Synthesis and Secretion of Retinol-Binding Protein and Transthyretin by Cultured Retinal Pigment Epithelium†

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ABSTRACT: Recent studies indicate that the retinal pigment epithelium (RPE) may serve as an extrahepatic source of retinol-binding protein (RBP) and transthyretin (TTR) for the retina by virtue of the fact that this cell layer is the exclusive retinal location for mRNA coding for these proteins [Herbert, J., et al. (1991) *Invest. Ophthalmol. Vis. Sci. 32*, 302–309; Cavallaro, T., et al. (1990) *Invest. Ophthalmol. Vis. Sci. 31*, 497–501], although the proteins themselves are present in a variety of retinal neurons. It is therefore necessary to determine whether these mRNAs are translated and whether their translated products are secreted like hepatic RBP and TTR. Metabolic labeling of cultured bovine RPE with [^{35}S]cysteine and [^{35}S]methionine and subsequent analysis of newly synthesized proteins in the conditioned medium by affinity chromatography, gel filtration, partial amino acid sequence analysis, and autoradiography of electrophoretograms indicate that both RBP and TTR are synthesized and secreted by the RPE. Moreover, for cells grown in chambers with permeable supports, the predominant direction for secretion was into the apical medium. The mean apical:basal ratio after 72 h of incubation was 9.2 for TTR and 4.5 for RBP. A function for these proteins in the neurosensory retina remains speculative. They could be involved in the delivery of *all-trans*-retinol to amacrine and Müller cells as a precursor for retinoic acid, since these cells are known to contain cellular retinoic acid binding protein [Gaur, V. P., et al. (1990) *Exp. Eye Res. 50*, 505–511; Milam et al. (1990) *J. Comp. Neurol. 296*, 123–129]. A role in the visual cycle remains a possibility, although the evidence is stronger that this function may be fulfilled by interphotoreceptor retinoid-binding protein (IRBP). Finally, since these proteins have been localized by immunocytochemistry to several neuronal cell types in the retina, they may play a role in cell differentiation.

Vitamin A (retinol) and its derivatives (retinoids) are fat-soluble compounds that require binding proteins for their solubility in aqueous environments (Bridges, 1984; Blomhoff et al., 1990). Within the circulating blood plasma, retinol is bound to retinol-binding protein (RBP),[1] which, in its holo form, is bound tightly to a tetrameric thyroxine-binding protein called transthyretin (TTR). These proteins are synthesized in the liver (Goodman, 1984; Fielding & Fex, 1982) and at several extrahepatic sites. The choroid plexus epithelium (Soprano et al., 1985; Dickson et al., 1985), visceral yolk sac (Soprano et al., 1986), and the retinal pigment epithelium (RPE) of the eye (Cavallaro et al., 1990) are additional sites of mRNA expression and protein distribution for TTR, whereas extrahepatic sites of expression for RBP mRNA expression and protein distribution include the yolk sac endoderm (Soprano et al., 1986; Sklan & Ross, 1987) the straight segment of the proximal renal tubule (Makover et al., 1989), perinephric adipose tissue (Makover et al., 1989), and the RPE (Herbert et al., 1991).

The discovery of TTR and RBP in the RPE of the eye is quite recent. Although both proteins are present in a variety of ocular cell types, *in situ* hybridization studies suggest that their mRNAs are found only in the RPE (Cavallaro et al., 1990; Herbert et al., 1991), suggesting that it is the unique ocular site for synthesis of these proteins, at least within the limits of sensitivity for this method.

Previous studies have suggested that neither RBP (Bok & Heller, 1976) nor TTR (Pino, 1986) is capable of crossing the blood–retinal barriers which are located at the level of tight junction between retinal vascular endothelium and the RPE (Peyman & Bok, 1972). RBP is reputed to deliver its cargo of *all-trans*-retinol to the retina via membrane receptors on the basolateral plasmalemma of the RPE (Heller & Bok, 1976; Bok & Heller, 1976; Båvick et al., 1991, 1993). High-resolution autoradiographic studies indicate that RBP does not enter the cell (Bok & Heller, 1976). Based on these observations, there was no apparent need for the presence of RBP in the neurosensory retina, particularly in view of the fact that another retinoid binding protein in the interphotoreceptor matrix, interphotoreceptor retinoid-binding protein (IRBP), is thought to subserve the shuttling of retinoids back and forth between RPE and photoreceptors during the visual cycle (Lai et al., 1982; Liou et al., 1982), which involves bleaching and regeneration of the photopigments (Dowling, 1960). However, the recent discovery of TTR and RBP mRNAs in the RPE and the fact that these are secreted proteins in nonocular tissues require further analysis of this issue for the RPE (Cavallaro et al., 1990; Herbert et al., 1991). It has recently been reported that cultured testicular Sertoli cells secrete RBP into their culture medium (Davis & Ong, 1992). Secretion of TTR was not examined in that study. We

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[1] Abbreviations: RPE, retinal pigment epithelium; IPM, interphotoreceptor matrix; TTR, transthyretin; RBP, retinol-binding protein; IRBP, interphotoreceptor retinoid-binding protein; MEM, Eagle's minimum essential medium; DEAE-cellulose, (diethylaminoethyl)cellulose; mRNA, messenger ribonucleic acid; CEM, Chee's essential medium.

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have used similar methods to look for the secretion of TTR and RBP by cultured bovine RPE. A preliminary report from another group has provided evidence from Western blot analysis of conditioned medium that cultured rat RPE secretes TTR (Defoe et al., 1992).

