THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 23, Issue of June 10, pp. 22135–22145, 2005
Printed in U.S.A.

The Canonical Intrinsic Mitochondrial Death Pathway Has a Non-apoptotic Role in Signaling Lens Cell Differentiation*

Received for publication, December 20, 2004, and in revised form, March 23, 2005
Published, JBC Papers in Press, April 12, 2005, DOI 10.1074/jbc.M414270200

Gregory F. Weber‡ and A. Sue Menko§

From the Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

The mitochondrial cell death pathway is known for its role in signaling apoptosis. Here, we describe a novel function for the mitochondrial cell death pathway in signaling initiation of differentiation in the developing lens. Most remarkably, we induced lens cell differentiation by short-term exposure of lens epithelial cells to the apoptogen staurosporine. Activation of apoptosis-related pathways induced lens epithelial cells to express differentiation-specific markers and to undergo morphogenetic changes that led to formation of the lens-like structures known as lentoids. The fact that multiple stages of differentiation are expressed at a single stage of development in the embryonic lens made it possible to precisely determine the timing of expression of proteins associated with the apoptotic pathway. We discovered that there was high expression in the lens equatorial epithelium (the region of the lens in which differentiation is initiated) of pro-apoptotic molecules such as Bax and Bcl-x_S and release of cytochrome *c* from mitochondria. Furthermore, we found significant caspase-3-like activity in the equatorial epithelium, yet this activity was far lower than that associated with lens cell apoptosis. These apoptotic pathways are likely regulated by the concurrent expression of prosurvival molecules, including Bcl-2 and Bcl-x_L; phosphorylation of Bad; and high expression of inhibitor of apoptosis proteins chicken IAP1, IAP3, and survivin. This finding suggests that prosurvival pathways allow pro-apoptotic molecules to function as molecular switches in the differentiation process without tipping the balance toward apoptosis. We call this process apoptosis-related Bcl-2- and caspase-dependent (ABC) differentiation.

Cell signaling mechanisms involving caspases, the cysteine protease effectors of apoptotic pathways, are responsible for the loss of organelles and nuclei in lens cells as they differentiate into mature fiber cells (1–3). This process is necessary to create an optically transparent lens. Interestingly, despite the activation of apoptotic signaling molecules and the loss of intracellular structures, differentiated lens fiber cells survive throughout an organism's lifetime. Caspases also play a requisite role in the differentiation of cell types that do not lose subcellular structures (4–7). This suggests that apoptosis-related signaling pathways may be required for lens epithelial cell differentiation, playing roles distinct from the removal of organelles and nuclei.

For secondary lens fiber cell differentiation to occur, undifferentiated epithelial cells from the anterior surface of the lens must enter the equatorial zone, where they first pass through a zone of proliferation (8) and then initiate their differentiation program (9). With the initiation of differentiation, these lens epithelial cells begin a remarkable process in which they change shape as they participate in the creation of the unique cellular organization that characterizes the lens and that is essential to its transparency. This dramatic morphogenesis involves molecular remodeling of the cytoskeleton and cell-cell adherens junctions (10). Only after these morphogenetic changes have occurred do the maturing lens fiber cells lose their organelles and nuclei in a wave that spreads outwards from the central nuclear fiber cell region (11–15). Although, in the chicken embryo, this organelle-free zone (OFZ)[1] is not observed until embryonic day (E) 12, mitochondria in the central fiber cell region begin to appear fragmented as early as E8 (14–16). This suggests that molecular mechanisms involving mitochondrion-dependent apoptosis-like pathways may be activated significantly earlier than the appearance of the OFZ. We have examined the involvement of apoptosis-like mechanisms in signaling the early stages of lens cell differentiation as well as the survival molecules that prevent these pro-apoptotic pathways from causing lens cell death.

In the canonical mitochondrial death pathway, pro-apoptotic Bcl-2 family members (*e.g.* Bax, Bcl-x_S, and Bad) mediate the release of cytochrome *c* from mitochondria (17), which then participates in initiating the caspase cascade with the activation of caspase-9 (18). Caspase-9 cleaves and activates the executioner caspases, including caspase-3, -6, and -7 (18), which cleave numerous intracellular components, leading to apoptosis. Although Bad, Bax, and Bcl-x_S promote apoptosis, other Bcl-2 proteins (phospho-Bad, Bcl-2, and Bcl-x_L) inhibit the apoptotic process (19). The balance between Bcl-2 family members defines whether a cell will live or die. Increased Bax expression is often correlated with apoptotic cell death (20–22). However, when Bax is bound to Bcl-2 or Bcl-x_L, it is prevented from mediating the release of cytochrome *c* from mitochondria (17). The overexpression of Bcl-2 or Bcl-x_L prevents the release

* This work was supported in part by National Institutes of Health Grants EY10577, EY014798, and EY014258 (to A. S. 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.

‡ Supported by National Institutes of Health Training Grant ES007282.

§ To whom correspondence should be addressed: Dept. of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 571 Jefferson Alumni Hall, 1020 Locust St., Philadelphia, PA 19107. Tel.: 215-503-2166; Fax: 215-923-3808; E-mail: sue.menko@jefferson.edu.

[1] The abbreviations used are: OFZ, organelle-free zone; E, embryonic day; TUNEL, terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling; IAP, inhibitor of apoptosis protein; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone; Ac-DEVD-cmk, acetyl-Asp-Glu-Val-Asp chloromethyl ketone; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor-1; ERK, extracellular signal-regulated kinase; TMRM, tetramethylrhodamine methyl ester perchlorate; ch-IAP1, chicken IAP1; ABC, apoptosis-related Bcl-2- and caspase-dependent.

of cytochrome *c* from mitochondria and subsequent cell death, and these survival factors are often highly expressed in cells resistant to apoptosis (23–25). When Bcl-2 and Bcl-x_L are bound and sequestered by Bad, apoptosis is promoted (26). For Bcl-2 and Bcl-x_L to be free to bind molecules such as Bax, they must be released from Bad. This occurs when Bad is phosphorylated at any of its serine residues (each targeted by multiple kinases), which releases Bcl-2 and Bcl-x_L so that they can perform their prosurvival functions (27). Relatively little is known about the mechanism by which Bcl-x_S induces apoptosis, although it has been suggested that Bcl-x_S can block the anti-apoptotic functions of Bcl-2 and Bcl-x_L (28). Bcl-x_S expression generally increases during apoptosis (22), and its expression causes a marked increase in sensitivity to apoptotic agents (29). Several Bcl-2-related proteins are expressed in the developing lens (2), but their differentiation stage-specific expression has not been determined.

In the canonical apoptotic pathway, caspases have multiple roles. They cleave proteins to disable repair mechanisms, disassemble organelles and nuclei, and reorganize the cell into apoptotic bodies (30–35). During lens development, caspases serve a non-apoptotic role. The major caspases (caspase-1, -2, -3, -4, -6, -7, -8, and -9) are all expressed in the lens, and caspase-1, -2, -4, -6, and -9 have been implicated in the process of lens fiber cell nuclear loss (1, 2). Caspase-6 activity in lens differentiation is precisely timed with the appearance of TUNEL-positive nuclei (1). DNA fragmentation in the lens, as detected by the TUNEL assay, occurs after the disappearance of organelles and the dismantling of the nuclear envelope (36). In addition to regulating nuclear and organelle loss in the developing lens, caspases cleave components of the membrane-associated cytoskeleton (37), thereby remodeling lens fiber cell plasma membrane structures. The activation of caspases within differentiation-specific regions of the embryonic lens at developmental times that precede the formation of the OFZ has not been studied.

Inhibitor of apoptosis proteins (IAPs) bind to caspases, block their proteolytic activity, and can target them for degradation. Thus, IAPs serve as important regulators of caspase function, acting as protectors against apoptosis. mRNAs for several IAPs have been detected in the lens. The adult human lens contains stable mRNA transcripts for livin (38), and mRNAs encoding survivin and IAP2 are present in neonatal murine lenses (1). IAPs likely play regulatory roles during differentiation, allowing caspases to perform crucial functions but preventing them from tipping the balance toward apoptosis. Nothing is known about IAP protein regulation during embryonic lens development.

In this study, we have tested the hypothesis that molecular mechanisms characteristically associated with apoptosis function as molecular switches early in lens cell differentiation, temporally preceding the signaling of nuclear and organelle loss. We have discovered that the activation of the canonical intrinsic mitochondrial death pathway can signal lens cells to initiate their differentiation. In addition, we present evidence that a molecular balance is maintained through the expression of prosurvival Bcl-2 family proteins and IAPs that could regulate the apoptosis-related pathway to prevent lens cell commitment to apoptosis.

EXPERIMENTAL PROCEDURES

Lens Microdissection—Lenses were isolated from E10 chicken eggs (Truslow Farms, Chestertown, MD) and microdissected as described previously (39) to yield four distinct regions of differentiation: the central anterior epithelium, the equatorial epithelium, the cortical fiber zone, and the central fiber zone (see Fig. 1*A*).

Preparation and Treatment of Primary Lens and Whole Lens Cultures—Differentiating lens cell primary cultures were prepared as described previously (40). Briefly, lens cells were isolated from E10 quail lenses by trypsinization and agitation. Cells were plated on laminin and cultured in Medium 199 with 10% fetal bovine serum. For whole lens cultures, chicken embryo lenses were removed at E10 with the vitreous attached and grown in serum-free Medium 199. Apoptotic pathways were induced with either anisomycin (Sigma) or staurosporine (BIOMOL Research Labs Inc., Plymouth Meeting, PA) as indicated. Caspases were inhibited with the general caspase inhibitor 50 μM benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (Z-VAD-fmk) (Promega Corp., Madison, WI) or the caspase-3-specific inhibitor acetyl-Asp-Glu-Val-Asp chloromethyl ketone (Ac-DEVD-cmk) (Calbiochem). For all experiments, control cultures were exposed to Me_2SO (Sigma), the solvent for anisomycin, staurosporine, Ac-DEVD-cmk, and Z-VAD-fmk. For growth factor experiments, primary lens cell cultures were exposed to either fibroblast growth factor (FGF) (Sigma) or insulin-like growth factor-1 (IGF-1) (LONG™ R^3IGF-I, GroPep, Adelaide, Australia) at 15 ng/ml for 24 h. To block the activation of ERK in cultures to be treated with growth factors, cells were exposed to the ERK inhibitor U0126 at a concentration of 10 μM beginning 24 h prior to addition of growth factors.

