Bim Expression Indicates the Pathway to Retinal Cell Death in Development and Degeneration

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Programmed cell death (PCD) during development of the mouse retina involves activation of the mitochondrial pathway. Previous work has shown that the multidomain Bcl-2 family proteins Bax and Bak are fundamentally involved in this process. To induce mitochondrial membrane permeabilization, Bax and Bak require that prosurvival members of the family be inactivated by binding of “BH3-only” members. We showed previously that the BH3-only protein BimEL is highly expressed during postnatal retinal development but decreases dramatically thereafter. The purpose of this study was to investigate a possible role for Bim, in retinal development and degeneration, upstream of Bax and Bak. *Bim*^{−/−} mice analyzed for defective retinal development exhibit an increase in retinal thickness and a delay in PCD, thereby confirming a role for Bim. We also demonstrate that in response to certain death stimuli, *bim*^{+/+} retinal explants upregulate BimEL leading to caspase activation and cell death, whereas *bim*^{−/−} explants are resistant to apoptosis. Finally, we analyzed Bim expression in the retinal degeneration (rd) mouse, an *in vivo* model of retinal degeneration. Bim isoforms, which decrease during development, are not reexpressed during retinal degeneration and ultimately photoreceptor cells die by a caspase-independent mechanism. Thus, we conclude that in cases in which BimEL is reexpressed during pathological cell death, developmental cell death pathways are reactivated. However, the absence of BimEL expression correlates with caspase-independent death in the rd model.

Key words: Bim; retina; development; apoptosis; caspase; rd

Introduction

The study of programmed neuronal cell death is an area of considerable interest given that it is implicated in both development and degeneration of the mammalian nervous system, and cells unable to make functional connections are efficiently removed by apoptosis. In addition, apoptosis is responsible for the removal of damaged cells in degenerative conditions of the retina such as retinitis pigmentosa (Li and Milam, 1995), glaucoma (Quigley et al., 1995), and age-related macular degeneration (Dunaief et al., 2002). It has been suggested that the molecular mechanisms of apoptosis may be different under circumstances of development and degeneration, but the identity of the key regulators remains unknown (Hsu et al., 2004; Bahi et al., 2006; Donovan et al., 2006).

The intrinsic or mitochondrial pathway is a pathway widely used by cells undergoing apoptosis in response to toxic stimuli. However, previous work has shown that this pathway is not activated in a number of animal models of retinal degeneration and consequently caspases are not involved (Donovan et al., 2001; Doonan et al., 2003; Chahory et al., 2004). Because Bcl-2 family proteins control mitochondrial integrity, it is possible that they play a key role in the decision between caspase-dependent and

-independent death (Danial and Korsmeyer, 2004). Members of this family fall into two broad categories, proapoptotic and anti-apoptotic, based on their ability to elicit opposing effects at the level of the mitochondria. Those proteins with proapoptotic functions are classified further into multidomain such as Bax and Bak (containing three BH domains) or “BH3-only” (possessing only the BH3 domain) such as Bim, Bid, Bad, Puma, Bik, and Noxa. The proapoptotic function of BH3-only proteins requires Bax or Bak (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001) and an intact BH3 domain (Wang et al., 1996; Zha et al., 1997).

Bcl-2-interacting mediator of cell death (Bim), of which there are at least three isoforms BimEL, BimL, and BimS, has become firmly established as a critical mediator of neuronal apoptosis through studies of Bim overexpression or inactivation (O'Connor et al., 1998; Putcha et al., 2001; Whitfield et al., 2001). The regulation of Bim activity is complex, involving both transcriptional and posttranslational mechanisms. Both JNK (c-Jun N-terminal kinase)/c-Jun and forkhead transcription factors contribute to transcription of Bim (Harris and Johnson, 2001; Dijkers et al., 2002; Gilley et al., 2003; Obexer et al., 2007), whereas posttranslational regulation involves rapid phosphorylation by Erk1/2 (extracellular signal-regulated kinase 1/2) resulting in its ubiquitylation, proteasomal targeting, and degradation (Biswas and Greene, 2002; Ley et al., 2003; Luciano et al., 2003).

