Temporal Regulation of VEID-7-amino-4-trifluoromethylcoumarin Cleavage Activity and Caspase-6 Correlates with Organelle Loss during Lens Development*

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Lens fiber cell differentiation involves extensive re-construction of the cell’s architecture, including the degradation and elimination of all membrane-bound organelles via a process that has been likened to apoptosis. Using caspase reporter assays under conditions in which nonspecific cleavage of the reporter peptides by the proteasome has been inhibited, we investigated whether any specific caspase activities are temporally correlated with this process of organelle loss. Extracts from neonatal mouse lenses contained strong VEID-7-amino-4-trifluoromethylcoumarin (AFC) and minor IETD-AFC and LEVD-AFC cleavage activities, but no DEVD-AFC cleavage activity. Further testing suggested that the VEID-AFC and IETD-AFC cleavage activities were likely due to the same enzyme. In lens extracts from rat embryos, VEID-AFC cleavage activity increased during the period when organelles are eliminated, between embryonic days 15.5 and 18.5, whereas procaspase-6 protein levels decreased, suggesting that this enzyme is responsible for VEID-AFC cleavage. By contrast, in extracts from αAE7 transgenic mouse lenses in which apoptosis was induced, strong DEVD-AFC cleavage activity and activated caspase-3 protein were detected. Thus, within the same tissue, different caspase activities can predominate depending on the context, normal differentiation versus apoptosis. These results highlight the difference between normal fiber cell differentiation and apoptosis and the capacity of the lens to differentially regulate these two processes.

Apoptosis is a tightly controlled process that results in the ordered disassembly of cells into small membrane-bound fragments that are efficiently eliminated from the organism. It is an important mechanism in the development of all multicellular organisms, being used for such processes as eliminating self-reactive immune cells, ensuring appropriate cell-tissue targeting, and defining organ morphology (1, 2). In addition to classical apoptosis, there appear to be a few cases in which a limited destruction of components of the cell occurs during terminal differentiation. In these cases, a restricted destruction of subcellular organelles is necessary to accommodate some unique cellular function. The classical examples of this phenomenon are found in the lens, epidermis, and mammalian erythrocytes (3–5). Although these differentiation events are distinct from classical apoptosis in that a highly specialized cell remains behind, these processes and apoptosis do share some characteristics, suggesting that a common set of effector molecules may be involved. In apoptosis, the effector molecules include members of a family of cysteine proteases, the caspases (6, 7). These enzymes play central roles in both the initiation and execution of the apoptotic pathway. Through their cleavage of a limited number of important cellular proteins, caspases alter specific enzymatic processes and structural properties of cells, ultimately resulting in the ordered dismantling of those cells. Caspases may also be important in initiating and executing discrete steps in cell differentiation such as intracellular organelle loss. In this study, we investigated which caspases are active during normal lens differentiation and compare the results with those found under conditions in which apoptosis is induced in the same tissue.

The vertebrate lens consists of a single cell type in two general states: a single cell layer called the anterior epithelium that, on the anterior surface, covers a large mass of differentiated cells referred to as fiber cells (8, 9). The process of fiber cell differentiation involves extensive reconstruction of the architecture of the cell. Lens cells begin as small cuboidal epithelial cells, but during the process of differentiation, they become extremely elongated, highly specialized cells that are ordered into very precise arrays. To accommodate the lens’ proper function in vision, the fibers must express large amounts of tissue-specific crystallin proteins as well as eliminate all membrane-bound organelles from the optical pathway. Failure to eliminate these organelles results in opacity in the lens due to disordered fiber cell packing and scattering of light by the organelles.

The manner in which lens cell nuclei are lost during lens fiber cell differentiation bears many similarities to the morphological and biochemical levels to that seen in apoptosis. These include a clumping and marginalization of chromatin (10), DNase I-like activity that leaves 3′-OH groups and fragmentation of DNA into integers of nucleosomal length (11), and kinase activities (12). Experimental evidence also suggests molecular links between apoptosis and the differentiation of fiber cells. Expression of transgenes that disrupt normal cell cycle control within the lens, such as the E7 oncogene from human papilloma virus type 16 and C-terminally truncated T antigen from simian virus 40, leads to massive apoptosis within the lens (13–16). Coexpression of human papilloma virus type 16 E6 with E7 can attenuate this apoptosis. Notably, when expressed alone, E6 also can inhibit nuclear loss during fiber cell differentiation (13). Similarly, overexpression of the anti-apoptotic gene bcl-2 in the lenses of transgenic mice severely disrupts normal lens development, resulting in defects in fiber formation and the retention of nuclei in spatially inappropriate
regions of the lens (17). Finally, in vitro lens epithelial cell differentiation models, the addition of cell-permeable caspase inhibitors to the culture medium results in the retention of nuclei (18, 19). Although suggesting a role for caspases in denucleation, these latter in vitro studies failed to provide direct evidence for specific caspase activities that correlate with organelle loss. Despite these similarities in organelle loss during fiber cell differentiation and apoptosis, there are key differences. In fiber cell differentiation, a functional cell body remains behind; cell membrane integrity and an actin cytoskeleton are maintained; and the overall period of time from the beginning of organelle loss to the DNA fragmentation at the end of denucleation is estimated to be several days rather than hours (20, 21). These differences in the organelle loss program between fiber cell differentiation and apoptosis suggest that they are distinct processes. If and how they overlap at the molecular level therefore remains to be elucidated.

