Caspase-7 Gene Disruption Reveals an Involvement of the Enzyme during the Early Stages of Apoptosis

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Caspases play a key role during apoptotic execution. In an attempt to elucidate the specific role of caspase-7 we generated a chicken DT40 cell line in which both alleles of the gene were disrupted. Viability assays showed that caspase-7−/− clones are more resistant to the common apoptosis-inducing drugs etoposide and staurosporine. Caspase-7−/− cells show a delay in phosphatidylserine externalization and DNA fragmentation as well as cleavage of the caspase substrates poly(ADP-ribose) polymerase 1 and lamin B1 and B2. Caspase affinity labeling and activity assays indicated that deficient cells exhibit a delay in caspase activation compared with wild type DT40 cells, providing an explanation for the differences in apoptotic execution between caspase-7 null and wild type DT40 cells. These results strongly suggest that caspase-7 is involved earlier than other effector caspases in the apoptotic execution process in DT40 B lymphocytes.

The first significant progress in understanding apoptosis came from studies of developmental cell death in the nematode Caenorhabditis elegans (1), where genetic analysis revealed that the worm CED-3 gene encodes an enzyme that is an essential executor of apoptosis (2). Mammalian counterparts of CED-3 have been identified as members of a family of intracellular proteases called caspases that are an integral part of the apoptotic machinery (3–5). Thirteen caspases have been identified to date. In vivo and in vitro studies have grouped them into two main categories, initiator caspases, which transduce various signals into proteolytic activity, and effector caspases, which are responsible for the bulk of the proteolytic cleavages observed during apoptosis (3–7). Among the initiator caspases (caspases-8, -9, -10, and possibly -2), mouse knockout studies showed that caspase-8 is essential for heart development, and caspase-9 is crucial for brain development in at least some genetic contexts (8–11). Mice lacking caspase-2 have only subtle developmental defects (12), but some caspase-2-deficient cells are resistant to induction of apoptosis by several DNA-damaging agents (12, 13).

The mammalian caspases with short prodomains, caspases-3, -6, and -7, are thought to be effector caspases that are activated by initiator caspases (3, 4, 6, 14). Among these three enzymes, caspase-3 has been shown to cleave numerous cytoplasmic and nuclear proteins (15–17). Most experiments indicate that caspase-6 is activated by caspase-3 in intact cells and under cell-free conditions (14, 18–20), although caspase-6 appears to be upstream of caspase-3 in at least one experimental system (21). We recently found that caspase-6−/− chicken DT40 B lymphocytes undergo a kinetically and morphologically normal apoptotic response to the topoisomerase II poison etoposide (22). Further analysis demonstrated that activation of caspase-3 was normal in the caspase-6−/− cells.

Although many studies point to a critical role for caspase-3 in mammalian cell apoptosis, caspase-3 knockout mice have developmental defects mainly in the central nervous system (23). The requirement for caspase-3 for apoptotic execution varies in a stimulus- and tissue-specific manner (10). In the MCF-7 breast carcinoma cell line, which lacks caspase-3 because of a functional deletion of the caspase-3 gene, staurosporine, tumor necrosis factor-α, and paclitaxel readily induce death, although the typical apoptotic morphology or DNA laddering is sometimes absent (20, 24). These studies in caspase-deficient cells have raised the possibility that another effector caspase might substitute for caspase-3 in its absence.

Several observations have suggested that caspase-7 replaces caspase-3 under certain circumstances. The two enzymes have a high degree of homology (15) and very similar activities against synthetic tetrapeptide-based substrates (15, 25). Activation of caspase-7 has been observed in MCF-7 cells lacking caspase-3 (20, 26) and has been implicated in poly(ADP-ribose) polymerase (PARP)1 cleavage when caspase-3 is absent (27).

Other studies have suggested that caspase-7 might have a unique role during apoptosis. Caspase-7 appears to be essential for the clonal deletion of autoreactive B cells by B cell receptor cross-linking (28). IgM treatment of immature B lymphocytes causes growth arrest and caspase-7 activation independently of caspase-8 activation and cytochrome c release (29). In addition,
B cell receptor cross-linking induces the selective activation of caspase-7 but not caspase-3 (30). More recently, caspase-7 activation was reported in T and B cells undergoing apoptosis under conditions in which the classical apoptosome/caspase-9 pathway was inactivated by targeted deletion of Apaf-1 (31).

