Insulin-like Growth Factor Receptor-1 and Nuclear Factor κB Are Crucial Survival Signals That Regulate Caspase-3-mediated Lens Epithelial Cell Differentiation Initiation*

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Background: Low levels of caspase-3 activity acts as a molecular switch in lens differentiation without causing apoptosis. Results: IGF-1R/NFκB-coordinated signaling pathway induces expression of Bcl-2 and IAP proteins to maintain caspase-3 activation at low levels that signal differentiation initiation. Conclusion: IGF-1R/NFκB signaling modulates caspase-3 activity for a non-apoptotic role in differentiation.

Significance: A novel differentiation initiation signaling pathway has been identified.

It is now known that the function of the caspase family of proteases is not restricted to effectors of programmed cell death. For example, there is a significant non-apoptotic role for caspase-3 in cell differentiation. Our own studies in the developing lens show that caspase-3 is activated downstream of the canonical mitochondrial death pathway to act as a molecular switch in signaling lens cell differentiation. Importantly, for this function, caspase-3 is activated at levels below those that induce apoptosis. We now have provided evidence that regulation of caspase-3 for its role in differentiation induction is dependent on the insulin-like growth factor-1 receptor (IGF-1R) survival-signaling pathway. IGF-1R executed this regulation of caspase-3 by controlling the expression of molecules in the Bcl-2 and inhibitor of apoptosis protein (IAP) families. This effect of IGF-1R was mediated through NFκB, demonstrated here to function as a crucial downstream effector of IGF-1R. Inhibition of expression or activation of NFκB blocked expression of survival proteins in the Bcl-2 and IAP families and removed controls on the activation state of caspase-3. The high level of caspase-3 activation that resulted from inhibiting this IGF-1R/NFκB signaling pathway redirected cell fate from differentiation toward apoptosis. These results provided the first evidence that the IGF-1R/NFκB cell survival signal is a crucial regulator of the level of caspase-3 activation for its non-apoptotic function in signaling cell differentiation.

Caspases play a crucial role in determining the balance between life and death of a cell. Although caspases were originally known as effectors of apoptosis, recent investigations have contributed to a deeper understanding of the full spectrum of caspase function in normal cells, which includes a non-apoptotic signaling role in cell differentiation (1–4). Although cell differentiation events are often tissue-specific, the caspase-3 protease is important to cell fate decisions across many tissue types including the differentiation of myoblasts to myotubes (5), lens epithelial cells to lens fiber cells (3), osteoblasts to form bone (6), and keratinocytes (7). Caspases also have non-apoptotic functions in terminal cell differentiation events including the loss of nuclei in erythrocytes (8) and keratinocytes (9). However, the pathways by which caspases execute their non-apoptotic roles in cells are still largely unclear.

The non-apoptotic versus apoptotic function of caspase-3 is related directly to its level of activation. One of the earliest studies noting this phenomenon examines the impact of the level of caspase-3 activation on a well known caspase-3 substrate, the signaling molecule RasGAP2 (10). That study shows that a low level of activated caspase-3 generates two fragments of RasGAP. The C-terminal fragment has an apoptosis-promoting function, and the N-terminal fragment has anti-apoptotic properties. A high level of active caspase-3 further cleaves the N-terminal fragment in two, and these fragments together with the C-terminal fragment potentiate a pro-apoptotic pathway (10). Although that study is not related to caspase-3 signaling in differentiation, it set the stage for understanding the importance of regulating the level of activation of caspase-3 for its non-apoptotic functions in the cell. Other studies have since shown such a non-apoptotic function for low level caspase-3 activation in cell differentiation through the limited cleavage of the caspase-3 substrate ICAD (inhibitor of caspase-activated DNase) (11). This pathway, discovered in skeletal muscle cells, reveals how caspase-3 can signal the initiation of cell differentiation. In this pathway, low level caspase-3

2 The abbreviations used are: RasGAP, Ras GTPase-activating protein; IGF-1, insulin-like growth factor-1 receptor; p-IGF-1R, phospho-IGF-1R; IκB, inhibitor of nuclear factor κB; E, embryonic day; IAP, inhibitor of apoptosis protein; X-IAP, X-linked inhibitor of apoptosis protein; ch-IAP-1/2, chicken IAP-1/2; Bcl-2, B-cell lymphoma-2; IκB, inhibitor of caspase-activated DNase; CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase; DMSO, dimethyl sulfoxide; PPP, picropodophyllin; PARP, poly(ADP-ribos)polymerase; cl-PARP-1, cleaved PARP-1 fragment.
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activity cleaves ICAD releasing CAD (caspase-activated DNase) at the low levels required for it to initiate a conserved genomic reprogramming that is required for differentiation initiation (4). In this instance, the cleavage of the p21 promoter (a critical differentiation regulator) by CAD (4, 11) induces p21 expression, altering cell fate. This mechanism emphasizes the importance of regulating the level of caspase-3 activity for its non-apoptotic functions in the cell, as high levels of caspase activation induces cell death through this same ICAD/CAD pathway by leading to high levels of CAD release (11). Consistent with the non-apoptotic role for caspase-3 in differentiation of the developing lens, its level of activation is far lower than when apoptosis is induced in these cells (3). The factors that control the level of caspase activation for cellular processes like differentiation initiation are not known. Our studies here are focused on determining the molecular components of the pathway that regulates the level of caspase-3 activation and makes it possible for this protease to play its non-apoptotic role in signaling differentiation initiation.

