Membrane Receptors For Retinol-Binding Protein in Cultured Human Retinal Pigment Epithelium

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The retinal pigment epithelium (RPE) is responsible for transport of retinol from the choroidal circulation to the photoreceptors. In the intact eye, this process is mediated by membrane receptors for plasma retinol binding protein (RBP) distributed basolaterally on the RPE cells. We have shown that cultured human RPE expresses this receptor. A binding curve exhibiting saturation was generated by incubating enzymatically detached epithelial sheets with increasing concentrations of 125I-labelled RBP. 125I-RBP binding experiments also show that the receptor is expressed at a high level in first passage subcultures, suggesting de novo synthesis, and that basally oriented receptors predominate over those associated with the apical surface, reflecting the polarization characteristic of RPE in vivo. Cultured RPE can internalize 3H-retinol carried by RBP, resulting in synthesis of labelled retinyl palmitate. Production of labelled retinyl ester is competitively inhibited when incubations include an excess of holo-RBP containing non-radioactive retinol. These results indicate that RBP not only binds to the receptor specifically, but also that this interaction is functional, effecting uptake of retinol by the RPE cells. The expression of this property of differentiated RPE favors the use of cultured RPE as a model system for studying vitamin A transport and metabolism. Invest Ophthalmol Vis Sci 27:1031-1040, 1986

Plasma retinol binding protein (RBP) is the transport vehicle for retinol (vitamin A alcohol) in the blood.1,2 It effectively maintains the plasma concentration of retinol at levels 1000-fold higher than would occur for the free molecule in aqueous solution,3,4 and binds retinol with an affinity such that the ligand is protected from enzymatic and oxidative breakdown.5 The only physiological mode for release of retinol by RBP is through interaction with target tissues, such as retinal pigment epithelium (RPE), which possess specific membrane receptors for the protein.6-8 Holo-RBP carries all-trans retinol in a 1:1 ratio and is also complexed with the tetrameric protein transthyretin (TTR) in a 1:1 ratio.9,10 (Transthyretin has recently been designated as the new name for prealbumin.11) This RBP-TTR complex diffuses from the choroidal circulation and binds to the basolateral surface of the RPE,7 where the receptor is thought to mediate transfer of the retinol from RBP to cellular retinol binding protein (CRBP).12,13 The apo-protein is not internalized during this process,6,7 but its affinities for both the receptor and TTR are reduced.6,14 The storage of vitamin A in RPE as retinyl esters, primarily retinyl palmitate,15 and its eventual use as retinal in photoreception are well established.16,17 However, the exact mechanisms of transfer of retinol from RBP to the RPE and the subsequent transport within and across the RPE are not understood.

A previous study18 showed that cultured human RPE cells are capable of esterifying exogenously supplied retinol delivered from bovine serum albumin (BSA), but the retinol was probably internalized in a non-specific manner. Since the binding of retinol to BSA is weak,3 this hydrophobic ligand would be expected to have a high affinity for the lipid bilayer of the RPE plasma membrane following its dissociation from the protein. The demonstration that RPE cells possess a functional membrane receptor for RBP under in vitro conditions would underscore the usefulness of the cultured RPE system for studying the physiological route of retinol transfer from RBP to the cell. We report here that long-term cultures of human RPE express the RBP receptor, a marker for RPE differentiation, as evidenced both by RBP binding to cells and by specific uptake and esterification of radiolabelled retinol delivered from RBP.

Materials and Methods

RPE Cell Culture

Cultures of human RPE cells were established from donor eyes ranging in chronological age from 8 months

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Supported by National Eye Institute Grants EY00444 and EY00331, EY03931, EY05624, and a National Retinitis Pigmentosa Foundation-Fighting Blindness Postdoctoral Fellowship.

Submitted for publication: July 12, 1985.