Cytosolic Fractionation for the Determination of Cytochrome c Release—Cytosolic fractions were prepared using a protocol modified from Yang *et al.* (23). Microdissected lens tissue was homogenized with a Teflon pestle in buffer containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotinin. Samples were clarified by centrifugation two times at 800 $\times$ *g*. The resulting supernatant was centrifuged twice, first at 10,000 $\times$ *g* for 20 min and then at 16,000 $\times$ *g* for 20 min, to remove mitochondria (41). The final supernatant was used as the cytosolic fraction.

Caspase-3 Activity Assay—The CaspACE colorimetric assay system (Promega Corp.) was used to measure caspase-3 activity. Samples were extracted in cell lysis buffer provided by the manufacturer. The caspase activity assay was performed as specified by the manufacturer. Note that the substrate used in this assay is a DEVD tetrapeptide, which also serves as the preferred substrate for caspase-7. Therefore, we refer to the activity detected by this assay as caspase-3-like. Protein concentrations were determined using a modified Bradford assay (Bio-Rad). To control for the possibility that the proteasome cleaves the caspase substrate DEVD, the assay was also performed in the presence of the specific proteasome inhibitor lactacystin (200 μM; BIOMOL Research Labs Inc.).

Immunoblotting—Samples were extracted in 44.4 mM *n*-octyl β-D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, and 10 mM imidazole containing 1 mM sodium vanadate, 0.2 mM H_2O_2, and Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using the BCA assay (Pierce) unless stated otherwise. 15 μg of protein extracts were subjected to SDS-PAGE on precast 8–16% Tris/glycine gels (Novex, San Diego, CA). For the determination of cytochrome *c* release, 30 μg of total protein from each sample were loaded on 10–20% Tris/glycine gels. Proteins were electrophoretically transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). Anti-Bax (Δ21), anti-Bcl-$x_{L/S}$ (L-19), anti-Bcl-2 (N-19), anti-Bad (C-7), anti-phospho-Bad Ser^{112}, anti-survivin (FL-142), and anti-cellular IAP1/2 (A-13), antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to X-linked IAP and cytochrome *c* were from BD Biosciences. Phospho-Bad Ser^{136} was obtained from BIOSOURCE (Camarillo, CA). Antibodies to filensin and CP49 were generous gifts from Dr. Paul FitzGerald (University of California, Davis, CA). Anti-δ-crystallin antibody was a generous gift from Dr. Joram Piatigorsky (NEI, National Institutes of Health, Bethesda, MD). Anti-aquaporin-0 (MP28) antibody was prepared as described previously (40). Secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were detected using ECL reagent (Amersham Biosciences). Immunoblots were scanned, and densitometric analysis was performed using Eastman Kodak 1D software. For all experiments with microdissected lenses, results were normalized to the undifferentiated cells of the central epithelium.

Fluorescence Detection of F-actin and Nuclei—Cultures and E10 lenses were fixed in 3.7% formaldehyde. Lenses were further processed to obtain 7-μm cryosections as described previously (42). Cells were permeabilized by exposure to 0.25% Triton X-100 and incubated with Alexa 488-conjugated phalloidin and 4′,6-diamidino-2-phenylindole (Molecular Probes, Inc. Eugene, OR) to detect F-actin and nuclei, respectively. Fluorescently stained samples were examined using either a Nikon Eclipse 80i or Nikon Optiphot microscope (Optical Apparatus,

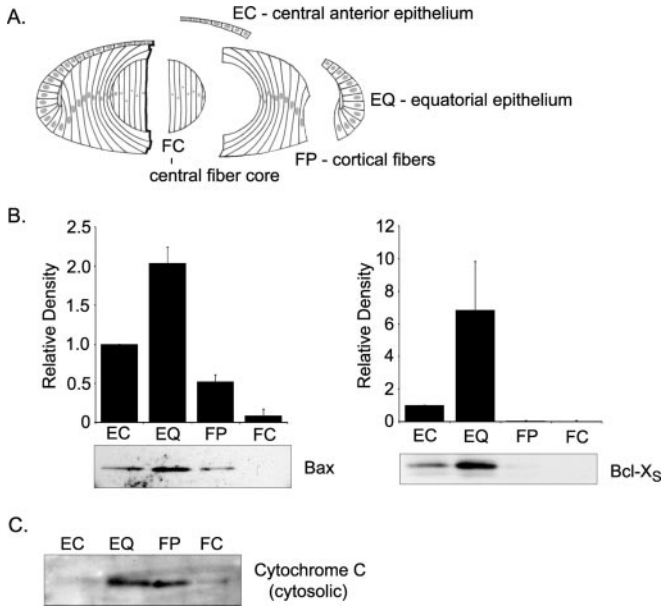

FIG. 1. **At the onset of lens cell differentiation, the expression of pro-apoptotic Bcl-2 proteins is increased concurrent with the release of cytochrome *c* from mitochondria.** Lenses from E10 chicks were microdissected to yield four differentiation-specific regions. *A*, the E10 chick lens is diagrammed as a sagittal section and also represented with the four distinct microdissected zones separated: the central anterior epithelium (*EC*), the equatorial epithelium (*EQ*), the cortical fiber zone (*FP*), and the central nuclear fiber zone (*FC*). *B*, protein extracts from microdissected E10 chick lens fractions were immunoblotted for the pro-apoptotic proteins Bax (*left panel*) and Bcl-x_S (*right panel*). Quantification of three independent experiments demonstrated that the expression of both Bax and Bcl-x_S was greatly increased in the EQ and decreased again in the FP. *C*, the release of cytochrome *c* into the cytosol of each of the microdissected regions at E10 was detected by immunoblotting following isolation of the cytosolic fraction by differential centrifugation. Cytochrome *c* was first released in the equatorial zone and continued to be released through the cortical fiber region.

Ardmore, PA), and images were acquired using Metamorph Version 6.2 (Universal Imaging Corp., Downingtown, PA).

Measurement of Mitochondrial Membrane Potential—Two dyes were used to track mitochondrial membrane potential, tetramethylrhodamine methyl ester perchlorate (TMRM) and JC-1 (Molecular Probes, Inc., Eugene, OR). TMRM is a classic tracker of mitochondrial membrane potential. JC-1 permits simultaneous measurement of mitochondria with and without active membrane potential. JC-1 forms aggregates in mitochondria with active membrane potentials that cause the dye to fluoresce red, whereas in mitochondria without a membrane potential, the dye remains as a monomer and fluoresces green. Chick lenses (removed at E10) were incubated with TMRM (3 h) or JC-1 (6 h) and observed live by confocal microscopy. Images were acquired using a Zeiss LSM510 META confocal microscope. Z-stacks were collected, and projection images were created from the Z-stack. Histograms were created using Zeiss LSM5 Image Examiner software.

RESULTS

Increase in Pro-apoptotic Bcl-2 Family Proteins as Lens Cells Initiate Their Differentiation—The embryonic lens provides a unique opportunity to study the role of apoptosis-like processes in the regulation of differentiation because, at a single time point in development, multiple stages of differentiation are present, from undifferentiated epithelial cells to terminally differentiating fiber cells (Fig. 1*A*). The ability to separate these stages by microdissection techniques makes it possible to examine the dynamic processes that regulate lens cell differentiation *in vivo*. For this study, E10 lenses were separated into four regions: the undifferentiated central epithelium, the equatorial epithelium (where lens cells initiate their differentiation), the cortical fiber zone (where much of lens cell morphological differentiation takes place), and the terminally differentiating core nuclear fiber region.

The Bcl-2 family member Bax, a potent inducer of apoptosis, leads to the release of cytochrome *c* from mitochondria (17, 43). The highest level of Bax expression occurred in the equatorial epithelium of the embryonic lens (Fig. 1*B*). Bax increased 2-fold in this region, where lens epithelial cells initiate their differentiation. Elevated Bax expression did not extend beyond the equatorial epithelium, dropping precipitously in the cortical fiber region. Bcl-x_S paralleled Bax and was most highly expressed in the equatorial zone. The expression of this pro-apoptotic molecule was enhanced 7-fold in equatorial epithelial cells (Fig. 1*B*). The increased expression of both Bax and Bcl-x_S in the equatorial epithelium and their subsequent suppression in the differentiating cortical fiber zone (2 days before organelles are lost in maturing lens fiber cells) suggest that these pro-apoptotic Bcl-2 family members provide an initiating signal in lens cell differentiation.

Cytochrome c Is Released at the Onset of Differentiation—Because Bax and Bcl-x_S induce the mitochondrial death pathway through the release of cytochrome *c* from mitochondria into the cytosol, we investigated the differentiation-specific release of cytochrome *c* into the cytosol of embryonic lens cells. The cytosolic fraction from each of the microdissected regions of the E10 chick lens was isolated and immunoblotted for cytochrome *c* (Fig. 1*C*). Cytochrome *c* was first released from mitochondria in the cells of the lens equatorial epithelium concurrent with high expression of Bax and Bcl-x_S. The release of cytochrome *c* from mitochondria continued to occur in the cortical fiber zone, the principle region of lens fiber cell morphogenetic differentiation, but was barely detected in the more mature central fiber zone.