In the intrinsic canonical mitochondrial death pathway, pro-apoptotic Bcl-2 family members facilitate the release of cytochrome c from mitochondria, triggering the “apoptotic” signaling cascade that activates caspase-3 (12). The reason that in the developing lens this pathway can signal lens epithelial cells to withdraw from the cell cycle and commit to fiber cell differentiation without causing apoptosis may be linked to the concomitant induction of survival proteins in both the Bcl-2 and IAP families (3), because these survival molecules have the potential to regulate the level of caspase-3 activation. We investigated likely upstream survival signals, such as insulin-like growth factor-1 receptor (IGF-1R), that have the potential to induce expression of Bcl-2 and IAP survival proteins (13, 14) during the initiation events of lens cell differentiation and thereby enable caspase-3 to act as a molecular switch in this differentiation process. IGF-1R, a classical survival-signaling protein, is highly expressed in the zone of differentiation initiation of the embryonic lens (15) and has been demonstrated to have a role in signaling lens differentiation (16–19). The transcription factor nuclear factor κB (NFκB) is also associated with cell survival signaling and can directly regulate expression of Bcl-2 and IAP family survival proteins (20). Although there is as yet little evidence that NFκB activation is a downstream effector of IGF-1R (13), NFκB is a key responder to various types of stimuli in the cell. It plays a critical role in the expression of proteins concerned with development and the immune response, as well as cell survival (21). The prototypical and ubiquitously expressed NFκB complex is the p65/p50 heterodimer, of which the RelA or p65 subunit is indispensable for cell survival (22). NFκB remains in the cytoplasm when complexed with the inhibitory protein IκB. In response to an activation stimulus, IκB is degraded leaving NFκB free to translocate to the nucleus and act as a transcription factor for expression of its target genes (21). After release from IκB, NFκB is phosphorylated by PKA, creating the active form of this transcription factor (23).

In this study we show that an IGF-1R-regulated NFκB survival signal regulates caspase-3 for its role as a differentiation-inducing signal in the lens, providing the first evidence of the mechanisms that control the level of caspase-3 activation in normal physiological processes. The results reveal that when the IGF-1R/NFκB survival signal is blocked, caspase-3 activation is elevated, blocking initiation of lens epithelial cell differentiation and inducing cell death by apoptosis.

EXPERIMENTAL PROCEDURES

Lens Microdissection—Lenses were isolated from embryonic day 10 (E10) chicken eggs (B&E Eggs, York Springs, PA) and microdissected into four distinct regions of differentiation as described previously (24) to yield the central anterior epithelium (EC), the equatorial epithelium (EQ), the cortical fiber zone (FP), and the central fiber zone (FC) (modeled in Fig. 3A).

Preparation of Primary Lens Cell Cultures—Primary lens cell cultures were prepared as described previously (25). Briefly, lens cells were isolated from E9 quail lenses by trypsinization and agitation. Cells were plated on laminin and cultured in complete medium (medium 199 with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin). These primary cultures mimic lens differentiation as it occurs in vivo (15). For inhibitor studies these cell cultures were treated with the IGF-1R inhibitor picropodophyllin (PPP; 1 μM) or the NFκB SN50 inhibitor peptide (18 μM) for 24 h in complete medium. For all inhibitor experiments, control cultures were exposed to 0.1% Me2SO (dimethyl sulfoxide (DMSO), Sigma), the vehicle for PPP and the SN50 inhibitor peptide.

Preparation of Ex Vivo Whole Lens Cultures—Embryonic E10 chicken lenses were removed and cleaned free of ciliary body. These lenses were cultured in complete medium, one lens/well in a 24-well plate at 37 °C. For PPP inhibitor studies the lenses were incubated for 2 h prior to the addition of the inhibitor or DMSO for the controls. Any lenses exhibiting damage or opacity at this time point were eliminated from the study.

siRNA Transfection—Cells were transfected with custom-made, chicken-specific NFκB p65 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to knock down NFκB p65 expression or with a control ON-TARGETplus non-targeting pool (Dharmacon RNAi Technologies, Thermo Scientific). Before transfection, complete medium was replaced with medium 199 without serum and antibiotics. Cells were exposed overnight at 37 °C to either the NFκB p65 siRNA or the non-silencing siRNA (60 nm) premixed with Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to manufacturer’s protocol. Then, the medium was changed, and the cells were incubated for the rest of the study in complete medium.