The purpose of this study was to examine the role of the BH3-only protein Bim in development and degeneration of the early postnatal retina. Our findings indicate that Bim is a key regulator of apoptosis during development, with cell death delayed signif-

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icantly in its absence. We also demonstrate that retinal explant cultures exposed to calcium stress, hydrogen peroxide, or staurosporine (STS), in which BimEL is expressed at very low levels, upregulate this proapoptotic protein before caspase activation and cell death. Furthermore, *bim*^{-/-} retinas are highly resistant to each of these apoptotic stimuli. Finally, we examine the expression pattern of BimEL in the retinal degeneration (rd) model during development and degeneration to try to gain an insight into the death mechanisms involved *in vivo*.

Materials and Methods

Animals. C57BL/6 (wt) and C3H/HEN (rd) animals were obtained from Harlan (Bicester, UK). Generation of Bim-deficient mice (266Del strain; C57BL/6 background) has been described previously (Bouillet et al., 1999). Animals were maintained in 12 h light/dark cycles and killed by cervical dislocation.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling. Enucleated eyes were fixed in 10% neutral buffered formalin overnight at 4°C, followed by cryoprotection in 25% sucrose overnight at 4°C. Retinal explants were treated in the same manner. Frozen sections (7 μm) were incubated with terminal deoxynucleotidyl transferase (MSC, Dublin, Republic of Ireland) and fluorescein-12-dUTP (Roche, Lewes, UK) according to manufacturers' instructions at 37°C for 1 h. Sections were mounted and viewed under a fluorescence microscope (Leica DM LB2; Leica, Nussloch, Germany) using an FITC filter.

Immunohistochemistry. Whole eyes or retinal explants were fixed as described above. After antigen retrieval in 10 mM citrate buffer, pH 6.0 (microwave at 650 W for 15 min), and quenching of endogenous peroxidase activity, frozen sections (7 μm) were incubated with anti-active caspase-3 (1:50) or anti-active caspase-9 (1:100) (Cell Signaling, Beverly, MA) overnight at 4°C. Washes in PBS with 0.01% Tween 20 were followed by incubation with secondary antibody for 1 h at room temperature. Antibody detection was achieved with VectorStain Elite ABC kit and DAB reagent (Vector Laboratories, Peterborough, UK). Sections were counterstained with hematoxylin to facilitate tissue orientation and mounted in DepeX.

Retinal explants. Eyes from C57BL/6 and C57BL/6 *bim*^{-/-} mice were removed and cleaned with 70% ethanol. The anterior segment, vitreous body, and sclera were removed and the retina mounted on Millicell nitrocellulose inserts (Millipore, Billerica, MA) photoreceptor-side down. Explants were cultured without retinal pigment epithelium (RPE) in 1.2 ml of R16 media (from Dr. P. A. Ekstrom, Wallenberg Retina Centre, Lund University, Lund, Sweden) without additional serum. Treated explants were cultured in medium containing 5 μM 5-methylamino-2-(2S,3R,5R,8S,9S)-3,5,9-trimethyl-2-(1-oxo-1-(1H-pyrrol-2-yl)propan-2-yl)-1,7-dioxaspiro(5.5)undecan-8-yl methyl)benzoxazole-4-carboxylic acid (A23187), 1 μM STS, or 600 μM H₂O₂ (Sigma, Dublin, Republic of Ireland), respectively, for 24 h.

Western blot analysis. After treatment, cells were scraped, washed once in PBS, and resuspended in radioimmunoprecipitation assay (RIPA) buffer. Alternatively, retinas were dissected in PBS and dissociated in RIPA buffer (Doonan et al., 2003). Equivalent amounts of protein were resolved using SDS-PAGE followed by transfer to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Antibodies reactive to Bim (Sigma) and GAPDH (Insight Biotechnology, Middlesex, UK) were used in this study. Membrane development was achieved using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

RT-PCR. Total RNA was isolated using Trizol reagent (Bio-Sciences, Dun Laoghaire, Republic of Ireland). cDNA was synthesized from RNA samples using Moloney murine leukemia virus reverse transcriptase and oligo-dT (MSC) according to manufacturers' instructions. The primers used were as follows: *bimEL*, forward, 5'-GACAGAACCGCAAG-GTAATCCCGACG-3', and reverse, 5'-TAGGATCCCCTCAATGCC-TCTCCCATACCAG-3'; *gapdh*, forward, 5'-ACCACAGTCCATGCC-ATCAC-3', and reverse, 5'-TCCACCACCTGTTGCTGTA-3' (MWG, Milton Keynes, UK). PCR products were separated on an agarose gel, stained with ethidium bromide, and visualized under UV light.