MATERIALS AND METHODS

Cell Culture. Retinal pigment epithelium (RPE) derived from 58 fetal bovine eyes was grown as confluent primary and secondary cultures by a modification of the method of Pfeffer et al. (1986) which has been previously described (Bok et al., 1992). Plastic culture dishes of approximately 100-mm diameter were seeded at an average density of 1.7×10^5 cells/cm^2. Culture dishes of bovine RPE were maintained 43–100 days prior to experiments.

In order to permit selective sampling of media from the apical and basal surfaces of RPE monolayers, fetal bovine RPE was also cultured on Millipore Millicell-PCF culture plate inserts of 12-mm diameter and 0.4-μm porosity (Millipore Corp., Bedford, MA). Millicell membranes were precoated with a dry film of mouse laminin (10 μg/Millicell; Collaborative Biomedical Products; Bedford, MA). Trypsinized cells were then seeded at a density of 6.3×10^5 cells/cm^2. Epithelial integrity was assessed by measurement of transepithelial resistance shortly before metabolic labeling, which was performed when the Millicells had been in culture 59–81 days. Resistances were recorded with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) and corrected by subtraction for the contribution of the laminin-coated membrane alone. Overall, the net resistance of the 30 Millicell cultures averaged 532 ± 39 (SEM) Ω·cm^2 with a range of 362–1148 Ω·cm^2.

Fetal human RPE was cultured as described in Flannery et al. (1990). A 100-mm culture dish was seeded at a density of 3.4×10^5 cells/cm^2, grown to confluence, and maintained up to a total of 81 days prior to experimentation. The gestational age of the donor was 23 weeks.

Cultures were fed with a 1:1 mixture of Chee's essential medium, modified (CEM; B & B Research Laboratories, Hope, RI) and Eagle's minimum essential medium (MEM; Sigma Chemical Co., St. Louis, MO). The CEM/MEM mixture was further supplemented as previously described (Frambach et al., 1990; Mircheff et al., 1990; Flannery et al., 1990). Heat-inactivated calf serum was included at the level of 1% for human RPE and 10% for bovine RPE.

Metabolic Labeling. Incubations were carried out with ^{35}S-labeled methionine or cysteine (Amersham, Arlington Heights, IL). L-[^{35}S]methionine (average specific activity 1184 Ci/mmol) was obtained as *in vivo* cell labeling grade stabilized with 0.1% 2-mercaptoethanol and 15 mM pyridine-3,4-dicarboxylic acid; L-[^{35}S]cysteine (specific activity 1300 Ci/mmol) was supplied with 20 mM potassium acetate and 5 mM dithiothreitol.

Complete growth medium was first replaced with a rinse of nonradioactive serum-free MEM deficient in methionine or cysteine. Cultures were then incubated at 37 °C, for 1.5–4 h prior to addition of radiolabeled medium. Methionine-deficient MEM was obtained from Sigma. MEM without cysteine and its dimer cystine was prepared from reagents supplied by Gibco (MEM Select-Amine Kit; Gibco Laboratories, Grand Island, NY). The pH of the medium used for incubating Millicells was further stabilized with 17.8 mM TAPSO, added as the sodium salt of 3-[[*N*-tris(hydroxymethyl)amino]methyl]-2-hydroxypropanesulfonic acid.

Experimental medium consisted of serum-free MEM deficient in the appropriate amino acid plus 1.0 μM *all-trans*-retinol and ^{35}S-labeled methionine or cysteine. Retinol was added by 1000-fold dilution of a freshly prepared 1.0 mM stock in absolute ethanol, the concentration of retinol having been determined from its absorbance in ethanol at 328 nm using an extinction coefficient of 52 000 M^{-1} cm^{-1}. Complete media were sterilized by filtration and all subsequent manipulations were done aseptically. Incubations were carried out at 37 °C in a moist atmosphere of 95% air/5% carbon dioxide.

Each culture dish of bovine RPE was given 100 μCi of [^{35}S]methionine or [^{35}S]cysteine and then incubated for 70–72 h. Medium from 10 dishes was pooled for each experiment. For human RPE, medium was recovered from one culture dish after an incubation of 72 h in the presence of 100 μCi of [^{35}S]methionine. The volume of medium was 10 mL for all dishes.

Polarity of secretion was studied in Millicell cultures of fetal bovine RPE which had been incubated with radioactive methionine for 6, 24, and 72 h. The initial transepithelial resistance for these groups averaged 399 ± 8 (SEM), 456 ± 9, and 742 ± 82 Ω·cm^2 respectively. Ten Millicells were included at each interval. Those with highest resistance were assumed most likely to resist leakage and were therefore assigned to the longest incubation period. Each Millicell received a total of 20 μCi of [^{35}S]methionine in 1.0 mL of medium: 10 μCi in 0.5 mL applied to the apical surface and 10 μCi in 0.5 mL to the basal surface. Apical and basal media samples were collected separately.

At the conclusion of incubations, individual media samples were pooled for each set of experimental conditions. After filtration through a 0.22-μm membrane, they were stored temporarily at −80 °C.

Isolation of Metabolically Labeled RBP and TTR by Affinity Chromatography. TTR and RBP were isolated from outdated human plasma as previously described (Shingleton et al., 1989). TTR and RBP were separately coupled to cyanogen bromide-activated Sepharose-4B (Pharmacia, Piscataway, NJ) according to the instructions of the supplier. Each was coupled at a ratio of approximately 20 mg of protein/g of support.

