Prosaposin Gene Expression in Normal and Dystrophic RCS Rat Retina

Loïc Van Den Berghe,1,2 Karine Sainton,1,2 Karin Gogat,1 Dominique Marchant,1 Eric Dufour,1 Sébastien Bonnel,1 Stéphanie Gadin,1 Maurice Menasche,1 and Marc Abitbol1

PURPOSE. To identify proteins secreted by the retinal pigment epithelium (RPE) and to analyze their cellular distribution in normal and pathologic rat retinas at various stages of eye development.

METHODS. A cDNA library was constructed with RNA isolated from porcine RPE sheets and screened by using the yeast signal sequence trap system. In situ hybridization, immunohistochemistry, and semiquantitative RT-PCR analysis were performed on rat retinas.

RESULTS. The cDNA encoding prosaposin was isolated. This is the first time this gene has been shown to be expressed in the retina. Prosaposin mRNA was detected in the rat RPE cell monolayer and in ganglion cells 14, 21, and 45 days after birth. The amount of prosaposin mRNA increased between days 14 and 45 after birth in normal retinas (rdy+/−), but not in the pathologic retinas (rdy+/−) of RCS rats.

CONCLUSIONS. Several techniques were used to determine the localization of prosaposin in rat retina. The increase in the amount of prosaposin mRNA in normal retinas coincided with the maturation of photoreceptor cells and the beginning of the phagocytosis process. In addition, the RCS rdyn RPE cells, characterized by the abrogation of the ingestion phase of the photoreceptor outer segments, are deficient in prosaposin expression. (Invest Ophthalmol Vis Sci. 2004;45:1297–1305) doi:10.1167/iovs.03-1048

The retinal pigment epithelium (RPE) is a neuroectoderm-derived cell monolayer located between the neuroretina and the choroid. The RPE helps to maintain homeostasis in the outer retina and assists the photoreceptors in phototransduction. RPE cells regulate the transport of retinoids and nutrients to the photoreceptors, the regeneration of the visual pigments, the phagocytosis and digestion of old rod outer segments, and the absorption of stray light and contribute to retinal adhesion. In addition, RPE cells are thought to play active roles in the immune response and in retinal wound healing. During the past decade, it has become clear that the critical role of RPE cells in maintaining visual functions depends on the production of many secreted proteins.1–7 For example, RPE cells produce interleukins (IL) such as IL-1α, -1β, -6, -8, and -15, which are involved in the regulation of ocular immunity and inflammation processes.7–8 The RPE also produces trophic agents, such as fibroblast growth factor 2 (FGF-2), pigmentation epithelium–derived factor (PEDF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin (NT)-3, which are involved in the development and functional maintenance of the neuroretina.6,9 The identification of several genes expressed by the RPE have greatly improved our understanding of the biology of the retina and vision; however, our knowledge is still incomplete.

RPE cells are also involved in several ocular diseases. Indeed, the RPE and the photoreceptors form a functional visual unit, meaning that the dysfunction of RPE cells can lead to photoreceptor degeneration. Mutations in at least nine genes expressed in RPE cells are associated with progressive photoreceptor degeneration (see RetNet,10,11). Mutations in the RPE65, RBP1, MERTK, LRAT, and RGR genes are associated with retinitis pigmentosa (for review, see Refs. 12,13). Mutations in the MVD2, TIMP3, MYO7A, and RDH5 genes are responsible for Best vitelliform macular dystrophy, Sorsby fundus dystrophy, Usher syndrome, and fundus albipunctatus, respectively. The MERTK (a tyrosine kinase receptor), TIMP3 (a tissue inhibitor of metalloproteinases), and RDH5 (11-cis retinol dehydrogenase) genes encode secreted proteins that carry a putative peptide signal sequence. Thus, the identification of new proteins secreted by the RPE cells may lead to the identification of additional genes involved in important retinal functions or retinal degenerative diseases.

In 1996, Klein et al.14 and Jacobs et al.15 described the “signal sequence trap” method, which can be used to identify genes encoding secreted proteins such as mitogenic, survival, or differentiation factors; neuropeptides; hormones; and membrane-bound proteins. This method is based on a single-step selection process in yeast.

