Inactivation of the Akt Survival Pathway during Photoreceptor Apoptosis in the Retinal Degeneration Mouse

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PURPOSE. Previous work has indicated that the serine-threonine protein kinase Akt is a general mediator of cellular survival signals and that loss of Akt-mediated signaling can lead to the activation of apoptosis. This study was conducted to establish whether regulation of the Akt survival pathway mechanisms is implicated in the induction of apoptosis during photoreceptor cell death in the rd mouse model of retinal degeneration.

METHODS. Quantitative Western blot analysis and immunocytochemistry were used to examine the status and localization of key components of the Akt signaling cascade (Akt, BAD, Forkhead [FKHR], HSP27, mitogen-activated protein [MAP] kinase kinase-3 and -6 [MKK3/6], the tumor-suppressor phosphatase PTEN, and the cytoplasmic protein-tyrosine kinase cSrc-p60), in the retina of the rd mouse in comparison with the control. The time points examined spanned the period of photoreceptor degeneration.

RESULTS. In the period up to the peak of photoreceptor apoptosis at postnatal day 15, dysregulation of the survival pathway was identified at several levels, including deactivation of both Akt itself and its downstream transcription factor target Forkhead (FKHR) and activation of the upstream negative regulator PTEN.

CONCLUSIONS. Taken in conjunction with previous studies, the data support a model in which photoreceptor cell death in the rd mouse is the result of combined inactivation of the Akt survival pathway and the activation of the two major apoptotic pathways. (Invest Ophtalmol Vis Sci. 2006;47:1620–1629) DOI:10.1167/iovs.05-1176

Apoptosis has been shown to be the final common pathway of photoreceptor cell death in several animal models of inherited retinal degeneration (for review, see Ref. 1). In the rd mouse strain, the best-characterized animal model of retinitis pigmentosa (RP), photoreceptors have been shown to die by apoptosis.1–4 due to a recessive mutation of the gene coding for the rod cGMP phosphodiesterase β-subunit. The two classical stress and cell death-receptor pathways have been reported to activate a caspase cascade during photoreceptor degeneration in this mouse model.5 A proapoptotic Bcl-2 family member, Bid, which allows crosstalk between the two pathways, was found to be activated in the apoptotic photoreceptor. Upstream of Bid, p38 mitogen-activated protein kinase (MAPK), an apoptosis regulator, was reported to be an activator of this key factor. As stress-activated kinases, p38 MAPKs regulate not only cell death but also cell survival (for review, see Ref. 6), suggesting that execution of the apoptotic multistep process could be modulated by survival pathways during photoreceptor degeneration. However, little is known about whether survival-signaling cascades are implicated in the regulation of photoreceptor cell death in this mouse model.

Death and survival signals converge to activate components of the posttranscriptional apoptotic machinery, such as the caspases or the Bcl-2 family of proteins. It has been suggested that the balance of pro- and antiapoptotic members of the Bcl-2 family determines life or death in a cell (for review, see Ref. 8). Indeed, the regulation of mitochondrial cytochrome c release and mitochondrial function by several Bcl-2 family members plays a large part in controlling cell death.9–11 BAD, one of the proapoptotic family members, is the first target of Akt, a major inhibitor of apoptosis (for review, see Ref. 12). By phosphorylating BAD, Akt precludes its binding to the Bcl-xL and Bcl-2 antiapoptotic family members leading to increased cell survival.13–15 Akt, a serine-threonine protein kinase, is a key component of cell survival pathways and is a major downstream target of phosphoinositide-3-kinase (PI3K), which has been shown to mediate growth factor-induced neuronal survival in a wide variety of circumstances (for review, see Ref. 14). Activation of PI3K leads to Akt activation by both phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates Akt at Thr-308, and PDK1-independent mechanisms, which result in Akt phosphorylation at Ser-473.12,14 Akt is also phosphorylated at Ser-473 by the p38 MAPK-activated protein kinase-2 (MAPKAPK-2) pathway.15,16 MAPKAPK-2 activates both Akt and the small heat shock protein 27 (HSP27).16–18 Moreover, there is evidence that the three components of the p38 MAPK, MAPKAPK-2, and HSP27 form a signaling complex with Akt.19 As a general mediator of cell survival, activated Akt catalyzes the phosphorylation of the Forkhead-related family of mammalian transcription factors (FKHRs; for review, see Ref. 12,14). FKHRs are inhibited when phosphorylated, which prevents them from stimulating the transcription of cell death genes, resulting in the inhibition of transcription-dependent apoptosis (for review, see Ref. 19). Further, the PI3K/Akt signaling pathway is negatively regulated by the tumor-suppressor phosphatase PTEN through the latter’s ability to dephosphorylate the lipid second messenger phosphatidylinositol.20,21 PTEN is thought to promote apoptosis by facilitating caspase-8 activation and Bid cleavage through a Fas-associated death domain (FADD)-dependent pathway.22 Its function has been shown to be inhibited by the Src protein-tyrosine kinases that efficiently induce PTEN tyrosine phosphorylation, leading to the activation of the PI3K/Akt signaling pathway.23