Because of their central role in apoptosis, we have chosen to determine whether caspases are expressed and active within normal murine lenses in a temporal pattern that could suggest a role in organelle loss during fiber cell differentiation. Multiple caspases and many of their regulatory genes were found to be expressed in the lens. A caspase-6-like VEID-AFC cleavage activity was detected within normal lenses, and this activity increased just prior to the onset of DNA degradation in primary fiber cells. Additionally, a decrease in the full-length inactive proenzyme form of caspase-6 was detected in this same time frame. Surprisingly, no DEVD-AFC cleavage or activated caspase-3 protein was detected in normal lenses. On the other hand, strong DEVD-AFC cleavage activity and activated caspase-3 were detected in extracts from lenses in which apoptosis was induced by the human papilloma virus oncogene E7. We conclude that lens cells are capable of the differential regulation of caspase activities and that only some of these activities are temporally correlated with organelle destruction during differentiation. These results are consistent with the hypothesis that some (but not all) caspases associated with apoptosis contribute to organelle loss during lens fiber cell differentiation.

**EXPERIMENTAL PROCEDURES**

**Animals**

FVB/n mice were used as the source for all non-transgenic mouse RNA, fixed sections, and extracts. αA7 transgenic mice, from line 75a described previously (13), were used as the source for apoptotic lens extracts. Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) were used as the source for all rat RNA, fixed sections, and lens extracts. For lenses isolated from embryos, the day of the vaginal plug was defined as day 0.5 in embryogenesis (E0.5).

**RT-PCR**

RT-PCR was carried out using 2.5 μg of total mRNA from lenses of neonatal FVB/n mice for the genes indicated in Table I. The identity of the PCR products was confirmed either by restriction digest analysis or by cloning into pGem®-T (Promega) or pBluescript SK II vectors, followed by dyeideo-XYA sequencing.

**In Situ hybridization**

In situ hybridization was performed as described previously (14). bam and bak were amplified by PCR from 5′-RACE products derived from an E12.5 whole mouse embryo and cloned into the pGem®-T-ESay vector to generate probes for in situ hybridization. A rat bcl-2 gene (provided by R. Nickells, University of Wisconsin, Madison, WI) was used for in situ hybridization of eye sections from neonatal mice. RNase protection was carried out with rat mRNA as described (22).

**Cell Culture**

15-mL cultures of THP-1 human monocytic leukemia cells were grown at 37 °C and 95% humidity in Iscove’s modified medium, 9% horse serum, 2 mM glutamine, 0.05 mM β-mercaptoethanol, and 1% penicillin/streptomycin. THP-1 cells were induced to undergo apoptosis (referred to as “activated” THP-1 cells) by incubation in 25 μM cycloheximide for 4 h prior to preparation of the extract (see below).

**Cytosolic Extract Preparation**

Whole lenses from neonatal mice or E15.5–18.5 rat embryos were excised, placed in hypotonic lysis buffer (25 mM HEPES (pH 7.5), 5 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μM pepstatin-A (acylguanidinyl) fluoride, and protease inhibitor mixture (13)), and sheared in a tight-fitting Dounce homogenizer. Cytosolic extracts were prepared as described previously (23). The extracts were dialyzed overnight at 4 °C using Slide-A-Lyzer® mini-dialysis units (M, 7000 cutoff, Pierce) against buffer containing 25 mM HEPES (pH 7.5), 10% sucrose, and 20 mM dithiothreitol. The pH was adjusted to 8–9.0 at 4 °C. Cytosolic lenses from two litters of neonatal mice were pooled, and three separate pools were analyzed for caspase activities. Likewise, lenses from two to four litters of mouse or rat embryos were pooled, and at least two separate pools were analyzed for caspase activity. For THP-1 cell extracts, cells were pelleted by centrifugation at 500 × g; the pellet was resuspended in hypotonic lysis buffer, and the lysate was placed in a hypotonic lysis buffer and heated for 3 min at 90 °C. Typically, lenses from two litters of mouse or rat embryos were pooled, and at least two separate pools were analyzed for caspase activity. For THP-1 cell extracts, cells were pelleted by centrifugation at 500 × g; the pellet was resuspended in hypotonic lysis buffer, and the lysate was placed in a hypotonic lysis buffer and heated for 3 min at 90 °C. Typically, lenses from two litters of mouse or rat embryos were pooled, and at least two separate pools were analyzed for caspase activity. For THP-1 cell extracts, cells were pelleted by centrifugation at 500 × g; the pellet was resuspended in hypotonic lysis buffer, and the lysate was placed in a hypotonic lysis buffer and heated for 3 min at 90 °C. Typically, lenses from two litters of mouse or rat embryos were pooled, and at least two separate pools were analyzed for caspase activity. For THP-1 cell extracts, cells were pelleted by centrifugation at 500 × g; the pellet was resuspended in hypotonic lysis buffer, and the lysate was placed in a hypotonic lysis buffer and heated for 3 min at 90 °C.