Targeted gene disruption studies have identified differential requirements for caspases-1, -2, -3, -8, -9, -11, and -12 during diverse apoptotic stimuli (28-33). However, at least one research group has reported an inability to determine the exact functions of caspase-7 because nullizygous embryos died early during embryogenesis (32, 34). We therefore exploited the powerful DT40 knockout system to elucidate the function of this caspase during the apoptotic process. We report here the derivation of a number of DT40 clones in which the caspase-7 gene has been disrupted. Our results show that, unexpectedly, caspase-7 is required for the early stages of apoptosis in these cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Chicken B lymphoma cell line DT40 was cultured as described previously (35). Cell viability was assessed using trypan blue staining under phase-contrast microscopy.

**Targeted Disruption of the Caspase-7 Gene**—A 19-kb fragment containing part of the caspase-7 gene locus isolated from a Φ FIX II DT40 genomic library using chicken caspase-7 cDNA 5' and 3' ends as probes was fully sequenced and used to construct the targeting vectors. A 3-kb arm upstream of the first codon found in the cloned genomic sequence and a 2-kb fragment downstream of the stop codon were amplified by PCR. The knockout vectors were obtained by inserting a blasticidin or histidinol resistance cassette between the 5' and 3' arms. The knock-out constructs (25 μg each) were linearized with XhoI and used to electroporate 2 × 106 DT40 cells (950 microfarads; 300 V on a Bio-Rad Gene Pulser system). Stable transfectants were selected by limiting dilution in 25 μg/ml blasticidin or 1 μg/ml histidinol for 5–7 days. After amplification of resistant clones, DNA was extracted and analyzed by Southern blotting. The 3'-genomic external probe was amplified by PCR.

**Southern Blot Analysis and Reverse Transcription-PCR**—Genomic DNA (5 μg) was digested with EcoRI, separated on an agarose gel, transferred to Hybond N nylon membrane (Amersham Biosciences), and subjected to Southern blotting. Hybridization was performed in 1M Na2HPO4/NaH2PO4, pH 7.4, SDS 7% at 65 °C using an external 3'-probe labeled by random primer extension (Amersham Biosciences). Caspase-7 mRNA expression was analyzed by reverse transcription-PCR using 1 μg of total RNA and oligonucleotide primers designed to amplify the caspase-7 open reading frame.

**Cell Viability, TUNEL and Annexin V Assays**—Cell viability was assessed after a 24-h treatment with etoposide or staurosporine using a colorimetric assay (WST-1 from Roche Applied Science). TUNEL (Roche Applied Science) and annexin V (BioVision) assays were performed at various times after treatment with etoposide or staurosporine and analyzed by FACScalibur flow cytometer (BD Biosciences). Data were acquired and analyzed using CellQuest software.

**DNA Fragmentation Analysis**—Cells or nuclei were pelleted and disrupted immediately in DNA lysis buffer (10 mM Tris-HCl, pH 8, 100 mM EDTA, 10 mM EGTA, 0.5% SDS). After DNase-free RNase was added to 20 μg/ml, lysates were incubated for 2 h at 37 °C and then treated with 100 μg/ml proteinase K at 37 °C overnight. The DNA was extracted with phenol, pelleted with 2 volumes of ethanol and 0.1 volume of 10 mM ammonium acetate, dissolved in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA), and separated on a 1.5% agarose gel.

**Preparation of Cell Extracts**—Cytosolic extracts were prepared from untreated and apoptotic wild type, heterozygote, and knockout DT40 cells (expressing or not expressing a rescue construct). Cells were treated with 10 μM etoposide for 30 min at 30 °C, pelleted, and washed once in PBS and once in KPM buffer (50 mM Pipes, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, 50 μM cytochalasin B, 0.02 mM methylthionyl fluoride, chymostatin, leupeptin, antipain, and pepstatin). Cells were then lysed by three freeze/thaw cycles, sonicated, and centrifuged at 190,000 g × 4 for 2 h at 4 °C. The clear supernatant was collected, aliquoted, and frozen at −80 °C.