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postnatal to 30 yr. The RPE was harvested by means of neutral protease \(^{19}\) (Dispase, Boehringer-Mannheim; Indianapolis, IN) treatment of globes from which pieces of sclera were removed, exposing the underlying choroid. After enzyme incubation, sheets of RPE were gently rinsed from Bruch’s membrane and seeded directly into either 35 mm tissue culture dishes (Lux; Naperville, IL) or 24-well clusters (Costar; Cambridge, MA). Primary cultures were established in Medium 199, with 1% calf serum, 1% bovine retina extract, and the following hormones and trace nutrients: insulin (10 \(\mu\)g/ml), transferrin (5 \(\mu\)g/ml), hydrocortisone (20 nM), triiodothyronine (1 nM), putrescine (0.3 \(\mu\)g/ml), and linoleic acid (0.3 \(\mu\)M; complexed to BSA). The final concentration of BSA in the medium was 10 \(\mu\)g/ml, and that of Ca\(^{++}\) was 0.1 mM. After maintenance in this medium for 2–3 weeks, the attached, confluent RPE cells yielded rounded-up cells that budded off into the medium in numbers large enough to harvest at each feeding interval (2–3 times per week). These "floaters" were seeded into new tissue culture dishes or frozen for future use. Cultures established in this way are here termed "first passage" cultures.

After several weeks of generating floaters, equivalent to approximately two population doublings, confluent primary and first passage cultures were switched to a 1:1 mixture of Medium 199 and Dulbecco’s modified Eagle’s medium (DMEM) with 0.9 mM Ca\(^{++}\), and the remainder of the above mentioned supplements. After 2 weeks in this medium, the RPE cells eventually developed the “cobblestone” morphology characteristic of an epithelium. The RPE could be maintained as a confluent monolayer in this latter medium for as long as 3 months.

Some first and second passage cultures were initiated from floaters that were originally generated in primary culture under reduced calcium ion conditions and then stored frozen in liquid nitrogen for 6 months to 1 yr. Frozen cells were cryoprotected and also thawed and plated using standard techniques.\(^{20}\)

**Release of Intact Epithelial Sheets**

Confluent cells were rinsed with balanced saline solution containing Ca\(^{++}\) and Mg\(^{++}\) (BSS-CM), pH 7.2, and the monolayer scored with a metal probe to provide the enzyme with more opportunity to diffuse beneath the basal side of the epithelium and to ensure that sheets of cells of uniform size were released. Monolayers were incubated at 37°C in a solution of 2.4% Dispase in basal DMEM, containing 100 mM sorbitol,\(^{31}\) requiring between 15 and 45 min, until a majority of sheets became detached by agitation. The sheets were pipetted into DMEM containing 10 \(\mu\)g/ml DNase I (Sigma Chemical Co.; St. Louis, MO), 0.2 \(\mu\)g/ml aprotinin, and 0.1% ovalbumin. After being centrifuged at 100 \(\times\) g for 5 min, the cells were resuspended in either BSS-CM (for the \(^{3}\)H-retinol-RBP incubations) or in phosphate-buffered saline, pH 7.5, plus 0.1% ovalbumin (for \(^{125}\)I-RBP binding).

**Binding Assay**

Dispase released RPE cells (3–5 \(\times\) \(10^5\)) were suspended in 1 ml BSS-CM with 0.1% ovalbumin. \(^{125}\)I-RBP (10–180 ng) was added, and cells were incubated at 24°C for 15 min. During incubation, cells were mixed every 30 sec. At the end of the incubation, the cells were collected by centrifugation (3000 \(\times\) g for 5 min) and washed twice with ice cold BSS without Ca\(^{++}\) and Mg\(^{++}\). Radioactivity associated with the cell pellet was quantitated in a Beckman Gamma 9000 counter with 52% efficiency.

