The Distribution of Retinol-Binding Protein and Its mRNA in the Rat Eye

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Although a constant supply of retinol is a critical requirement for the visual cycle, the molecular mechanisms underlying retinol delivery, uptake, storage, and transport in the eye are not well understood. Previously the synthesis of serum retinol-binding protein (RBP) in the mammalian eye was reported. Now the distribution of RBP and RBP mRNA in the rat eye has been studied by immunohistochemical and in situ hybridization techniques. The RBP mRNA was present only in the cytoplasm of retinal pigment epithelial (RPE) cells, terminating abruptly at the pars plana. On the other hand, RBP immunoreactivity was more widespread. The most intense immunostaining was present in retinal ganglion cells, the corneal endothelium, and under certain conditions of tissue fixation, the corneal epithelium. Consistent but less intense immunoreactivity was detected in the photoreceptors, Müller cells, inner plexiform layer, ciliary epithelium and stroma, iris epithelium, retinal pigment epithelium, lacrimal glandular epithelium, and periorbital soft tissues. These findings suggest that RBP synthesized by the RPE may be secreted to various ocular locations. However, at present, uptake from plasma cannot be excluded as another possible source of ocular RBP.

In the plasma, holo-RBP (the retinol–RBP complex) is transported in complex with another plasma protein, transthyretin (TTR). This substance is also synthesized by the RPE and its distribution in the eye is similar to that described for RBP. Taken together, these findings support the proposal that ocular RBP and TTR may function cooperatively in the intraocular translocation of retinol. Invest Ophthalmol Vis Sci 32:302–309, 1991

Retinol-binding protein (RBP) is a 21-kD polypeptide which plays an important role in the plasma transport of retinol (vitamin A). Plasma RBP is synthesized by liver hepatocytes; other extrahepatic sites of RBP synthesis include the yolk sac endoderm, the straight segment of the proximal renal tubule, and perinephric adipose tissue. Although the physiologic role of RBP in the latter locations has not yet been determined, it is possible that extrahepatic RBP functions in the storage and local transport of tissue retinol.

The retinol–RBP complex, holo-RBP, is secreted from the liver into the blood, where it is complexed with another plasma protein, transthyretin (TTR, prealbumin), for transport to vitamin A-requiring tissues. This substance is synthesized in the liver, yolk sac endoderm, and choroid plexus epithelium. In addition, we showed abundant TTR synthesis in the retinal pigment epithelium (RPE) of the rat eye. Considering the role of the RPE in ocular retinoid metabolism, we suggested that RPE TTR may function in the intraocular cycling of retinol. If ocular TTR plays a role in the local translocation of retinol, this suggests, by analogy with plasma TTR, the concomitant presence in the eye of RBP. However, in the rat, plasma RBP does not cross the blood–retina barrier. Recently we presented evidence, obtained from northern analysis and immunoblotting, for de novo synthesis of RBP in the eye. We now describe the distribution of RBP and its mRNA in the rat eye. Although RBP mRNA is localized only in the RPE, RBP immunoreactivity in the rat eye is more widespread.

Materials and Methods

Materials

Rabbit polyclonal antiserum to rat RBP was generously provided by D. S. Goodman (Dept. of Internal Medicine, Columbia University, New York). Rabbit polyclonal antiserum to rat RBP was generously provided by D. S. Goodman (Dept. of Internal Medicine, Columbia University, New York).
ylated peroxidase were from Vector (Burlingame, CA). A partial-length cDNA to rat RBP subcloned into plasmid pGEM4 was generously provided by D. R. Soprano and D. S. Goodman. Restriction enzymes were obtained from New England Biolabs (Beverly, MA), placental RNase inhibitor (RNasin), SP6 and T7 polymerase from Promega Biotech (Madison, WI), (alpha-32P-thio)-uridine triphosphate (35S-UTP) from New England Nuclear (Boston, MA), OTC embedding compound from Miles Laboratories (Elkhart, IN), M1 embedding compound from Lipshaw (Detroit, MI), NTB2 nuclear track emulsion, D19 developer, fixer, and XAR x-ray film from Kodak (Rochester, NY), ultrapure DNase from Cooper Biomedical (Malvern, PA), ultrapure sucrose from Boehringer, and all other reagents from Sigma (St. Louis, MO).