After incubation of RPE cells with [^{35}S]methionine in methionine-deficient medium for 70 h, the medium was removed and combined and 10 mL was applied to a 1.0-mL TTR–Sepharose column prepared in a 9-inch (22.86-cm) Pasteur pipette. Before chromatography, unlabeled human RBP–retinol (300 μg) was added to the medium so the fluorescence of RBP–retinol could be followed. Also, 1 mL of 10-fold concentrated phosphate-buffered saline (PBS) was added to ensure sufficient ionic strength for RBP to bind tightly to the TTR–Sepharose. After the conditioned medium had passed through the column, the column was washed with 5 mL of PBS (pH 7.5) and then with 5 mL of PBS containing an additional 0.5 M NaCl. The RBP was then eluted with deionized water (>18 MΩ), which reverses the binding of RBP to TTR. Fractions of 300 μL were collected and the radioactivity and fluorescence were measured on each fraction.

The unretained material from the above procedure was then applied to an RBP-Sepharose affinity column to isolate any TTR that may have been synthesized and secreted. Again, a 1.0-mL column was used and eluted as described above. The radioactivity was determined for each fraction. The elution volume of TTR was determined by adding authentic TTR to the same affinity column using identical volumes equal to those employed for the experimental samples.

Sephadex G-75 Chromatography. A 16- × 300-mm column of Sephadex G-75 (Pharmacia, Piscataway, NJ) was prepared

and washed initially with PBS and then with 18-MΩ H_2O. Its void volume (26 mL) was determined using blue dextran 2000, and authentic samples of RBP and TTR (3 mg each) were separately eluted in 1.0-mL fractions at a flow rate of 0.75 mL/min in order to determine elution volumes. The radioactive, fluorescent peak eluted from the TTR–Sepharose column by deionized H_2O was submitted to gel filtration on the G-75 column and eluted with 18-MΩ H_2O; 1.0-mL fractions were collected. The radioactivity and the absorbance at 330 nm were determined.

In a similar manner, the radioactive peak eluted from the RBP–Sepharose column by deionized H_2O was submitted to the same G-75 column and eluted in the same way. The radioactivity and absorbance (280 nm) were determined on each fraction.

SDS–PAGE. The radioactive, fluorescent peak isolated from a TTR–Sepharose affinity column as well as the radioactive peak isolated from an RBP–Sepharose column were subjected to SDS–PAGE. The samples were prepared by boiling the proteins with an SDS solubilizing buffer containing 5% 2-mercaptoethanol and bromophenol blue as the tracking dye. Minigels containing 0.1% SDS, 15% polyacrylamide resolving gel, and 6% stacking gel (1.5 mm) were used. Approximately 5–10 μg of protein were applied per lane of the gel. The tank buffer was Tris–glycine (0.02 M Tris and 0.192 M glycine) buffer (pH 8.3) containing 0.1% SDS. Electrophoresis was carried out for 1 h at 10 mA/gel and then for approximately 2 h at 20 mA/gel. The gels were stained with Coomassie Blue R-250, destained, dried, exposed to a sensitized PhosphorImager plate (Molecular Dynamics, Sunnyvale, CA) overnight, and then scanned.

Sequencing of Protein Isolated by TTR-Affinity and RBP-Affinity Chromatography. [^{35}S]Cysteine (100 μCi) was incubated with each dish of RPE cells for 72 h and 10 mL of the medium was applied to a TTR–Sepharose column as described. The fractions containing the radioactive–fluorescent peak eluted by deionized water were pooled and lyophilized. The sample was submitted to the Core Protein Laboratory at Vanderbilt University for sequencing. The *S*-(pyridylethyl) derivative of the protein cysteines was prepared (Andrews & Dixon, 1987) and the protein was sequenced through the tenth residue. Samples of the 10 residues were collected as separate fractions and the radioactivity of each was determined.

The radioactive peak (from medium of cells incubated with [^{35}S]methionine) from the RBP–Sepharose column was treated in a similar manner, sequenced through 20 residues and counted.

Antibody Titration of TTR. The radioactive peak isolated from the RBP–Sepharose column was titrated with an anti-human antibody (IgG fraction) raised in rabbits against human TTR (Boehringer Mannheim Biochemicals, Indianapolis, IN). A constant amount of radioactivity (ca. 20 000 cpm) was titrated against an increasing amount of IgG. The antibody-bound radioactivity was precipitated with Pansorbin cells (Calbiochem, San Diego, CA) and counted by liquid-scintillation spectrometry.

RESULTS

To determine initially if RPE cells might synthesize and secrete RBP and TTR, cells were provided high specific activity [^{35}S]methionine and the medium were collected after 70 h of incubation. A small amount of nonradioactive holo-RBP was added to the medium so that the elution of RBP could be followed in subsequent steps by monitoring fluorescence. The initial isolation of the putative RBP and TTR from the medium took advantage of the specific TTR–RBP interaction. This interaction is sensitive to ionic strength and is reversed when the ionic strength is decreased. TTR immobilized on Sepharose has been used previously for the purification of RBP (Vahlquist et al., 1971). In a similar manner, RBP immobilized on Sepharose has been used for the purification of TTR (Navab et al., 1977). When the medium from RPE cells cultured with [^{35}S]methionine was passed over a TTR–Sepharose column, a small amount of radioactivity bound to the column and was retained, despite extensive elution with PBS and with 0.5 M NaCl. When the column was eluted with deionized water (18 μΩ), which reverses the RBP–TTR interaction, a peak of radioactivity coeluted with the fluorescent peak of added, nonradioactive human RBP–retinol (Figure 1). When the same medium was passed over an RBP–Sepharose column, a peak of radioactivity could also be isolated by the same procedure at the position established for human TTR (Figure 2).