Specific binding of \(^{125}\)I-RBP was determined by incubating parallel samples of cells with \(^{125}\)I-RBP in the presence of a 300-fold excess of nonradioactive (native) holo-RBP. Specific binding was defined as the quantity of \(^{125}\)I-RBP bound in the absence of excess cold RBP minus the quantity bound in the presence of excess cold RBP. Where specific binding is expressed in relation to mg of cellular protein, protein was assayed according to the method of Lowry et al.\(^{22}\)

For measuring differential \(^{125}\)I-RBP binding to apical and basal RPE surfaces, confluent cells cultivated in well clusters were first incubated, after a short rinse with BSS-CM, with the iodinated reagent in situ for 15 min. In this case, the incubation medium contained Ca\(^{++}\) and Mg\(^{++}\). During this step, the \(^{125}\)I-RBP was available to the apical RPE membrane only. The cells were quickly rinsed before being released enzymatically, rinsed again and pelleted as above, counted, and then reincubated as a suspended sheet with \(^{125}\)I-RBP for 15 min. After being washed and pelleted, the cells were recounted. The difference in the two values for specific counts represented the specific counts associated with the basal membrane.

**Incubations With \(^{3}\)H-Retinol-RBP**

Single sheets of RPE containing 5 \(\times\) \(10^5\) cells, obtained as for the binding assay (see above), were incubated at 37°C in 250 \(\mu\)l of BSS-CM containing 5 \(\mu\)g of holo-RBP in which the endogenous retinol was replaced with \(^{3}\)H-retinol. Cell counts were made using a hemocytometer. The effective molar concentration of \(^{3}\)H-retinol was 1.0 \(\mu\)M. Incubations were carried out in duplicate for either 20 or 60 min, with one additional sample at each time point containing a 250-fold molar excess of nonradioactive holo-RBP. At the end of the incubations, the samples were flooded with 1 ml of ice cold BSS-CM and placed on ice. The sheets were pelleted by centrifugation at 100 \(\times\) g, and the
pellets were washed three times, the last wash containing 0.5 mg/ml ascorbic acid (as an antioxidant). The cells were then broken by freezing and thawing before being stored under nitrogen in 1.5 ml conical snap-cap plastic vials at −80°C, protected from light until further processing.

Lyophilization and Extraction

Cell pellets were lyophilized in darkness and the retinoids were extracted under dim red light by homogenizing the tissue first with methanol:water (99:1) followed by a separate homogenization in hexane.23 Extractions were performed three times for each solvent (HPLC grade, as were all solvents utilized) with the aid of a Teflon pestle. The methanol:water and hexane extracts were separately evaporated to dryness under a stream of nitrogen, the residues dissolved in methanol:chloroform (1:1)24 combined, and spun in a Beckman (Fullerton, CA) Microfuge at 12,000 rpm to remove insoluble particulates before injection into the HPLC column.

Culture media were extracted by the addition of absolute ethanol in a 1:1 ratio, followed by centrifugation of the mixture and partitioning of the retinoids from the supernatant into hexane.25 The upper hexane phase was dried and the residue prepared for HPLC injection as above.

Analytical HPLC

Reverse phase high pressure liquid chromatography (HPLC) was used to analyze the cell extracts for radiolabeled retinoids. A 4.6 mm I.D. × 250 mm Vydac TP silica C18 column with 5 μm particles (Separations Group; Hesperia, CA) was used at a flow rate of 0.25 ml per minute for separation of retinyl esters. The mobile phase solvent was acetonitrile:tetrahydrofuran:water (55:37:8). All-trans retinyl palmitate for use as a chromatographic standard was the generous gift of Dr. P. F. Solter, Hoffman-LaRoche Co., Nutley, NJ. All-trans retinyl acetate was obtained from Sigma Chemical Co., St. Louis, MO.