Data analysis. After terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining, slides were coded

and analyzed by an independent observer. For each developmental stage, at least three animals were used and three fields (40× magnification) per section of at least three different sections were evaluated. Images of the retinal sections were obtained using a Leica fluorescence microscope. In Figure 1, the number of TUNEL-positive cells in each field was counted and calculated as a percentage over the total number of cells in the same field. To determine the total number of cells in each layer, a gridline was established. Twenty percent of the squares in each retinal layer were selected at random, and the cells in each grid were counted. This value was extrapolated to the number of squares covering each layer in the entire field. In Figure 1, we have expressed the results as relative values, because cell numbers in each retinal layer are constantly changing as a result of neuronal pruning. In Figure 4, in which only one retinal age was examined, the total cell numbers in the outer nuclear layer (ONL) would be approximately the same, and thus relative and absolute values would be the same. Therefore, the results presented in Figure 4 are presented as absolute values.

Retinal thickness was assessed at each developmental stage in at least three animals comparing three fields (40× magnification) per section of at least three different sections. Thickness of the ONL, inner nuclear layer (INL), and ganglion cell layer (GCL) was measured with a calibrated scale of the objective of the light microscope. Comparisons between *bim*^{-/-} and wt were performed using unpaired, two-tailed *t* test analysis.

Results

Developmental programmed cell death is delayed in retinas of *bim*^{-/-} mice

We have previously shown that BimEL is highly expressed at the peak of developmental programmed cell death (PCD) and is rapidly downregulated thereafter (Donovan et al., 2006). This suggests a requirement for Bim during postnatal retinal development when cells are primed to die. We assessed the potential involvement of this protein in apoptosis in the developing mouse retina *in vivo* using *bim*^{-/-} mice. TUNEL staining of retinal sections from *bim*^{-/-} mice shows that the peak of death is delayed considerably in each layer (Fig. 1A). The first wave of retinal PCD, occurring normally at postnatal day 2 (P2) (Pequignot et al., 2003) in the GCL of wt retinas is delayed by 5 d, occurring in *bim*^{-/-} retinas at P7. A second peak of GCL death occurring at P8 is also delayed, which may explain why a number of TUNEL-positive cells are detected in the adult *bim*^{-/-} GCL (Fig. 1B). The waves of postnatal cell death that eliminate certain cell types from the INL were also significantly delayed. Apoptosis in this layer peaks at approximately P7/P8 in the wt compared with two separate peaks at P11 and P15 in the *bim*^{-/-} retina. Although the individual cell types undergoing apoptosis were not identified in this study, excess amacrine cells (P3–P5), bipolar cells (P8–P11), and Müller glia (P8–P11) are eliminated during normal retinal development (Young, 1984). Furthermore, INL rods that fail to migrate successfully into the ONL remain temporarily in the INL where they undergo apoptosis (Young, 1984; Hahn et al., 2003). Finally, we show that the percentage of TUNEL-positive cells peaks in the wt ONL at P15 corresponding with a previous report (Pequignot et al., 2003). The percentage of TUNEL-positive cells in the ONL of P15 *bim*^{-/-} retinas is much less than in wt, and significantly more dying cells were counted in the ONL of adult *bim*^{-/-} retinas compared with wt, suggesting that the wave of death is also delayed.

In addition, the thickness of each of the nuclear layers was measured (Fig. 2A). Our results show that the *bim*^{-/-} ONL is significantly thicker from P7 through P15 and into adulthood. In the INL, the largest differences were detected from P5 to P8, whereas the most significant difference in the GCL is present at P0 (Fig. 2B). Furthermore, *bim*^{-/-} retinas are already thicker than their wt counterparts at P0, implying a role for Bim in cell

Figure 1. Developmental apoptosis in the retina is delayed in *bim*^{-/-} mice. **A**, TUNEL staining in retinal sections from P2, P5, P8, and P15 mice demonstrates the pattern of cell death in wt versus *bim*^{-/-} mice. NBL, Neuroblastic layer. **B**, The number of TUNEL-positive cells in each field was counted and calculated as a percentage over the total number of cells in the same field. To determine the total number of cells, a gridline was established. Twenty percent of the squares in each retinal layer were selected at random, and the cells in each grid were counted. This value was extrapolated to the number of squares covering each layer in the entire field. Error bars represent $\pm$ SD (* p < 0.05; ** p < 0.01). The ONL is not represented until P5 when the NBL separates, yielding the ONL and INL.