**Enzyme Activity Assays**

**General Caspase Activity Assay Conditions—** Assays were performed in 100-μl reaction mixtures containing 100 mM HEPES (pH 7.5), 10 mM dithiothreitol, 1% CHAPS, and 10 μM dithiothreitol. The volume of stock solutions was adjusted to yield concentrations of 1–10 μM peptide substrates indicated in the figure legends. Each reaction mixture was incubated with 50 μg of extract and 1 μM peptide substrate (HIAD-AMC, HIAE-AMC, EVD-AMC, LEHD-AMC, MAEL-AMC, NED-AMC, YVAD-AMC, LEVD-AMC, or VEID-AMC) in a total volume of 50 μl for 20 min at 37 °C.

**Caspase Reporter and Inhibitor Peptides—** Caspase activity was measured using the following reporter peptides and inhibitors: YVAD-AFC/YVAD-CHO, IETD-AFC/IETD-CHO, and LEHD-AFC/LEHD-fmk, DEVD-AFC/DEVD-CHO, LEVD-AFC/LEVD-CHO, VEID-AFC/VEID-CHO, IETD-AFC/IETD-CHO, and LEHD-AFC/LEHD-fmk (Calbiochem).

**Proteasome Activity Assays and Proteasome Inhibitors—** Proteasome activity was measured by monitoring cleavage of 300 μM LLVY-AMC (Promega) as described for caspase activity. Lactacystin (1 μM) was used to control for proteasome-specific activity. The proteasome inhibitors tested for their effects on caspase activity were lactacystin (100 μM), 4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-vinylsulfone (100 μM), epoxomicin (50 μM), and clasto-lactacystin β-lactone (50 μM) (all from Calbiochem). The effect of proteasome inhibitors on caspase activity was calculated as the percentage of caspase reporter peptide cleavage by the recombinant caspase in the absence of inhibitor. All recombinant caspases were obtained from Calbiochem.

**Western Blot Analysis**

Lenses were lysed in radioimmunoprecipitation buffer; lysates were prepared; and protein concentrations were measured as described previously (13, 24). 300 μg of extract was separated on 15% SDS-polyacrylamide gels and transferred to nitrocellulose, and the membranes were blotted overnight with anti-caspase-6 or anti-caspase-3 antibody (9762 and D175, respectively; Cell Signaling Technology). Primary antibody was detected using horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences), and signal was detected using the ECL Plus...
Table I
Expression of caspases and their regulators in the mouse lens

| Gene         | Expression | Method of detection |
|--------------|------------|---------------------|
| Caspase-1    | Yes        | RT-PCR              |
| Caspase-2    | Yes        | RT-PCR              |
| Caspase-3    | Yes        | RT-PCR              |
| Caspase-5    | Yes        | RT-PCR              |
| Caspase-6    | Yes        | RT-PCR              |
| Caspase-7    | Yes        | RT-PCR              |
| Caspase-8    | Yes        | RT-PCR              |
| Caspase-9    | Yes        | RT-PCR              |
| bcl-2        | Yes        | RT-PCR              |
| bcl-x        | Yes        | In situ/RNase protection |
| bak          | Yes        | In situ             |
| TNFR1        | Yes        | RT-PCR              |
| fas          | Yes        | RT-PCR              |
| favd         | Yes        | RT-PCR              |
| tradd        | Yes        | RT-PCR              |
| apaf1        | Yes        | RT-PCR              |
| AIF          | Yes        | RT-PCR              |
| survivin     | Yes        | RT-PCR              |
| iap1         | No         | RT-PCR              |
| iap2         | Yes        | RT-PCR              |

Both long and short forms were detected.
Caspe-6 is grouped with Group III caspases as done by Thornberry et al. (31).
RNase protection was performed with rat lens RNA.

RESULTS
Expression of Caspases and Regulatory Molecules in the Lens—Many caspases and caspase regulatory molecules have been reported to be expressed in lenses from a range of species (3, 19, 25–27). Although some studies have examined caspase and caspase regulator expression in murine lenses (28–30), a broad survey of the spectrum of molecules involved in the caspase pathway for mouse and rat has not been conducted. To determine the extent to which caspases and their regulators are expressed in murine lenses, we conducted RT-PCR, RNase protection, and in situ hybridization experiments on lens RNA or lens sections from neonatal mice. Caspases can be divided roughly into three groups based on their substrate affinities (31). Group I caspases are primarily involved in the generation of an immune response. Group II caspases are thought to mediate the majority of changes that are associated with the apoptotic phenotype. Group III caspases are thought to be primarily involved in the activation of the group II caspases. RNA transcripts from a wide range of caspase genes and genes that encode proteins that can regulate caspases were detected in neonatal mouse lenses (Table I). Intact receptor-associated pathways were present for both tumor necrosis factor receptor-1 and fas. Transcripts for the Group I caspase, caspase-1, and for several Group II and III caspases were detected. Finally, transcripts for many caspase regulatory molecules also were detected, indicating that multiple levels of control of caspase activity might be in place within the lens. Thus, it would appear that lens cells should be capable of regulating and promoting a variety of caspase responses.