**Preparation of HeLa Nuclei and in Vivo Apoptosis Induction**—HeLa nuclei were prepared as described previously (36). To induce apoptosis under cell-free conditions, nuclei were washed twice in MCB buffer (10 mM Pipes, pH 7.0, 50 mM NaCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol) before the addition of apoptotic extracts and an ATP regenerating system (2 mM ATP, 20 mM creatine phosphate, 50 μM creatine kinase). Mixtures were incubated at 37 °C for 3 h then lysed in Laemmli buffer for immunoblotting (see below).

**Immunoblotting and Antibodies**—Cytosolic extracts (30 μg of protein) or whole cell lysates (from 1 million cells) were boiled for 5 min in Laemmli buffer containing β-mercaptoethanol, subjected to SDS-PAGE, blotted onto nitrocellulose membranes (Amersham Biosciences), and stained with Ponceau S (Sigma). Membranes blocked with 5% skim milk in PBS were incubated with specific antibody diluted in PBS and 2% milk for 3 h at 25 °C. After washing, membranes were incubated with horseradish peroxidase-linked secondary antibody (Amersham Biosciences) for 30 min at 25 °C. Each step was followed by three washes for 10 min in PBS and 5% milk. Bound antibody was detected using ECL (Amersham Biosciences).

Monoclonal antibody to chicken lamin B2 was obtained from Pr. Reimer Stick. Monoclonal antibody to chicken lamin B1, polyclonal antibody to human FARP (9542), and monoclonal antibody to human α-tubulin (clone B512) were purchased from Zymed Laboratories Inc., Cell Signaling Technology, and Sigma, respectively. Rabbit polyclonal antibody to chicken caspase-7 (R1106) was raised against the large subunit of caspase-7 expressed in Escherichia coli as a glutathione S-transferase fusion protein.

Immunoprecipitations were performed from cytosolic extracts (5 mg of protein) using the anti-caspase-7 (R1106) cross-linked to sheep antirabbit IgG-covered beads according to the manufacturer’s instructions (Dynal Biotech).

**Caspase Affinity Labeling and Activity Analysis**—Cytosolic extracts (30 μg of protein) were incubated in 1 μM EK(bio)D-omk (Peptide Institute, Kyoto, Japan) for 15 min at 37 °C before being boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE. After transfer and blocking in PBS and 5% milk, membranes were incubated with horse-radish peroxidase-linked streptavidin (Sigma) for 3 h at 25 °C, washed, and reacted with ECL.

Clavage of DEVD-AFC (Biomol, Plymouth Meeting, PA), VEID-AFC, and LEHD-AFC (Enzyme Systems Products, Dublin, CA) was assayed as described previously (17, 37). In brief, 25 μg of cytosolic protein was incubated with 50 μM of unlabeled DEVD-AFC (2.5 mM Tris-HCl, 1 mM EGTA, 1 mM methylthionyl fluoride, 10 μM ϕ-lysine A, and 10 μM ϕ-lysine O) at 37 °C for 2 h at 37 °C. After reactions were terminated by addition of 1.225 ml of ice-cold buffer B, fluorescence was measured using an excitation wavelength of 360 nm and emission wavelength of 475 nm. Standards containing 0–15 nmol of AFC were utilized to determine the amount of fluorochrome released.

**RESULTS**

**Generation of Caspase-7-deficient DT40 Clones**—An EST clone obtained from the Delaware University chicken EST bank and containing the complete cDNA sequence of chicken caspase-7, was used to generate different probes to screen a genomic DT40 phage library. After a phase clone containing part of the caspase-7 locus was isolated and sequenced, the positions of the exons were determined by comparison with the cDNA sequence. The phase sequence covers the last 5 exons of the gene locus, excluding the 5'-end of the gene (Fig. 1A). To disrupt the caspase-7 gene, we constructed targeting vectors in which a resistance cassette (blasticidin or histidinol) was flanked by a 5'-genomic arm situated upstream of the first exon found in the phase clone and a 3'-genomic arm situated downstream of the stop codon (Fig. 1A). Targeted integration of these constructs, which were termed Casp7blast and Casp7his, respectively, would remove a 3,700-bp gene fragment containing the majority of the open reading frame (786 of 930 bp). After insertion of these vectors, only the first 13 amino acids of the coding region potentially be expressed, giving a peptide highly unlikely to be functional.