The radiolabeled retinyl esters were eluted from the column and their absorbance detected at 326 nm in a Kratos detector (Kratos GmbH; Karlsruhe, FRG), and fractions were collected every minute in a Pharmacia Frac 100 collector (Pharmacia; Uppsala, Sweden). The fractions were mixed with 4 ml Aqueous Counting Scintillant (Amersham, Arlington Heights, IL), and dpm determined for a 10-min counting period in a Beckman (Fullerton, CA) LS 7500 scintillation counter.

Preparation of Reagents

Human RBP and TTR were purified from outdated human plasma using previously described methods26,27 and stored at −50°C until used. RBP was labeled with 125I by the lactoperoxidase method of Roth.28 Apo-RBP was prepared by irradiating holo-RBP at 335 nm in a Farrand (Valhalla, NY) Mark I spectrofluorimeter. The conversion of holo-RBP to apo-RBP was monitored fluorimetrically by the loss of specific fluorescence at 460 nm,29 and confirmed spectrophotometrically by the decline in the ratio of absorbance at 330 nm to that at 280 nm.

3H-retinol was prepared by sodium borohydride reduction of 3H-retinal, a gift from Dr. Wayne Hubbell. The radiolabeled retinol had a specific activity of 0.55 Ci/mmol, based on a molar extinction coefficient of 53,000 at 326 nm.30 The tritium label was predominantly at the 13-methyl position.31 After reduction to the alcohol, all-trans 3H-retinol was separated from contaminating 9-, 11-, and 13-cis isomers by preparative HPLC on a reverse-phase column identical to the one used for separating retinyl esters. In this case, the solvent was 60 parts acetonitrile to 40 parts aqueous solution of 0.015 M ammonium acetate, pH 4.7, with a flow rate of 1.0 ml/min. Pure all-trans retinol standard was prepared from all-trans retinal (Sigma Chemical Co., St. Louis, MO) by borohydride reduction. The eluted fraction containing the all-trans isomer of 3H-retinol was extracted with hexane, evaporated to dryness under nitrogen, and redissolved in ethanol for storage at −80°C.

Apo-RBP was incubated with a two-fold molar excess of purified all-trans 3H-retinol to reconstitute 3H-retinol-RBP, and the unbound 3H-retinol was removed from the mixture by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden). The 3H-retinol-holo-RBP was further purified by affinity chromatography on a TTR-Sepharose column,26 yielding holo-protein in which the A330 to A280 ratio was close to unity. TTR was bound to cyanogen bromide activated Sepharose (Pharmacia, Uppsala, Sweden) following the manufacturer's protocol.

The final specific activity of the 3H-retinol-RBP after affinity chromatography was 0.3 Ci/mmol, somewhat lower than that of the initially synthesized 3H-retinol (see above). This diminished value probably was due to dilution by residual nonradioactive retinol, endogenously bound to the original holo-RBP, that was not destroyed by irradiation in the spectrofluorimeter.

Electron Microscopy

RPE cells were fixed either as enzymatically detached sheets (see above) or in situ as an attached monolayer. The primary fixative was 2% formaldehyde and 2.5% glutaraldehyde in BSS-CM (1 hr), pH 7.2. After several rinses with BSS-CM, the cells were postfixed in 1% OsO₄ in 0.1 M sodium phosphate buffer, pH 7.4 (30 min), dehydrated in ethanol, infiltrated and finally
embedded in Araldite 502. Thin sections were cut on a Reichert (Vienna, Austria) ultramicrotome, stained with uranyl acetate and lead citrate, and viewed and photographed in a Siemens Elmiskop IA electron microscope.

Results

Ultrastructural Appearance of Cells

Human RPE cells cultured on tissue culture plastic, fixed and embedded in situ, have the ultrastructural appearance of a polarized epithelium (Fig. 1A). Not only do the cells exhibit well-developed apical microvilli, melanosomes, and apico-lateral junctions, but they possess basal membrane convolutions reminiscent of the basal infoldings characteristic of the tissue in vivo (Fig. 1B). An extracellular matrix is also deposited between the cell layer and the tissue culture plastic (Fig. 1B). These fine structural attributes are preserved in the sheets of RPE released from the culture dish enzymatically, although only traces of the extracellular matrix remain (Fig. 2). The RPE cells in the freed epithelial sheets do undergo an accordion-like contraction, resulting in a more columnar, as opposed to cuboidal, morphology of the cells.