Tissue Preparation

Animal experimentation was performed in accordance with the principles of the ARVO Resolution on the Use of Animals in Research.

Adult male Sprague-Dawley albino rats were killed by intraperitoneal injection of a lethal dose of pentobarbital, rapidly thoracotomized, and the systemic circulation perfused by intraaortic administration of 4% paraformaldehyde in sterile phosphate-buffered saline (PBS), pH 7.4, at 4°C for 20 min, with exit through the right atrium. Whole eyes and livers were removed and immersed either in the same fixative or through the right atrium. Whole eyes and livers were removed and immersed either in the same fixative or in Bouin’s fixative at 4°C overnight.

For in situ hybridization studies and for some immunohistochemical studies, frozen sections were prepared by immersing paraformaldehyde-fixed tissue in 15% sucrose solution in sterile PBS for 24 hr. Tissue specimens were then embedded in OTC or M1 compound in plastic boats and frozen rapidly in isopentane. Tissue blocks were sectioned at 6–8 μm on a cryostat at −20°C and mounted on polylysine-coated glass slides. The slides were stored at −70°C in the presence of a dessicant until use.

In addition immunohistochemistry was done on paraformaldehyde- or Bouin’s-fixed tissue which was dehydrated through alcohols and xylene, embedded in paraffin, sectioned at 8 μm on a microtome, and mounted on gelatin-coated slides.

Immunohistochemistry

The antiserum used in this study was previously characterized by western blotting and its specificity reported. In total rat eye homogenates, this antiserum recognized a single protein band which comigrated at 21 kD with purified rat RBP. No cross-reactivity with other ocular proteins was detected.

Immunohistochemistry was done by a previously described modification of the avidin-biotin-peroxidase complex technique. Optimal dilution for the primary antiserum was determined as 1:1000. Each experiment included, as controls, adjacent sections in which nonimmune serum was substituted for the primary antiserum and additional section in which primary antiserum was omitted from the first incubation. In each case, these procedures entirely eliminated staining. Liver sections served as positive controls.

Preparation of Probes

One microgram of plasmid DNA was linearized with BamHI and transcribed according to the manufacturer’s specifications in a 10-μl reaction containing 25 μM 35S-UTP, 500 μM each of guanosine triphosphate, cytidine triphosphate, and adenosine triphosphate, 20 units of RNasin, and 20 units of either SP6 or T7 polymerase to a specific activity of approximately 108 cpm/μg DNA template. After transcription, template DNA was removed by treatment with 20 μg of ultrapure DNase. The 35S-labeled cRNA transcripts were purified on a Sephadex G-100 column and stored under ethanol.

In Situ Hybridization

In situ hybridization was done as described previously. Briefly, sections underwent further fixation by exposure to formaldehyde vapors for 30 min, and then they were prehybridized with a solution containing 50% formamide, 10% dextran sulfate, 600 mM NaCl, 10 mM Tris (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 0.12% bovine serum albumin, 0.5% salmon sperm DNA, 0.5 mg/ml total yeast RNA, and 50 μg/ml yeast tRNA at 50°C for 2 hr. The 35S-labeled cRNA probe was added to the same solution containing 10 mM dithiothreitol to a final activity of 3 × 106 cpm per ml (HB solution). After prehybridization, sections were overlaid with 100 μl of HB solution and hybridized overnight at 50°C. The following day sections were washed in 2× SSC (standard saline citrate; SSC = 0.15 M NaCl + 0.015 M Na citrate), treated with 30 μg/ml RNase A at 37°C for 45 min, and then washed for 60 min in 0.5× SSC at 50°C, followed by 3 hr at 50°C in 0.1× SSC containing 0.05% sodium pyrophosphate and 14 mM 2-mercaptoethanol. Sections were then dehydrated, vacuum-dried in a dessicator, exposed to x-ray film for 4–7 days, dipped in NTB2 emulsion, and stored in the dark for 3–4 weeks. After development in D19 developer, slides were counterstained with hematoxylin and eosin, mounted, and viewed under combined bright-field/epiluminescent illumination on a Leitz Orthoplan microscope.
Fig. 1. RBP immunoreactivity in rat eye. Primary antiserum diluted 1:1000. (A) Cornea. Paraformaldehyde fixation, frozen section. No counterstain. en, endothelium; s, stroma; ep, epithelium (original magnification ×275). (B) Cornea. Bouin’s fixation, paraffin section. No counterstain (orig. mag. ×275). (C) Iris. Paraformaldehyde fixation, frozen section. No counterstain. Note intense immunoreactivity of epithelial surface (arrow) adjacent to cornea (c) relative to that adjacent to lens (l) (orig. mag. ×110). (D) Ciliary body. Bouin’s fixation, paraffin section. No counterstain. Note epithelial staining (orig. mag. ×165).
Results