The deletion was performed by homologous recombination in the chicken B cell lymphoma line DT40. After wild type DT40 cells were transfected with the Casp7his construct, histidinol-
Caspase-7 Gene Knockout

Fig. 1. Characterization of the Gallus gallus caspase-7 gene knockout. A, partial structure of the chicken caspase-7 gene and the knockout constructs containing the 5′- and 3′-arms separated by a resistance cassette (blasticidin or histidinol). The strategy to differentiate between the wild type (WT) allele and homologous recombinants using a 3′-external probe is shown. B, Southern blot analysis of EcoRI-digested DNA from wild type, caspase-7+/−, and caspase-7−/− cells, using the 3′-genomic external probe. C, immunoblotting analysis of caspase-7 expression in wild type (lane 1), caspase-7+/− (lane 2), caspase-7−/− (lane 3), and caspase-7−/−-casp-7-EGFP cells (lane 4). Caspase-7 was immunoprecipitated from 5 mg of protein from cytosolic extracts of the indicated cells 5 h after the addition of etoposide. D, reverse transcription-PCR analysis for caspase-7 mRNA expression. Total RNA was extracted from wild type (lane 2), caspase-7−/− (lane 3), caspase-7+/− (lanes 4 and 5), caspase-7−/−-casp-7 (lane 6), and caspase-7−/−-casp-7-EGFP cells (lane 7). Lanes 4 and 5 represent two independent caspase-7−/− clones. Lane 1, cell extract was omitted (negative control for the PCR).

Resistant clones were obtained. Targeted events were recognized by Southern blot analysis of EcoRI-digested DNA and the use of a 3′-external probe. The probe recognizes an 11-kb band corresponding to the wild type allele and a 4.3-kb band after targeted integration of the Casp7blasto or Casp7his construct (Fig. 1B). Putative targeting events were further verified by Southern blot analysis using an external genomic 5′-probe (data not shown).

Several of these heterozygous clones were then transfected with the Casp7blasto construct to delete the second allele. The targeting efficiency for the first allele of 1.4% was similar for both knockout constructs. Because the targeting efficiency for the second allele was an unexpectedly high 10%, many independent caspase-7-deficient clones were isolated. The loss of caspase-7 expression was then confirmed by immunoblotting and reverse transcription-PCR (Fig. 1, C and D). Fig. 1D is representative of three experiments in which we detected the caspase-7 mRNA in wild type DT40 cells. We could never detect caspase-7 mRNA in heterozygous cells. This suggests that caspase-7 mRNA is expressed at very low levels in DT40 cells, and this was confirmed by comparison with other chicken cell lines (data not shown). The caspase-7 mRNA was easily detectable in caspase-7−/− cells overexpressing either untagged chicken caspase-7 or the chicken caspase-7 cDNA fused at its C terminus to EGFP (Fig. 1D, lanes 6 and 7).

To confirm the effect of the gene knockout on caspase-7 protein levels, we performed the immunoblotting experiment shown in Fig. 1C. Procaspase-7 was observed very faintly in immunoblots of DT40 cells after immunoprecipitation from cytosolic extracts containing 5 mg of protein from apoptotic cells (lane 1). The antibody recognizes the cleaved form better than the pro-form of caspase-7 and could not detect the protein in nonapoptotic DT40 cells. Importantly, the protein could not be detected in lysates from caspase-7 knockout cells.