To evaluate the extent to which the balance between prodeath and prosurvival pathways is dysregulated during photoreceptor degeneration, we have investigated the modulation of different players implicated in the Akt survival pathway in the rd mouse model of retinal degeneration.
METHODS

Animals

The animals were maintained in a 12-hour light-dark cycle. The C57BL/6J (control) animals were obtained from Harlan UK (Bicester, UK). Homozygous rd mice, in which the rd locus is closely linked to the light ear (le) character, were maintained in an inbred colony at St. Thomas' Hospital. The animals were killed by cervical dislocation and the eyes enucleated during the light phase. All animal procedures were performed in compliance with UK Home Office regulations and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunocytochemical Analysis

The eyes were fixed in 4% paraformaldehyde, followed by embedding, freezing, and cryosectioning, as previously described. Antibodies were obtained from Cell Signaling Technology (Beverly, MA): polyclonal rabbit anti-mouse phospho-Akt Ser-473 (9271; 1:200), polyclonal rabbit anti-mouse phospho-FKHR Ser-256 (9461; 1:1000), polyclonal rabbit anti-human PTEN (9552; 1:400), monoclonal anti-human phospho-BAD Ser-112 (9296; 1:400), and from Santa Cruz Biotechnology (Santa Cruz, CA): polyclonal goat anti-human phospho-HSP27 (Ser-82; sc-12923; 2 μg/mL), polyclonal rabbit anti-human Src-p60 (sc-19-G; 2 μg/mL), and monoclonal anti-human phosphorylated MKK3/6 (B-9; sc-8407; 2 μg/mL). The antibodies were visualized as appropriate using goat anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG conjugated to fluorescein (F-9887, F-4018, F-7367, respectively; Sigma-Aldrich Co., Ltd., Dorset, UK) at concentrations in accordance with the manufacturer’s recommendations.

Western Blot Analysis

Retinas were dissected and pooled from 10 to 12 animals for each time point studied. Subcellular fractionation was performed to obtain a heavy membrane fraction enriched for mitochondria and a soluble fraction, as previously described. The soluble fraction was analyzed by Western blot. Equal quantities of protein were loaded (15 μg per lane), and loading and transfer to the blot were assessed by subsequent stripping and detection of actin. Antibodies were obtained from Cell Signaling Technology: polyclonal rabbit anti-mouse phospho-Akt Ser-473 (9271; 1:1000), polyclonal rabbit anti-mouse phospho-mTOR (Ser-2448; 15 μg/mL), polyclonal rabbit anti-human phospho-FKHR Ser-256 (9461; 1:1000), polyclonal rabbit anti-human PTEN (9552; 1:400), monoclonal anti-human phospho-BAD Ser-112 (9296; 1:400), polyclonal rabbit anti-human phospho-HSP27 (Ser-82; sc-12923; 2 μg/mL), polyclonal rabbit anti-human Src-p60 (sc-19-G; 2 μg/mL), and monoclonal anti-human phosphorylated MKK3/6 (B-9; sc-8407; 2 μg/mL). The antibodies were visualized as appropriate using goat anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG conjugated to horseradish peroxidase. The blots were scanned with a Molecular Dynamics Storm 860 phosphorimager, and quantitation was performed using ImageQuant software (Molecular Dynamics). The densitometric values of the bands were normalized to the levels of actin seen in each fraction.