Immunohistochemistry

In general, staining of frozen sections was considerably more intense than that of paraffin-embedded sections. In sections prepared by either technique, prominent staining was detected in the corneal endothelium (Figs. 1A-B) and the retinal ganglion cells (Figs. 2A, 4A). Moderately intense immunostaining was present in the inner plexiform layer of the retina (Fig. 2A), the Müller cells (Fig. 2B) at the junction of the outer nuclear and outer plexiform layers, the photoreceptor layer (Figs. 2A–B), RPE (Fig. 2A), ciliary epithelium (Fig. 1D), iris epithelium (Fig. 1C), and lacrimal glandular epithelium (not shown). Weak staining was present in the sclera, choriocapillaris, and extraocular muscles (Fig. 2A). The corneal stroma was consistently unstained; however, the corneal epithelium stained intensely in paraffin-embedded sections (Fig. 1B) but not in frozen sections (Fig. 1A).

In Situ Hybridization

Multiple sections were examined from two rat eyes sectioned in coronal and transverse planes. Liver sec-

Fig. 2. RBP immunoreactivity in rat retina. Paraformaldehyde fixation, frozen section. Primary antiserum diluted 1:1000. No counterstain. cc, choriocapillaris; gc, ganglion cell layer; ip, inner plexiform layer; in, inner nuclear layer; is, photoreceptor inner segments; on, outer nuclear layer; op, outer plexiform layer; os, photoreceptor outer segments; pr, photoreceptor layer; pe, retinal pigment epithelium. (A) Low-power montage of whole retina (original magnification X157). (B) High-power view to demonstrate inner segments of photoreceptors and Müller cells and their processes (orig. mag. X940).
Fig. 3. In situ hybridization demonstrating RBP mRNA in rat eye. Hematoxylin and eosin counterstain. Abbreviations as in Figure 2. (A) Outer retina. Anti-message sense cRNA probe. Exposure time 21 days. Note concentration of silver grains over pigment epithelium (pe) (original magnification ×514). (B) Retina, full thickness. Control section using message sense cRNA probe. Note absence of silver grains (orig. mag. ×180). (C) Cornea. Anti-message sense probe. Same section as in (A). Note absence of epithelial (ep) or endothelial (en) silver grains (orig. mag. ×342).
tions served as positive controls. In each case, immediately adjacent sections were hybridized with probes prepared in the message- and antimesseage-sense orientation.

After exposure and development, specific hybridization was detected microscopically in sections hybridized with the antimesseage-sense probe. Silver grains in hybridizing sections were distributed uniformly, albeit at relatively low abundance, in the cytoplasm of the RPE cells (Fig. 3A). Silver-grain density was highest at the equator and diminished toward the pars plana. Control sections hybridized with the massage-sense probe revealed no hybridization signal above background (Fig. 3B). In hybridizing sections, no silver grains were detected over the ciliary or iris epithelium or in any other ocular structure, including the lens, cornea (Fig. 3C), neurosensory retina (Fig. 4B), sclera, choriocapillaris, ciliary body, optic nerve, or lacrimal gland.

Discussion

This study extends previous observations concerning the synthesis and localization in the eye of two plasma proteins involved in retinol transport, RBP and TTR. The former is the principle plasma transport protein for retinol. Holo-RBP circulates in the plasma in a 1:1 molar complex with TTR and delivers retinol to vitamin A-requiring tissues. Retinol may be delivered to the eye by holo-RBP through putative receptors on RPE cell membranes, but neither RBP nor TTR crosses the blood-retina barrier.

