snRNP.

Caspase-3 Is Essential for Proteolysis of DFF45, Rb, Vimentin, Topoisomerase I, RIP, STAT1, and X-IAP—To explore further the contributions of caspase-3, -6, and -7 to the execution...
phase of apoptosis, we extended our analysis to several more caspase substrates (34–42). Fig. 3 shows that removal of caspase-6 or -7 failed to have any impact on cytochrome c-initiated proteolysis of DFF45, Rb, vimentin, topoisomerase I, RIP, X-IAP, and STAT1. A, Jurkat extracts, either mock-depleted or depleted of the indicated caspases, were incubated for 2 h at 37 °C with or without cytochrome c (50 μg/ml) and dATP (1 mM), as indicated. Reactions were then analyzed by SDS-PAGE and immunoblotted for the indicated caspase substrate proteins. Note that calcium/calmodulin-dependent protein kinase IV (CaMK IV) did not undergo proteolysis under any conditions and serves as a useful loading control for samples where degradation products were not detectable (DFF45, vimentin, STAT1). Results are representative of a minimum of three separate experiments. B, absence of cytochrome c/dATP-inducible substrate cleavage in caspase-3-deficient MCF-7 extracts. MCF-7 extracts, prepared as described under “Experimental Procedures,” were incubated for 2 h at 37 °C with or without cytochrome c (50 μg/ml) and dATP (1 mM), as indicated. Caspase substrate proteolysis was assessed by Western blot. Note that the cytochrome c-inducible proteolytic fragment of fodrin detected in MCF-7 cells has previously been attributed to calpain activity (30, 31).

Caspase-3, but not caspase-6 or -7, is required for cytochrome c-inducible cleavage of DFF45, Rb, vimentin, topoisomerase I, RIP, X-IAP, and STAT1. A, Jurkat extracts, either mock-depleted or depleted of the indicated caspases, were incubated for 2 h at 37 °C with or without cytochrome c (50 μg/ml) and dATP (1 mM), as indicated. Reactions were then analyzed by SDS-PAGE and immunoblotted for the indicated caspase substrate proteins. Note that calcium/calmodulin-dependent protein kinase IV (CaMK IV) did not undergo proteolysis under any conditions and serves as a useful loading control for samples where degradation products were not detectable (DFF45, vimentin, STAT1). Results are representative of a minimum of three separate experiments. B, absence of cytochrome c/dATP-inducible substrate cleavage in caspase-3-deficient MCF-7 extracts. MCF-7 extracts, prepared as described under “Experimental Procedures,” were incubated for 2 h at 37 °C with or without cytochrome c (50 μg/ml) and dATP (1 mM), as indicated. Caspase substrate proteolysis was assessed by Western blot. Note that the cytochrome c-inducible proteolytic fragment of fodrin detected in MCF-7 cells has previously been attributed to calpain activity (30, 31).

Caspase-6 is widely regarded to be responsible for lamin A/C cleavage during apoptosis; however, this remains to be proven conclusively (47, 48). Furthermore, it is unclear whether lamin B is also targeted by caspase-6 during apoptosis (49, 50). To explore this, we used the caspase-depleted extracts and assessed the impact of caspase-3, -6, or -7 depletion on lamin A and B proteolysis.

Fig. 4 shows that depletion of caspase-7 had no affect on proteolysis of either lamin species. In contrast, depletion of caspase-3 completely abrogated proteolysis of both lamin A and B. The latter result can be readily explained due to the fact that caspase-3 is required for activation of caspase-6 in these extracts (Fig. 1; Ref. 22). However, analysis of extracts immuno-depleted of caspase-6 revealed that, whereas proteolysis of lamin A was very substantially inhibited under these conditions, proteolysis of lamin B was unaffected by removal of caspase-6 from the extracts (Fig. 4, A and B). These data confirm that lamin A breakdown is largely mediated by caspase-6 during the execution phase of apoptosis but suggest that lamin B degradation is probably achieved through caspase-3 activity.

Nuclear Condensation, Chromatin Margination, and DNA Fragmentation Require Caspase-3 but not Caspase-6 and -7—Some of the most striking cellular changes that occur during apoptosis affect the cell nucleus (51). Upon activation, caspases are thought to translocate to the nuclear compartment, where they trigger substrate proteolysis and other changes that trig-
ger chromatin condensation, margination of chromatin to the periphery of the nuclear envelope, and fragmentation of DNA. Therefore, it was of considerable interest to explore the contributions of caspase-3, -6, and -7 to the latter events, all of which are cytochrome c/dATP-inducible in cell-free extracts. As illustrated in Fig. 5, depletion of caspase-3 had a very substantial impact on the appearance of nuclei with apoptotic features in response to cytochrome c/dATP. In contrast, depletion of caspase-6 or caspase-7 had little detectable impact on these events (Fig. 5, A and B).