death during embryonic development of the mouse retina, which peaks at embryonic day 10.5 (Pequignot et al., 2003). Finally, we analyzed *bim*^{-/-} and wt retinas for caspase-9 activation to determine whether or not dying cells still activate the mitochondrial pathway in the absence of Bim, given that cell death in the absence of caspase activation has been readily detected in dying retinal cells (Donovan et al., 2001; Doonan et al., 2003; Lohr et al., 2006). We found that caspase-9 cleavage [and caspase-3 cleavage (data not shown)] was evident both in wt and *bim*^{-/-} retinas (Fig. 2A) and that the peak of caspase activation was also delayed (data not shown). This is consistent with activation of the mitochondrial pathway, implying redundancy within the BH3-only family.

Bim-deficient retinal explants are resistant to calcium overload

Having identified an important role for Bim in developmental apoptosis, we extended our study to include models of degeneration. Retinal explants from P10 and P15 mice were used, because our previous data showed a rapid decrease in BimEL expression between these ages (Donovan et al., 2006). Treatment with 5 μ M calcium ionophore (A23187), which is relevant to models of retinal disease (Bouillet et al., 1999; Donovan et al., 2001; Read et al., 2002; Doonan et al., 2003; Fox et al., 2003; Takano et al., 2004),

for 24 h induced cell death in both P10 and P15 explants, mostly in the ONL containing the photoreceptor cells. Cells of the INL and GCL were also affected but to a lesser degree. Our results show that P15 retinal explants are consistently more resistant than P10 retinal explants to cell death and caspase-9 processing (Fig. 3A). Protein expression of BimEL increased in response to ionophore at both P10 and P15, indicating that Bim is an important sensor of calcium stress in retinal explants (Fig. 3B). To confirm that BimEL expression is required for ionophore-induced cell death, retinal explants from *bim*^{-/-} mice were used. The amount of cell death in each panel 24 h after calcium treatment mirrors exactly the level of BimEL (Fig. 3C). Western blot data show induction of Bim expression in +/+ (wt) P15 retinal explants in response to calcium stress as described above, whereas the level of BimEL is greatly reduced in +/- explants and undetectable in -/- explants (Fig. 3D). Caspase-9 processing is detected in the -/- panel suggesting that the remaining TUNEL-positive cells die in a caspase-dependent manner (Fig. 3E).

Bim-deficient retinal explants are also resistant to oxidative stress and STS

We showed that induction of Bim expression in response to calcium overload plays an important role in activation of the mitochondrial pathway and cell death. Given that distinct BH3-only proteins seem to induce apoptosis in response to specific death stimuli, we wanted to determine whether or not Bim is a sensor for death in response to other stimuli. Hence we exposed retinal explants from *bim*^{-/-} mice to oxidative stress (600 μ M hydrogen peroxide) (Fig. 4A) or kinase inhibition (1 μ M STS) (Fig. 4B). H₂O₂-induced cell death was almost completely inhibited in *bim*^{-/-} explants. Figure 4C illustrates in graph format the dramatic protection observed in -/- explants, whereas +/- explants exhibit an intermediate effect in response to both A23187 and H₂O₂. Interestingly, there is a statistically significant difference between the number of TUNEL-positive cells in untreated wt explants compared with the number in untreated -/- explants (p < 0.01).

STS treatment induced apoptosis in the both the ONL and INL, giving us the opportunity to observe the effect in both layers. We discovered that the absence of Bim affords protection in both layers (Fig. 4B). The GCL is also protected in the STS model, but optic nerve transection also contributes to cell death in this retinal layer. Therefore, the absence of Bim could be affording protection against STS, optic nerve transection, or both. This laboratory previously showed that the adult GCL is protected from axotomy in the absence of Bim (McKernan and Cotter, 2007); therefore, counts of TUNEL-positive cells were compared in untreated wt and *bim*^{-/-} P15 retinal explants. Results suggest that there is 50–60% protection of the GCL in P15 explants, and thus Bim contributes to death by axotomy (data not shown).