Caspase-7-deficient DT40 Cells Show Increased Resistance to Apoptosis—Caspase-7+/− and caspase-7−/− cells proliferated at a rate similar to wild type DT40 cells (Fig. 2A). To examine whether caspase-7 deletion affected resistance of the cells to proapoptotic stimuli, we assayed cell viability 24 h after initiating treatment with different concentrations of etoposide in wild type cells and two independent clones each of caspase-7+/− and caspase-7−/− cells (Fig. 2B). Both types of caspase-7-deficient clones were 3.5 to 4.5 times more resistant to the drug than wild type cells tested in parallel. The IC50 values for the wild type, caspase-7+/−, and caspase-7−/− cells were 1.35 ± 0.10, 5.25 ± 0.15, and 7.75 ± 0.15 μM, respectively (Fig. 2B). Resistance of caspase-7+/− and caspase-7−/− cells was likewise observed after treatment with staurosporine, another inducer of apoptosis in DT40 cells (data not shown). Although we were surprised that the heterozygous clones showed a phenotype similar to the knockout cells, this resistance could explain the higher targeting efficiency obtained for the second allele if heterozygous cells were also more resistant to apoptosis during electroporation. Four independent heterozygous clones were
**Fig. 2.** Caspase-7−/− cells show an increased resistance to apoptosis. A, growth curves of wild type (WT), caspase-7−/−, caspase-7+/−, and caspase-7−/−-casp-7-EGFP DT40 cells. B and C, viability of different DT40 clones 24 h after incubation with increasing doses of etoposide. Each point represents a triplicate value; error bars are not visible. B, comparison of two different caspase-7−/− clones (a and b) and two different caspase-7−/− clones (a and b) with the wild type DT40 cells. C, comparison of caspase-7−/−, caspase-7−/−-casp-7-EGFP clones with wild type cells.
tested in viability assays similar to those shown in Fig. 2 with comparable results. This appears to confirm that caspase-7 is haploinsufficient and to rule out the possibility of clonal variability.

To confirm that this drug resistance phenotype was a direct result of the caspase-7 gene deletion, caspase-7−/− cells were transfected with a construct containing the chicken caspase-7 cDNA fused at its C terminus to EGFP and under the control of a cytomegalovirus promoter. After cells containing the cDNA were selected, clones expressing the transgene were identified based on fluorescence of the caspase-7-EGFP fusion protein. Caspase-7-EGFP appears to be overexpressed roughly 5–10-fold in these cells but is fully processed during apoptosis (Fig. 1C). We have shown elsewhere that EGFP is cleaved very near its N terminus in apoptotic cells, and this explains why the caspase-7 has a normal mobility in these extracts (22). Cell viability assays were performed by comparing caspase-7−/−, casp-7-EGFP clones with the wild type cells and a third caspase-7−/− clone (Fig. 2C). In all cases, transfection with caspase-7−/−-casp-7-EGFP restored drug sensitivity to levels observed in wild type cells. This rescue of the phenotype by expression of an exogenous caspase-7 in caspase-7−/− cells confirmed that the drug-resistance phenotype arose as a result of the deletion of the caspase-7 locus.

Caspase-7-deficient Cells Show Delayed DNA Fragmentation during Apoptotic Execution—Because caspase-7 has been viewed as an effector caspase, we examined terminal events in the apoptotic process in parental and caspase-7−/− cells. At various times after treatment with etoposide, cellular DNA was extracted and examined for internucleosomal cleavage (Fig. 3A). DNA nucleosomal laddering analysis during an etoposide time course on wild type (WT) cells and two different caspase-7−/− clones. B, quantitative analysis of DNA fragmentation by TUNEL staining measured by flow cytometry. A comparison of wild type, caspase-7−/−, and caspase-7−/−-casp-7-EGFP cells after exposure to 10 μM etoposide for varying lengths of time is shown. Data represent three independent experiments. For each sample, 15,000 cells were counted.
Our results show that caspase-7 is required for normal kinetics and the extent of lamin B1 and B2 cleavage in DT40 cells (Fig. 4A). In wild type cells, both lamins were totally cleaved after 3 h of etoposide treatment. However, in the caspase-7-/- cells, lamin B1 was partially cleaved at 3 h, and lamin B2 cleavage began only at 4 h. For both lamins, we observed an uncleaved pool remaining for as long as 5 h. These results could reflect either a direct involvement of caspase-7 in lamin cleavage, or they could be a result of delayed activation of other caspases that normally function downstream of caspase-7 in DT40 cells. In the former case, we would expect that the slower cleavage might be a result of suboptimal recognition of the binding site by a redundant caspase. In the latter case, we would expect that once the downstream caspase were activated, the cleavage would proceed with normal kinetics.