Establishing Caspase-specific Assays for the Lens: Eliminating the Contribution of the Proteasome in Caspase Activity Assays—To determine which caspases are active in cell extracts from the lens, we used caspase activity assays. These assays take advantage of a panel of short peptide substrates, each of which contains the preferred sequence for cleavage by a given caspase (32). For use in these activity assays, the short peptide substrates are conjugated to fluorochromes and used as reporter molecules. When mixed with extracts containing caspases, the reporter peptide is recognized by caspases in an amino acid sequence-specific manner, and the fluorochrome is cleaved from the peptide, generating fluorescence that can be measured by fluorometry. The same peptides, when conjugated to reactive chemical moieties such as CHO and fmk are potent inhibitors of caspases (33). Preincubation of an extract with an inhibitor prior to the addition of the corresponding reporter peptide arguably controls for non-caspase-mediated cleavage of the reporter peptides. The difference between fluorescence generated in the absence and presence of inhibitor is taken to be reflective of the “caspase-specific” activity. However, for the calculation to be accurate, these peptides must be highly specific substrates for caspases. If any other enzyme cleaves the reporter and is also inhibited by the caspase inhibitor, then caspase activity will be overestimated. This error could become quite pronounced if there is a large amount of nonspecific enzyme activity and/or a small amount of intrinsic caspase activity within the extracts being tested.

The proteasome is a cytosolic multisubunit complex containing a number of discrete proteolytic activities that degrade specifically targeted proteins. The proteasome contains an activity capable of cleaving caspase reporter peptides (34, 35). This capacity of the proteasome most likely is due to the post-glutaryl-peptide hydrolase activity of this enzyme complex (36). Of note, this proteasome activity was also found to be sensitive to preincubation with YVAD-CHO and YVKD-CHO, two caspase peptidyl inhibitors. Bovine, rat, and human lenses have been shown to contain significant levels of proteasome activity (37–39). To assess proteasome activity levels in murine lens extracts ourselves, we tested extracts from whole lenses of neonatal mice for their capacity to cleave the proteasome chymotrypsin-like substrate LLVY-AMC. The extracts contained an activity of 15.88 ± 1.82 fmol μg⁻¹ min⁻¹. Additionally, the LLVY-AMC cleavage activity was inhibited by the caspase inhibitors VEID-CHO and IETD-CHO (data not shown). Finally, YVAD-AFC cleavage activity was abolished by preincubation with the proteasome inhibitor lactacystin. Thus, not only can the proteasome recognize caspase substrates, as previously noted (34, 35), but also at least four different caspase inhibitory peptides can significantly inhibit the proteasome. From these data, we conclude that proteasome activity must be inhibited to measure accurately caspase activity in lens extracts.

To identify a proteasome inhibitor that would not cross-react with caspases, we tested four common proteasome inhibitors for their ability to inhibit caspases. Recombinant caspase-1, -3, -6, and -8 were examined for activity against their preferred reporter peptides after preincubation with the proteasome inhibitors listed in Table II. Each of these proteasome inhibitors...
TABLE II

| Inhibitor | Caspase-1 | Caspase-3 | Caspase-6 | Caspase-8 |
|-----------|-----------|-----------|-----------|-----------|
| NIP-L-S | 60        | ND        | 12        | 81        |
| Epoxomicin | 61        | 91        | 72        | ND        |
| β-Lactone | 72        | 92        | 69        | ND        |
| Lactacystin | 112       | 112       | 108       | 91        |

has been demonstrated to inhibit the trypsin-like, chymotrypsin-like, and post-glutamyl-peptide hydrolase activities of the proteasome (40–42). As shown in Table II, the inhibitors varied in their effect on the recombinant caspases, ranging from minimal to almost complete inhibition. Of the four inhibitors studied, lactacystin was the only one to show minimal effects on the activity of all caspases tested. Therefore, we chose to use lactacystin to inhibit the proteasome in subsequent caspase activity assays.

We next determined the concentration of lactacystin needed to inhibit the proteasome activity found in lens extracts. Consistent with previous studies in the lens (39), proteasome activity was fully inhibited by 100 μM lactacystin (data not shown), a concentration ~10-fold higher than that typically used to inhibit this activity in extracts from other tissues.

Caspase Activity in Normal Lens Extracts—Having determined the appropriate conditions for reliably measuring caspase activities in mouse lens extracts, we next tested lens extracts for caspase peptidyl cleavage activities. As a wide range of caspases were found to be transcribed in the lens (Table I), cleavage of eight different reporter peptides that are the preferred substrates for eight different caspases (31) was tested. First, to verify that all peptides could be cleaved, cleavage of all the peptides was tested in an extract generated from THP-1 cells that had been induced to undergo apoptosis. In preliminary experiments, there was very little cleavage of the WEHD-AFC and VEID-AFC peptides by the THP-1 extract. To ensure that the preferred enzymes for cleaving these substrates were present in the extract, recombinant caspase-1 and caspase-6 were added to the extract. Fig. 1A shows that all eight substrate peptides could be cleaved by an activated THP-1 extract supplemented with recombinant caspase-1 and caspase-6.