Reduced Bim expression correlates with a switch to caspase-independent apoptosis in the rd model

Publications from this laboratory and others have shown that cell death in the retinal degeneration (rd) model of retinitis pigmentosa, in which calcium stress is an apical event, does not require caspase activation (Zeiss et al., 2004; Doonan et al., 2005; Paquet-Durand et al., 2006). Figure 5A shows the pattern of cell death at specific time points during postnatal development (P7 and P8) and degeneration (P12 and P14) of the rd mouse retina. Caspase-9 activity was detected by immunohistochemical analysis with an antibody specific to cleaved caspase-9 during development of the rd retina but not during degeneration. These data clearly demonstrate that cells of the retina are capable of using different cell death programs. Protein lysates from rd retinas analyzed for BimEL, demonstrated that expression of this protein decreased markedly during development of the rd retina between P8 and P9 and was not re-expressed during degeneration (Fig. 5B). In fact, BimEL was downregulated in the rd retina even earlier than wt, P8–P9 compared with P11–P12. Transcription of the *bim* gene had also ceased at P8 and up to P14 had not resumed (Fig. 5C). Therefore, the absence of Bim expression correlates with the absence of cytochrome *c* release (Doonan et al., 2003) and caspase-dependent apoptosis in the rd mouse. To determine whether or not caspase mechanisms remain intact in the rd mouse, we attempted to force Bim expression in rd retinal explants by addition of calcium. Rd retinas were explanted at P11 and either left untreated or treated for 24 h with 1 μ M A23187. Cleaved caspase-9 was detected by immunohistochemistry in A23187-treated rd retinas but not in untreated retinas despite the presence of TUNEL-positive cells (Fig. 5D). We also observed an increase in Bim expression by Western blot, indicating that the presence of Bim correlates with caspase activation (Fig. 5E).

These data combined with the *bim*^{-/-} studies suggest that Bim plays an important role in caspase-dependent retinal cell death and may thus explain why death in the rd retina does not involve caspase activation. We are certain from our work with cell-free extracts that the mitochondrial pathway can be activated, but is not activated because of a block at the level of cytochrome *c* release (Doonan et al., 2003). This supports the evidence presented here that the downregulation of Bim and potentially other BH3-only proteins is an important checkpoint for activation of mitochondrial pathway.

Discussion

An important role for Bim in many neuronal paradigms and in lymphoid cell death has been described in the literature (Bouillet et al., 1999; Putcha et al., 2001; Linseman et al., 2002; Gilley et al., 2003; Erlacher et al., 2005; Hutcheson et al., 2005). Here, we describe, for the first time, an important role for Bim during

Figure 2. *bim*^{-/-} retinas are thicker than their wt counterparts. **A**, Retinal sections from wt and *bim*^{-/-} mice at P2, P5, P13, and P60 were stained with an antibody reactive to cleaved caspase-9. Caspase-9 processing is detectable in wt and *bim*^{-/-} retinas, as indicated by the arrows. **B**, Thickness of the ONL, INL, and GCL in micrometers measured with a calibrated scale of the objective of the light microscope. NBL, Neuroblastic layer. Comparisons between *bim*^{-/-} and wt were performed using unpaired, two-tailed *t* test analysis. Error bars represent $\pm$ SD (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

postnatal development of the mouse retina. From birth, *bim*^{-/-} retinas mice exhibit a clear increase in thickness, which is still evident in the ONL at later stages of development. We showed that increased thickness of *bim*^{-/-} retinas is a result of decreased developmental apoptosis at early postnatal ages. This is not unexpected given the potent proapoptotic function of this gene. The first wave of retinal PCD, occurring in the GCL, is delayed by a number of days in *bim*^{-/-} retinas as are the subsequent waves of postnatal cell death eliminating cells of the INL and ONL. A transient delay such as this, is consistent with a report describing a temporary delay in developmental apoptosis in dorsal root ganglion cells from *bim*^{-/-} mice *in vivo* (Putcha et al., 2001).

Thus, our data indicate a dependence on Bim for the progression of normal retinal development, through the removal of excess cells by apoptosis, in both the embryonic and postnatal phase. We hypothesize that Bim is a critical BH3-only protein on which developmental death signals converge in retinal neurons, and the absence of this protein significantly delays apoptosis. However, the protection conferred by Bim deficiency is transient, and retinal cells in the *bim*^{-/-} mouse still die, albeit at a slower rate, via the intrinsic pathway (verified by caspase-9 cleavage). Therefore, alternate mechanisms seem to exist that allow resumption of normal development, presumably involving recruitment of other BH3-only proteins upstream of the Bax/Bcl-2 checkpoint that are capable of responding to the death stimulus