Immunoblotting—Cells were extracted in 44.4 mM n-octyl-β-D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl2, 5 mM EDTA, and 10 mM imidazole containing 1 mM sodium vanadate, 0.2 mM H2O2, and protease inhibitor mixture (Sigma). The concentration of protein was determined using the BCA assay (Thermo Fisher Scientific). For most studies 15 μg of protein from each sample was subjected to SDS-PAGE on precast 8–16% Tris/glycine gels (Novex, San Diego, CA). For detection of cl-PARP-1, X-IAP (X-linked inhibitor of apoptosis protein), p-Bad Ser-112, Bad, and GAPDH in the PPP inhibitor studies, 50 μg was loaded on the gels, and in the SN50 inhibitor and siNFκB p65 knockdown experiments 30 μg was loaded on the gels. Proteins were transferred electrophoretically onto Immobilon-P membranes (Millipore Corp., Bedford, MA).
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IGF-1R (C-20), Bcl-X<sub>L</sub> (L-19), Bcl-2 (N-19), NFκB p65 (C-20), p-NFκB p65 Ser-276 (SC-101749), NFκB p50 (SC-114), GAPDH (FL-335), Oct-1 (C-21), survivin (FL-142), p-Bad Ser-112 (sc-7998-R), Bad (C-7), PARP-1/2 (H-250), and c-IAP-1/2 (A-13) antibodies were purchased from Santa Cruz Biotechnology. Antibodies to phospho-IGF-1R (44-806G) and β-actin (A5441) were purchased from Invitrogen and Sigma, respectively. Antibody to cleaved caspase-3 (catalog No. 9664), which recognizes the 17/19-kDa subunit produced by cleavage at Asp-175, was purchased from Cell Signaling (Danvers, MA). Antibody to X-IAP (catalog No. 610716) was purchased from BD Transduction Laboratories. Antibodies to filensin and CP49 were generous gifts from Dr. Paul FitzGerald (University of California, Davis). Secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were detected using ECL reagent (Thermo Fisher Scientific) and ECL Plus reagent (GE Healthcare). Immuno-blots were scanned, and densitometric analysis was performed using Eastman Kodak 1D software.

Cell Fractionation—Nuclear and cytoplasmic fractions were prepared from primary lens cell cultures using the NE-PER cell fractionation kit from Thermo Scientific according to the manufacturer’s protocol.

Immunoprecipitation—Immunoprecipitation studies were performed on extracts of cells from the equatorial region of the lens incubated sequentially at 4 °C with primary antibody to p65 and TrueBlot immunoprecipitation beads from eBiosciences (San Diego, CA). To determine co-immunoprecipitation of p50 with p65, the p65 immunoprecipitates were subjected to SDS-PAGE as described previously (15). Proteins were transferred electrophoretically onto Immobilon-P membranes (Millipore) and p50 detected using standard Western blot techniques (15).

Immunostaining—For immunostaining, cells were fixed in 3.7% formaldehyde for 10 min and permeabilized with a 0.25% Triton X-100 solution. Cells were then incubated sequentially in primary antibody followed by fluorescence-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). F-actin was localized with Alexa 448-conjugated phalloidin (Invitrogen-Molecular Probes). Nuclei were stained with TO-PRO-3 (Invitrogen-Molecular Probes). Annexin V staining was performed using annexin V-Alexa Fluor 488 (V13241) from Invitrogen. Briefly, 10 μl of annexin V-488 dye was added directly in culture dishes with 1 ml of complete medium and incubated at 37 °C for 15 min, according to manufacturer’s instructions, prior to live imaging by confocal microscopy.

RESULTS

Blocking IGF-1R Signaling Removes Constraints on the Level of Caspase-3 Activation and Inhibits Lens Cell Differentiation—Previous studies from our laboratory have shown that a low level of caspase-3 activation is necessary for the induction of lens epithelial cell differentiation (3). However, there exists very little knowledge as to how caspase-3 can be regulated so that it is activated only to the low level required for its non-apoptotic role in normal developmental processes such as lens differentiation initiation. In the current study we examined whether IGF-1R was a required upstream signal in the pathway that regulates caspase-3 activation in differentiating lens epithelial cells. If so, then in the absence of IGF-1R signaling, caspase-3 activation would be unregulated and would signal cell death instead of differentiation. For this analysis, primary lens cell cultures were treated with the IGF-1R inhibitor PPP (1 μM) or its vehicle, DMSO, prior to differentiation initiation, and the cells were examined after 24 h in the presence of the inhibitor. This IGF-1R inhibitor specifically inhibits phosphorylation of a critical tyrosine residue, Tyr-1136, in the activation loop of IGF-1R preventing its activation with no off-target effects (26–28). Immunoblot analysis confirmed that PPP effectively blocked activation of IGF-1R (phospho-IGF-1R (p-IGF-1R)) (Fig. 1B) with a minimal effect on IGF-1R expression and caused dramatic morphogenetic changes in the primary lens cell cultures (Fig. 1A). To investigate the link between IGF-1R and caspase-3 activation, primary lens cell cultures grown in the presence of the IGF-1R inhibitor PPP for 24 h were analyzed for the level of activation of caspase-3 with an antibody biomarker for cleaved caspase-3 (Fig. 1C). This antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage at Asp-175 and does not bind to either full-length caspase-3 or any other cleaved caspases. These cultures were