Loss of Mitochondrial Membrane Potential as Lens Cells Differentiate—Because cytochrome *c* is essential to mitochondrial function, we examined whether its release from mitochondria as lens cells initiate their differentiation results in a parallel decrease in mitochondrial membrane potential. E10 chick lenses were incubated with TMRM or JC-1 and observed live by real-time confocal microscopy (Fig. 2). For experiments with the JC-1 tracking dye, histogram analysis demonstrated the proportion of active (red) to inactive (green) mitochondria (Fig. 2*E*).

In the central epithelium of the E10 lens, the mitochondria had active membrane potentials, as seen with both TMRM and JC-1 (Fig. 2, *A* and *F*). Histogram analysis of the JC-1 results showed that the mitochondria in these undifferentiated lens epithelial cells were almost exclusively red, evidence that most of the mitochondria had high activity. Dye tracking in the equatorial zone revealed that the release of cytochrome *c* as these cells initiated their differentiation was accompanied by decreased mitochondrial membrane potential (Fig. 2, *B* and *G*). Mitochondrial membrane potential was increasingly diminished as lens cells in the equatorial epithelium became more differentiated and moved toward the cortical fiber zone.

In the cortical fiber region, where mitochondria are localized primarily in the apical tips of the cells (44) and cytochrome *c* release remained high (Fig. 1), there was a continued loss of mitochondrial membrane potential (Fig. 2, *C* and *H*). Tracking in this region for mitochondria with and without membrane potential using JC-1 showed that, although some mitochondria in the outermost region of the cortical zone still maintained a potential, the labeling of mitochondria without membrane potential (green) continued to increase with differentiation (Fig. 2*C*).

Most of the mitochondria in the differentiated cells of the central nuclear fiber region had lost their membrane potential at E10, even though this was 2 days before their degradation.

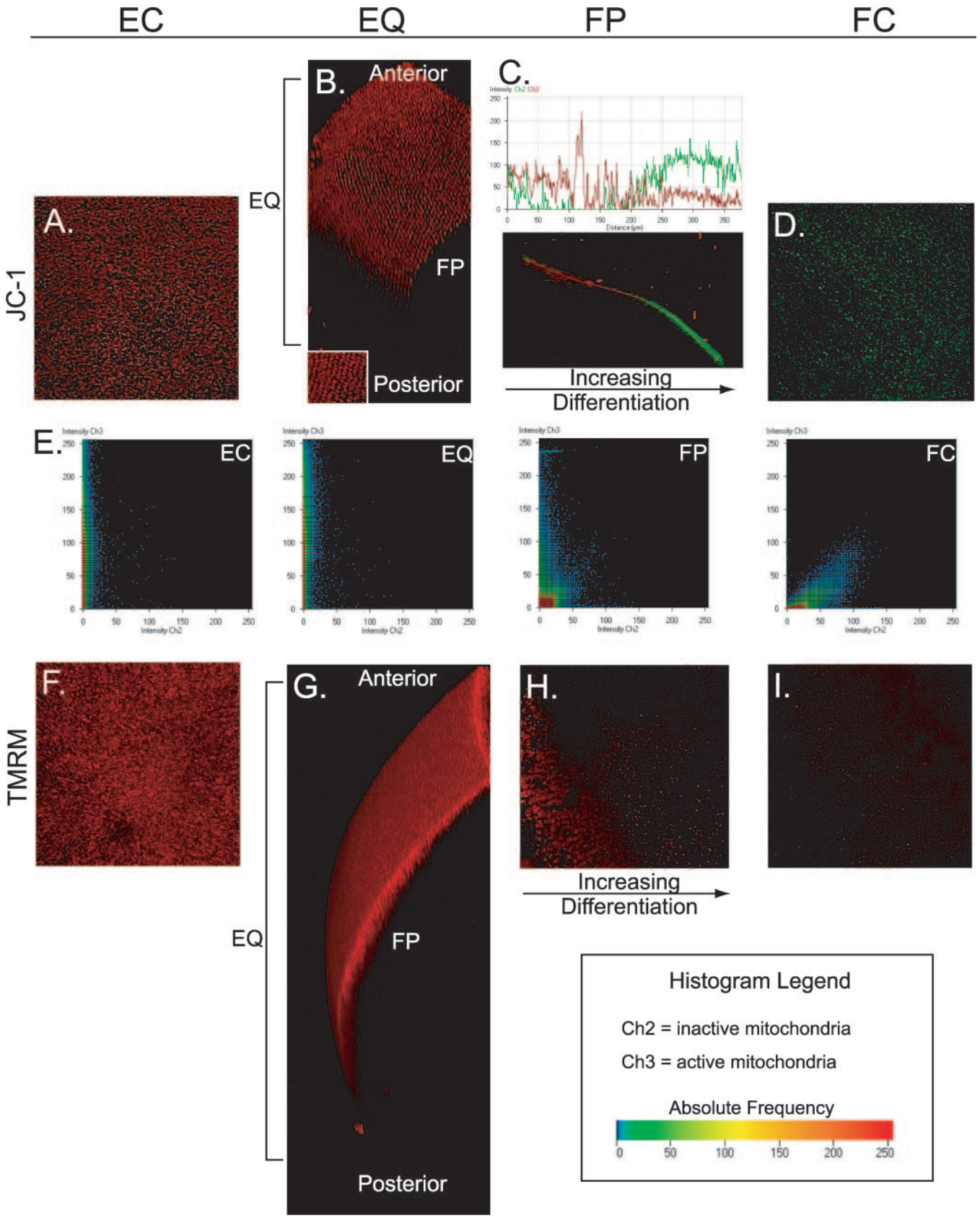

FIG. 2. **Release of cytochrome *c* from mitochondria as lens cells initiate their differentiation results in decreased mitochondrial membrane potential.** Mitochondrial membrane potential was assessed with two tracking dyes, JC-1 (*A–E*) and TMRM (*F–I*). *A*, *B*, *F*, and *G*, whole lenses; *C* and *H*, fiber cell mass (after removal of epithelial cells); *D* and *I*, central fiber cells. *A* and *F* are projection images of the central epithelium viewed *en face*. *B* and *G* are projection images of the EQ rotated to view the curvature of the lens equator and the underlying cortical fiber cells. Note that mitochondrial membrane potential decreased as lens cells moved through the equatorial zone. The *inset* in *B* is an alternative view that demonstrates the periodic alignment of mitochondria in the EQ. *C* is a projection image of a cross-sectional view of the cortical fiber region shown with increasing differentiation from left to right. Note that mitochondria were observed primarily in the apical tips of these differentiating fiber cells. The switch from active (*red*) to inactive (*green*) mitochondria as the cortical fiber cells differentiated is represented graphically. The

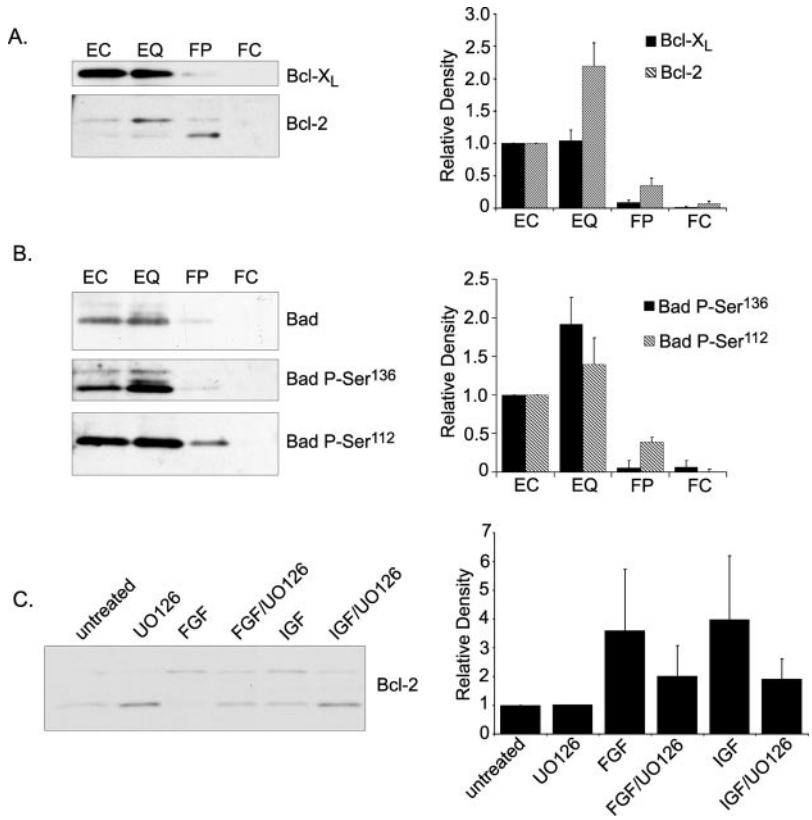

FIG. 3. **Expression of anti-apoptotic Bcl-2 proteins in the EQ to protect the lens from apoptotic pathways may be regulated by FGF and IGF-1.** *A*, extracts of microdissected E10 chick lens fractions were immunoblotted for the anti-apoptotic Bcl-2 family proteins Bcl-x_L and Bcl-2. Both were highly expressed in lens epithelial cells. An alternative, faster migrating form of Bcl-2 was present in the FP. *EC*, central anterior epithelium; *EQ*, equatorial epithelium; *FP*, cortical fiber zone; *FC*, central nuclear fiber zone. *B*, phosphorylation of Bad at Ser^{112} and Ser^{136}, both anti-apoptotic signals, was examined in microdissected E10 lens fractions by immunoblot analysis with phospho (*P*)-specific antibodies. Total Bad expression also was determined. Phosphorylation of Bad was high in the region of the EQ. *C*, in lens epithelial cell cultures, exposure to either FGF or IGF-1 induced the expression of Bcl-2 in an ERK-dependent manner. Data were normalized to untreated cultures.