To distinguish between these two models, we used cell-free extracts to examine the cleavage of PARP, a classical substrate of both caspases-3 and -7 in vitro (15, 45, 46). PARP cleavage takes place before lamin cleavage (44, 47). Because we were unable to identify any antibody that recognized chicken PARP1, we used a cell-free system in which HeLa nuclei were induced to undergo apoptotic biochemical changes in extracts prepared from apoptotic wild type and caspase-7-/- DT40 cells. HeLa nuclei were added to cytosolic extracts prepared from wild type and caspase-7-/- DT40 cells after exposure to etoposide for 1, 3, and 6 h. After a 3-h incubation at 37 °C in vitro, PARP cleavage was assessed by immunoblotting (Fig. 4B). PARP was >50% cleaved in extracts prepared after incubation of DT40 cells with etoposide for 1 h and was completely cleaved in 3-h and 6-h extracts. In contrast, when using caspase-7-/- extracts, only a small fraction of PARP1 was cleaved in the 1-h extract, and some uncleaved protein could still be seen after incubation in the 3-h extract. PARP1 cleavage was complete in the 6-h extract. The fact that PARP was cleaved when caspases were activated in caspase-7-/- cells implies that caspase-7 is not essential for this cleavage, but instead suggests that caspase-7 might be involved directly or indirectly in activating another caspase that is responsible for PARP processing.

Caspase-7-deficient Cells Show Delayed Loss of Plasma Membrane Asymmetry during Apoptotic Execution—Loss of phosphatidylserine asymmetry is an early marker of apoptotic execution (48, 49). Although some studies have suggested that this occurs in a caspase-dependent manner (50, 51), others have suggested that it is independent of caspase activation (52). Caspase-7 involvement in this event was assessed by measuring annexin V labeling after the addition of etoposide to wild type or caspase-7-/- DT40 cells. In contrast to wild type cells, annexin V labeling was delayed by 3 h in the caspase-7-/- cells. The fact that annexin V binding is delayed in the caspase-7-/- cells implies that caspase-7 is not essential for this cleavage, but instead suggests that caspase-7 might be involved directly or indirectly in activating another caspase that is responsible for PARP processing.
caspase-7 in the knockout cells gave only a partial rescue of the phenotype, suggesting that the caspase-7-EGFP fusion protein may not be fully functional. These results demonstrate that caspase-7 is required for timely progression of the membrane reorganization events in DT40 cells.

Caspase Activation Is Delayed in Caspase-7/−/− Cells—To assess further the possibility that caspase-7 deficiency results in altered activation of other caspases, cytosolic extracts (47) were prepared from wild type (WT), caspase-7/−/−, and caspase-7/−/−-casp-7-EGFP DT40 cells at varying times after the addition of 10 μM etoposide (Fig. 6). Caspase activity was first assayed in extracts prepared 3 and 6 h after the addition of etoposide (Fig. 6, A and B). These two time points were used because FACS analysis indicated a delay in TUNEL staining over this time frame (Fig. 3), and, when checked using the inverted phase-contrast microscope, wild type DT40 cells did not begin to display apoptotic morphological changes until at least 3 h after the etoposide addition.

Extracts were characterized in terms of overall caspase activation and also for specific caspase activity. Broad spectrum labeling of caspases using zEK(bio)D-aomk showed the activation of several caspase species in these extracts, with a major

![Graph A](image)

**Fig. 5.** Caspase-7-deficient cells show a delay in annexin V staining. Quantitative analysis of phosphatidylserine exposure at the cytoplasmic membrane by annexin V staining measured by flow cytometry is shown. Data represent three independent experiments. For each sample, 15,000 cells were counted. Wild type (WT), caspase-7/−/−, and caspase-7/−/−-casp-7-EGFP cells were compared after exposure to etoposide at 10 μM for up to 6 h (A) or staurosporine at 1 μM for up to 9 h (B).
labeled band appearing at ~20 kDa (Fig. 6A). Wild type and caspase-7−/− cells exhibited no significant differences in caspase labeling at 3 and 6 h after the addition of etoposide. In an independent approach, activities that cleave DEVD-AFC, VEID-AFC, and LEHD-AFC (classical substrates for caspases-3/7, -6, and -9, respectively) were assessed in these extracts (Fig. 6B). Again, no major differences in activity were detected between wild type and caspase-7−/− cells at the 3- and 6-h time points.