TUNEL Assay—A cell death detection kit (In Situ Cell Death Detection, TMR red, Roche Applied Science) was used to label the fragmented DNA of apoptotic cells. For the TUNEL assay of cell cultures, the cells were fixed for 15 min in 3.7% formaldehyde. For the TUNEL assay of tissue sections, E10 chicken lenses were isolated, fixed, and then permeated with a 30% sucrose solution prior to freezing. Cryosections of 20 μm were cut. TUNEL staining was performed in cultured cells and tissue sections according to the manufacturer’s instructions. Nuclei were stained with TO-PRO-3 (Invitrogen-Molecular Probes). TUNEL and TO-PRO-3 staining was imaged by confocal microscopy. Quantification was performed by analyzing five randomly selected fields from each sample over three independent studies, represented as the percent of total nuclei (stained with TO-PRO-3) that were TUNEL-positive.

Annexin V Assay—Annexin V assay was performed to analyze cell death by apoptosis using the Dead Cell Apoptosis Kit with annexin V-Alexa Fluor 488 (V13241) from Invitrogen. Briefly, 10 μl of annexin V-488 dye was added directly in culture dishes with 1 ml of medium and incubated at 37 °C for 15 min, according to manufacturer’s instructions, prior to live imaging by confocal microscopy.
FIGURE 1. Inhibition of IGF-1R signaling leads to high levels of caspase-3 activation and blocks lens epithelial cell differentiation. Primary lens cell cultures at a stage just prior to differentiation initiation were exposed to the IGF-1R inhibitor PPP (1 μM) or to DMSO (control) for 24 h. A, phase contrast microscopy showed that PPP treatment altered lens cell morphology. B, immunoblot analysis with an antibody to p-IGF-1R showed that PPP effectively inhibited IGF-1R activation and had a minimal effect on total IGF-1R expression during the 24-h treatment period. Densitometric analyses of the immunoblots were plotted as the ratio of p-IGF-1R to IGF-1R. C, primary lens cell cultures that were exposed to the IGF-1R inhibitor PPP (1 μM) or to DMSO (control) for 24 h were immunostained for the cleaved (activated) caspase-3 17-kDa fragment (red) and co-stained with TO-PRO-3 for nuclei (blue) and F-actin (green) (shown here as an overlay with nuclei (blue)), and images were acquired by confocal microscopy. Images represent a single optical plane of 0.5 μm. Caspase-3 activation was quantified by creating fluorescence intensity line scans across the field of cells (panels i and ii), revealing increased levels of caspase-3 activation when IGF-1R activation was inhibited by PPP. PPP treatment also induced formation of pyknotic nuclei and reorganization of actin filaments in lens cells. D, the extent of caspase-3 activation also was determined by analyzing cl-PARP-1, a direct substrate of caspase-3. Immunoblot analysis showed that PARP-1 cleavage, plotted as a ratio to GAPDH loading control, increased significantly when IGF-1R activation was inhibited in lens epithelial cells. E, inhibition of IGF-1R activation blocked induction of lens differentiation-specific proteins CP49 and filensin, plotted here as a ratio to actin, demonstrating that deregulation of caspase-3 blocked its ability to function as a differentiation initiation signal. Results are representative of at least three studies. Error bars represent S.E. *, p < 0.05, t test.
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A