This was observed in whole lenses and when the nuclear fiber region was dissected free of the rest of the lens prior to incubation with the tracker dyes (Fig. 2*D*), ensuring that the lack of mitochondrial membrane potential was not a dye penetration problem. It is important to note that there was a small subset of mitochondria in this region that maintained membrane potential and therefore the capacity to function (Fig. 2*I*). These results demonstrate that there is a tight correlation between loss of cytochrome *c* from mitochondria and loss of membrane potential in the developing lens.

Anti-apoptotic Bcl-2 Family Members Are Expressed Early in Lens Cell Differentiation—One mechanism of regulating the quantity of cytochrome *c* released and the resultant induction of apoptosis-like pathways involves the action of prosurvival Bcl-2 proteins such as Bcl-2 and Bcl-x_L. By immunoblot analysis of microdissected E10 chick lens fractions, we found Bcl-x_L to be highly expressed in the epithelial regions of the embryonic lens (Fig. 3*A*). For Bcl-2, two bands were detected. The uppermost Bcl-2 band, which comigrated with authentic Bcl-2, exhibited the greatest expression in lens epithelial cells. The lower band, perhaps a splice variant, cleavage product, or post-translationally modified form of Bcl-2, was most highly expressed in the cortical fiber region. The expression of Bcl-2 and Bcl-x_L alone does not mean that they are functioning as prosurvival molecules. When Bcl-2 and Bcl-x_L are bound by Bad, apoptosis is promoted. Only when Bad is phosphorylated at one of its serine sites (each targets of multiple kinases) are Bcl-2 and Bcl-x_L released from Bad and available to bind pro-apoptotic Bcl-2 family members such as Bax to block apoptosis (27). To determine whether Bcl-x_L and Bcl-2 are available for regulation of Bax, we examined the phosphorylation state of Bad by immunoblot analysis. Using an antibody that recognizes all forms of Bad, we found Bad to be expressed primarily by cells in the lens epithelium, increasing 1.3-fold between the central epithelium and the equatorial epithelium. Consistent with our hypothesis, Bad was most highly phosphorylated in the equatorial epithelium at both its Ser^{112} and Ser^{136} sites (Fig. 3*B*). These results suggest that the anti-apoptotic proteins Bcl-2 and Bcl-x_L are released from Bad as lens differentiation is initiated to regulate the action of apoptosis-like molecules such as Bax, allowing for controlled release of cytochrome *c* from mitochondria. This could allow Bax to signal a transition in lens cell differentiation without tipping the balance toward apoptosis.

FGF and IGF-1 Promote the Expression of Bcl-2 in an ERK-dependent Pathway—FGF and IGF-1 play a crucial role in lens cell differentiation (45–47), and both of these growth factors have been identified as upstream inducers of cell survival pathways (48, 49). Therefore, it seemed likely that one of the functions of FGF and IGF-1 in the induction of lens cell differentiation may involve regulation of the mitochondrial cell death pathway. Treatment of lens epithelial cells with either FGF or IGF-1 signaled an increase in the expression of the prosurvival protein Bcl-2 (Fig. 3*C*). Because both FGF and IGF-1 are well characterized activators of the ERK mitogen-activated protein kinase signaling pathway, we investigated whether the induction of Bcl-2 by FGF and IGF-1 is dependent on the activation of ERK. For these experiments, lens epithelial cells were exposed to FGF and IGF-1 in the presence of the ERK inhibitor U0126. Inhibiting ERK activation was sufficient to block the induction of Bcl-2 by either of these growth factors. These

region along which the graph was generated is indicated with a *red line*. The decreased membrane potential as cortical fiber cells differentiated is also seen in an *en face* view of the cortical fiber region (*H*). *D* and *I* are projection images of *en face* views of the FC. Mitochondrial membrane potential was greatly decreased in this region. Histogram analysis of the data collected with JC-1 is presented in *E*. Intensity of labeling for active mitochondria is on the *y axis* (*Ch3*), and intensity of labeling for inactive mitochondria is on the *x axis* (*Ch2*). Colors in the histogram represent pixel frequency of intensity values. *EC*, central anterior epithelium; *EQ*, equatorial epithelium; *FP*, cortical fiber zone; *FC*, central nuclear fiber zone.

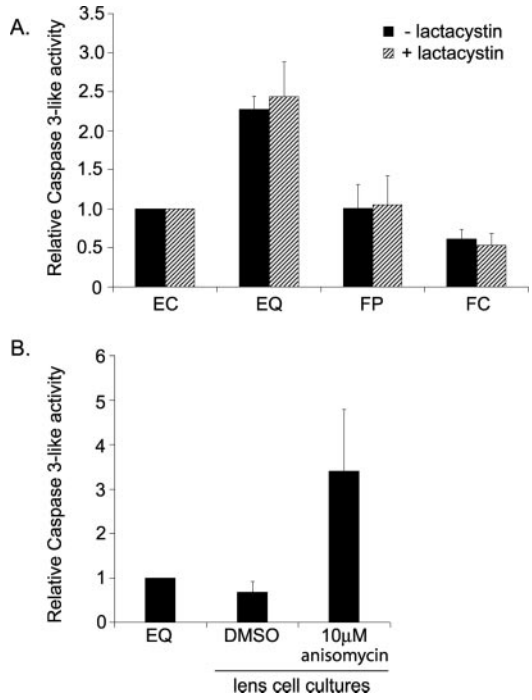

FIG. 4. **Caspase-3-like proteases are active in the early stages of lens cell differentiation.** *A*, caspase-3-like activity assays performed on microdissected E10 chick lens fractions (*black bars*) showed that caspase-3-like activity was greatest in the EQ, where lens cells initiate their differentiation. The proteasome was not responsible for the measured caspase-3-like activity, as the results were unaffected by addition of the proteasome inhibitor lactacystin to the assay (*hatched bars*). *EC*, central anterior epithelium; *EQ*, equatorial epithelium; *FP*, cortical fiber zone; *FC*, central nuclear fiber zone. *B*, the level of caspase-3-like activity in cells of the equatorial region at E10 was compared with that in primary differentiating lens cells cultured in the presence or absence of 10 μM anisomycin (18 h) to induce apoptosis. Although caspase-3-like activity in the differentiating lens cultures was similar to that which occurred during lens cell differentiation *in vivo*, this protease activity was far below the levels that occurred during apoptosis. *DMSO*, dimethyl sulfoxide.

results indicate that both FGF and IGF-1 could function as modulators of the apoptosis-like cell death pathways that are required for the initiation of lens cell differentiation and that this process is mediated by an ERK signaling pathway.

Executioner Caspase-3-like Proteases Are Activated as Lens Cells Initiate Their Differentiation—Caspase-3 is one of the major executioner caspases in the Bax/cytochrome *c* apoptotic pathway. Although it is expressed in the lens, it is not responsible for signaling organelle and nuclear loss (1, 2). Therefore, it seemed a likely candidate caspase for activation by the apoptosis-like pathway that is induced during the early stages of lens cell differentiation. Indeed, we found that caspase-3-like activity was high only in the equatorial zone (Fig. 4*A*) concurrent with high expression of Bax and release of cytochrome *c* from mitochondria. Caspase-3-like activity decreased in the cortical fiber zone and was even lower in the central fiber region. The fact that caspase-3-like activity peaked and then decreased 2 days before the loss of organelles and nuclei supports our hypothesis that caspase-3-like proteases play a regulatory role during the early stages of lens cell differentiation and not in the process of organelle loss. Because the substrate used in the caspase-3 activity assay (DEVD) has also been reported to be a proteasome target (1), we also performed the caspase activity experiments in the presence of the proteasome inhibitor lactacystin. Inhibition of proteasome activity in the microdissected embryonic lens fractions did not affect the results of the caspase-3-like activity assay (Fig. 4*A*).

The activation of caspases in the developing lens does not lead to apoptosis, providing evidence that caspase activity is tightly regulated during lens cell differentiation. This suggests that the level of caspase activity in the embryonic lens, even at its peak, is far lower than that in apoptotic cells. To examine this question, we utilized a differentiating lens cell culture system in which apoptosis can be readily induced by exposure to apoptogenic agents such as anisomycin. As a control for the level of caspase-3-like activity in differentiating lens cell cultures, we used lens cells isolated from the equatorial region of the E10 lens. Although caspase-3-like activity in differentiat-

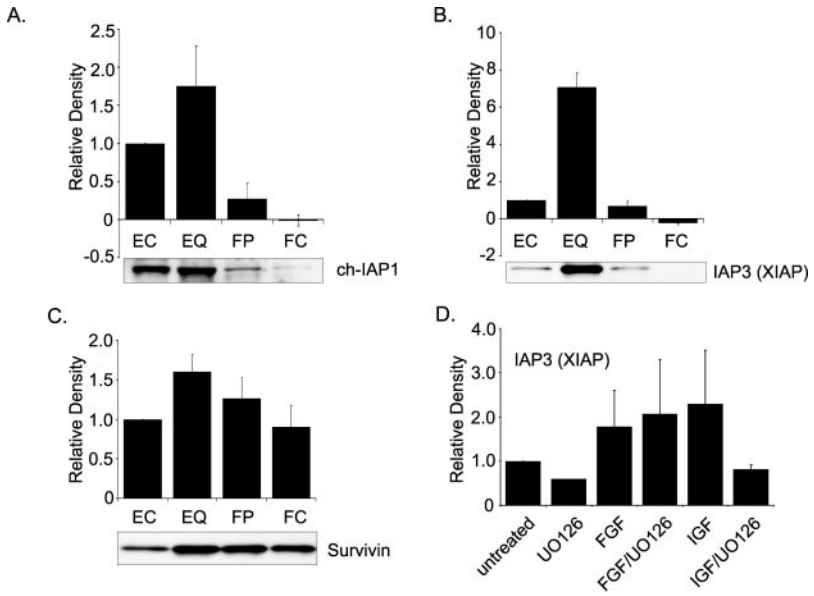

FIG. 5. **IAPs are expressed to regulate caspase activity in differentiating lens cells and prevent their commitment to apoptosis.** Extracts of microdissected E10 chick lens fractions were immunoblotted with antibodies to the three known chicken homologs of mammalian IAPs: ch-IAP1, IAP3, and survivin. *EC*, central anterior epithelium; *EQ*, equatorial epithelium; *FP*, cortical fiber zone; *FC*, central nuclear fiber zone. ch-IAP1 expression occurred primarily in lens epithelial cells (*A*). The expression of IAP3, the most potent IAP, specifically increased in the EQ, where lens differentiation is initiated, and was almost completely down-regulated in the cortical fiber region (*B*). *XIAP*, X-linked IAP. The expression of the IAP survivin increased in the equatorial region and remained high in the fiber zones (*C*), suggesting that survivin may provide prolonged caspase inhibition during lens cell differentiation. In lens epithelial cell cultures, exposure to either FGF or IGF-1 induced the expression of IAP3 (*D*); however, only IGF-1 induction of IAP3 was ERK-dependent. In *D*, data were normalized to untreated cultures.

ing lens cells in culture was similar to that in the cells in the equatorial region *in vivo*, exposure of the cultures to 10 μM anisomycin for 18 h increased caspase-3-like activity by ~5-fold (Fig. 4*B*). The level of caspase activity available to signal events in lens cell differentiation is far lower than that which induces apoptosis, indicating that caspase activity in development is tightly controlled.