Upon closer inspection, a slight decrease of about 15% was observed for the DEVD-AFC cleavage activity in the caspase-7−/− cells compared with the wild type. We hypothesized that this might result from a delay in caspase activation occurring earlier than the caspase-7−/− cells. Therefore, we repeated the caspase activity and caspase labeling assays using apoptotic extracts from cells treated with 10 μM etoposide for 30 min or 1 h. Interestingly, both the caspase labeling and activity assays showed a delay in caspase activation in caspase-7-deficient cells (Fig. 6C and D). Affinity labeling demonstrated caspase activation within 1 h of etoposide treatment in wild type cells, but no active caspases were labeled in caspase-7−/− cells (Fig. 6C). Quantitative assays of caspase-3/7, -6, and -9-like activity confirmed this result. Within 1 h of treatment with 10 μM etoposide, activity capable of cleaving DEVD-AFC, VEID-AFC, and LEHD-AFC cleavage was readily detectable in cytosol. In contrast, activity cleaving these substrates was almost totally absent from caspase-7−/− extracts (Fig. 6D).

**DISCUSSION**

It is widely believed that caspase-7 is an effector caspase with a role in the later stages of apoptotic execution. Indeed, a variety of studies have raised the possibility that caspase-3 and caspase-7 might have redundant roles. This view is based on the similar ability of these two enzymes to cleave tetrapeptide-based synthetic substrates (15, 25) and on the ability of caspase-9 to activate caspases-3 and -7 directly (18, 53). On the other hand, differences in the abilities of caspases-3 and -7 to cleave polypeptide substrates have been described extensively (20, 54–57). Moreover, the caspase-3 gene deletion results in viable mice that have neurological defects in some genetic backgrounds, whereas the caspase-7 gene deletion reportedly is lethal in mice (32, 34). These results raise the possibility that caspase-3 and caspase-7 have separate and distinct functions in a cellular context.

The present results provide new insight into the role of caspase-7 during apoptotic execution. In particular, our results in DT40 cells show that caspase-7 functions early in the apoptotic pathway induced by drugs such as etoposide and staurosporine. Because caspase-7 deficiency results in a delay in activation of caspase-3 and caspase-6, our results place
Caspase-7 is required for timely execution of late events of apoptotic DNA fragmentation in DT40 cells—Caspase-7−/− cells exhibit a delay in both TUNEL labeling and DNA fragmentation after treatment with apoptotic stimuli. These effects must reflect a delay in the activation of DFF40/CPAN following cleavage of DFF40/CAD/CPAN, as we have shown previously that endonuclease G has no significant role in apoptotic DNA fragmentation in DT40 cells (19). These conclusions were supported by studies of other caspase substrates. For example, cleavage of lamin B1 and B2 was delayed for at least 3 h after exposure of caspase-7−/− cells.

We also found that the surface exposure of phosphatidylserine (48) was delayed at least 90 min after exposure of caspase-7−/− cells to etoposide or staurosporine. Published evidence has disagreed over whether caspases are (58) or are not (52) required for phosphatidylserine exposure. Our results clearly demonstrate that caspase-7 is required for the timely execution of this important stage of the apoptotic pathway.

The observed delays could be caused by slower rates of cleavage by a caspase with a lower affinity for each substrate if, for example, this cleavage were normally executed by caspase-7. To assess this possibility, nuclei were exposed to lysates prepared from wild type and caspase-7−/− cells after exposure to etoposide for 3 h, at which time the caspase cascade appeared to be equally active in both cell types (Fig. 4, A and B). If caspase-7 were particularly effective at cleaving certain substrates, we would have observed diminished cleavage in caspase-7-deficient extracts in this experiment. Although previous findings have indirectly implicated caspase-7 in PARP cleavage (10, 27), our observations revealed no difference in PARP cleavage (Fig. 4B) or DNA ladder production (data not shown) in the 3-h extracts. Our results support and extend a previous study in which caspase-7 was immunodepleted from Jurkat extracts (53). Results of both studies argue against the possibility that caspase-7 is required for apoptotic cleavages of the substrates tested thus far. Instead, our further experiments suggest that the delayed cleavage of substrates and CAD activation observed in caspase-7−/− cells reflect a delay in activation of the effector caspases.