To improve our understanding of the role of RPE cells in fundamental mechanisms of vision, we constructed a porcine RPE cDNA library and screened it using the signal sequence trap method to identify new proteins specifically secreted by the RPE. This led to the identification of a secreted protein, prosaposin, which is produced in the retina, mainly in RPE and ganglion cells.

Prosaposin is synthesized as a 53-kDa precursor protein that is posttranslationally modified to a 65-kDa form that is associated with the Golgi membranes and targeted to lysosomes. Proteolytic cleavage of prosaposin in the lysosome releases four smaller active peptides of between 8 and 11 kDa, known as saposin-A, -B, -C, and -D.16,17 The dissociated mature pro-
tease, cathepsin D, participates in the maturation of prosaposin.16 The saposins activate specific lysosomal hydrolases including cerubrosidas, ceramidas, sphingomyelinase, galactosidase and arylsulfatase A, which are necessary for the in vivo degradation of various glycosphingolipids, components of the plasma membrane.16,17,19,20–25 A significant portion of prosaposin is glycosylated, leading to a 70-kDa secreted form that is found in several extracellular fluids, such as cerebrospinal fluid, maternal milk, seminal plasma, and pancreatic secretions.24–28 and in the human29 and rat30 brain, where it is predominantly found in neurons.28 This secreted form can act as a neurotrophic, neuroprotective, reparative, and myelinothrophic factor.31–37 Prosaposin stimulates neurite outgrowth and prevents programmed cell death of a variety of neuronal cells.31,38,39 Moreover, prosaposin can protect neurons against ischemic damage.40,41 Direct application of prosaposin to transected sciatic nerves promotes nerve regeneration and/or prevents retrograde neuronal peripheral cell death after injury.32–34 These data suggest that prosaposin is an endogenous modulator of neuronal sprouting and regeneration. Prosaposin is also thought to have specific effects on the development, maintenance, and differentiation of the male reproductive organs and may play a role in lysosomal residual body degradation in Sertoli cells.32,43

The physiological in vivo importance of saposins has been demonstrated, thanks to the identification of several mutations in the saposin gene that are responsible for lysosomal disorders such as metachromatic leukodystrophy and Gaucher disease, both of which are characterized by the accumulation of undigested glycosphingolipids in cells.44–46 Furthermore, the total inactivation of the prosaposin precursor leads to death during fetal development or early childhood in humans.

We used in situ hybridization, immunohistochemistry, and RT-PCR to detect and to localize prosaposin mRNA and protein within the retina. We found that prosaposin mRNA and protein are mainly present in the RPE layer and that the amounts present differ between normal (RCS red) and dystrophic (RCS red–) rat retinas.

Materials and Methods

Animals

All animals were handled in strict accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. The animals were kept at 21°C with a 12-hour light (100 lux)–dark cycle and fed ad libitum. Normal pigmented (p–) and unpigmented (p+) Royal College of Surgeons (RCS) rats with RCS red+ for retinal dystrophy) and without (RCS red–) retinal dystrophy were kindly provided by Matthew M. LaVail (University of California San Francisco). Adult Wistar rats were obtained from Iffa Credo (L’Arbresle, France) and were used between the ages of 12 and 14 weeks.

Construction of the Porcine RPE cDNA Library

Pigs obtained from the SOCOPA slaughterhouse (Evron, France) were killed by electrocution and the eyes enucleated. The anterior parts of the eyeballs were discarded. After removal of neural retina tissues, RPE cells were scraped from the posterior eye cups into a lysis buffer from an RNA extraction kit (RLE buffer; RNeasy RNA; Qiagen, Valencia, CA), which was used to purify total RNA. mRNA was then extracted by use of a kit (Oligofect; Qiagen), according to the manufacturer’s instructions. Samples were treated with DNase I to remove genomic DNA before the washing steps. PolyA+ RNA (1 μg) was used to synthesize cDNA (SuperScript Choice System; Invitrogen-Gibco, Grand Island, NY). For the reverse transcription step, the degenerate primer 5’-CGATTGAATTCCTGGACTCCTGGACTGNNNNNNNN-3’ (where N denotes G, A, T, or C and the Xbol restriction enzyme site is italic) was used. The cDNAs obtained after second-strand synthesis were ligated to EcoR I adapters and digested with Xbol. Products of length between 300 and 900 bp were selected. The double-stranded cDNAs were then ligated into the EcoR I and Xbol restriction enzyme sites of the pUC21U7M13ORI vector2 to be fused to the invertase gene lacking its signal sequence. E. coli DH10B cells (ElectroMax; Invitrogen-Gibco) were transformed with the resultant cDNA library.