Receptor Binding Assays

When a fixed number of human RPE cells (3 x 10^5) were incubated with increasing concentrations of ^125^I-RBP, a typical receptor binding curve exhibiting saturation kinetics were generated (Fig. 3). Saturation of available specific binding sites occurred at approximately 100 ng/ml of ^125^I-RBP. Of the total counts obtained from the cell pellets, after incubation and washing to remove unbound radioactivity, 30% typically accounted for nonspecific binding of the ligand to the cells. If the concentration of ^125^I-RBP was fixed at a
Fig. 2. A, Dispase-released epithelial sheet of human RPE in first passage culture. Same donor as in Figure 1. Arrow, junctional complex. Bar = 5 μm. B, Detail showing the basal membrane convolution retained by the enzyme-released cells. Bar = 1 μm.
level above saturation, it could be demonstrated that the amount of specifically bound $^{125}\text{I}}$-RBP increased linearly as the number of cells in the incubation medium was increased (Fig. 4). In order to determine the extent to which human RPE cells could maintain expression of the RBP receptor in culture, we tested $^{125}\text{I}}$-RBP binding to long-term primary and first passage cultures. Specific binding in a primary culture established from a 21-yr-old donor and maintained for 85 days amounted to 50,451 counts per mg protein. By comparison, a first passage subculture of the same cell line, whose in vitro life was 79 days, exhibited only a slight decrease in specific binding; 42,550 counts per mg protein. In keeping with the definition of a passage used in this report (see Materials and Methods), this in vitro age included the time required for the preceding primary culture to yield the suspended cells (floaters) which ultimately were seeded and gave rise to the first passage culture used for the receptor binding assay.

In an experiment designed to determine the polarity of expression of the RBP receptor, attached, confluent monolayers of RPE grown in wells were incubated with radioiodinated RBP. The specific counts per mg protein recorded from this incubation, which would have favored binding to any sites existing on the apical plasma membranes of the cells, totaled 5,940. After enzymatic detachment and reincubation of the same epithelial sheets, the specific counts per mg protein, representing specific binding to both surfaces, numbered 21,906. The ratio of basal to apical counts was 2.69, indicating that the majority of the receptors were associated with the basal surface.

**HPLC Analysis of Endogenous Retinoids in Media and Cultured Cells**

The standard formulation of Medium 199 includes 0.43 nmol/ml retinyl acetate. In light of this, and also because it was expected that calf serum and bovine retinal extract could contribute additional amounts of retinoid compounds to our final growth medium, we analyzed our complete medium by HPLC to determine the levels of vitamin A to which the RPE cells were exposed. It was found that these levels were below the limits of detection at the highest sensitivity we employed for the HPLC detector, i.e., 0.001 absorbance units full scale (a.u.f.s.). RPE cultures, grown in parallel with those that were incubated with $^3\text{H}}$-retinol-RBP, were also extracted to determine the amount and forms of vitamin A contained endogenously by these cells in second passage subculture. No detectable endogenous retinoids were found in the sample ($5 \times 10^5$ cells). Analysis of retinyl ester standards also indicated that our reverse-phase system would not separate $11\text{-cis}$ from $\text{all-trans}$ compounds.