RESULTS

This study focused on the peak of photoreceptor degeneration in rd mice between postnatal days 13 and 20. During this period, histomorphologic analysis of retinas confirmed the degenerative process in comparison with age-matched control, as previously reported. Akt Activation

Because Akt has been shown to be essential for neuronal survival, we investigated its expression during photoreceptor degeneration in the rd mouse model. Antibodies recognizing the total and the phosphoactivated form of Akt were used to monitor Akt activation by Western blot analysis and immunocytochemistry. Total Akt levels were found to be similar in control and rd retinas up to postnatal day 17, but a significantly lower level of expression (~20% of control) was observed in rd retinas at 20 days (Fig. 1A). However, evaluation of its activation showed a biphasic phenomenon: first, a markedly lower level of phosphorylated Akt was observed up to the peak of photoreceptor degeneration in rd relative to control retinas (~50% at postnatal day 14, and ~25% at postnatal day 13) (Fig. 1B); and from postnatal day 16, a significant increase of phosphorylated Akt (from approximately two to four times higher levels of expression) was detected in the rd compared with control retinas (Fig. 1C). The levels of nonphosphorylated or phosphorylated Akt immunoreactivity was detected at the photoreceptor outer segments, inner nuclear layer, and ganglion cell layers at 14 days (Fig. 1C), but was confined mainly to the photoreceptor outer segments at 20 days of age (Fig. 1E). In contrast, in rd retinas, only a slight immunostaining was observed at the ganglion cell layer level at 14 days (Fig. 1D), but intense immunolabeling of the ganglion cell layer, diffusing into the inner plexiform layer, and increased staining of the inner nuclear layer were detected at postnatal day 20 (Fig. 1F). No nonspecific immunofluorescence was observed in sections without primary antibody in this or any other of the immunocytochemical analyses described later herein (data not shown).

BAD Activation

To determine whether Akt activation correlates with the inactivation of BAD proapoptotic functions, we evaluated BAD phosphorylation status in control and rd retinas using antibodies that recognize the total and phosphorylated forms. At all time points investigated, the levels of nonphosphorylated or phosphorylated Ser-112 BAD were essentially similar in control and rd retinas (Figs. 2A, 2B). Comparable results were obtained with the phosphorylated Ser-136 BAD antibodies (data not shown). These results were supported by the detection of phosphorylated Ser-112 BAD immunoreactivity at the retinal pigment epithelium and ganglion cell layer levels in both control and rd retinas (Figs. 2C, 2D). The immunolabeling signal arising from the ganglion cell layer was slightly more intense in rd than in control retinas at 15 days (Figs. 2C, 2D).

FKHR Activation

To establish whether the FKHR member of the Forkhead transcription regulator family is activated by phosphorylated Akt, we evaluated the phosphorylation state of FKHR at Ser-256, one of the three potential Akt phosphorylation sites on the FKHR protein present in the Forkhead domain common to all the family members. Antibodies recognizing either the total or the phosphorylated form of FKHR were used for Western blot analysis and immunocytochemistry in control and rd retinas. Total FKHR levels were approximately two times higher in rd than in control retinas at 13 and 16 days (Fig. 3A). In contrast, at postnatal day 15 in rd, levels were approximately one-fifth those of control retinas (Fig. 3A). Variations in phosphorylated FKHR levels were also observed (Fig. 3B). The levels were significantly higher in control than in rd retinas at postnatal days 13 and 14, but from postnatal days 16 to 20 they were significantly lower in control than in rd retinas. These results were supported by detection of phosphorylated FKHR immunoreactivity, intense at the photoreceptor outer segments and ganglion cell layer levels and less so at the inner nuclear and inner plexiform layers in control retina (Fig. 3C). A weak signal from the inner parts of the rd retina was observed at postnatal day 13 (Fig. 3D). By day 20, the immunolabeling of the inner
nuclear layer and inner plexiform layer had disappeared in control retinas (Fig. 3E), but the whole residual rd retina was immunoreactive, with a stronger signal arising mainly from the ganglion cell layer (Fig. 3F).