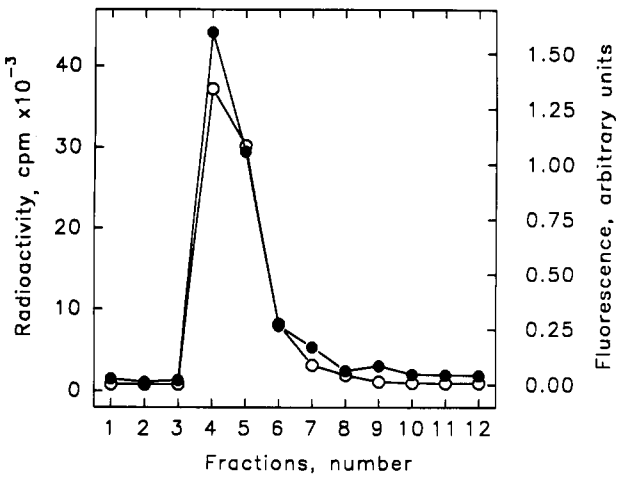

FIGURE 1: Elution of radioactivity and retinol-like fluorescence from a TTR–Sepharose affinity column. Medium from bovine RPE cells cultured with [^{35}S]methionine for 70 h, after addition of 300 μg of hRBP, was applied to the column and washed with PBS, then PBS + 0.5 M NaCl, before beginning elution with deionized water (>18 MΩ). Fractions of 300 μL were collected and radioactivity (●) and fluorescence (○) were determined as described in Materials and Methods.

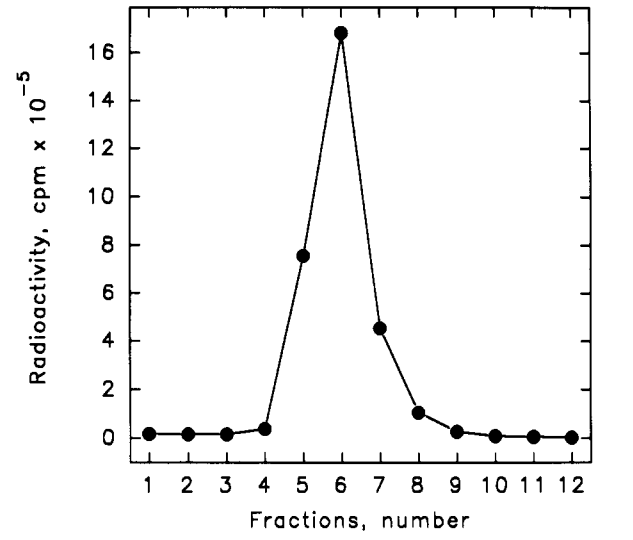

FIGURE 2: Elution of radioactivity from an RBP–Sepharose affinity column. Medium from bovine RPE cells cultured with [^{35}S]-methionine, after being passed over a TTR-affinity column, was passed over an RBP–Sepharose column as described in Figure 1. Fractions of 300 μL were collected and the radioactivity was determined as described in Materials and Methods.

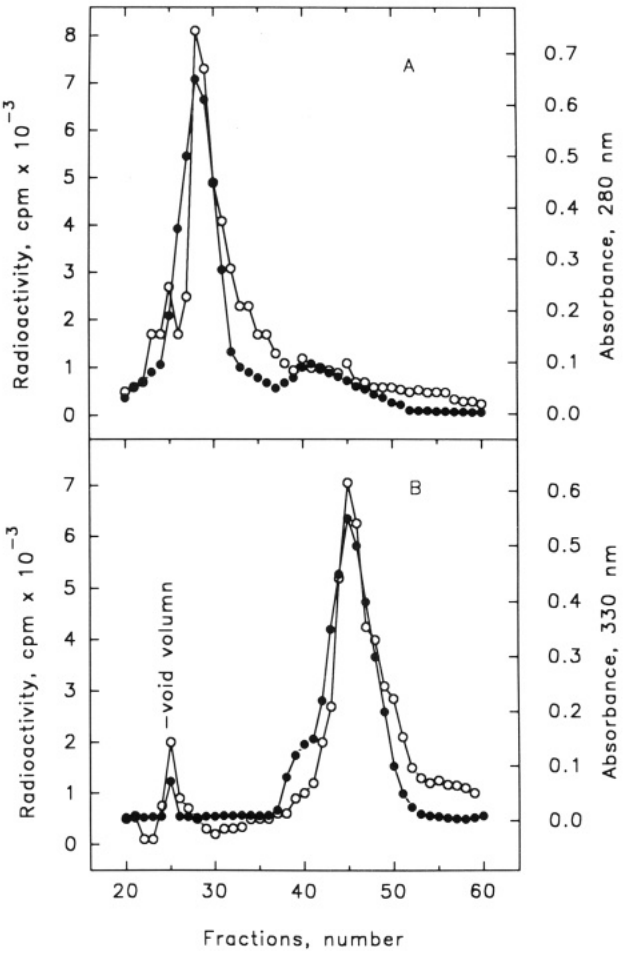

FIGURE 3: Gel filtration of material obtained from the RBP-affinity column (A) and the TTR-affinity column (B). The radioactive peak obtained from the RBP-affinity column after adding pure TTR was applied to a Sephadex G-75 column (16 × 300 mm) and eluted with deionized water. Fractions of 0.75 mL were collected and the radioactivity (●) and absorption at 280 nm (○) were determined as described in Materials and Methods. The radioactive–fluorescent peak obtained from the TTR-affinity column was treated similarly as described above after adding pure RBP-retinol. The radioactivity (●) and absorbance at 330 nm (○) were determined on the fractions.