**Analysis of Labelled Retinyl Ester Produced by Cultured Cells**

Besides binding to cultured RPE, it was found that RBP labelled with $^3\text{H}}$-retinol could interact with receptors resulting in uptake of $^3\text{H}}$-retinol by the cells. Sheets of RPE in second passage culture were incubated for either 20 or 60 min with $^3\text{H}}$-retinol-RBP, and the presence of labelled retinyl ester in the cells was taken as a measure of the internalization of $^3\text{H}}$-retinol. Two extra incubations that included a 250-fold molar excess...
of unlabelled retinol bound to RBP were carried out at both time points to test whether incorporation of \(^3\)H-retinol into ester would be suppressed by competitive inhibition at the level of the receptor. Since the intention was to extract labelled retinyl esters from cells after incubation, to collect fractions eluted from the HPLC column, and finally to analyze them for radioactivity, purified retinyl palmitate standard was first chromatographed, and its elution time of 33 min was determined by absorbance (Fig. 5A). When the experimental samples were chromatographed, it was found that peaks of radioactivity appeared with elution times coincident with retinyl palmitate standard only in those extracts obtained from cells incubated with \(^3\)H-retinol-RBP alone (Figs. 5B, 5C). For both exposure times, the palmitate peak was reduced to background when the incubation medium included an excess of holo-RBP containing unlabelled retinol (Figs. 5B, 5C).

The counts in all the peak fractions were added to determine the amounts of retinyl palmitate synthesized, and they totaled 535 dpm for the 20 min time point and 1,108 dpm for the 60 min time point. Taking into account the specific activity of the \(^3\)H-retinol bound to the holo-RBP (0.3 Ci/mmole), it was calculated that for the 20 min and 60 min incubations, respectively, 0.5 ng and 1.0 ng of \(^3\)H-retinyl palmitate were synthesized per cell. The analytical HPLC separation was therefore efficient enough to yield sharply defined peaks of radioactivity which were detectable by liquid scintillation counting, even though the actual amounts present were below the limits of detection by absorbance.

**Discussion**

The RPE constitutes part of the blood–retina barrier, and, as such, mediates the transfer of important components such as retinol from the choroidal circulation to the photoreceptors. Additionally, retinol metabolism by the RPE is an integral part of the visual cycle. In order to assess more completely the role of the RPE in these processes, it would be desirable to study the isolated RPE in culture, under conditions such that the retinol transport, metabolism, and storage functions are fully expressed. In this study, we demonstrate the presence, in cultured human RPE, of the RBP receptor, a prerequisite for physiological uptake of retinol into these cells.

The experiments using \(^125\)I-RBP showed that binding of the labelled protein to RPE cells was linearly dependent on the number of cells and, thus, the available binding sites. More importantly, by measuring specific binding with respect to the concentration of \(^125\)I-RBP, saturation kinetics typical for a receptor-ligand interaction could be observed. This result depends on competitive displacement of labelled molecules by unlabelled ones and indicates that a finite number of binding sites exist per cell. By using saturating levels of the iodinated protein, it could be shown that first passage cells had a specific binding capacity of the same order of magnitude as primary cultures. It is not known what the in vivo or in vitro turnover time is for the RBP receptor, but when the proliferation of the cells and
their in vitro age are taken into account, our results suggest that the cells are capable of producing receptors de novo in culture. Not only were the confluent first passage cultures the product of a tenfold amplification in cell number from the initial seeding, but their total chronological age in culture was 3 months.

Autoradiographic studies have established that, in keeping with the presumed spatially oriented role of the RPE in retinol transport, the RBP receptor is confined to the basolateral membrane of the RPE both in the intact eye and in freshly harvested epithelial sheets. This apical-basal polarization is a fundamental property of epithelia, and may be dependent in part on the integrity of tight junctions. In the current study, there were more RBP receptors, as judged by specific binding of I\textsubscript{25}I-RBP, on the basal membrane of the cultured RPE cells than on the apical membrane, demonstrating that these cultured cells are functionally polarized. Specific binding to the apical surface was unexpected, but the levels found could be explained in several ways. During the incubation of attached RPE cells in well clusters, the I\textsubscript{25}I-RBP may have diffused around the edges of the monolayers and come into contact with the basal membrane RBP receptors of cells near the rim of the epithelium. This has been observed to be the mechanism of lactoperoxidase-catalyzed I\textsubscript{25}I labelling of the basal surface of cultured rat RPE cells. Furthermore, some of the I\textsubscript{25}I-RBP may have been internalized at the apical surface through endocytosis unrelated to the vitamin A transport function of RBP. Certainly, the phagocytic capability of the apical membrane of RPE cells is well-documented, and cultured epithelial cells also display endocytic function in vitro.