B

C

D

Annexin V

Annexin V/DIC overlay

DMSO

PPP

Survivin

ch-IAP1

p-BadS112

X-IAP

Nuclei TUNEL

Nuclei/TUNEL overlay

DMSO

PPP
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IGF-1R mediates expression of pro-survival molecules and maintains caspase-3 at low levels that signal differentiation initiation but prevent apoptosis in lens epithelial cells. A, immunoblot analysis was performed to determine the effect of inhibiting IGF-1R activation (1 μM PPP, 24 h) on the expression of survival proteins in the Bcl-2 and IAP families including Bcl-2, Bcl-X<sub>L</sub>, survivin, X-IAP, and ch-IAP-1. Expression of these survival proteins was quantified and plotted as a ratio to total Bad and GAPDH, respectively. The expression of these survival proteins was suppressed when the IGF-1R signaling pathway was blocked. B, a live annexin V assay, an early apoptosis marker, was used to examine whether inactivation of IGF-1R by PPP induced cell death by apoptosis. Confocal image analysis of annexin V staining showed the appearance of annexin V (green)-positive cells following exposure of the lens cell cultures to the IGF-1R inhibitor PPP. The fluorescence image of annexin V was overlaid with a differential interference contrast (DIC) image to show the annexin V-positive cells (green). C, lens cell cultures exposed to PPP were also examined for the induction of apoptosis by TUNEL assay (red), a late apoptotic marker. These cultures were co-stained with TO-PRO-3 to detect nuclei (blue) and evaluated by confocal microscopy. PPP treatment induced an increase in TUNEL-positive cells as compared with controls. D, E10 chicken lenses were treated in ex vivo culture with DMSO (i–iii) or PPP (iv–vi) for 24 h. 20-μm-thick cryosections of these lenses were examined following a TUNEL assay (red) and co-stained with TO-PRO-3 to detect nuclei (blue) at low and high magnification. The black-boxed area (top) in the model represents the equatorial zone (EQ) of the embryonic lens sections shown in the confocal images. The white-boxed areas in the low magnification images of DMSO-treated (i) and PPP-treated (iv) lenses represent the region in the EQ zone that is shown at a higher magnification in panels ii and v, respectively. PPP treatment induced apoptosis of cells in the equatorial zone of the lens, which is the region of differentiation initiation in the developing lens. Arrows in panels i–iii and iv–vi denote the same cells. All confocal imaging within each study was performed using the same settings. Z-stacks were collected, and images represent a single optical plane of 0.3 μm. Results are representative of at least three independent studies. Error bars represent S.E. *, p < 0.05, t test. Scale bars, 20 μm.
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**FIGURE 3. NFκB expression and activation is dependent on IGF-1R signaling in differentiating lens epithelial cells.** A, E10 chicken lenses were microdissected into four distinct zones of differentiation: undifferentiated cells of the central epithelium (EC); the zone of differentiation initiation (equatorial epithelium (EQ)); cortical fiber cells (FP), where lens cell morphogenetic differentiation occurs; and nuclear fiber cells (FC), the region of fiber cell maturation. Each fraction was analyzed by immunoblot (lower panel) for expression of NFκB subunits p65 and p50 and for the activation state of NFκB p65 (p-NFκB p65 Ser-276). Samples were immunoblotted for actin as a control. A high level of expression of NFκB subunits p65 and p50 and NFκB activation occurred in the lens epithelium, with the highest in the equatorial zone of the lens. B, co-immunoprecipitation (IP) studies (immunoprecipitate for the NFκB p65 subunit and immunoblot for the NFκB p50 subunit) showed that these subunits formed a heterodimeric NFκB p50/p65 complex in the equatorial zone. WCL, whole cell lysate. C, prior to differentiation initiation, primary lens cell cultures were exposed to the IGF-1R inhibitor PPP (1 μM) or DMSO (control) for 24 h, and protein extracts were immunoblotted for active p-IGF-1R, total IGF-1R, NFκB, transcriptionally active NFκB (p-NFκB p65 Ser-276), and actin. Blocking IGF-1R activation inhibited NFκB expression and its transcriptional activity. D, the effect of exposure of lens cell cultures to PPP on translocation of the transcription factor NFκB to the nucleus was examined in lens cell cultures by confocal microscopy imaging following immunostaining for the NFκB p65 subunit (red) and co-staining with TO-PRO-3 to detect nuclei (blue). White-boxed areas show the area magnified in the inset. Images shown are of a single 0.5-μm optical plane. Inhibition of IGF-1R activation blocked the nuclear translocation of the NFκB p65 subunit. E, similarly, inhibition of the nuclear translocation of NFκB p65 was observed when IGF-1R signaling was blocked by immunoblot analysis following cell fractionation into nuclear (N) and cytoplasmic (C) cell compartments. Effectiveness of the cell nuclear/cytoplasmic fractionation was demonstrated by immunoblotting for the cytoplasmic marker GAPDH and the nuclear marker Oct-1. Densitometric analyses were performed on the level of nuclear NFκB p65 relative to Oct-1 in PPP and DMSO-treated cultures. Error bars represent S.E. *, p < 0.05, t test. Results are representative of at least three studies. Scale bar, 10 μm.