HSP27 Activation

To determine whether HSP27, an Akt substrate, is involved in photoreceptor degeneration, we analyzed its activation in the rd mouse model. Antibodies recognizing the total forms and activated phosphorylated form of the heat shock protein were used to monitor its activation by Western blot analysis and immunocytochemistry. Increased expression of total HSP27 was detected in rd compared with control retinas at 16 days only (Fig. 4A). Variations in phosphorylated HSP27 (Ser-82) levels were observed (Fig. 4B). They were significantly lower in rd than in control retinas at 13, 15, 17, and 20 days (Fig. 4B). Phosphorylated HSP27 immunoreactivity was detected at the photoreceptor outer segment and ganglion cell layer levels in control retinas at 13 days (Fig. 4C). Similar distribution and immunolabeling of the inner plexiform and the inner nuclear layers, with a slightly more intense signal coming from the inner and outer parts of this layer, were observed in 20-day-old retinas (Fig. 3F).

FIGURE 1. Analysis of Akt and phosphorylated Akt expression in the retina of control (C57BL/6) and rd mice by Western blot and immunocytochemistry. Densitometric analyses of (A) the ratio of total Akt to actin and (B) of phosphorylated Akt to actin. Data are the mean ± SEM (n = 3). *For total Akt: 20 days, P < 0.03. For phosphorylated Akt: 13 days, P < 0.05; 14 days, P < 0.07; 16 days, P < 0.08; 17 days, P < 0.13; and 20 days, P < 0.0005, unpaired t-test. Equal quantities of protein were loaded in all lanes, and representative exposures of enhanced chemiluminescence detection of the immunoreactive proteins are shown. Phospho-Akt immunostaining in control (C, E) and rd (D, F) mouse retina at postnatal ages 14 (C, D), and 20 (E, F) days. Arrowheads: thickness of outer nuclear layer (F). PR: photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 100 μm.

FIGURE 2. Analysis of total BAD and phosphorylated BAD expression in the retina of control (C57BL/6) and rd mice by Western blot and immunocytochemistry. Densitometric analyses of (A) the ratio of total BAD to actin and (B) of phosphorylated BAD to actin. Data are the mean ± SEM (n = 3). Phospho-BAD immunostaining in control (C) and rd (D) mouse retinas at postnatal day 15. Abbreviations are defined in Figure 1. Bar, 100 μm.
control retina (Fig. 4 E). However, weak immunosignal was detected in rd retinas (Figs. 4D, 4F). At postnatal day 13, extremely weak immunoreactivity was observed in the ganglion cell layer (Fig. 4D). The retinal pigment epithelium and a few cells in the ganglion cell layer were immunolabeled in 20-day-old rd retinas (Fig. 4F).

**Figure 3.** Analysis of total FKHR and phosphorylated FKHR expression in the retina of control (C57BL/6) and rd mice by Western blot and immunocytochemistry. Densitometric analyses of (A) the ratio of total FKHR to actin and (B) of phosphorylated FKHR to actin. Data are the mean ± SEM (n = 3). *For total FKHR: 13 days, P < 0.05; 15 days, P < 0.001; and 16 days, P < 0.003. For phosphorylated FKHR: 13 days, P < 0.01; 14 days, P < 0.02; 16 days, P < 0.03; 17 days, P < 0.02; and 20 days, P < 0.04, unpaired t-test. Equal quantities of protein were loaded in all lanes, and representative exposures of enhanced chemiluminescence detection of the immunoreactive proteins are shown. Phospho-FKHR immunostaining in control (C, E) and rd (D, F) mouse retinas at postnatal days 13 (C, D), and 20 (E, F). Arrowheads: thickness of outer nuclear layer (F). Abbreviations are defined in Figure 1. Bar, 100 μm.

**Figure 4.** Analysis of total HSP27 and phosphorylated HSP27 expression in the retina of control (C57BL/6) and rd mice by Western blot and immunocytochemistry. Densitometric analyses of (A) the ratio of HSP27 to actin and (B) of phosphorylated HSP27 to actin. Data are the mean ± SEM (n = 3). *Total HSP27: 15 days, P < 0.06; phosphorylated HSP27: 13 days, P < 0.004; 15 days, P < 0.03; 17 days, P < 0.05; and 20 days, P < 0.03, unpaired t-test. Equal quantities of protein were loaded in all lanes, and representative exposures of enhanced chemiluminescence detection of the immunoreactive proteins are shown. Phospho-HSP27 immunostaining in control (C, E) and rd (D, F) mouse retina at postnatal ages 13 (C, D), and 20 (E, F) days. Arrowheads: thickness of outer nuclear layer (F). Abbreviations are defined in Figure 1. Bar, 100 μm.
MKK3/6 Activation