Caspase-7 Is Required for a Normal Time Course of Caspase Activation in DT40 Cells—Examination of the kinetics of caspase activation (Fig. 6) revealed a delay in activation of caspases-3 and -6 in caspase-7−/− cells. This delay was evident when active caspases were analyzed by affinity labeling and when individual caspase activities were assayed using tetrapeptide-based substrates. In particular, activities that cleave DEVD-AFC, VEID-AFC, and LEHD-AFC, classical substrates for caspases-3, -6, and -9, were all elevated by <1 h in parental cells but not caspase-7−/− cells. The delay in activation of caspases-3 and -6 clearly places caspase-7 upstream of the abundant effector caspases; and the delay in detection of caspase-9-like activity would seem to place caspase-7 upstream of caspase-9 activation as well. Consistent with this possibility, Marason et al. (31) recently suggested that active caspase-7 can be detected by affinity labeling as Apaf-1-deficient cells, which lack the ability to activate caspase-9, undergo apoptosis. On the other hand, the fact that the level of active caspase in caspase-7−/− cells is ultimately reached that in wild type cells (Fig. 6, A and B) reveals that a slower alternative mechanism of caspase activation remains intact in these cells.

An Emerging View of the Role of Caspase-7 in Apoptosis—A combination of direct experimental evidence and primary structure analysis has led to the view that there are two classes of caspases. Initiator caspases contain long prodomains with protein-protein interaction motifs that are responsible for targeting them to scaffolding complexes, where binding-induced conformational changes (59, 60) or induced proximity (4) leads to activation of the weakly active zymogens. Effector caspases lack extended prodomains and have been thought to be activated en masse by the initiator caspases. Our experiments show that caspase-7 may function closer to the apex of the cascade than implied by this dogma.

After 24 h of etoposide treatment, at least half of the caspase-7−/− cells were still viable at drug concentrations where >90% of wild type cells and cells expressing a caspase-7-EGFP construct were dead. Caspase-7−/− and caspase-7+/− cells also seemed to survive electroporation and cloning better than their wild type counterparts. These observations raise the possibility to caspase-7 might be involved in biochemical changes that determine whether cells will live or die.

To our surprise, caspase-7−/− cells showed the same phenotype as caspase-7−/− cells in all assays. This is probably explained by the extremely low levels of caspase-7 expression in DT40 cells (Fig. 1, C and D). Importantly, the phenotypes observed in caspase-7−/− and caspase-7+/− cells were reversed upon transfection with a cDNA encoding chicken caspase-7 fused to EGFP.

The observation that caspase-7 deletion results in delayed caspase-3 activation provides genetic evidence that caspase-7 functions upstream of the most abundant effector caspases. These results provide a framework for understanding several prior observations. It has been reported that caspase-7 is activated prior to caspase-3 in irradiated MOLT-4 T cell leukemia cells (61). After B cell receptor cross-linking, B lymphocytes selectively activate caspase-7 but not caspase-3. Likewise, caspase-7 (but not caspase-3) has been implicated in the cleavage of caspase-12 during endoplasmic reticulum stress (62) and in cell death occurring in the absence of Apaf-1 and caspase-9 activation (31). Finally, a form of the caspase cascade has recently been proposed in which the down-regulation of the anti-apoptotic protein Bel-2 leads to initiator caspase activation followed by a selective activation of caspase-7 and dismantling of the cell (63). The present studies, which suggest that caspase-7 has an important role upstream of caspase-3 during apoptosis in DT40 B lymphocytes, provide a context for understanding these results.

Although our data suggest that caspase-7 is involved earlier than other short prodomain caspases in the apoptotic execution process in DT40 cells, it is important to emphasize that caspases-3 and -6 are ultimately activated even in the absence of caspase-7 (Fig. 6, A and B). These observations led us to hypothesize that DT40 cells may have two parallel pathways leading to caspase activation in response to etoposide and staurosporine. The first pathway leads to the activation of caspase-7, and this leads to activation of the other caspases. In the absence of caspase-7, the second pathway is eventually activated, with a 1–2-hour delay in the amplification of the caspase cascade. Further studies are required to determine the presence and relative importance of these two pathways in other cell types.

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