Similarly, caspase-3-depleted extracts failed to support DNA fragmentation, whereas extracts devoid of caspase-6 or -7 remained competent in this regard (Fig. 5C). The latter observations are in line with previous observations that caspase-3-mediated DFF45/inhibitor of caspase-activated DNase (ICAD) proteolysis, which results in activation of DFF40/caspase-activated DNase (CAD), plays an essential role in apoptosis-associated DNA fragmentation (34, 44, 52–54). However, the failure of caspase-6 and -7 depletion to impact on these events further underscores our observations that caspase-3 carries out proteolytic cleavage events during the execution phase of apoptosis that neither caspase-7 nor caspase-6 can substitute for, or contribute to, in any significant way.

**DISCUSSION**

Here we have used a cell-free system comprised of cytoplasmic extracts prepared from viable Jurkat cells to emulate events that occur after the release of mitochondrial cytochrome c during apoptosis. Using extracts immuno-depleted of caspase-3, caspase-6, caspase-7, or all three caspases, we assessed the role played by each caspase in the cleavage of a panoply of proteins known to be targeted for proteolysis during apoptosis. We also assessed the contributions made by caspase-3, -6, and -7 to other apoptosis-associated events, such as chromatin condensation, chromatin margination, and DNA fragmentation. Rather surprisingly, our results suggest that caspase-6 and -7 play relatively minor or highly specialized...
roles during the execution phase of apoptosis. In contrast, caspase-3 appears to act globally, as required for multiple proteolytic events, suggesting that it is the primary executioner caspase (Fig. 6).

These data call into question the hypothesis that caspase-6 and -7 play a major role in the terminal phase of apoptosis. This hypothesis has been founded largely on the basis that caspase-6 and -7 have short prodomains and have significant sequence similarity to caspase-3. However, it is possible that these caspases may play a more upstream role as regulators of caspase activation within the caspase cascade rather than as destructive caspases in the demolition phase of death. Alternatively, the major caspase substrates that are targeted by caspase-6 and -7 during the execution phase of apoptosis, with the exception of lamin A and PARP, may not have been identified thus far. We do recognize the possibility that the majority of the substrate cleavage events that we have assessed may, by sheer coincidence, happen to be caspase-3-dependent since there are many more caspase substrates than we have examined. However, we did not choose substrates with any particular bias and also included several substrates (lamin A, lamin B, STAT1, RIP, gelsolin, PARP) that are reported to have cleavage motifs that would not be expected to be targeted by caspase-3. One intriguing possibility that we have not been able to examine using the cell-free approach is that caspase-6 or -7 may be responsible for triggering the membrane changes that promote phagocytosis of apoptotic cells. It has previously been shown that cells from CASP-3 null mice are normal with respect to phosphatidylserine externalization (16). Thus, it remains possible that caspase-6 or caspase-7 plays a role in promoting redistribution of this phospholipid to the outer leaflet of the plasma membrane, as this event has been shown to be caspase-dependent (55, 56).

Because caspase-3 and -7 exhibit very similar substrate specificities in peptide hydrolysis assays in vitro, it is often assumed that caspase-7 can substitute functionally for caspase-3 (32). However, despite the fact that caspase-7 is abundant in our experimental system (and is activated by caspase-9 at the same point in the hierarchy as caspase-3), caspase-7 clearly cannot substitute for caspase-3 for the majority of the substrate cleavage events measured. The single exception we have found appears to be PARP (Fig. 2). The latter observation is supported by data obtained using cells from CASP-3 knockout animals, where PARP was still cleaved in these cells under a variety of conditions (17, 57). The present study also suggests that the use of recombinant caspases at nonphysiological levels to determine the optimal substrate for a given caspase has its limitations. For example, PARP has long been considered to be a major substrate of caspase-3 due to the fact that the latter cleaves it very efficiently in vitro. Factors that are likely to influence substrate targeting in vivo include the configuration of the caspase catalytic pocket, the relative abundance of the caspases, the point in the hierarchy at which individual caspases become activated, the sub-cellular localization of the caspases relative to potential substrates, and the abundance of caspase inhibitory proteins (such as the IAPs) present in particular cell types. Although sub-cellular partitioning (with the exception of the nuclear compartment) is clearly disrupted in cell-free extracts, the relative ratios of the caspases and their inhibitors are maintained.

Clearly, cell-free systems are unlikely to perfectly reproduce the cellular context; thus, it will be important to establish whether our observations hold true using cells from CASP-6 and CASP-7 null mice when these become available. However, our data are in agreement with studies that have already been performed using cells from CASP-3 null mice, where cleavage of several substrates also examined in the present study (α-fodrin, lamin B, gelsolin) as well as nuclear condensation and DNA fragmentation were found to be impaired (14–17). As mentioned above, we also found that PARP cleavage was not impaired in the absence of caspase-3, which is also in agreement with previous studies using caspase-3-deficient cells (15, 17, 57). Moreover, preliminary reports indicate that apoptosis-associated cleavage of several caspase substrates (α-fodrin, DFF45, lamin B, gelsolin) proceeds normally in CASP-6+/− or CASP-7+/− cells (58), suggesting that our observations are not an artifact of the cell-free context. Furthermore, the early embryonic lethal phenotype of CASP-7−/− mice is at odds with the perceived functional redundancy between the latter and caspase-3 (58).