IAPs, Regulators of Caspase Activity, Increase Their Expression as Lens Cells Begin to Differentiate—The most likely candidates for regulators of the apoptosis-like caspase pathways during normal lens differentiation are the IAPs. These regulatory molecules inhibit the proteolytic activity of both initiator and executioner caspases. Three homologs to mammalian IAPs have been identified in the chicken: chicken IAP1 (ch-IAP1; cellular IAP1/2 homolog), IAP3 (X-linked IAP homolog; GenBank™ accession number AAL47170), and survivin (50, 51). Immunoblot analysis of microdissected chicken embryo lenses showed that ch-IAP1, an endogenous inhibitor of initiator and executioner caspase function (52), was expressed by lens epithelial cells (Fig. 5*A*). IAP3, which also inhibits both initiator and executioner caspases (52), was expressed almost exclusively in the equatorial zone (Fig. 5*B*) coordinated with the release of cytochrome *c*, high caspase-3-like activity, and the initiation of differentiation. The expression of survivin, an inhibitor of executioner caspases such as caspase-3 (53), increased in the equatorial epithelium and was maintained at high levels through the cortical fiber zone (Fig. 5*C*). The high expression of all three of these chicken IAPs in the equatorial zone is consistent with a role for them in the regulation of caspase-3-like proteases that are activated as lens cells initiate their differentiation.

FGF and IGF-1 Promote the Expression of IAP3—FGF and IGF-1, as regulators of both lens cell differentiation (45–47) and cell survival (48, 54), were investigated for their potential role in regulating the expression of the prosurvival molecule IAP3. IAP3 was chosen for this analysis because its expression is significantly increased in the equatorial region of the lens as lens cells initiate their differentiation. Exposure of lens epithelial cells to either FGF or IGF-1 resulted in an increase in IAP3 expression (Fig. 5*D*). To investigate the involvement of the ERK signaling pathway in growth factor induction of IAP3, we exposed lens epithelial cells to FGF and IGF-1 in the presence of the ERK inhibitor U0126. Interestingly, although IGF-1 induction of IAP3 expression was ERK-dependent, there was little effect of ERK inhibition on FGF-directed IAP3 expression.

Caspase Activity Is Required for Lentoid Formation—To determine the specific role of caspases in the early stages of lens cell differentiation, we exposed lens epithelial cells growing in a differentiating primary culture system to the caspase inhibitor Z-VAD-fmk. We chose Z-VAD-fmk for this study because it is a general caspase inhibitor that blocks both initiator and executioner caspases. The culture system in this study is unique in that it mimics normal lens cell differentiation as it occurs *in vivo* (40). As the cultured lens epithelial cells initiate their differentiation, they not only express differentiation-specific proteins, but also undergo dramatic reorganization to form the multicellular, multilayered lens-like structures known as lentoids. The control cultures (grown in the presence of the vehicle Me_2SO) differentiated with extensive lentoid structures present by culture day 8. The caspase inhibitor Z-VAD-fmk interfered with normal lens morphogenesis *in vitro* as evidenced by suppression of lentoid formation (Fig. 6*A*). Similar results were obtained with the caspase-3-specific inhibitor Ac-DEVD-cmk (Fig. 6*B*), demonstrating that the result observed with Z-VAD-fmk is likely to be specific to caspase-3 function. To distinguish between a role for caspases in signaling the initiation of differentiation-specific

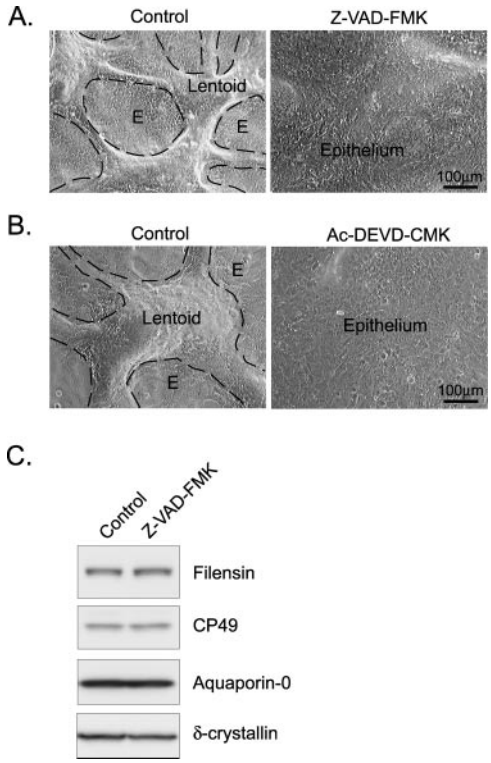

FIG. 6. **Caspase activity is required for lens fiber cell morphogenetic differentiation.** At 2 days after plating, primary embryonic lens cell cultures were exposed for 6 days to the general caspase inhibitor Z-VAD-fmk (50 μM), the caspase-3-specific inhibitor Ac-DEVD-cmk, or the vehicle Me_2SO (*Control*). *A* and *B*, although normal lens cultures formed extensive, multicellular, multilayered, differentiated lentoid structures, lentoid formation in the presence of either Z-VAD-fmk or Ac-DEVD-cmk was markedly reduced, with most cells still present as an epithelium (*E*). *C*, to distinguish between a role for caspases in the initiation of lens differentiation-specific gene expression and in lens cell morphogenetic differentiation, we examined the effect of the caspase inhibitor on the expression of the lens fiber cell proteins filensin, CP49, aquaporin-0 (MP28), and δ-crystallin. Although caspases were not found to regulate the expression of these lens differentiation-specific genes, they were required for lens fiber cell morphogenetic differentiation.

gene expression and in signaling events specific to lens morphogenetic differentiation, we examined whether the caspase inhibitor affects the expression of the lens differentiation-specific proteins filensin, CP49, aquaporin-0, and δ-crystallin. The results demonstrate that caspase function was not required for the expression of these lens-specific differentiation markers (Fig. 6*C*) and suggest that the principal role of caspases in the early stages of lens cell differentiation is in regulating the morphogenesis of the embryonic lens.

Activation of Apoptosis-related Pathways Is Sufficient for Induction of Lens Cell Differentiation—Our results show that components of the canonical mitochondrial death pathway are expressed as lens cells initiate their differentiation *in vivo*. We speculated that stimulation of the mitochondrial death pathway in undifferentiated lens epithelial cell cultures by exposure to an apoptogenic agent may induce lens cell differentiation. To examine this hypothesis, we treated primary lens epithelial cell cultures for 4 h with 250 nM staurosporine, a broad-spectrum kinase inhibitor; removed the apoptogen; and then cultured the treated cells for an additional 6 days in normal culture medium. Exposure to staurosporine dramatically altered lens cell morphology, with many cells appearing contracted (Fig. 7*A*). This short-term exposure to staurosporine did not commit these lens cells to apoptosis. The lens cells quickly recovered following the removal of the staurosporine-containing medium. Remarkably, after only 6 days in normal