Lens extracts were subjected to a two-step analysis. First, an initial screen was performed at relatively low stringency so that cleavage of any peptide by a caspase would not be overlooked. The cleavage of all eight caspase reporter peptides by the lens extract was tested using the corresponding caspase inhibitors (10 μM) and lactacystin (10 μM). Next, peptides that were cleaved in this initial screen were subjected to testing at a much higher stringency using 500 nM to 1 μM caspase inhibitor and 100 μM lactacystin to determine accurate caspase activities for cleavage of these peptides. The VEID-AFC, IETD-AFC, LEVD-AFC, and YVAD-AFC peptides were all cleaved by lens extract in the initial screen (Fig. 1B). No caspase-specific cleavage of the other four peptides, including DEVD-AFC, was detected. In the high stringency assays (Fig. 1C), the cleavage rate for VEID-AFC, the preferred substrate for caspase-6, was highest in the extracts at 18.75 ± 0.32 fmol μg⁻¹ min⁻¹, the IETD-AFC and LEVD-AFC cleavage rates were 5.34 ± 0.13 and 3.23 ± 0.11 fmol μg⁻¹ min⁻¹, respectively. YVAD-AFC cleavage activity was zero under these high stringency conditions. These results indicate that normal lens extracts contain a subset of possible caspase cleavage activities.

Peptide Cleavage by One Versus Multiple Caspases—Although each of the reporter peptides we tested for cleavage by lens extracts is the preferred peptide for one specific caspase, it is known that an individual caspase can also cleave at least some other peptides, albeit at suboptimal rates (31). The reporter peptides that were cleaved by lens extracts (VEID-AFC, IETD-AFC, and LEVD-AFC) are the preferred peptides for caspase-6, -8, and 4, respectively. These caspases are known to be the most promiscuous caspases in terms of their substrate specificity. We had determined previously that recombinant caspase-6 and caspase-8 will cleave both their preferred reporter peptides (VEID-AFC and IETD-AFC, respectively) as well as the other reporters, albeit at differing rates (data not shown). It is therefore possible that the cleavage of IETD-AFC

![FIG. 1. Caspase activities in lens extracts from neonatal FVB mice.](image-url)
and LEVD-AFC by the lens extracts was due to suboptimal cleavage of these peptides by the enzyme that cleaves VEID-AFC rather than the activity of additional enzymes. This possibility seemed likely because LEVD-AFC is considered the preferred peptide for human caspase-4, an enzyme for which no mouse homolog has been identified. We therefore asked whether the cleavage of VEID-AFC and IETD-AFC might be due to a single caspase.

To address this question, we investigated whether the VEID-CHO and IETD-CHO inhibitors could help us distinguish between caspase-6 and caspase-8 activities. Specifically, we measured the relative efficiency with which each of these peptide inhibitors could inhibit the peptide cleavage activities of recombinant caspase-6 and caspase-8. As shown in Fig. 2A, both the cleavage of VEID-AFC by caspase-6 and the cleavage of IETD-AFC by caspase-8 were efficiently inhibited by 500 nM VEID-CHO. However, although the cleavage of IETD-AFC by caspase-8 was completely inhibited by 500 nM IETD-CHO, the cleavage of VEID-AFC by caspase-6 was only partially inhibited by 500 nM IETD-CHO. Because the IETD-CHO peptide discriminates between caspase-6 and caspase-8 activities, we used it to help determine whether the cleavage of VEID-AFC and IETD-AFC by lens extracts was due to caspase-6 and/or caspase-8.

The VEID-CHO and IETD-CHO inhibitors were tested for their ability to inhibit cleavage of VEID-AFC and IETD-AFC by lens extracts from neonatal mice. Fig. 2B shows that the degree of inhibition of the peptide cleavage reactions by the two inhibitors paralleled that seen with recombinant caspase-6, i.e. IETD-CHO only partially inhibited the cleavage of these two reporters, whereas VEID-CHO efficiently inhibited the cleavage of both. This result is consistent with caspase-6 in lens extracts being the enzyme mainly responsible for cleaving both VEID-AFC and IETD-AFC. Were caspase-8 in lens extracts responsible for cleavage of IETD-AFC, we should have seen efficient inhibition of its cleavage by IETD-CHO, as seen for the recombinant caspase-8.

Timing of DNA Degradation in Rat Primary Fiber Nuclei—In late stage murine embryos, the fiber cell compartment of the lens consists of two populations of cells referred to as primary and secondary fiber cells. The primary fiber cells are the first fiber cells to form and arise from elongation of cells at the posterior of the hollow lens vesicle. The vesicle is fully occluded by about E13.5 in mice and E14.5 in rats (43). These primary fiber cells become a scaffold upon which secondary fiber cells are concentrically laid down throughout the life of the organism. Due to the relatively synchronous differentiation of the primary fibers, these cells represent a large population of cells in approximately the same state of differentiation at any given time. The secondary fiber cells differentiate asynchronously; therefore, there are fewer cells in any one state of differentiation at a given time. Thus, we reasoned that primary fiber cell differentiation is the stage of lens differentiation where it would be possible to determine whether caspase cleavage activities are temporally regulated in a manner that correlates with organelle loss (44). DNA degradation is a convenient marker for the end stages of organelle loss, as the fragmented ends of the DNA are easily labeled by TUNEL analysis. To determine whether VEID-AFC cleavage activity is temporally correlated with primary fiber denucleation, we elected to use rat embryos as the source of lens tissue due to their relatively large size compared with mouse lenses. It has been shown previously that primary fiber cell denucleation begins at ~17 days of embryonic development in the Wistar strain of rats (45). To confirm that the timing of denucleation is similar in the Sprague-Dawley rats, we performed TUNEL analysis on lens sections from E15.5, E17.5, E18.5, and E19.5 rat embryos. As shown in Fig. 3, at E15.5, primary fiber cell nuclei were not labeled by TUNEL. At E17.5, a small percentage of nuclei were TUNEL-positive in most (but not all) lenses examined. By E18.5 (data not shown) and E19.5, TUNEL-positive primary fiber cell nuclei were widespread in all of the lens sections examined. In addition, the intensity of the TUNEL labeling was less at E17.5 than at E18.5 (data not shown) and E19.5. Overall, these results confirm the previous findings of He et al. (45) and define the temporal window for primary fiber cell DNA degradation as beginning sometime between E15.5 and E17.5 and being widespread by E19.5.