Previously, we reported RBP mRNA and protein in homogenates of rat and bovine eyes. We now have established that the RPE is the unique site of localization of RBP mRNA in the rat eye, and RBP immunoreactivity in the eye is widespread. These findings suggest that RBP may be synthesized in the RPE for export to these various ocular locations. Although consistently present, RBP immunostaining in the RPE was never intense, suggesting that this export may occur rapidly. However, while RBP reportedly does not cross the choriocapillaris into the retina, the possibility of another anterior source of ocular RBP cannot be excluded. Further studies will be necessary to determine what proportion of ocular RBP is derived from the RPE and whether ocular RBP may also be derived from serum by uptake from plasma filtrates in aqueous, vitreous, or tears.

The distribution of RBP and its mRNA reported here parallels to a large extent that reported previously by us for TTR. This substance is also synthesized only in the RPE, and TTR immunoreactivity is present in many of the same structures as RBP.

![Fig. 4. Comparison of immunohistochemical (A) and in situ hybridization (B) studies of the outer retina. (A) is the same section as in Figure 2A (no counterstain). (B) is the same section as in Figure 3A (H&E counterstain), in which RBP mRNA was present in the RPE. Note absence of mRNA from retinal layers which are rich in RBP: ganglion cell layer (gc); Müller cells in the outer plexiform layer (op); and photoreceptor (pr) inner segments (original magnifications ×382).](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933159/)
such as the retinal ganglion cells, the inner plexiform layer, the corneal endothelium, and the lacrimal, ciliary, and iris epithelia. We postulated that ocular TTR may play a role in the transport of retinol between the RPE and the retina or other ocular structures. If true, this would necessitate, by analogy with the plasma, the concomitant presence of RBP in these structures. The current report confirms that prediction.

Colocalization of RBP and TTR in the eye provides strong circumstantial evidence that RBP and TTR may function cooperatively in the intraocular translocation of retinol. In the retina, this may represent one mechanism by which retinol is supplied to the photoreceptors for use in the visual cycle. In other ocular locations, however, retinol probably plays a different role, possibly in cellular development and differentiation.

The major difference between the distribution of immunoreactivity reported previously for TTR and that reported here for RBP is in the pattern of staining seen in the cornea. Whereas TTR immunoreactivity was detected only in the corneal endothelium, intense RBP immunostaining was detected in both the corneal endothelium and, under certain conditions, the corneal epithelium. Epithelial staining was dependent on the method of tissue preparation; immunoreactivity was lost from the epithelia of frozen sections, suggesting that RBP in the corneal epithelium may be present in a different physicochemical state from RBP elsewhere in the eye. A similar phenomenon, albeit involving different ocular structures, was reported by us with respect to TTR immunoreactivity.

The mechanisms underlying ocular retinol transport and storage are still poorly understood. Several retinoid-binding proteins have been described in the eye, all of which are biochemically distinct from RBP. Cellular RBP (CRBP) binds all-trans retinol and is found in both RPE and retina.

Cellular retinoic acid-binding protein is found in the retina. Cellular retinaldehyde-binding protein occurs in the same locations as CRBP and binds 11-cis retinal and 11-cis retinol. Interphotoreceptor retinoid-binding protein is synthesized by the retina and secreted into the interphotoreceptor matrix, and its contribution to the choroidal plexus of rat brain. Purpurin is a retinal-adhesion molecule synthesized by the photoreceptors which is capable of binding hydrophobic molecules including retinol.

The RPE is the primary site of pathology in some hereditary retinal dystrophies in animals and possibly also in humans. An abnormality of retinoid metabolism, as yet undefined, has been suspected in the pathogenesis of retinitis pigmentosa (RP). However, plasma RBP levels in some forms of RP have been reported as normal. Our demonstration of a separate source of RBP synthesis within the ocular barrier suggests that plasma RBP levels may not reflect ocular RBP levels accurately. By virtue of their ocular locations and known plasma functions, both RBP and TTR appear to be promising candidate proteins for study in the pigmentary retinopathies.

**Key words:** retinol-binding protein, retinal pigment epithelium, in situ hybridization, retinol transport, transthyretin

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**References**