Figure 3. Bim-deficient retinal explants are resistant to calcium overload. **A**, TUNEL staining and immunohistochemistry for cleaved caspase-9 in retinal sections from untreated (unt) and calcium ionophore-treated (A23187; 5 μ M) P10 and P15 explants after 24 h. P10 explants are more susceptible to cell death and caspase activation than P15 explants. **B**, Western blot analysis of Bim expression in untreated and treated (A23187; 5 μ M) P10 and P15 retinal explants after 24 h. Blots were reprobed with GAPDH to demonstrate equal protein loading. **C, D**, TUNEL staining of retinal sections and Western blot analysis of Bim expression from untreated wt and A23187-treated wt, *bim*^{+/+} (+/+), and *bim*^{-/-} (-/-) explants after 24 h. **E**, Immunohistochemistry for cleaved caspase-9 in a retinal section from A23187-treated *bim*^{-/-} (-/-) explants.

in the absence of Bim. This has been described in DNA-damaged lymphocytes *in vivo* in which both Puma and Bim contribute significantly to cell death (Erlacher et al., 2005). Similarly, spermatogenesis is arrested in adult mice lacking both Bik and Bim, whereas mice lacking only one gene exhibit no adverse effects (Coultas et al., 2005; Erlacher et al., 2005). Interestingly, retinas from Bax/Bak double knock-outs remain markedly thicker at 2 months (and 8 months) of age, whereas there is very little discernable difference in *bim*^{-/-} retinas. This indicates an absolute dependence on the mitochondrial pathway for normal retinal development and certainly supports the theory that BH3-only proteins other than Bim may be involved (Hahn et al., 2003; Pequignot et al., 2003).

Despite the apparent redundancy in BH3-only proteins during retinal development, retinas of Bim knock-out mice are protected against 80–90% of cell death in response to diverse stimuli. There have been many reports in the literature suggesting that the involvement of individual BH3-only proteins in the initiation of apoptosis is stimulus-specific. For example, Bim is activated in

response to cytokine withdrawal and calcium flux (Bouillet et al., 1999; Puthalakath et al., 1999), Bim is activated by loss of cellular attachment (anoikis) (Puthalakath et al., 2001), and both Puma and Noxa are activated in response to DNA damage (Erster et al., 2004; Wytenbach and Tolksky, 2006). This laboratory has previously demonstrated that Bim expression is rapidly induced in a photoreceptor cell line in response to serum starvation and UV light resulting in caspase-dependent cell death (Gomez-Vicente et al., 2006). However, this study is the first to show that retinal explants from *bim*^{-/-} mice are resistant to caspase-dependent apoptosis in response to a variety of death stimuli, including calcium overload, protein kinase inhibition, and oxidative stress. Bim certainly does appear to be the limiting factor for activation of cell death in retinal explants because a gene dosage effect was consistently observed in heterozygotes, which died at an intermediate rate between that of wt and *bim*^{-/-} (Fig. 3C). However, for each set of data with the exception of STS-treated ONL, the level of cell death in treated *bim*^{-/-} explants remains slightly higher than in untreated explants and those cells that are dying stain positive for caspases (Fig. 3E) (data not shown for hydrogen peroxide and STS). Whether or not caspase activation in the remaining TUNEL-positive cells is attributable to other BH3-only proteins remains unclear.

Thus, Bim appears to play a dominant role in stimulus-induced death in retinal explants, despite the fact that numerous BH3-only proteins have been described. The particular potency of Bim as a death inducer can probably be attributed to its ability to strongly bind and neutralize all prosurvival Bcl-2 proteins (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1). The same is true for Puma and tBid, whereas other BH3-only proteins exhibit clear selectivity (Chen et al., 2005). For example, the complementary binding profiles of Bad (Bcl-2, Bcl-x_L, and Bcl-w) and Noxa (Mcl-1 and A1) suggest that alone they are inefficient killers but in combination work very effectively to induce apoptosis (Willis et al., 2005). In this model, BH3-only proteins activate Bak (and possibly Bax in the same manner) simply by sequestering the prosurvival Bcl-2 family proteins to which Bak is bound. An alternative model suggests that BH3-only proteins can be divided into two subclasses; “sensitizers” (e.g., Bik), which inactivate the prosurvival proteins, and “activators” (Bim and tBid), which directly bind and activate Bax and Bak (Letai et al., 2002). However, the observations leading to the conception of this model could equally be explained by the superior ability of Bim and tBid to inactivate prosurvival proteins present at the mitochondria.