Fig. 3A) revealed that RelA/p65, a crucial NFκB transcription factor subunit, and the NFκB p50 subunit with which RelA/p65 forms the prototypical NFκB heterodimer (21) were highly expressed in the equatorial epithelium, which is the differentiation initiation zone of the lens (Fig. 3A, EQ). In contrast, little expression of either the p65 or p50 NFκB subunits was detected in differentiating lens fiber cells (Fig. 3A, FP and FC). Paralleling its pattern of expression, NFκB RelA/p65 was most highly activated (phosphorylated) in the lens equatorial zone (Fig. 3A, p-NFκB p65S276). That the p65 and p50 NFκB subunits exist as a heterodimer in the equatorial epithelial region (EQ) was shown by their co-immunoprecipitation (Fig. 3B, IP: p65/blot: p50). These results indicate that the prototypical NFκB p65/p50 heterodimer may have a crucial role during lens cell differentiation initiation.

To determine whether NFκB is activated downstream of IGF-1R in differentiating lens cells, IGF-1R activation was blocked by exposing lens epithelial cell cultures to the IGF-1R inhibitor PPP for 24 h from a time prior to differentiation initiation. In the presence of PPP both expression and activation of NFκB were suppressed (Fig. 3C). Because a hallmark of NFκB activation is its translocation to the nucleus (35, 36), we examined the effect of blocking IGF-1R activation on the localization of NFκB by immunofluorescence analysis. Whereas in control cultures NFκB freely transited between the cytoplasm and the nucleus, there was an absence of NFκB RelA/p65 in the nuclei...
of PPP-treated lens cultures (Fig. 3D). This result was supported by biochemical analysis of the subcellular distribution of NFκB p65 in lens epithelial cell cultures grown in the presence and absence of the inhibitor PPP. Immunoblot analysis following fractionation into nuclear and cytoplasmic compartments showed decreased nuclear localization of NFκB p65 after inhibi-

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bition of IGF-1R activation (Fig. 3E). Together these results demonstrated that NFκB RelA/p65 was activated downstream of IGF-1R in differentiating lens cell cultures, suggesting that the IGF-1R survival signal associated with lens differentiation initiation was mediated through NFκB RelA/p65.

Blocking Activation of NFκB Removes Constraints on Level of Activation of Caspase-3, Prevents Differentiation, and Induces Apoptosis of Lens Epithelial Cells—Previous studies in the literature have shown that the RelA/p65 NFκB subunit is essential for the expression of anti-apoptotic proteins and for cell survival (22, 37). Here we analyzed whether NFκB activation was required to regulate the level of caspase-3 activity so that it is maintained at the low levels required for signaling the initiation of lens cell differentiation. For these studies we used both inhibitor and siRNA knockdown approaches. For the inhibitor studies, lens cell cultures were exposed for 24 h to the NFκB inhibitor peptide SN50, which blocks the nuclear localization signal of the p50 subunit of the prototypical p65/p50 NFκB complex. This inhibitor blocked the nuclear translocation of both the p50 (Fig. 4A) and p65 (Fig. 4B) subunits of this NFκB heterodimer. Consistent with NFκB RelA/p65 self-regulating its own expression, this block in nuclear translocation of p65 by the SN50 inhibitor also resulted in decreased expression of RelA/p65 (Fig. 4C). Thus SN50 effectively blocked the translocation of both the p50 and p65 subunits of NFκB in lens cell cultures.

Exposure of lens cell cultures to SN50 resulted in increased levels of caspase-3 activation as shown by confocal microscopy imaging of cultures immunostained for the cleaved 17-kDa fragment of activated caspase-3 (Fig. 4D), quantified with line scan intensity analysis (Fig. 4D, i and ii). Immunoblot studies for PARP-1, a direct caspase-3 substrate, showed increased proteolysis of PARP-1 when NFκB p50 activation was blocked in lens epithelial cells (Fig. 4E). Therefore, blocking the activation of NFκB p50 deregulated caspase-3, leading to much greater levels of caspase-3 activation than was associated with lens differentiation initiation.

Next, to examine whether blocking the activation of NFκB p50 could induce a block in lens epithelial cell differentiation, lens cell cultures were exposed to the SN50 inhibitor for 24 h and immunoblotted for lens differentiation-specific proteins CP49 and filensin. Immunoblotting results showed a block in the initiation of lens cell differentiation, as determined by the suppression of expression of CP49 and filensin (Fig. 4F). Consistent with these results, SN50 blocked the expression of Bcl-2 and IAP family survival molecules including Bcl-XL, Bcl-2, ch-IAP-1, X-IAP, and survivin (Fig. 4G). Decreased phosphorylation of Bad on Ser-112 was also observed, with no change in total Bad expression (Fig. 4G). The block in NFκB p50 activation not only resulted in increased activation of caspase-3 but also induced cell death by apoptosis as shown by annexin V (Fig. 4H) and TUNEL (Fig. 4I) assays.