Importantly, whereas previous studies using caspase-3-deficient cells could not rule out the possibility that certain events in apoptosis were impaired due to a failure to activate caspases downstream of caspase-3 in the cytochrome c/Apaf-1 pathway (caspase-6, -2, -8, -10; Ref. 22), our approach enabled us to eliminate the majority of these. Thus, caspase-6-depleted extracts enable us to eliminate caspase-6 in addition to caspase-8 and -10, as activation of the latter are controlled by caspase-6 in this context (Ref. 22; see Fig. 1). The only exception to this is caspase-2, since its activation in our system requires caspase-3 (Fig. 1; Ref. 22). Several attempts were made to deplete caspase-2 from the extracts to determine whether the latter was required for any of the events that were impaired in the absence of caspase-3; however, none of the commercially available caspase-2 antibodies could immuno-deplete this protease. Therefore, it remains possible that some of the defects seen in caspase-3-depleted extracts are due to the failure to activate caspase-2. However, it is seems unlikely that caspase-2 acts as an executioner caspase, since cells from CASP-2 null mice were not reported to exhibit any defects in this regard (59).

In summary, we have presented evidence that caspase-3 is the dominant executioner caspase and is not functionally re-

---

2 E. A. Slee and S. J. Martin, data not shown.
The Role of Executioner Caspases in Apoptosis

dundant with other executioner caspases, such as caspase-6 and -7. We suggest that caspase-6 and -7 play much more specialized roles in apoptosis than was initially thought. The phenotypes of the CASP-6 and CASP-7 knockout mice will be very interesting in this regard and should help to resolve the precise contributions that each make to the cell death program. Clearly, much work remains to be done with respect to linking caspase substrate cleavage events with the overt morphological and plasma membrane alterations that occur during apoptosis.

Acknowledgments—We thank Drs. Doug Green and Eng Tan for provision of antibodies and Professor Bill Earnshaw for providing green fluorescent protein (GFP)-lamin A cDNA.

REFERENCES

1. Cohen, G. M. (1997) Biochem. J. 326, 1–16
2. Salvesen, G. S. and Dixit, V. M. (1997) Cell 91, 443–446
3. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
4. Nichols, D. W. (1999) Cell Death Differ. 6, 1028–1042
5. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052
6. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
7. See, E. A., Aadrin, C., and Martin, S. J. (1999) Cell Death Diff. 6, 1067–1074
8. Stennicke, H. R., and Salvesen, G. S. (1999) Cell Death Diff. 6, 1054–1059
9. Green, D. R. (1998) Cell 94, 695–698
10. Kumar, S. (1999) Cell Death Diff. 6, 1066–1066
11. Salvesen, G. S. and Dixit, V. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10964–10967
12. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352
13. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
14. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) J. Biol. Chem. 273, 9357–9360
15. Neamati, N., Fernandez, A., Wright, S., Kiefer, J., and McConkey, D. J. (1995) J. Biol. Chem. 270, 1304–1314
16. Ja¨nicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 92, 9042–9046
17. Acknowledgments—We thank Drs. Doug Green and Eng Tan for provision of antibodies and Professor Bill Earnshaw for providing green fluorescent protein (GFP)-lamin A cDNA.

REMARKS

1. Cohen, G. M. (1997) Biochem. J. 326, 1–16
2. Salvesen, G. S. and Dixit, V. M. (1997) Cell 91, 443–446
3. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
4. Nichols, D. W. (1999) Cell Death Differ. 6, 1028–1042
5. Wolf, B. B., and Green, D. R. (1999) J. Biol. Chem. 274, 20049–20052
6. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
7. See, E. A., Aadrin, C., and Martin, S. J. (1999) Cell Death Diff. 6, 1067–1074
8. Stennicke, H. R., and Salvesen, G. S. (1999) Cell Death Diff. 6, 1054–1059
9. Green, D. R. (1998) Cell 94, 695–698
10. Kumar, S. (1999) Cell Death Diff. 6, 1066–1066
11. Salvesen, G. S. and Dixit, V. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10964–10967
12. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352
13. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
14. Janicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) J. Biol. Chem. 273, 9357–9360
15. Neamati, N., Fernandez, A., Wright, S., Kiefer, J., and McConkey, D. J. (1995) J. Biol. Chem. 270, 1304–1314
16. Ja¨nicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 92, 9042–9046
17. Acknowledgments—We thank Drs. Doug Green and Eng Tan for provision of antibodies and Professor Bill Earnshaw for providing green fluorescent protein (GFP)-lamin A cDNA.