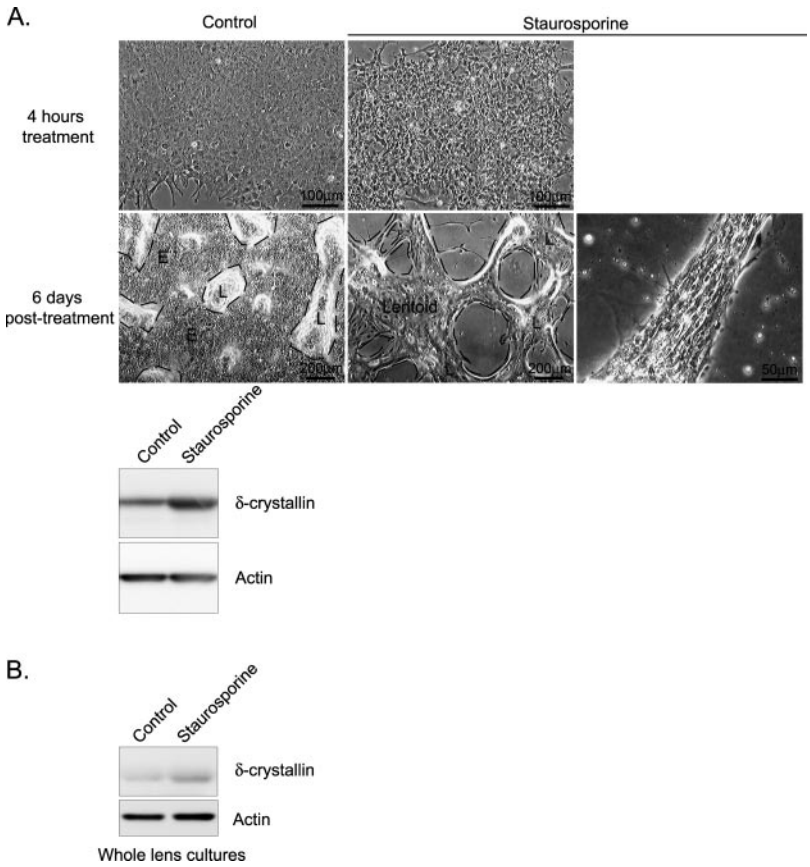

FIG. 7. **Induction of the mitochondrial cell death pathway signals the initiation of lens cell differentiation.** *A*, primary lens epithelial cell cultures were exposed to 250 nM staurosporine or the vehicle Me_2SO (*Control*) for 4 h and then cultured for an additional 6 days in normal culture medium. After 4 h, the lens epithelial cells exhibited morphological changes consistent with cell contraction. At the end of the culture period (6 days post-treatment), the control lens epithelial cells had differentiated normally, with ~50% of the culture area containing differentiated lentoid (*L*) structures (within *dashed areas*). *E*, epithelium. Exposure to staurosporine induced 100% of the lens epithelial cells to differentiate and to form lentoid structures. Note the extensive elongation of the differentiating lens fiber cells when the lentoids induced by staurosporine were observed at higher magnification. Immunoblot analysis demonstrated that exposure of the lens epithelial cell cultures to 250 nM staurosporine induced the expression of the lens differentiation-specific protein δ-crystallin. *B*, E10 chick lenses were exposed *ex vivo* to 250 nM staurosporine for 4 h and then cultured for an additional 5 days under serum-free conditions. Immunoblot analysis demonstrated that exposure of whole lenses to this apoptogen induced the expression of δ-crystallin in the cells of the lens epithelium.

culture medium, 100% of the cells had differentiated, forming extensive lentoid structures (Fig. 7*A*). Individual cells within these lentoid structures were highly elongated. This is in contrast to control cultures, in which <50% of the cells formed differentiated lentoid structures and many cells still remained as undifferentiated epithelial cells (Fig. 7*A*). In lens cultures treated with staurosporine, not only were the cells highly elongated and organized into branching lentoid structures, but they expressed high levels of the differentiation-specific protein δ-crystallin (Fig. 7*A*). The expression of actin remained unaffected by staurosporine treatment.

To examine whether exposure of whole lenses to staurosporine would induce differentiation of lens epithelial cells, we developed an *ex vivo* model. For these experiments, E10 chick lenses were removed with the vitreous attached and placed in culture wells in the presence of serum-free medium. Lenses were exposed to 250 nM staurosporine for 4 h and grown for 5 days in serum-free medium, and the expression of differentiation markers was determined. Exposure to staurosporine induced the expression of the differentiation-specific proteins δ-crystallin (Fig. 7*B*) and aquaporin-0 (data not shown). These results demonstrate that induction of the mitochondrial death pathway in cells of the lens epithelium promotes the initiation of lens cell differentiation.

Although exposure to 250 nM staurosporine signaled the initiation of differentiation of lens epithelial cells in culture, this concentration of staurosporine initially had a detrimental effect on cell morphology. As a result, it was not possible to observe the differentiation-specific effects on lens cell morphology following the activation of the mitochondrial death pathway until after the cells had time to recover from the apoptogen. To better simulate the low level induction of the mitochondrial death pathway that our study has demonstrated to occur *in vivo*, lens epithelial cells were treated with a low concentration of staurosporine (50 nM). The majority of cells in these cultures began to elongate and align after only 2 h (data not shown), and lens cell elongation was quite distinctive by 18 h of exposure to this low dose of the apoptogen (Fig. 8*A*). In contrast to the characteristic cortical actin filaments in control cultures, actin in the highly elongated staurosporine-treated cells was reorganized into filamentous structures that spanned the length of the cells (Fig. 8*B*). The nuclei of 50 nM staurosporine-treated lens cells had a distinct elongated shape (Fig. 8*B*, *arrows*) that was very different from the smaller rounded shape of nuclei in control untreated lens epithelial cells. Interestingly, cell elongation, filamentous actin extension, and elongated nuclei are characteristics evocative of differentiating lens fiber cells in the cortical fiber zone of the developing lens (Fig. 8*B*). To confirm that these elongated lens fiber-like cells induced by short-term exposure to 50 nM staurosporine had initiated the lens differentiation pathway, we examined δ-crystallin expression. δ-Crystallin expression was induced in lens cell cultures treated with 50 nM staurosporine (Fig. 8*C*). Collectively, our results show that low level short-term activation of the mitochondrion-mediated apoptotic pathway is able to initiate the cell signaling pathways required for lens cell differentiation.

DISCUSSION

Apoptosis-related Bcl-2- and Caspase-dependent (ABC) Differentiation—Differentiating lens cells undergo a process we call ABC differentiation, in which molecules characteristic of the canonical intrinsic mitochondrial death pathway signal early events in lens cell differentiation (Fig. 9). In lens development, non-apoptotic functions for signaling components of the mitochondrial death pathway are likely to be tightly regulated by anti-apoptotic molecules that are expressed concurrently. Classic cell survival molecules such as Bcl-2 and IAPs can modulate the effects of molecules in the Bax/mitochondrial/caspase pathway, which would allow them to act as molecular

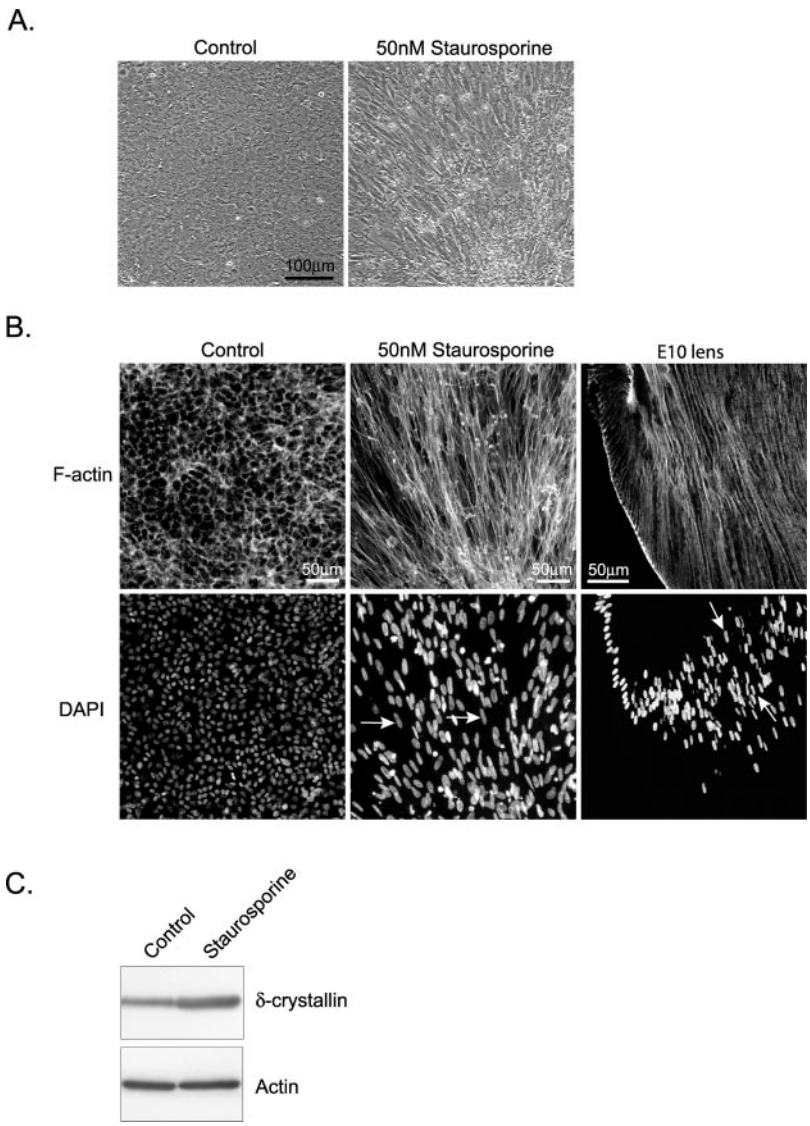

FIG. 8. **Lens cell differentiation can be initiated by low level activation of apoptotic pathways through staurosporine.** At 2 days after plating, primary embryonic lens cell cultures were exposed for 18 h to 50 nM staurosporine or Me_2SO (*Control*). *A*, this low concentration of staurosporine induced elongation of lens cells, whereas control cultures maintained classic epithelial cell morphology. *B*, fixed samples were labeled with 4′,6-diamidino-2-phenylindole (*DAPI*) and phalloidin to further characterize the morphological effects of induction of the mitochondrial death pathway. The highly elongated nuclei (*arrows*) and actin filament organization found in lens cells treated with 50 nM staurosporine were strikingly similar to those in cortical fiber cells of an E10 lens. *C*, the expression of the differentiation-specific marker δ-crystallin was examined in lens cultures treated for 18 h with 50 nM staurosporine or Me_2SO (*Control*) and then maintained for 6 days in normal culture medium. The increased expression of δ-crystallin was induced in the staurosporine-treated lens cell cultures.

switches in lens cell differentiation. We suggest that the apoptotic process "hijacks" these normal cell signals by interfering with the functioning of their regulatory anti-apoptotic controls, pushing the balance toward cell death. There are many other examples of signaling molecules that, when unregulated, have a detrimental effect on cell function, usually leading to a pathophysiological state. A classic example of this is the Src tyrosine kinase. Although originally discovered as an oncogene, Src was later found to play a major role in normal cell signaling pathways, where its activity is tightly regulated by phosphatases and other kinases (55). Thus, we suggest that the mitochondrial death pathway has an important physiological role in many non-apoptotic cells, as we have shown here for the developing lens.