Semiquantitative RT-PCR

Total RNA was extracted from rat RPE cells and cerebellum at different postnatal (P) ages (P14, P21, and P45) with extraction reagent (TRIZol; Invitrogen-Gibco) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed by using an oligodT primer with reverse transcriptase (SuperScript II; Invitrogen-Gibco), according to the manufacturer’s instructions. For semiquantitative PCR, the number of cycles, amount of cDNA, and annealing temperature were optimized (data not shown). Cyclophilin was coamplified with the target gene as an internal control for comparative purposes. PCRs were conducted in a 20-μL volume containing 1 μL cDNA, 1 μL dimethyl sulfoxide (DMSO), 1 μL 10× PCR buffer (Promega, Madison, WI), 50 pM each 5’ and 3’ prosaposin primer, 25 pM each 5’ and 3’ cyclophilin primer, 0.2 mM dNTP, 1.5 mM MgCl2, and 0.5 U Tag DNA polymerase. The initial denaturation step at 92°C for 2 minutes was followed by 25 cycles of 15 seconds at 92°C, 1 minute at 55°C, and 1 minute 30 seconds at 72°C. The prosaposin primers (5’-TCAAGGACGCGGTTTGAC-3’ and 5’-CGGGTTGGCAGAACAGAG-3’) amplified an 858-bp product, and the cyclophilin primers (5’-TGTGCACCCCCAGCGTGTCCCTG-3’ and 5’-TCCAGCATTGCCCCATGGGACAAGAG-3’) amplified a 311-bp product.

Signal Sequence Trap Screening

The yeast signal sequence trap screening method was performed as previously described.15

Plasmid Construction

To construct the plasmid NA-PROSAP, the full-length prosaposin cDNA was isolated by RT-PCR (Superscript kit; Invitrogen-Gibco) from total rat RPE RNA. The primers used were PROSAP5 (5’-AAAAAGAATTCAGTATGCTCTCCTCCTCT-3’, where the EcoR I restriction enzyme site is in bold and the ATG start codon is in italic) and PROSAP3 (5’-TTTCTGGAGCTAGTCTCCACATGGGCTT-3’ where the Xbol restriction enzyme site is in bold and the TAG stop codon is in italic). The 1684-bp PCR product was subcloned into the EcoR I restriction enzyme sites of pCDNA3 (Invitrogen, San Diego, CA).

In Situ Hybridization

After CO2 asphyxiation, eyes were enucleated from rats at different postnatal ages (P14, P21, and P45) and immediately fixed in 4% paraformaldehyde for 3 hours at 4°C before being embedded in paraffin. Five-micrometer sections were cut and mounted on precoated slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA). Sense and antisense riboprobes were synthesized from the pCDNA-PROSAP vector linearized with Bgl II. The probes were labeled using the digoxigenin (DIG) RNA labeling kit (Roche Diagnostics, Indianapolis, IN). The transcription reaction was performed with SP6 (antisense) and T7 (sense) RNA polymerases according to the manufacturer’s instructions. Sections were deparaffinized and rehydrated, and in situ hybridization was performed as previously described,15 except that sections were treated for 10 minutes with 0.2 M HCl before proteinase K digestion. The prehybridization and the hybridization steps were performed at 65°C. We used 15 μL of DIG-labeled antisense or sense probes for 150 μL of hybridization buffer.

Immunohistochemistry

Saposin was detected by incubating 5-μm paraffin-embedded retina sections from red+ and red– RCS rats with the SGP-1 antibody.24 A kit (ChemMate peroxidase/DAB Rabbit/Mouse detection kit; Dako

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/932924 on 06/22/2017
The intensity of labeling in the RPE cells increased considerably after birth and the loss of vision.\(^5\) The prosaposin protein is one of the shed photoreceptor outer segments (POS) by the RPE. In normal animals, POS fragments accumulate, leading to the degeneration of photoreceptor cells between 18 days and 3 months postnatal stage.\(^5\) As expected, no specific labeling was detected in sections hybridized with the prosaposin sense probe (Fig. 2B, for example).