Because mitogen-activated protein (MAP) kinase kinases 3 and 6 (MAPK kinase 3/6 or MKK3/6) have been shown to activate Akt through the MAPK kinases cascade, we investigated their expression by Western blot analysis and immunocytochemistry, using an antibody specific to the phosphorylated forms of these proteins, in control and rd retinas. Increased levels of phosphorylated MKK3/6 were observed in rd compared with control retinas (1.8 times) at 13 and 14 days (Fig. 5 A). By day 15, levels of expression in rd retinas were approximately one third those in the control (Fig. 5 A). These results were confirmed by immunocytochemistry. Phosphorylated MKK3/6 immunostaining was localized mainly to the retinal pigment epithelium, ganglion cell, and inner nuclear and plexiform layers at 14 days and also to the photoreceptor outer segments at 15 days in control retinas (Figs. 5B, 5D). In 14-day-old rd mice, the immunolabeling was localized to the retinal pigment epithelium; the photoreceptors; and the inner nuclear, inner plexiform, and ganglion cell layers (Fig. 5C). By day 15, the immunoreactivity remained only at the retinal pigment epithelium and ganglion cell layer levels in rd retinas (Fig. 5E).

PTEN Expression

To explore the potential mechanisms by which Akt activation is dysregulated in rd retinas, expression of the tumor-suppressor phosphatase PTEN (named for its homology to phosphatases and tensin and the deletion of its gene on chromosome 10 in human cancers), which negatively regulates the PI3K/Akt pathway, was assessed by Western blot analysis and immunocytochemistry. Levels of PTEN were significantly higher in rd retinas at post-natal day 13 (3.3 times) and 14 (4.1 times; Fig. 6 A). PTEN immunoreactivity was mainly localized to the inner plexiform and ganglion cell layers at 13 days, and also to the photoreceptor outer segment and inner nuclear layers at 17 days in control retinas (Figs. 6B, 6E). The immunolabeling signal, localized at the photoreceptor outer segment, inner nuclear, inner plexiform, and ganglion cell layers, was stronger in 13-days-old rd retinas than in age-matched controls (Figs. 6B, 6C). By postnatal day 17, all remaining retinal layers were immunopositive in rd mice (Fig. 6E).

Src Kinase Expression

Because PTEN function can be inhibited by activated Src kinases, leading to alterations in signaling through the PI3K/Akt pathway, we examined the expression of Src-p60, one member of the Src family required for the activation of the PI3K-dependent survival pathway, by immunoblot analysis and immunocytochemistry in control and rd retinas. Whereas in both rd and control retinas there was a general developmental decline in Src-p60 expression, the levels of expression in rd retinas at postnatal day 15 were one third of those in the age-matched control (Fig. 7A). Immunocytochemistry showed Src-p60 localized mainly at the photoreceptor outer segment and inner nuclear layer, and diffusely in the outer nuclear and ganglion cell layers in control retinas at postnatal day 15 (Fig. 7B). A similar pattern was observed in rd retinas, except that no immunoreactivity was detected at the photoreceptor outer segments at the corresponding age (Fig. 7C). By 20 days, most of the immunolabeling was concentrated in the inner nuclear, inner plexiform, and ganglion cell layers in both strains (Figs. 7D, 7E).

DISCUSSION

The evidence from the present study, taken in conjunction with our previous findings, indicates that the network of
interactions at the molecular level in the rd mouse model of retinal degeneration comprises both the activation of death-promoting pathways and the suppression of the PI3K-Akt survival-promoting pathway. Akt plays a central role in cell survival and has been shown to block apoptosis. The ability of Akt to promote cell survival depends on its activation by PI3K. Once activated, Akt may phosphorylate and inactivate several proapoptotic proteins, including BAD, and the Forkhead family transcription factor FKHR, leading to inhibition of caspase-3 activation. Because photoreceptors have been shown to die by a caspase-dependent process in the rd mouse model, it was essential to ask if and how survival pathways might be implicated in these mechanisms.