Temporally coordinated with the initiation of lens cell differentiation is the increased expression of Bax and Bcl-x_S, proteins that function in the release of cytochrome *c* from mitochondria and the activation of the mitochondrial death pathway. We suggest that these signaling mechanisms allow the permeabilization of mitochondria during the initiation of lens differentiation, but that the phosphorylation of Bad, which releases Bcl-2 and Bcl-x_L, tightly controls the release of cytochrome *c*. This results in the prolonged release of cytochrome *c* throughout the early stages of lens cell differentiation and the low level activation of caspase-3-like proteases. Previous studies have shown that disrupting the balance between Bcl-2 and Bax results in incomplete differentiation or apoptosis in the developing lens. The overexpression of the survival factor Bcl-2 causes reduced caspase-9 activity, prevents cleavage of the caspase-3 substrate DFF45, interferes with proper fiber cell elongation, and blocks both chromatin condensation and nuclear loss (56, 57). Conversely, lens fiber cells overexpressing the transcription factor E2F1 or E2F2 exhibit increased expression of Bax and increased apoptosis (20).

FGF and IGF-1 have long been studied for their role in lens cell differentiation (45–47), yet there is much evidence in the literature that both of these growth factors also have an important function in signaling cell survival (48, 49, 54). Our study provides a link between the role of growth factor signaling in cell survival and differentiation. We found that Bcl-2, a cell survival protein most highly expressed in the equatorial zone of the embryonic lens, was induced in lens epithelial cells by both IGF-1 and FGF through their activation of the ERK signaling pathway. Similarly, the expression of IAP3 was induced by these growth factors in lens epithelial cells. These results indicate that, although the mitochondrial cell death pathway is induced to initiate lens cell differentiation, FGF and IGF-1 activate the cell survival pathways that protect the cells from apoptosis.

Our finding that cytochrome *c* was released at E10 to initiate

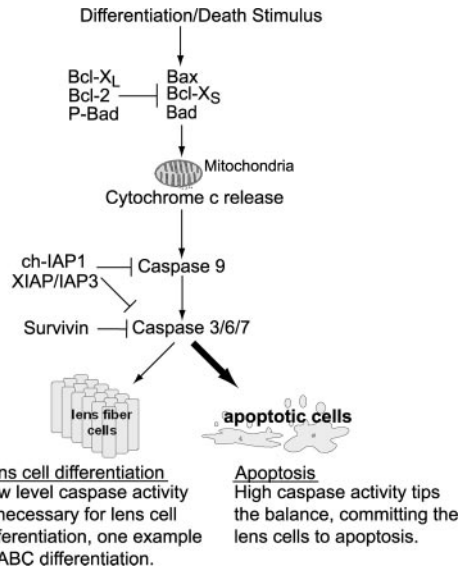

FIG. 9. **Model of the function of pro- and anti-apoptotic molecules in the regulation of lens cell differentiation *versus* their role in the canonical mitochondrial death pathway.** In response to differentiation or apoptotic signals, members of the Bcl-2 family such as Bax and Bcl-x_S regulate the release of cytochrome *c* from mitochondria. In lens cell differentiation, the release of cytochrome *c* is slow and prolonged because it is regulated by the anti-apoptotic molecules Bcl-2 and Bcl-x_L. Although the exit of cytochrome *c* from mitochondria in differentiating lens cells activates the caspase cascade, caspase activity is kept low because the caspases are regulated by the IAPs. This low level of caspase activity is necessary to remodel lens epithelia as they differentiate to form the lens fiber cells. In contrast, when cytochrome *c* is released rapidly, caspase activity reaches high levels, and the cells commit to the apoptotic program. *P-Bad*, phospho-Bad; *XIAP*, X-linked IAP.

pathways required for the early stages of lens cell differentiation and significantly earlier than the appearance of the OFZ at E12 is consistent with previous observations that lens cell mitochondria at E10 are heavily fragmented, with only remnants of cristae remaining in the central fiber zone (14). A previous study that examined total cytochrome *c* at E12 found none in the cortical fiber zones of the lens, which still maintain organelles (58), demonstrating that cytochrome *c* release from mitochondria is complete before the appearance of the OFZ. The loss of cytochrome *c* from mitochondria in the equatorial and cortical fiber zones at E10 resulted in a loss of mitochondrial membrane potential. When the lens cells reached the central fiber zone, only a small subset of the mitochondria in these differentiated fiber cells were active. These results indicate that mitochondria are an unlikely energy source for lens cells as they differentiate. In addition, our results suggest that alternative pathways to cytochrome *c* must be responsible for the activation of caspases that are involved in the loss of nuclei and organelles at later stages of lens cell differentiation. We speculate that mitochondrial factors that can activate caspases (such as Omi/HtrA2) could be released from the mitochondria of lens fiber cells at later stages of lens development.

Cytochrome *c* release leads to the activation of the caspase cascade. Caspases have been shown to control nuclear loss in differentiating lens cells. Using a general caspase inhibitor, Ishizaki *et al.* (3) prevented nuclear loss in rat lens explant cultures. Wride *et al.* (2) extended these experiments using chicken embryo lens cell cultures and showed that nuclear loss during lens cell differentiation specifically requires the function of caspase-1, -2, -4, -6, and -9, but not caspase-3 and -8. We have shown for the first time that caspases also function in signaling the initiation of lens cell differentiation. Our *in vivo* experiments demonstrated that caspase-3-like proteolytic activity increased temporally and spatially as lens cells began their differentiation and was attenuated long before the appearance of the OFZ. The highest level of caspase-3-like activity occurred in the equatorial epithelium, the region of differentiation initiation. By inhibiting caspase activity in our differentiating lens culture system, we were able to establish that caspases play a requisite role in lens fiber cell morphogenesis without affecting the expression of lens differentiation-specific proteins, including the water channel protein aquaporin-0 (MP28), δ-crystallin, and the beaded filament proteins filensin and CP49. This finding is supported by a previous study in which the caspase inhibitor had no effect on the expression of α- or β-crystallin (3). Most significantly, we have shown that the activation of the intrinsic apoptotic pathway by limited exposure to the apoptogen staurosporine was sufficient to induce lens cells to begin their differentiation program. Activating apoptotic pathways in lens epithelial cell cultures induced both differentiation-specific gene expression and morphogenesis (lentoid formation). In whole lenses, exposure to staurosporine induced differentiation of cells in the lens epithelium. We are the first to demonstrate that lens cell differentiation can be initiated through controlled activation of the intrinsic apoptotic pathway.

Our study also presents the first analysis of IAP protein expression in the lens. IAPs, which modulate caspase activity, were highly expressed in the equatorial epithelium, where caspase-3-like proteases are active and cells initiate their differentiation program. ch-IAP1 and IAP3 are inhibitors of both initiator and executioner caspases (52). Their restricted expression to the lens epithelium is consistent with a role in regulating the apoptosis-related pathways that initiate lens differentiation. Survivin is a more specific inhibitor of executioner caspases (53). Interestingly, survivin was the only IAP whose protein expression persisted throughout the later stages of differentiation. Its prolonged expression suggests that it may also promote survival during terminal differentiation of lens fiber cells. The pattern of IAP expression suggests that, in the developing lens, IAPs tightly regulate caspase activity at multiple steps in the caspase cascade, allowing caspases to function as molecular switches in lens differentiation without inducing apoptosis.

ABC Differentiation Is Distinct from Apoptosis—The cellular effects associated with apoptosis, whether stimulated *in vitro* or observed *in vivo* during development, usually occur within a time period of <24 h (59). Following induction of apoptosis, perturbation of mitochondria followed by release of cytochrome *c* is typically a rapid event (~5 min) (60) that is immediately followed by condensation of the cell, loss of organelles and nuclei, and finally reorganization of the cell into apoptotic bodies (59). In the developing lens, a period of 4 days passes from the time that fragmentation of mitochondria is first observed (E8) to the time of organelle loss (E12) (14). Our observation of cytochrome *c* in the cytosol of cells in both the equatorial region and the cortical fiber zone clearly demonstrates that, in the differentiating lens, the release of cytochrome *c* extends over a significantly prolonged period of time compared with that which occurs during apoptosis. Also in contrast to apoptosis, phosphatidylserine does not flip to the outer leaflet of the plasma membrane during lens development (36, 61). Because phosphatidylserine flipping is an early event in apoptosis, its absence supports the finding that apoptotic pathways are controlled and are activated only at low levels for signaling lens cell differentiation. Furthermore, some typical caspase substrates such as actin are protected from cleavage during lens cell differentiation, whereas other components of the membrane-associated cytoskeleton, including α- and β-spectrins, are targeted by caspase cleavage (37, 62). Components of focal adhesion complexes in the lens such as focal

adhesion kinase and paxillin also appear to be protected from caspase digestion.[2] Our results show that caspase-3-like activity levels were significantly lower during lens cell differentiation than during apoptosis. It is probable that the subcellular compartmentalization of caspases and IAPs may protect certain proteins from caspase-mediated cleavage, whereas other proteins are targeted for degradation.

ABC Differentiation Occurs in Diverse Cell Types—The regulation of differentiation by Bcl-2 family members, cytochrome *c*, and caspases is not unique to lens cells or to other cells that lose organelles and nuclei. The overexpression of Bcl-2 or BHRF1 (a viral homolog of Bcl-2) in keratinocytes inhibits terminal differentiation (63, 64). The overexpression of Bcl-2 in monocytes inhibits cytochrome *c* release, caspase activation, and differentiation into macrophages (6). In addition, Bcl-x_L and Bcl-2 play critical roles in the differentiation process and survival of erythroid cells (65, 66). Caspases are involved in the differentiation of keratinocytes, erythroblasts, macrophages, skeletal muscle cells, and osteoblasts (4–7, 67), but the stage of differentiation affected by caspases is specific to the cell type. Interestingly, in developing erythroid progenitors, the highest level of caspase activity occurs 4–9 days prior to the actual appearance of enucleated reticulocytes (68), and inhibition of caspases early in erythrocyte differentiation prevents later nuclear loss (67).