Temporal Regulation of VEID Cleavage Activity—To determine whether caspase activity is correlated with the onset of nuclear labeling by TUNEL analysis, we tested lens extracts from E15.5–18.5 rat embryos for VEID-AFC, IETD-AFC, and DEVD-AFC cleavage activities. As shown in Fig. 4, at E15.5, weak VEID-AFC was detected (3.36 ± 1.83 fmol μg⁻¹ min⁻¹). At E16.5, the cleavage activity was substantially higher and continued to increase incrementally at E17.5 and E18.5, at which point it had reached 20.61 fmol μg⁻¹ min⁻¹. This level of activity was similar to that found in extracts from neonatal
The cleavage activities (expressed in fmol \( \mu g^{-1} \) min \(^{-1} \)) generated from E15.5, E16.5, E17.5, and E18.5 rat embryos were tested for activity against 30 \( \mu \)M VEID-AFC (■) or IETD-AFC (▲) peptide. The corresponding CHO inhibitor was used at 500 nM, and lactacystin was used at 100 \( \mu \)M. The cleavage activities (expressed in fmol \( \mu g^{-1} \) min \(^{-1} \)) represent the difference in the rate of fluorescence generated by the cleavage of the reporter peptide in the absence of the corresponding CHO inhibitor and the rate of fluorescence generated by the cleavage activity in the presence of the corresponding CHO inhibitor. The average activity ± S.D. from two independent experiments is shown. The values obtained for VEID-AFC cleavage activity at E17.5 \( (p = 0.05) \) and E18.5 \( (p = 0.01) \) are significantly different from the value obtained for VEID-AFC cleavage activity at E15.5. There was no statistically significant difference between the values obtained for IETD-AFC cleavage activity at E16.5 \( (p = 0.51) \), E17.5 \( (p = 0.26) \), and E18.5 \( (p = 0.34) \) and the value obtained at E15.5.

Therefore, the inhibitory activities of the DEVD-CHO and VEID-CHO peptides on cleavage activities in lens extracts from rat and mouse embryos were compared in tests analogous to those described for caspase-6 and caspase-8 (Fig. 2). The results from these tests suggest that a single enzyme could account for most of the activity (data not shown).

Curiously, no DEVD-AFC cleavage activity was detected in any of the extracts from rat or mouse embryos. Caspase-3 activity has been associated with activation of DNases and degradation of nuclei in apoptotic cells (46, 47). To determine whether a DEVD-AFC activity might be associated with the organelles that were removed during preparation of the extracts, we tested the pellets generated during the extract preparation for DEVD-AFC and VEID-AFC activities. No DEVD-AFC activity was detected; however, a low level of VEID-AFC cleavage activity was detected (data not shown). This activity may represent an enzyme activity that is associated with the organelles, a residual enzyme activity resulting from the incomplete removal of the supernatant from the pelletted fractions, or a combination of both.

**Temporal Regulation of Caspase-6 Protein Levels**—VEID is the preferred consensus cleavage sequence for caspase-6 (31, 48). To determine whether the increase in the VEID-AFC activity correlates with changes in caspase-6 protein levels, extracts from rat embryos were subjected to Western blot analysis with an anti-caspase-6 antibody that can recognize procaspase-6 (Fig. 5A). Procaspase-6, the inactive precursor form of the enzyme, was detected at E15.5–18.5. Changes in the levels of procaspase-6 during this developmental window were assessed by comparing the levels with those of glyceraldehyde 3-phosphate dehydrogenase (Fig. 5A) and β-actin (data not shown). Between E15.5 and E18.5, procaspase-6 levels decreased by 5.9-fold (i.e. the levels at E18.5 were 17% of those at E15.5). This decrease correlated with the 6.1-fold increase in VEID-AFC cleavage activity (Fig. 4) detected over the same time frame. Furthermore, the fold decreases in procaspase-6 levels from E15.5 to E16.5 (3.1-fold) and from E15.5 to E17.5 (5.4-fold) correlated with the fold increases in VEID-AFC cleavage activity from E15.5 to E16.5 (3.7-fold) and from E15.5 to E17.5 (4.5-fold). A second antibody, anti-Mch2 antibody, which recognizes full-length caspase-6 and the alternatively spliced β-isofrom (49) of the protein, detected both of these isoforms in extracts from neonatal FVB mice (data not shown).

These results demonstrate that multiple isoforms of caspase-6 are expressed in lenses and that a decrease in procaspase-6 levels (Fig. 5) during the developmental window tested is inversely correlated with the increase in VEID-AFC cleavage activity (Fig. 4). In contrast, consistent with our failure to
detect DEVD-AFC cleavage activity, Western blot analysis of extracts from E15.5–17.5 rat embryos using an antibody specific for the cleaved activated form of caspase-3 failed to detect cleaved caspase-3 (Fig. 5B). Two other anti-caspase-3 antibodies also failed to detect cleaved caspase-3 in these extracts (data not shown). Together, these results suggest that caspase-6 may be responsible for the VEID-AFC cleavage activity detected in lenses.