We first evaluated the phosphorylation state of Akt at Ser-473, the site of phosphorylation by MAPKAPK-2, which has been shown to be critical for the generation of high levels of Akt enzyme activity. Several other kinases have also been reported to phosphorylate Akt at Ser-473, including integrin-linked kinase (ILK), 3-phosphoinositide-dependent kinase-1 (PDK-1), and DNA-dependent protein kinase (DNA-PK). Our data showed two distinct phases of Ser-473 Akt modulation during photoreceptor degeneration: the first one was characterized by the inactivation of Akt, and extended from early onset (13 days) to the peak of photoreceptor apoptosis (15 days), and the following one displayed a striking Akt activation during the period when most photoreceptors have degenerated. The fact that Akt activation levels were decreased and no immunoreactivity of the active form was detected at the photoreceptor level, strongly suggests that the Akt survival signaling pathway was inhibited during the induction of photoreceptor apoptosis in rd retinas. Because both caspase-independent and caspase-dependent apoptotic cell death have been shown to be invariably and causally correlated with the deactivation of the Akt neuronal survival pathway in various degenerative cellular models, our results indicate that Akt inhibition most probably contributes to photoreceptor cell death. There is evidence that Akt signaling may protect photoreceptor cells during normal developmental apoptotic cell death in the retina. However, in the present case of disease-related apoptosis, Akt is clearly not implicated in the protection of photoreceptor cells. Instead, Akt activation is most likely essential for the protection and survival of the remaining neurons of the inner layers of the residual rd retina, especially the ganglion cells, consistent with studies of cell survival in retinal ischemia. Akt phosphorylation is reduced at Ser-473, suggesting that at least one of the regulatory kinases is inactivated during the induction of photoreceptor apoptosis. Evaluation of the activation states of these kinases will be necessary to ascertain which one is implicated. Among these kinases, MAPKAPK-2 is activated by the p38 mitogen-activated protein kinase (p38-MAPK) cascade. The fact that Akt activation correlates with p38 MAPK activation after the peak of photoreceptor degeneration in the rd mouse model suggests that Akt inactivation most probably results from inhibition of the MAPK cascade during the induction phase of apoptosis. We investigated this hypothesis by evaluating the activation of the upstream kinases MKK3 and MKK6 which have been shown to activate specifically p38 MAPK (for review, see Refs. 6,46).
receptor cells suggested that the p38 MAPK inactivation is unlikely to be the result of the inactivation of these two kinases in the induction phase of apoptosis. Because mitogen-activated protein kinase phosphatases (MKPs) inactivate MAPKs by dephosphorylation (for review, see Ref. 47), and MKP-1 has been shown to regulate p38 MAPK negatively, it will be essential to ascertain whether MKP-1 participates in the inhibition of p38 MAPK during photoreceptor degeneration in the rd mouse model.

Furthermore, a signaling complex formed of Akt, p38 MAPK, MAPKAPK-2, and HSP27 has been reported. HSP27 has been shown to be necessary for Akt activation and may act as a scaffolding protein between Akt and MAPKAPK-2 proteins in this complex, facilitating activation of Akt by MAPKAPK-2. Absence of HSP27 and Akt immunoreactivities at the photoreceptor level supports the hypothesis that the Akt survival pathway is not activated in these cells during apoptosis.

An important mechanism of phospho-Akt’s cytoprotective effect is thought to come from its ability to inhibit BAD pro-apoptotic functions by phosphorylating BAD at Ser-136. In addition, BAD is phosphorylated at Ser-112 by several kinases, thereby blocking apoptosis. Therefore, despite evidence indicating activation of apoptotic pathways in the retinal ganglion cells in rd mice, the slight increase of phosphorylated Ser-112 BAD immunostaining in this layer in 15-day-old rd retina could contribute to the survival of these cells after photoreceptor degeneration. However, no quantitative differences in the total retinal level of phosphorylated BAD at either site of phosphorylation were observed at any time point during photoreceptor degeneration in rd in comparison with control animals. Assessment of quantitative BAD expression in the ganglion cell layer is needed, to ascertain whether BAD is implicated in the survival of these cells after photoreceptor apoptosis. In accordance with previous studies in c-fos knock-out mice, and in drug-induced cell death of retinal tissue, BAD is most probably not implicated in neuronal survival of the residual inner retinal cells, apart possibly from the ganglion cells, after photoreceptor degeneration.

Akt can also regulate the activity of other transcription factors that control cell death genes. Members of the Forkhead transcription factor family have been shown to be the target of Akt phosphorylation, suppressing the transcription of death genes such as the Fas ligand genes, and thereby promoting cell survival (for review, see Ref. 14). Strikingly, we found that activation of FKHR paralleled Akt activation, showing a biphasic reduction in activation during the induction phase of photoreceptor apoptosis followed by an increased activation after the peak of photoreceptor cell death. These results strongly indicate that FKHR is one of the Akt pathway’s targets implicated in the survival of the remaining neurons of the retina after the induction of photoreceptor disease-induced apoptosis.