The material isolated from both the TTR-affinity column and the RBP-affinity columns was further characterized by gel filtration on Sephadex G-75. In the case of the TTR-retained material the radioactive peak coeluted with the fluorescence and absorbance (330 nm) peaks of the added human RBP and approximately where a 21-kDa protein would elute (Figure 3B). The radioactive peak which was isolated from the RBP–Sepharose column was likewise submitted to gel filtration on Sephadex G-75. A quantity of pure nonradioactive TTR was added for cochromatography with the radioactive material. The nonradioactive TTR (followed by absorbance at 280 nm) coeluted with the radioactive peak (Figure 3A) and at a position consistent with the molecular weight of the TTR tetramer.

As an additional determination of the molecular size of the recovered, labeled material, the samples were submitted to SDS–PAGE and compared to pure human RBP and TTR. After the SDS–polyacrylamide gels were dried and exposed to a sensitized PhosphorImager plate overnight, the image generated by the scan of the plate was compared to the dried gel. Figure 4 shows that the major radioactive bands shown in lanes 1 and 5 moved identically with the Coomassie-stained RBP and TTR respectively (shown in lanes 2 and 4). The molecular weight standards are shown in lane 3. The position of the radioactive bands in lanes 1 and 5 compared favorably

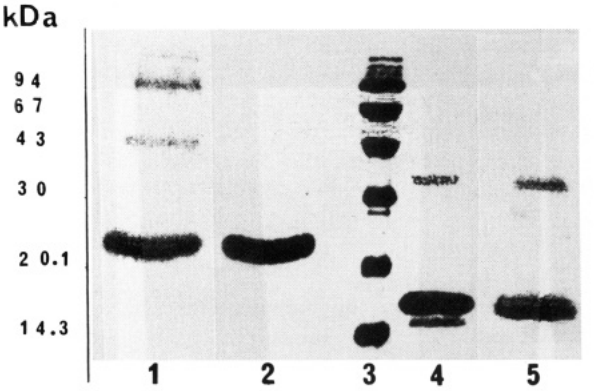

FIGURE 4: SDS–PAGE and PhosphorImager scans of material isolated by TTR and RBP-affinity chromatography. Lanes 2 and 4 are Coomassie-stained pure RBP (2) and TTR (4), which was also added to the radioactive material isolated from a TTR-affinity column and an RBP-affinity column, respectively. Lanes 1 and 5 are PhosphorImager scans whose major bands superimpose upon the Coomassie-stained bands. The major band of lane 1 contains 81% of the total activity of lane 1, while the major band of lane 5 contains 97% of the total activity of lane 5. Lane 3 contains molecular weight standards; in descending order, phosphorylase, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme.

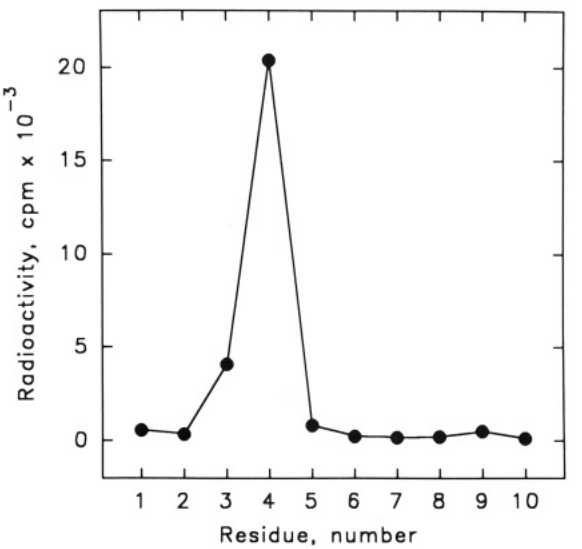

FIGURE 5: Presence of radioactivity in residue 4 of the protein recovered from TTR–Sepharose column. After [^{35}S]cysteine was incubated with the RPE cells, the radioactive peak that eluted from the TTR–Sepharose column was isolated, the *S*-(pyridylethyl) derivative of the protein cysteines was prepared, and the sample was sequenced through the tenth residue.

with the mass of RBP (21 kDa) and the monomeric mass of TTR (14 kDa). The radioactive bands overlaid exactly the Coomassie-stained bands of the added RBP or TTR.

Finally, when [^{35}S]cysteine was incubated with the RPE cells, a radioactive peak coeluted from the TTR–Sepharose column with the fluorescent peak of added human RBP–retinol. This peak was lyophilized and the *S*-(pyridylethyl) derivative of the protein cysteines was prepared by the Protein Core Laboratory before sequencing through the tenth residue. The bulk of the activity was found in the fourth residue (Figure 5), which is the position of the only cysteine residue found in the first 10 residues of bovine RBP (Berni et al., 1990).