In a parallel approach, the function of the RelA/p65 subunit of NFκB in lens epithelial cell cultures was blocked through siRNA knockdown. Transfection of primary lens cell cultures with siRNA for RelA/p65 effectively knocked down RelA/p65 (Fig. 5A) and the expression of its active form, p-NFκB p65 Ser-276 (Fig. 5B). Like the SN50 inhibitor, the knockdown of p65 inhibited lens differentiation, shown by a block in the expression of lens differentiation-specific proteins CP49 and filensin (Fig. 5C). Inhibition of NFκB p65 induced an increase in the activation of caspase-3 (Fig. 5D) as shown by immunostaining for the cleaved 17-kDa fragment of activated caspase-3 (Fig. 5D), quantified with line scan intensity profiles (Fig. 5D, i and ii); these results were consistent with the PPP inhibitor studies. Knockdown of NFκB p65 also caused increased cleavage of the caspase-3 substrate PARP-1 (Fig. 5E). These results indicated that regulation of caspase-3 activation was dependent on NFκB p65. Knockdown of p65 also caused a block in the expression of the survival molecules Bcl-2, X-IAP, ch-IAP-1, and survivin (Fig. 5F), decreased phosphorylation of Bad on Ser-112, and induced apoptosis as shown by annexin V (Fig. 5G) and TUNEL (Fig. 5H) assays in lens cell cultures. These studies illustrated that NFκB RelA/p65 is a transcriptional regulator of the survival proteins that regulate the lens differentiation initiation signal and that it is necessary for lens epithelial cells to differentiate and form fiber cells.

DISCUSSION

Caspase-3 has become recognized as a crucial differentiation-inducing signal. This protease is activated downstream of the canonical mitochondrial death pathway, a pathway originally discovered for its function in signaling apoptosis but now known to have many non-apoptotic roles in the cell. We propose that the association of this signaling pathway with cell death is, in fact, an example of its induction in the absence of regulatory pathways that otherwise control its level of activation. Such are the circumstances that result in the activation of caspase-3 to the high levels at which it executes the DNA cleav-
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A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Image D](image)

E

![Graph E](image)

F

![Graph F](image)

G

![Image G](image)

H

![Image H](image)
IGF-1R/NFκB Control of Caspase-3-induced Lens Differentiation

Caspase-3 is activated and pushes cell fate toward differentiation by creating line scans (Z slices) and analyzing the data as a single optical plane of 0.5 μm. Results are representative of at least three studies. Error bars represent S.E., *p < 0.05, t test. Scale bar, 20 μm.

The dependence of cell differentiation initiation on caspase-3 activation is widespread and includes diverse cell types such as myoblasts (5), keratinocytes (7), macrophages (39), neural stem cells (5, 40, 41), and from our own studies, the lens (3). However, it is studies with the muscle differentiation model that have provided the most substantial evidence of how the role of caspase-3 is executed in cell differentiation. Those investigations show that low level cleavage of ICAD induced by caspase-3, for which in itself activation is maintained at low levels, releases CAD to affect specific cleavage in the p21 promoter (4, 11). This action induces expression of p21, in effect initiating a conserved genomic reprogramming event essential to the initiation of muscle cell differentiation (4). In our own studies of caspase-3 as a differentiation-inducing signal in lens epithelial cells, we provide evidence that survival signals in the Bcl-2 and IAP families are induced concurrent with caspase-3 activation (3), suggesting a likely regulatory pathway. Other signaling cascades have been investigated that could also be involved in the non-apoptotic modulation of caspases including the DIAP-1 (Drosophila inhibitor of apoptosis-1) protein, which facilitates the differentiation of neurons and sheath cells from the sensory organ precursor lineage (42), and the anti-apoptotic protein ARC (apoptosis repressor with caspase recruitment domain) in myogenic differentiation (11). Notwithstanding these examples, prior to our current studies the pathway that temporally regulates the expression of survival proteins so that they can modulate the degree to which caspase-3 is activated and push cell fate toward differentiation was not known. In the current study, we show that an IGF-1R/NFκB signaling pathway is responsible for induction of Bcl-2 and IAP family survival protein expression and for controlling the level of caspase-3 activation (modeled in Fig. 6). The function of NFκB as a downstream effector of IGF-1R survival signaling had not been identified previously. In the absence of this survival pathway the control on caspase-3 activation is lost, and instead of signaling differentiation initiation, which requires a low level of caspase-3 activation, caspase-3 is deregulated and activated to high levels that induce apoptosis.

A non-apoptotic role for caspase family proteases also has been identified in differentiation processes other than differentiation initiation, including the syncytial fusion of human trophoblast cells in placental development (43) and removal of cytoplasmic bridges between spermatids during sperm development (40). The activation of caspase-3 during development often is a localized event as seen in the pruning of dendrites in arborizing sensory neurons (40) or in the redistribution of caspase-3 to the perinuclear region in myoblast differentiation (44). Previous studies from our laboratory show that the induction of caspase-3 activation is specific to the equatorial region of the lens, the zone of differentiation initiation (3). Here, the level of caspase-3 activity is five times lower than in lens epithelial cells in which apoptosis is induced in response to an apoptogen, now known to result from its tight regulation by an IGF-1R/NFκB survival-signaling pathway.