Despite the evidence that Bim and the mitochondrial pathway play an essential role in both development and stimulus-induced cell death in explants, degenerating photoreceptors in

the rd model execute caspase-independent death. We showed previously that caspase-9 and -3 are not activated during degeneration in the rd mouse possibly because of a block at the level of cytochrome *c* release (Doonan et al., 2003; Donovan et al., 2006). In this study, our results demonstrate that the expression of Bim decreases rapidly in the developing rd mouse retina and is not reexpressed during degeneration. Forced reexpression of Bim *ex vivo* converts the pathway to one in which caspases are active (Fig. 5*D,E*), indicating that caspase-dependent mechanisms are intact in the rd mouse *in vivo* but perhaps cannot respond to an initiation signal at the level of the mitochondria. This could be attributable to absence of Bim, and other BH3-only proteins preventing mitochondrial pore formation, and release of proapoptotic factors. Interestingly, differentiated cardiomyocytes demonstrate resistance to caspase-dependent cell death because elements of the caspase-dependent pathway are silenced during heart development. Using ischemia as a trigger, caspase activation was shown in embryonic cardiomyocytes and proliferating fibroblasts but not in neonatal and adult cardiomyocytes (Bahi et al., 2006). Similar findings have been reported in *Drosophila* carrying a mutation in the *norpA* gene, causing retinal degeneration (Hsu et al., 2004). IAP (inhibitor of apoptosis) antagonists *grim*, *rpr*, and *hid*, which are common inducers of developmental PCD, are not necessary for adult retinal degeneration. In addition, expression of the caspase inhibitor p35 does not alter the progression of apoptosis, indicating that the pathways typically responsible for developmental PCD do not play an essential role in *norpA*-induced degeneration. Thus, the same neuronal population can use different developmental and pathologic cell death pathways. Therefore, we propose that proteins such as Bim, abundantly expressed for the purpose of developmental apoptosis, are downregulated to avoid rapid caspase activation and cell death, which could have serious implications for the maturing retina. Hence, two options are available to photoreceptors in response to apoptotic stimuli. First, components required for caspase-dependent apoptosis may be reexpressed; for instance, Bim and Hrk are upregulated in axotomized retinal ganglion cells before apoptosis (Wakabayashi et al., 2002; Napankangas et al., 2003). Alternatively, a caspase-independent death pathway, which we observed in a number of models of retinal degeneration, may be activated.

In conclusion, the present findings indicate that caspases are the primary executioners of developmental retinal cell death and indeed most of the stimulus-induced apoptosis in early postnatal retinas *in vitro*. However, termination of developmental apoptosis in the retina appears to initiate downregulation of the Bcl-2 family member Bim, a key intermediary in caspase-dependent death. Reexpression of Bim in retinal explants appears to reactivate developmental cell death pathways. However, in certain *in vivo* models, such as the rd

Figure 4. Bim-deficient retinal explants are also resistant to H_2O_2 and STS. **A**, TUNEL staining of retinal sections from untreated (unt) wt and H_2O_2 -treated wt, $Bim^{+/-}$ (+/-), and $Bim^{-/-}$ (-/-) P15 explants after 24 h. **B**, TUNEL staining of retinal sections from untreated wt and STS-treated wt, $Bim^{+/-}$ (+/-), and $Bim^{-/-}$ (-/-) P15 explants after 24 h. **C**, For each stimulus, at least three animals were used, and the number of TUNEL-positive cells in three fields (40 $\times$ magnification) per section of at least three different sections was counted. Error bars represent $\pm$ SD.

model in which this does not occur, photoreceptor cells are capable of dying independently of caspases.

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Figure 5. Caspase-independent cell death in the rd mouse correlates with Bim downregulation. **A**, TUNEL staining (arrowheads) and immunohistochemistry for cleaved caspase-9 (arrows) in the rd mouse retina during development (P7 and P8) and degeneration (P12 and P14). **B**, Western blot analysis of Bim expression in the rd retina from P8 to P15. Blots were reprobed with GAPDH to demonstrate equal protein loading. **C**, Semiquantitative RT-PCR of *bimEL* in the rd retina with *gapdh* as a loading control. **D**, TUNEL staining and immunohistochemistry for cleaved caspase-9 in retinal sections from untreated (24 h) and A23187-treated (1 μ M for 24 h) rd P12 explants. **E**, Western blot analysis of Bim expression in untreated and A23187-treated (1 μ M for 24 h) rd retinas at P12.

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