Similarly, when RPE cells were incubated with [^{35}S]-methionine, a radioactive peak was isolated (Figure 2). This peak after concentration and addition of a small quantity of nonradioactive TTR was sequenced in like manner to the RBP. It was noted that radioactive residues appear in positions 10 and 13 (Figure 6) where a cysteine and a methionine are

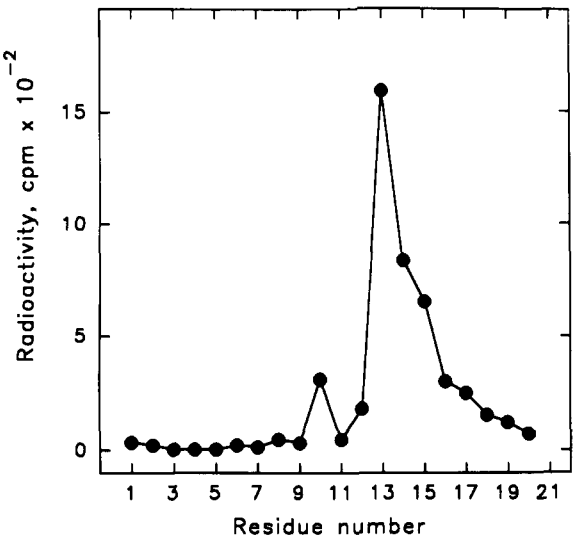

FIGURE 6: Presence of radioactivity in residues 10 and 13 of the protein recovered from an RBP–Sepharose column. After RPE cells were incubated with [^{35}S]methionine, the radioactive peak that eluted from the RBP–Sepharose column was isolated for sequencing as described in the legend of Figure 5.

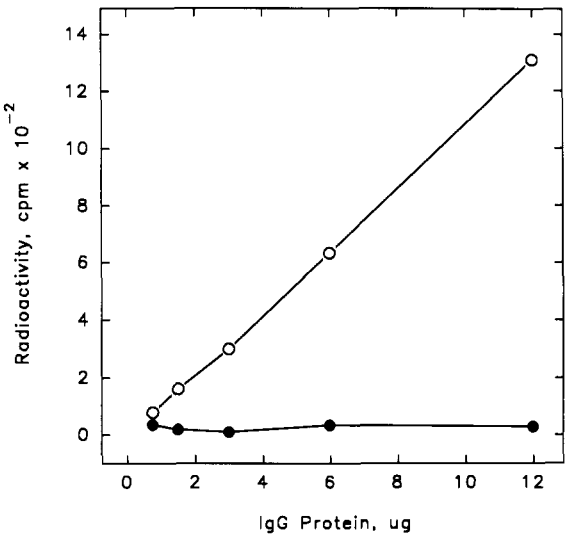

FIGURE 7: Antibody titration of the radioactive material isolated by the RBP–Sepharose affinity column. A constant amount of radioactivity was titrated with an increasing amount of antibody (IgG fraction). The radioactivity that bound to the antibody was precipitated with Pansorbin and counted (○). Little activity (●) was bound when control IgG was substituted for the specific IgG fraction.

found in human TTR (Fex & Lindgren, 1977). Bovine TTR was isolated by affinity chromatography of bovine serum on an RBP–Sepharose column and sequenced. Cysteine and methionine were found in positions 10 and 13, respectively, confirming the sequence similarity with human TTR and consistent with the recovery of radioactivity. The occurrence of label in the position for cysteine can be attributed to the fact that the added [^{35}S]methionine can be used for the synthesis of cysteine within the cell.

As a final test of protein identity, reaction with a specific IgG preparation was examined. Since the preparation was raised against human TTR, it was not known if it would cross-react with the bovine TTR. However, Figure 7 shows that as the antibody was added in increasing amounts to the radioactivity recovered from the RBP-affinity column, the precipitated radioactivity increased. Antibody against human RBP (Boehringer Mannheim Biochemicals, Indianapolis, IN) did not precipitate any radioactivity recovered from the TTR-affinity column, indicating that it recognized epitopes unique to human RBP. However, the other characterizations are sufficient to establish that this material was indeed bovine RBP.

The recovery of [^{35}S]methionine in the respective RBP and TTR fractions indicated that approximately 50 times more TTR tetramer had been synthesized and released by the RPE cells than RBP. This is based on the assumption that the bovine TTR amino acid content of methionine and cysteine is the same as for human TTR (there are four methionines in the tetramer of human TTR and there are four methionines in the human RBP molecule).

We performed a similar but limited analysis on cultured human RPE. In this case, having already established the identity of secreted RBP and TTR by partial sequencing of the bovine proteins, we limited this analysis to affinity chromatography. Results for human RPE were similar to those described for bovine RPE; both RBP and TTR were secreted into the culture medium. In this instance, the recovered radioactivity was 30 000 cpm for RBP and 850 000 cpm for TTR, an approximately 30-fold excess for TTR secretion.

Having determined that cultured bovine and human RPE synthesize and secrete RBP and TTR, we sought to determine whether this secretion took place preferentially from the apical or basolateral surface of the cells. This was tested by growing bovine RPE cells in chambers with microporous support membranes until they reached transepithelial resistances of 360–1150 Ω·cm^2. Cultures with resistances of this magnitude would be expected to prevent the paracellular diffusion of RBP and TTR from one side to the other. The cells where then metabolically labeled with [^{35}S]methionine by addition of the precursor to the culture media bathing the apical and basal sides and, following incubation periods of 6, 24, and 72 h, basal and apical media were analyzed by affinity chromatography for the presence of RBP and TTR. The results of this experiment are shown in Table 1.