Caspase Activities in Apoptotic Lenses—Lens fiber cell de-nucleation has often been characterized as an apoptosis-like event (3). However, the caspase activities found in extracts from normal murine lenses (Figs. 1C, 2B, and 4) are atypical of a classical apoptotic profile because caspase-3-like DEVD-AFC cleavage was not detected. To determine whether the lens is restricted in its potential to VEID-AFC caspase activities, we examined the caspase profile in lenses that had been induced to undergo classical apoptosis.

We previously characterized transgenic mice that express the human papilloma virus type 16 E7 gene within the lens, referred to as aAE7 transgenic mice (13, 14). Expression of E7 results in dysregulation of cell cycle control and concomitant apoptosis of lens fiber cells. We used lenses from neonatal aAE7 transgenic mice as a source of apoptotic lens cells. Extracts prepared from the lenses of these mice were screened at low stringency for activity against the panel of caspase reporter peptides listed in Fig. 1A. Positive peptides were then subjected to retesting at high stringency. Fig. 6A shows that lens extracts from neonatal aAE7 transgenic mice contained a high level of cleavage activity for DEVD-AFC (38.16 ± 3.47 fmol µg⁻¹ min⁻¹), the preferred reporter peptide for caspase-3. Consistent with this result, we detected activated caspase-3 protein in extracts from neonatal aAE7 transgenic mice by Western blot analysis (Fig. 6B), suggesting that this enzyme is responsible for the DEVD-AFC cleavage activity in these lenses. As seen in rat lenses (Fig. 5B), activated caspase-3 was not detected in lens extracts from neonatal FVB non-transgenic mice (Fig. 6B). In lens extracts from aAE7 transgenic mice, the cleavage rate for VEID-AFC was 21.84 ± 1.64 fmol µg⁻¹ min⁻¹, which was slightly higher than that seen in extracts from neonatal FVB non-transgenic mice. The inhibitory activities of DEVD-CHO and VEID-CHO against reporter peptide cleavage by the extracts from the aAE7 mice were compared in tests analogous to those described for caspase-6 and caspase-8 (Fig. 2). These tests showed that the enzyme responsible for cleavage of the DEVD-AFC peptide (i.e. caspase-3) in the aAE7 lens extract contributed partially (but not fully) to the cleavage of the VEID-AFC peptide (data not shown). Low and highly variable cleavage activity of IETD-ABC was also observed. Thus, our results are consistent with both activated caspase-3 and activated caspase-6 being present in extracts of lens cells undergoing apoptosis.

DISCUSSION

Multiple lines of evidence suggest that caspases might be involved in the elimination of organelles from lens fiber cells in vertebrates (3). In this study, we determined whether caspase activities are present in normal murine lens tissue and if these activities correlate with organelle loss. We showed that a VEID-AFC activity predominates in normal lenses (Fig. 1C). This activity most likely arises from caspase-6 given that VEID...
is the preferred peptide for caspase-6 and given the observed decrease in the levels of full-length procaspase-6 that was temporally and inversely proportional to the increase in VEID-AFC cleavage activity (compare Figs. 4 and 5A). In addition, the lens VEID-AFC activity resembles caspase-6 in its sensitivity to VEID-CHO and IETD-CHO inhibition. However, lens organelle loss does not appear to be affected in mice carrying a null mutation of caspase-6. The absence of a phenotype in caspase-6-null mice may be due to compensation for the loss of caspase-6 by other caspases, influences of the genetic background of the mutant mice that modify the effect of loss of caspase-6 function, or the presence and activity of a previously unidentified caspase-6-like protein in murine lenses. Our data suggest that the IETD-AFC cleavage activity is due mainly to the same enzyme that cleaves VEID-AFC (Fig. 2 and data not shown). However, it is possible that a minor part of the IETD-AFC cleavage activity is due to caspase-8 given the divergence of VEID-AFC and IETD-AFC cleavage rates seen at later embryonic time points (Fig. 4) compared with earlier time points.

Previous work has suggested that organelle loss occurs over a period of as long as 3 days, beginning with a rapid loss of mitochondria and endoplasmic reticulum over 2–4 h and ending with a protracted process of nuclear loss that occurs over 2–3 days (20, 21). Using the strong TUNEL staining in rat primary fibers as a marker for the DNA fragmentation occurring during denucleation (Fig. 3), this time window appears to encompass E16.5–19.5. We detected a sharp rise in VEID-AFC cleavage activity on E16.5 (Fig. 4). That the VEID-AFC cleavage activity rises 2–3 days before widespread extensive DNA degradation suggests that this caspase activity may play a role not only in DNA degradation, but also in earlier aspects of organelle loss. Alternatively, these data may suggest that the caspase activity is involved only in denucleation, but that denucleation is a lengthy and highly regulated process.