Although regulation of caspase-3 activity is required for non-apoptotic signaling pathways in normal developmental processes that do not involve cell death, caspase-3 also is activated without regulation to eliminate cells during development or to remove abnormal or diseased cells in various pathological conditions (45, 46). Examples where caspase-3-induced apoptosis as a programmed cell death occurs during normal development include neural tube formation and the terminal differentiation of the neural network (40) as well as the development of the
limb for removal of interdigital cells (46). In relation to neural development, caspase-3-deficient mice (CPP32/H11002/H11002) show severe defects in the brain cortex, including protrusions of the neuroepithelium in the retina, accumulation of supernumerary cells in the cerebellum, and ectopic cell masses in the cerebral cortex, hippocampus, and striatum, all due to lack of apoptosis (40). Hence, although regulation of caspase-3 activity is crucial for its non-apoptotic roles in development, the apoptotic function of caspase-3 is also vital to normal developmental processes. In the reverse, in cancer, tumor cells are often able to survive by developing resistance to apoptosis, activating fundamental survival molecules including IGF-1R (47), NFkB (48), and Bcl-2 and IAP family survival proteins (45). These mechanisms prevent the activation of apoptotic pathways that would lead to their demise. Hence, current therapeutic strategies to kill tumor cells include blocking activation of the same survival-signaling proteins that we show function in development, to dampen the level of caspase-3 activation. Therefore, on many levels, our studies here have added an important new understanding to the full spectrum of regulating apoptotic signaling in the cell.

The importance of IGF-1R in cell survival has been well documented in the literature (26, 47, 49), and in the lens the expression of IGF-1R is up-regulated in the zone of differentiation initiation (or equatorial epithelium) (15, 50). Although IGF-1 is known to be important in lens cell survival (16–18, 51), the mechanism by which IGF-1R was required for signaling lens epithelial cell differentiation had previously remained unclear. Interestingly, however, and in support of our current findings, the overexpression of an IGF-1 transgene in the embryonic lens delays lens cell differentiation, moving the transition zone of differentiation initiation to more posterior aspects of the lens (52). This result is consistent with the function that we propose for IGF-1R as a modulator of the caspase-3 differentiation initiation signal. The overactivation of IGF-1R would be predicted to suppress induction of the lens differentiation program by suppressing the caspase-3 signal to a level too low to effectively initiate differentiation. Here, we show for the first time that this IGF-1R signal is essential for the differentiation of lens epithelial cells through its regulation of the level of caspase-3 activation (Fig. 6).

Future studies will examine the signaling intermediates between IGF-1R and the activation of the NFkB transcription factor. IGF-1R can signal cell survival through the PI3K/Akt and ERK/MAPK pathways (53), both of which are able to phosphorylate and activate the IkB kinase complex (IKK), releasing NFkB for its transcription of essential survival genes (21). The PI3K/Akt and ERK signaling pathways have been implicated in lens cell survival and differentiation (15, 54) and both are inhibited when IGF-1R activation is blocked in lens epithelial cells.

**FIGURE 6. Proposed model for the role of the IGF-1R/NFkB signaling pathway in regulating the caspase-3 differentiation initiation signal.** In the differentiation of lens epithelial cells, low level activation of caspase-3 has an essential role in differentiation initiation. Caspase-3 activation for this differentiation pathway was shown previously to be downstream of the intrinsic canonical mitochondrial death pathway. Here, we show now that caspase-3 activation was maintained at the low levels at which it functions as a differentiation initiator through an IGF-1R signal that activates NFkB, a transcription factor required for the expression of survival proteins in the Bcl-2 (Bcl-2, p-Bad Ser-112, and Bcl-XL) and IAP (ch-IAP-1, X-IAP, and survivin) families. These survival proteins can regulate caspase-3 activity to maintain it at the low level required to signal lens differentiation initiation; in their absence caspase-3 is deregulated and instead signals apoptotic cell death. This is a novel role for the IGF-1R/NFkB signaling pathway in lens epithelial cell differentiation.
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(data not shown). Therefore, it is likely that in the developing lens the IGF-1R differentiation regulatory signal will be found to activate NFκB through its activation of ERK and/or PI3K.

In summary, we have discovered a novel IGF-1R-dependent NFκB survival signal in differentiating lens cells that is responsible for the expression of proteins in the Bcl-2 and IAP families. These survival factors regulate the activation of the canonical mitochondrial death pathway so that it leads to the low level activation of caspase-3 that acts as a molecular switch in lens cell differentiation initiation (Fig. 6).

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