Radioactivity due to RBP and TTR was found in both the basal and apical culture media. However, the total radioactivity recovered was higher for the apical compartment at all times tested. This difference increased with time, reaching an apical:basal ratio of 4.5 and 9.2 at 72 h for RBP and TTR, respectively.

DISCUSSION

Previous work by others has established the presence of mRNAs for RBP and TTR (Cavallaro et al., 1990; Herbert et al., 1991) in the RPE and the presence of the proteins (as assessed by immunocytochemistry) at various sites within the retina (Herbert et al., 1991; Dwork et al., 1990). Here we have extended those observations by demonstrating that those mRNAs present in the RPE are indeed translated and that the proteins are then secreted in a vectorial manner, primarily from the apical surface of the cell. The identities of the secreted proteins were rigorously confirmed by several biochemical criteria. The demonstration of preferential apical release indicates that, if it also occurs *in vivo*, these proteins would be released preferentially to the extracellular matrix (the interphotoreceptor matrix or IPM) between the RPE and rod and cone photoreceptor outer segments and that they would also be available to all cells of the retina. The immunocytochemical study of Herbert et al. (1991) did not reveal immunoreactivity within the IPM, but this may be due to the limit of detection for this method.

The RPE may well be the source of all ocular TTR and RBP. Plasma RBP does interact with RPE on its basolateral

Table 1: Comparison of the Amount of RBP and TTR Secreted into the Apical and Basal Media by Bovine RPE Cells Cultured on Microporous Membrane Supports[a]

incubation time (h)	apical RBP (cpm × 10^{-3})	basal RBP (cpm × 10^{-3})	apical/basal RBP	apical TTR (cpm × 10^{-3})	basal TTR (cpm × 10^{-3})	apical/basal TTR	apical TTR/RBP	basal TTR/RBP
6	22.3	9.3	2.4	870	194	4.5	39	21
24	27.5	15.9	1.7	1172	240	4.9	43	15
72	41.2	9.1	4.5	1030	112	9.2	25	12

[a] Media were collected after addition of [^{35}S]methionine at the times indicated. The radioactivity present as RBP or TTR was determined by affinity chromatography as described in Materials and Methods.

(blood) side for the delivery of *all-trans*-retinol by a mechanism thought to involve a membrane receptor (Heller, 1975; Heller & Bok, 1976; Båvik et al., 1991, 1993). However, the RBP moiety is not internalized in this process. Therefore, plasma RBP cannot be the source of ocular RBP. Cytoplasmic RBP (Herbert et al., 1991) and TTR (Dwork et al., 1990), as shown by immunocytochemistry, have been observed in many cells of the neurosensory retina, suggesting possible synthesis of these secreted proteins within those cells. However, the corresponding mRNA levels are below the level of detection by *in situ* hybridization. Thus these proteins would appear to be acquired by an extracellular route. Receptor-mediated uptake and internalization of both TTR and RBP have been described for hepatocytes (Divino & Schussler, 1990; Senoo et al., 1990), and such internalization of RPE-derived TTR and RBP may also occur for cells of the neurosensory retina.

It is of interest to consider whether RBP might play a role in the trafficking of retinoids that occurs during the visual cycle (Dowling, 1960). That cycle involves a complex series of interactions between membrane receptors, retinoid binding proteins, and enzymes (Bok, 1990). Events include the uptake of *all-trans*-retinol by the RPE, its processing of *all-trans*-retinol into 11-*cis*-retinal, and the release of 11-*cis*-retinal into the IPM. The 11-*cis*-retinal thus must be delivered to the photoreceptors for utilization in the production of photopigments from rod and cone opsins. Subsequent bleaching of photopigments in the rods and cones by light involves the isomerization of 11-*cis*-retinal to *all-trans*-retinal, its enzymatic conversion to *all-trans*-retinol, and its release into the extracellular matrix from the photoreceptors. The *all-trans*-retinol then returns to the RPE, entering through the apical plasmalemma, and there is some evidence that, like the delivery of *all-trans*-retinol through the basolateral membrane, this process is receptor-mediated (Okajima et al., 1989, 1990; Carlson & Bok, 1992).

RBP can bind not only *all-trans*-retinol but also, *in vitro*, 11-*cis*-retinal (Horwitz & Heller, 1973). Consequently, it could be considered as a candidate for transport not only of *all-trans*-retinol from the photoreceptors to the RPE for isomerization to 11-*cis*-retinal but also for transport of that 11-*cis*-retinal back to the photoreceptors. However, secretion of newly synthesized RBP has been shown to require binding of *all-trans*-retinol (Goodman, 1984) and there is no evidence yet that 11-*cis*-retinal can also be exported from the cell bound to RBP. Because the photoreceptors apparently do not synthesize RBP, transport of *all-trans*-retinol from those cells to the RPE by RBP would require that extracellular apoRBP be present in the IPM and able to acquire the *all-trans*-retinol after release of the latter from the photoreceptor, a possibility that must be considered.