At P45 and P60, the distribution of prosaposin mRNA was similar to that observed at P21 (data not shown). To confirm that the signals detected are not specific to the RCS strain, we repeated these experiments on retina sections from Wistar rats. The distribution of prosaposin mRNA in the Wistar rat retina was generally similar to that observed in the RCS rat retina, with very large amounts of prosaposin in the cytoplasm of RPE cells (Figs. 4A, 4B). Again, no specific labeling was detected in sections hybridized with the prosaposin sense probe (Fig. 4C).

These results indicate that the pigment epithelial cell layers and the ganglion cells of \(rd^y\) and Wistar rats at different developmental stages contain large amounts of prosaposin mRNA.

**Prosaposin Protein Expression**

To confirm the in situ hybridization results, we used immunohistochemistry to determine the prosaposin protein expression pattern in retinal sections from normal unpigmented RCS \(rd^y\) rats at P45, by using the SGP-1 antibody. Prosaposin immunostaining was detected in the cytoplasm of ganglion (Fig. 5C) and RPE (Fig. 5B) cells. In the RPE, the prosaposin immunostaining seemed to be concentrated on the apical side that faces the photoreceptor cells. Weak staining was detected in the outer segment layer, near the RPE cells (Fig. 5B, arrowheads). A very weak prosaposin signal was also observed in the inner nuclear layer (Fig. 5A). No signal was observed with nonimmune serum, confirming that the immunostaining was specific to prosaposin (Fig. 5D).

The same analysis was performed on retinal sections from unpigmented dystrophic RCS \(rd^y\) rats, the first spontaneous animal model of inherited retinal pigment epithelium defect to be described.\(^5\) This is one of the major animal models for human retinitis pigmentosa. In this rat strain, the phagocytosis of shed photoreceptor outer segments (POS) by the RPE is defective because of a deletion in the \(c\)-Mer gene.\(^5\)\(^,\)\(^6\) Consequently, POS fragments accumulate, leading to the degeneration of photoreceptor cells between 18 days and 3 months after birth and the loss of vision.\(^5\) The prosaposin protein expression pattern was similar to that observed in the retinas of normal \(rd^y\) rats (Fig. 5E). Although the immunohistochemical analysis is not quantitative, it appeared that the staining was lighter in the RCS \(rd^y\) retina than in the \(rd^y\) rat retina. Moreover, the prosaposin immunostaining was stronger in the outer segment layer, near the RPE cells, of the RCS \(rd^y\) rat.
retina than in the equivalent layer of the rdy<sup>−/−</sup> rat retina (compare Fig. 5E with Fig. 5B).

These data confirm the results of the in situ hybridization analysis and demonstrate that prosaposin is principally localized in the RPE and ganglion cells of the rat retina.

**Differential Prosaposin mRNA Analysis in Normal and Dystrophic RCS Rat Retina**

We used semiquantitative RT-PCR to compare the amounts of prosaposin mRNA in RPE cells of normal rdy<sup>−/−</sup> and pathologic rdy<sup>−/−</sup> RCS rats at various developmental ages (Fig. 6A). As a control, the same experiment was performed on mRNA extracted from the cerebellums of the two rat strains at the same ages (Fig. 6B). Cyclophilin mRNA was used as an internal control. As expected, an 818-bp band corresponding to prosaposin mRNA was observed in all samples tested (Fig. 6). In the normal rdy<sup>−/−</sup> retina, the amount of prosaposin mRNA in pigment epithelial cells was clearly higher at P21 and P45 than at P14 (Fig. 6A). Thus, in normal physiological conditions, the prosaposin mRNA levels appear to increase with age. In contrast, in RPE cells from the dystrophic RCS rdy<sup>−/−</sup> rat strain, the prosaposin mRNA level remained constant over time (Fig. 6A). The amounts of prosaposin mRNA in the control cerebellums of the two strains remained stable over time (Fig. 6B).

**DISCUSSION**

We used the yeast sequence signal trap method to identify proteins secreted by RPE cells. This system allowed us to select the cDNA encoding for prosaposin. Using in situ hybridization, RT-PCR, and immunohistochemistry on the whole retina, we confirmed that prosaposin is present in RPE cells and, to a lesser extent, in ganglion cells. Prosaposin mRNA was also detected in the INL at P14 and in the INL and ONL at P21. At later stages (P45 and P60), the pattern was identical with that observed at P21. The distribution of prosaposin mRNA in the retina was essentially the same in the two rat strains studied.
This is the first time that the distribution of prosaposin in the retina has been reported. Our semiquantitative RT-PCR analysis demonstrated that the amount of prosaposin in the RPE of normal rdy+/H11001 rats progressively increases with age. This is specific to RPE cells and was not observed in the cerebellums of the two rat strains (Fig. 6).