Surprisingly, we detected neither DEVD-AFC cleavage activity (Fig. 1B) nor activated caspase-3 protein (Figs. 5B and 6B) in normal lenses despite the fact that both were readily detected in apoptotic lenses of aAE7 transgenic mice (Fig. 6). Consistent with our observations, recent studies suggest that caspase-3 activity is not associated with epidermal differentiation (50, 51), a process that is also characterized by organelle loss. However, because it may be possible that the caspase-3 is rapidly turned over in cells, we cannot rule out the possibility that activated caspase-3 is present at levels below our detection limits. Even if this were the case, the biological significance of an undetectable caspase-3 activity is unclear, given that targeted deletion of caspase-3 in mice does not affect lens development despite causing developmental defects in other tissues (52).

Two previous studies examining the possibility that caspases are involved in fiber cell differentiation led to the conclusion that caspase-3 is involved in fiber cell denucleation, a conclusion that differs from ours. Ishizaki et al. (18) reported that the pan-caspase inhibitor benzoyloxycarbonyl-VAD-fmk inhibits loss of nuclei and poly(ADP-ribose) polymerase cleavage in rat lens explant cultures. The inhibition of poly(ADP-ribose) polymerase cleavage by benzoyloxycarbonyl-VAD-fmk led them to conclude that caspase-3 or a caspase-3-like caspase may be involved in the loss of nuclei. Wride et al. (19) reported that multiple activated caspase family members, including caspase-3, are present at the protein level in the developing chick lens. They also reported that multiple caspase inhibitor peptides, but significantly, not the caspase-3 inhibitor DEVD-CHO, reduce the loss of nuclei in a chick lens cell culture differentiation assay. Given that these two studies did not measure caspase activities directly, poly(ADP-ribose) polymerase can be cleaved by multiple caspases (53, 54), and DEVD-CHO did not inhibit chick lens cell differentiation in culture, it is unclear whether caspase-3 activity accounts for the inhibition of denucleation these authors observed. Interestingly, Wride et al. found that the most effective caspase inhibitor in their system was VEID-fmk, a result that is consistent with our findings and that supports our conclusion that a caspase-6 or caspase-6-like enzyme is required for denucleation.

We have shown that the caspase activity in normal lenses is regulated in a temporal fashion that correlates with denucleation, suggesting that this activity promotes the DNA degradation during lens fiber cell differentiation (Fig. 4). How this proteolytic activity might promote DNA degradation is not known. In apoptosis, caspase-3 has been shown to target ICAD (inhibitor of caspase-activated DNase; DNA fragmentation factor) and thus promote internucleosomal fragmentation (55). Although it has been documented that cleavage of DNA into internucleosomal-length fragments occurs during fiber cell differentiation in chicks (11), the DNA in differentiating mouse and rat lens fibers appears to be more random in size (13, 14).

Thus, the primary mechanism of DNA fragmentation in the murine lens may not be internucleosomal cleavage. In support of a non-ICAD-related mechanism, we found no caspase-3 activity in normal lenses, and targeted deletions of caspase-3 or ICAD in mice do not lead to lens phenotypes (52, 56). Caspase-6 might promote the activation of a DNase, other than CAD (caspase-activated DNase), within the lens. Nishimoto et al. (57) recently reported that targeted deletion of the DLAD (DNase II-like acidic) gene results in denucleation defects in the mouse lens. Unlike that seen with CAD, hydrolysis of DNA by DLAD results in 3’-phosphates. Why 3’-OH groups are detected by TUNEL on DNA within degenerating lens nuclei is unclear, but might be the result of phosphatase activity secondary to DLAD-dependent DNA hydrolysis. Alternatively, caspase-6 might promote DNA degradation via an indirect mechanism. Bassnett and Mataic (20) have shown that DNA degradation in chick lenses is a relatively late event, occurring after nuclear envelope breakdown. A caspase-6-mediated breakdown of the nuclear envelope could facilitate access of a DNase that is activated via a non-caspase mechanism.

The profile of caspase activities in normal lenses differs from that seen in the classically apoptotic situation within the same tissue. These differences underscore the fact that organelle loss and apoptosis are distinct processes and suggest that a cell can use an apoptotic enzyme(s) for more than one purpose. How lens cells might accomplish the differential regulation of caspase activities is an interesting question. Kamradt et al. (58) have demonstrated that α-crystallin, a major protein species in lens fiber cells, can bind to partially processed but inactive caspase-3 and block its maturation to a fully active enzyme. These findings provide a plausible explanation for the ability of lens cells during normal fiber cell differentiation to contain an activated caspase capable of VEID-AFC cleavage activity and, at the same time, prevent activation of caspase-3. How procaspase-3 is activated in lens cells when apoptosis is induced remains to be elucidated.

The role of caspases in initiating and effecting the events of apoptosis has been well established. Their function in dismantling cells, along with their combination of strictly circumscribed targets and tight regulation, makes them ideal candidates for effecting the more limited destruction of organelles that occurs during differentiation of tissues such as the lens, epidermis, and mammalian erythrocytes. We have demonstrated that a small subset of caspase activities exists within

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S. Bassnett, personal communication.
the lens and that these activities can exist without cross-activating caspase activities found in a typical apoptotic response. This suggests that the disassembling role of caspases can be parcelled out into discrete packages, i.e., that activities can be controlled in such a way that some structures are eliminated while others remain intact. Indeed, intact actin filaments within the lens attest to the ability of the lens to protect some structures that are known caspase targets during apoptosis (59). If caspase-6 is involved in organelle loss and determination of the mechanisms by which caspase activity is regulated are important areas for future study.

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