The better candidate for retinoid transport within the extracellular matrix during the visual cycle is the most abundant soluble protein within this matrix, IRBP (Adler & Evans, 1985), which is capable of binding several different retinoids (Liou et al., 1982; Adler et al., 1985; Saari et al., 1985). This protein is synthesized and secreted by the photoreceptor cells (Gonzalez-Fernandez et al., 1985; van Veen et al., 1986; Porrello et al., 1991). The predominant retinoid bound to IRBP from light-adapted animals is *all-trans*-retinol (Adler & Evans, 1985; Liou et al., 1982; Adler et al., 1985; Bunt-Milam et al., 1985; Saari et al., 1985) whereas the predominant retinoid in dark-adapted animals is 11-*cis*-retinal (Lin et al., 1989). Thus, it is generally believed that IRBP serves as an important, protective, binding protein in the IPM and that it facilitates the diffusion of retinoids to and from the photoreceptors and RPE along concentration gradients (Ho et al., 1989; Chader, 1989; Lin et al., 1989; Saari, 1990). Providing further credence for a functional role for IRBP in the visual cycle is the observation that IRBP promotes the release of 11-*cis*-retinal from the apical surface of amphibian (Okajima et al., 1989, 1990) and cultured mammalian RPE (Carlson & Bok, 1992). Furthermore, when 11-*cis*-retinal is the predominant ligand, IRBP promotes rhodopsin regeneration in amphibian photoreceptors (Jones et al., 1989). Thus IRBP appears to serve all needs of retinoid transport for the visual cycle but perhaps not all retinoid transport that occurs within the IPM.

There are undoubtedly roles for retinoids in the retina in addition to serving as chromophores for the photopigments. Retinal amacrine and Müller cells contain cellular retinoic acid-binding protein (Gaur et al., 1990; Milam et al., 1990) and presumably require *all-trans*-retinol as a precursor for the production of retinoic acid. It is reasonable to consider a role for RBP in the delivery of *all-trans*-retinol to amacrine cells in light of the fact that IRBP, due to its mass, cannot easily penetrate the adhaerens junctions between the apical region of Müller cells and photoreceptor inner segments (the so-called outer limiting membrane of the neurosensory retina; Bunt-Milam et al., 1985), a passage that must be breached in order for RBP to reach those cells. RBP, by virtue of its lower mass, could readily pass through these junctions. The RBP–TTR complex is somewhat larger but still within the range of proteins that can pass this potential barrier since the cutoff for filtration is not absolute.

The Müller cells of the retina contain cellular retinol-binding protein (Bok et al., 1984), whose ligand is *all-trans*-retinol; cellular retinal-binding protein (Bunt-Milam & Saari, 1983), which binds 11-*cis*-retinol and 11-*cis*-retinal specifically (Saari et al., 1982); and cellular retinoid acid binding protein (Milam et al., 1990), whose ligand is retinoic acid. It is likely then that these cells also acquire retinoids, but since the apical surface of the Müller cell is in contact with IRBP, this binding protein could potentially serve as the vehicle for delivery. However, because uptake of retinoids from both IRBP and RBP appears to be mediated by different cell-surface receptors, the receptor that is expressed by a particular cell could determine which protein serves as the delivery vehicle. Cell-specific expression of receptor type would then have the effect of determining ability to tap the two pools of retinoid within the same space, that bound to RBP and that bound to IRBP, and could permit a differential regulation of uptake, perhaps depending on the ultimate fate or use intended for the retinoid.

The multifunctional nature of the retinoids should be stressed, since they provide not only the visual chromophore but also serve as hormones that can directly interact with nuclear receptors to cause ligand-dependent activation or repression of gene expression (Wolf, 1990).

It should be noted that earlier studies that detected RBP receptors on the basolateral plasmalemma of the isolated RPE did not reveal binding sites for RBP on cells of the isolated neurosensory retina (Heller & Bok, 1976). Thus the delivery of retinoids to cells of the neurosensory retina may differ from that of the basolateral surface of the RPE. Indeed, the action of RBP and TTR in the neurosensory retina could be quite different from plasma RBP and TTR which bathe the basolateral surface of the RPE but do not enter the RPE (Bok & Heller, 1976; Pino, 1986). On the other hand, this negative result could be due to low receptor density and lack of appropriate sensitivity of the methods that were used at the time.

A particularly unexpected observation for us was the surprisingly high ratio (as high as 50:1) of TTR to RBP secretion. To our knowledge, the rate of secretion of these two proteins by a single cell type has not been previously determined. The ratio of the steady-state levels of TTR and RBP in plasma is 2 for both man and rat (Goodman, 1974; Peterson et al., 1974). Because RBP has a shorter half-life than TTR (Socolow et al. 1965; Vahlquist et al., 1973; Peterson et al., 1974), it has been calculated that the ratio of the overall rate of secretion of TTR and RBP into plasma (from all sources) is about 1 (Peterson et al., 1974). Studies with HeLa cells transfected with both the RBP and TTR gene have suggested that association of TTR with RBP–retinol may occur within the lumen of the endoplasmic reticulum prior to secretion (Melhus et al., 1991) rather than in the plasma as previously suggested (Navab et al., 1977). This would provide a physiologic rationale for the calculated 1:1 secretion ratio. However, association with TTR is not required for RBP secretion from transfected cells (Melhus et al., 1991) nor is it required for secretion of RBP from Sertoli cells in primary culture (Davis & Ong, 1992), because Sertoli cells do not secrete TTR (Davis and Ong, unpublished observations). Thus, TTR must be serving some function in the retina which is unrelated to its ability to form a ternary complex with RBP–retinol. One might then suspect that the ability of TTR to bind and transport thyroxine could be the important property of TTR that leads to its relatively greater secretion rate from the RPE. That has been suggested to be a possible reason for the very high rate of secretion of TTR into the cerebrospinal fluid by the choroid plexus (Herbert et al., 1986) but other, unknown roles could also be important.

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