In situ hybridization also showed more intense labeling at P21 than at P14 (compare Figs. 3D and 2E). This increase in the
amount of prosaposin mRNA in normal RPE cells coincided with the maturation of the photoreceptor cells, especially the elongation of the POS and the beginning of the phagocytosis process. In contrast, in the pathologic RCS 
rdy- rat strain, which is characterized by the abrogation of the ingestion phase of POS, the amount of prosaposin mRNA remained the same between P14 and P45 (Fig. 6A). These data are also consistent with the results of differential display analysis, which showed that the pathologic RCS 
rdy- RPE contains less prosaposin mRNA than the 
rdy+ RPE at P14 and P21.62 These results suggest that the RPE cells of adult RCS 
rdy- rats present a deficiency in prosaposin expression and that this deficiency is closely associated with the RCS rat disease.

The retinal pigment epithelium produces large amounts of many cysteine proteases such as cathepsin S and D, which are the main proteases involved in the proteolytic processing of diurnally shed POS.63-65 We hypothesize that prosaposin, which activates the hydrolysis of sphingolipids by lysosomal hydrolases, plays a complementary role in photoreceptor outer segment degradation. This is supported by the fact that, as with cystatin C, which is a regulator of the cathepsin activities, the increase in prosaposin mRNA concentration in the RPE cells coincides with the opening of the eyelids in rat pups (P14),66 which is associated with an increase in phagocytic activity in the RPE cells.

Mice with targeted disruptions of the genes encoding the tyrosine kinase receptors Tyro3, Axl, and Mer are blind due to the postnatal degeneration of rods and cones. The retinal phenotype of the mer knockout mice has been reported recently.68 It is morphologically and functionally identical with the phenotype of RCS retinal degeneration.57,58 A potential role for prosaposin in the c-Mer signaling pathways remains to be investigated.

Mutations in the prosaposin precursor-encoding gene result in phenotypes that are similar to metachromatic leukodystro-
phy (MLD) or variants of Gaucher disease.44–51,69–72 Saposin-deficient patients have various ophthalmic disorders. Some patients with sap-B deficiencies have pallid optic disks. The eyes of adult sap-B-deficient patients sometimes display a macular grayness, consistent with an RPE abnormality. Some sap-C-deficient patients have irregularly pigmented retinas. The first patients to be described with prosaposin precursor deficiencies consistently display precocious optic nerve atrophy.17 Thus, there is clearly a link between the ophthalmic clinical manifestations of saposins and prosaposin deficiencies and the retinal sites found to express the prosaposin gene in our study: RPE and ganglion cells. Prosaposin and/or saposin deficiencies may alter the function of these cells, thus resulting in retinal pigment alterations and optic nerve atrophy. Moreover, some sap-C-deficient patients store massive amounts of lipids, including lipofuscin granules intraneuronally.73 These observations suggest that prosaposin is a potential candidate gene for ocular diseases.

Prosaposin, a neurotrophic factor and a lysosomal hydrolase activator, may be a new therapeutic agent for the treatment of ocular diseases associated with alterations of the RPE phagocytic process or with some inherited diseases characterized by neuroretinal degeneration.

Acknowledgments

The authors thank Jean-Jacques Frayssinet, President, Retina France; Patrick Berche, Dean of the Necker Medical School; and Professor Philippe Even, President of Necker Institute, for their continuous support. The authors also thank Michael Griswold, Dean of Washington State University College of Sciences, for generously providing the antibody recognizing rat prosaposin.

References

10. Daiger SP, Sullivan LS, Bowne SJ. Cloned and/or mapped genes causing retinal diseases. RetNet is available at http://www.sph.uth.tmc.edu/RetNet/ provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX.


52. Hukova H, Cervenkovska M, Ledvinova J, et al. A novel mutation in the coding region of the prosaposin gene leads to a complete deficiency of prosaposin and saposins, and is associated with a