Given the wide spectrum of cell types that use ABC mechanisms during differentiation, it is clear that apoptosis-related pathways provide essential non-apoptotic signals for cell differentiation, and it is only when they are activated unchecked that apoptosis ensues. Here, we have shown, in the developing lens, a novel role for components of the mitochondrion-induced apoptotic pathway as molecular switches for the initiation of differentiation. We suggest that this function for apoptotic molecules in the lens will serve as a model for understanding the mechanisms of differentiation initiation in many developing systems.

Acknowledgments—We thank Dr. Marilyn Woolkalis for critical reading of this manuscript, Dr. Jan Hoek for advice on mitochondrial studies, and Ni Zhai and Iris Wolff for excellent technical assistance. We appreciate the generous gifts of antibodies to filensin and CP49 from Dr. Paul FitzGerald and antibody to δ-crystallin from Dr. Joram Piatigorsky.

REFERENCES

1. Foley, J. D., Rosenbaum, H., and Griep, A. E. (2004) *J. Biol. Chem.* **279,** 32142–32150
2. Wride, M. A., Parker, E., and Sanders, E. J. (1999) *Dev. Biol.* **213,** 142–156
3. Ishizaki, Y., Jacobson, M. D., and Raff, M. C. (1998) *J. Cell Biol.* **140,** 153–158
4. Fernando, P., Kelly, J. F., Balazsi, K., Slack, R. S., and Megeney, L. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11025–11030
5. Weil, M., Raff, M. C., and Braga, V. M. (1999) *Curr. Biol.* **9,** 361–364
6. Sordet, O., Rebe, C., Plenchette, S., Zermati, Y., Hermine, O., Vainchenker, W., Garrido, C., Solary, E., and Dubrez-Daloz, L. (2002) *Blood* **100,** 4446–4453
7. Mogi, M., and Togari, A. (2003) *J. Biol. Chem.* **278,** 47477–47482
8. Persons, B. J., and Modak, S. P. (1970) *Exp. Eye Res.* **9,** 144–151
9. Menko, A. S. (2002) *Exp. Eye Res.* **75,** 485–490
10. Ferreira-Cornwell, M. C., Veneziale, R. W., Grunwald, G. B., and Menko, A. S. (2000) *Exp. Cell Res.* **256,** 237–247
11. Kuwabara, T. (1975) *Exp. Eye Res.* **20,** 427–443
12. Modak, S. P., and Perdue, S. W. (1970) *Exp. Cell Res.* **59,** 43–56
13. Kuwabara, T., and Imaizumi, M. (1974) *Investig. Ophthalmol.* **13,** 973–981
14. Bassnett, S., and Beebe, D. C. (1992) *Dev. Dyn.* **194,** 85–93
15. Bassnett, S. (1995) *Investig. Ophthalmol. Vis. Sci.* **36,** 1793–1803
16. Bassnett, S. (1992) *Curr. Eye Res.* **11,** 1227–1232
17. Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4997–5002
18. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
19. Cory, S., Huang, D. C., and Adams, J. M. (2003) *Oncogene* **22,** 8590–8607
20. Chen, Q., Hung, F. C., Fromm, L., and Overbeek, P. A. (2000) *Investig. Ophthalmol. Vis. Sci.* **41,** 4223–4231
21. Merio, G. R., Cella, N., and Hynes, N. E. (1997) *Cell Growth & Differ.* **8,** 251–260
22. Heermeier, K., Benedict, M., Li, M., Furth, P., Nunez, G., and Hennighausen, L. (1996) *Mech. Dev.* **56,** 197–207
23. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132
24. Minn, A. J., Rudin, C. M., Boise, L. H., and Thompson, C. B. (1995) *Blood* **86,** 1903–1910
25. Manon, S., Chaudhuri, B., and Guerin, M. (1997) *FEBS Lett.* **415,** 29–32
26. Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) *Cell* **80,** 285–291
27. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87,** 619–628
28. Lindenboim, L., Yuan, J., and Stein, R. (2000) *Oncogene* **19,** 1783–1793
29. Mercatante, D. R., Mohler, J. L., and Kole, R. (2002) *J. Biol. Chem.* **277,** 49374–49382
30. Rao, L., Perez, D., and White, E. (1996) *J. Cell Biol.* **135,** 1441–1455
31. Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A., and Rosen, A. (2000) *J. Cell Biol.* **149,** 603–612
32. Chiu, R., Novikov, L., Mukherjee, S., and Shields, D. (2002) *J. Cell Biol.* **159,** 637–648
33. Lane, J. D., Lucocq, J., Pryde, J., Barr, F. A., Woodman, P. G., Allan, V. J., and Lowe, M. (2002) *J. Cell Biol.* **156,** 495–509
34. Casciola-Rosen, L., Nicholson, D. W., Chong, T., Rowan, K. R., Thornberry, N. A., Miller, D. K., and Rosen, A. (1996) *J. Exp. Med.* **183,** 1957–1964
35. Rudel, T., and Bokoch, G. M. (1997) *Science* **276,** 1571–1574
36. Bassnett, S., and Mataic, D. (1997) *J. Cell Biol.* **137,** 37–49
37. Lee, A., Morrow, J. S., and Fowler, V. M. (2001) *J. Biol. Chem.* **276,** 20735–20742
38. Wistow, G., Bernstein, S. L., Wyatt, M. K., Behal, A., Touchman, J. W., Bouffard, G., Smith, D., and Peterson, K. (2002) *Mol. Vis.* **8,** 171–184
39. Walker, J. L., and Menko, A. S. (1999) *Dev. Biol.* **210,** 497–511
40. Menko, A. S., Klukas, K. A., and Johnson, R. G. (1984) *Dev. Biol.* **103,** 129–141
41. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) *EMBO J.* **17,** 37–49
42. Zhou, J., and Menko, A. S. (2002) *Investig. Ophthalmol. Vis. Sci.* **43,** 2293–2300
43. Pastorino, J. G., Chen, S. T., Tafani, M., Snyder, J. W., and Farber, J. L. (1998) *J. Biol. Chem.* **273,** 7770–7775
44. McNulty, R., Wang, H., Mathias, R. T., Ortwerth, B. J., Truscott, R. J., and Bassnett, S. (2004) *J. Physiol. (Lond.)* **559,** 883–898
45. Lovicu, F. J., and McAvoy, J. W. (2001) *Development (Camb.)* **128,** 5075–5084
46. Beebe, D. C., Silver, M. H., Belcher, K. S., Van Wyk, J. J., Svoboda, M. E., and Zelenka, P. S. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2327–2330
47. Le, A. C., and Musil, L. S. (2001) *Dev. Biol.* **233,** 394–411
48. Sell, C., Baserga, R., and Rubin, R. (1995) *Cancer Res.* **55,** 303–306
49. Pardo, O. E., Arcaro, A., Salerno, G., Raguz, S., Downward, J., and Seckl, M. J. (2002) *J. Biol. Chem.* **277,** 12040–12046
50. You, M., Ku, P. T., Hrdlickova, R., and Bose, H. R., Jr. (1997) *Mol. Cell. Biol.* **17,** 7328–7341
51. Johnson, A. L., Langer, J. S., and Bridgham, J. T. (2002) *Endocrinology* **143,** 3405–3413
52. Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S., and Reed, J. C. (1998) *EMBO J.* **17,** 2215–2223
53. Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., and Reed, J. C. (1998) *Cancer Res.* **58,** 5315–5320
54. Pardo, O. E., Lesay, A., Arcaro, A., Lopes, R., Ng, B. L., Warne, P. H., McNeish, I. A., Tetley, T. D., Lemoine, N. R., Mehmet, H., Seckl, M. J., and Downward, J. (2003) *Mol. Cell. Biol.* **23,** 7600–7610
55. Hunter, T. (1995) *Cell* **80,** 225–236
56. Fromm, L., and Overbeek, P. A. (1997) *Dev. Genet.* **20,** 276–287
57. Sanders, E. J., and Parker, E. (2003) *Differentiation* **71,** 425–433
58. Sanders, E. J., and Parker, E. (2002) *J. Anat.* **201,** 121–135
59. Goldstein, J. C., Kluck, R. M., and Green, D. R. (2000) *Ann. N. Y. Acad. Sci.* **926,** 132–141
60. Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., and Green, D. R. (2000) *Nat. Cell Biol.* **2,** 156–162
61. Wride, M. A., and Sanders, E. J. (1998) *Exp. Eye Res.* **66,** 371–383
62. Lee, A., Fischer, R. S., and Fowler, V. M. (2000) *Dev. Dyn.* **217,** 257–270
63. Harada, H., Mitsuyasu, T., Seta, Y., Maruoka, Y., Toyoshima, K., and Yasumoto, S. (1998) *J. Oral. Pathol. Med.* **27,** 11–17
64. Dawson, C. W., Eliopoulos, A. G., Dawson, J., and Young, L. S. (1995) *Oncogene* **10,** 69–77
65. Motoyama, N., Kimura, T., Takahashi, T., Watanabe, T., and Nakano, T. (1999) *J. Exp. Med.* **189,** 1691–1698
66. Hafid-Medheb, K., Augery-Bourget, Y., Minatchy, M. N., Hanania, N., and Robert-Lezenes, J. (2003) *Blood* **101,** 2575–2583
67. Zermati, Y., Garrido, C., Amsellem, S., Fishelson, S., Bouscary, D., Valensi, F., Varet, B., Solary, E., and Hermine, O. (2001) *J. Exp. Med.* **193,** 247–254
68. Carlile, G. W., Smith, D. H., and Wiedmann, M. (2004) *Blood* **103,** 4310–4316

[2] G. F. Weber and A. S. Menko, unpublished data.