Akt is indirectly negatively regulated by the tumor-suppressor phosphatase PTEN, which dephosphorylates PI(3,4,5)P3 (for review, see Ref. 54). During the induction phase of photoreceptor apoptosis, PTEN was increased especially in the degenerative cells, strongly suggesting that the Akt survival pathway was inhibited by PTEN in rd retinas. After the peak of photoreceptor cell death, PTEN expression decreased to control levels, and Akt activation increased in the remaining surviving retinal cells. Taken together, these results provide evidence that the inhibition of the Akt survival pathway by PTEN plays a role in photoreceptor cell death in the rd mouse model, consistent with the report that PTEN promotes apoptotic

**Figure 7.** Analysis of Src-p60 expression in the retina of control (C57BL/6) and rd mice by Western blot and immunocytochemistry. (A) Densitometric analysis of the ratio of Src-p60 to actin. Data are the mean ± SEM (n = 3). *Significant difference at 15 days: P = 0.03, unpaired t-test. Equal quantities of protein were loaded in all lanes, and representative exposures of enhanced chemiluminescence detection of the immunoreactive proteins are shown. Src-p60 immunostaining in control (B, D) and rd (C, E) mouse retina at postnatal days 15 (B, C), and 20 days (D, E). Arrowheads: indicate thickness of outer nuclear layer (E). Abbreviations are defined in Figure 1. Bar, 100 μm.
death in hippocampal neurons. PTEN has been shown to be inhibited by the Src family protein-tyrosine kinases. The low level of Src-p60 expression observed at the photoreceptor level during degeneration supports the hypothesis that PTEN is indeed activated and contributes to the inhibition of Akt survival pathways in rd retinas. Considering that Src-p60 is required for the activation of the PI3K-dependent survival pathway by glial cell line–derived neurotrophic factor (GDNF) family ligands, and that the neurotrophic factor neurturin is upregulated in rd retinas, it could be postulated that growth factor upregulation cannot counteract photoreceptor apoptosis due to the inhibition of the Akt survival pathway by PTEN in the rd mouse. Accordingly, The findings in the present study could help to explain the limited ability of growth factors to rescue photoreceptors from apoptosis in the rd mouse model.

In summary, our data show two successive modulation phases of the Akt survival pathway in rd retinas: inactivation, affecting mostly the apoptotic photoreceptor cells, and activation associated with the remaining retinal neurons after the peak of photoreceptor degeneration. The present study provides a framework with which to integrate the various signaling molecules implicated in the survival and apoptotic pathways, and to understand their relationship during photoreceptor cell death in the rd mouse model. Based on our previously published work and the present study, we propose that during the induction phase of apoptosis, Akt is inactivated by at least two processes (Fig. 8). First, Akt is not phosphorylated at Ser-473 by MAPKAPK-2 or other kinases. Because p38 MAPK is not activated, it cannot recruit MAPKAPK-2 to the complex formed from Akt, p38 MAPK, and HSP27. Although MKK3/6 is activated and can phosphorylate p38 MAPK, dephosphorylation of p38 MAPK most probably occurs due to MKP-1 activity. Akt does not modulate the proapoptotic or prosurvival functions of BAD in the apoptotic photoreceptor cells. Second, PTEN inhibits Akt activation by prohibiting PIP2-mediated activation of PDK1. PTEN remains active due to inactivation of its negative regulator Src-p60. The low levels of Src-p60 may also contribute to direct attenuation of PDK1 activity. These effects will result in inactivation of Akt by loss of phosphorylation at Thr-308 and Ser-473. The net output during apoptosis is inactivation of FKHR signaling and inhibition of the survival pathway. In conclusion, the Akt survival pathway is blocked at several levels during the induction of photoreceptor cell death. Therefore, photoreceptor degeneration appears to be the result of combined inactivation of the Akt survival pathway and activation of the two major apoptotic pathways in the rd model. Much remains to be clarified about the interconnection between these apoptotic and survival pathways and how therapeutic interventions in one or the other will affect photoreceptor degeneration.

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