Not only was there specificity of binding of RBP to the cultured cells, but the receptor was also biologically functional, in that it effected internalization of retinol, as measured by incorporation into retinyl ester. The single retinyl ester species detected after both 20-min and 60-min incubations with I\textsuperscript{3}H-retinol-RBP was retinyl palmitate. On the other hand, Flood et al detected roughly equivalent amounts of all-trans retinyl stearate, -palmitate, and -oleate in first passage cells that had attained 50 days in vitro and had been incubated for 24 hr with exogenously supplied retinol. The two studies employed different conditions, however, and therefore cannot be directly compared. Flood et al supplied I\textsuperscript{3}H-retinol bound to serum albumin, thereby bypassing membrane RBP receptor-mediated internalization of retinol. It is possible that the distribution of various acyl species of retinyl esters is affected by the mode of uptake. The pathway leading to esterification could be more direct when retinol is delivered through a process that is not mediated by RBP receptors. Although linoleic acid, an essential fatty acid, was supplied to our cultured cells exogenously, retinyl linolate, which is known to occur to a small extent in the vertebrate retina, was not detected.

In agreement with a similar study of RBP receptors in intestinal epithelial cells, we found that holo-RBP containing non-radioactive retinol competitively inhibited the uptake of labelled retinol by cultured RPE cells incubated with I\textsuperscript{3}H-retinol RBP. Given the stability of the retinol-RBP complex, it is unlikely that physiologically significant amounts of retinol can enter target cells simply by diffusing from the RBP. We therefore conclude that this competitive inhibition occurred at the level of the receptor. In our study, we used the appearance of labelled retinyl palmitate as a measure of uptake, since any conversion of retinol to retinyl ester must take place intracellularly under our mild incubation conditions, and, as far as is known, retinyl esters are not secreted by RPE cells. Interestingly, in spite of the levels of non-specific binding that we observed in our receptor binding assays even in the presence of ovalbumin, we could almost completely suppress esterification of labelled retinol by competitive inhibition with holo-RBP carrying unlabelled retinol. If we assume that there was significant non-specific binding of I\textsuperscript{3}H-retinol-RBP to RPE cells in the latter experiments, it can be deduced that this non-specific interaction in and of itself is not sufficient for internalization of retinol.

Along with freeing the cells from the tissue culture plastic and making the basal surface more accessible to the RBP, the treatment of the RPE cells with Dispase may also have enhanced the interaction of RBP with its receptor as a result of enzymatic digestion of the extracellular material associated with the basal surface of the freed cells. The enzyme is apparently more specific in its choice of substrate and less disruptive of the cells than trypsin. Trypsin-dissociated cells customarily lack intact intercellular junctions. It has not been established that the RBP receptor, even if proteinaceous in nature, is protease sensitive. Rask and Peterson found that trypsin had no effect on specific RBP binding to the presumed receptor, whereas one of us has noted that I\textsubscript{125}I-RBP binding to porcine RPE was not altered by treatment of fresh, nonenzymatically isolated cells with trypsin and an entire spectrum of other proteases, including Dispase (Clark VM, unpublished observations).

CRBP or some other specific cytosolic carrier may be a necessary factor in the receptor-mediated internalization of retinol, in contrast to the nonspecific uptake of retinol and its esterification observed in certain retinoblastoma cell lines lacking CRBP. While apor-RBP loses affinity for the receptor after retinol transfer to target cells, the holoproduct, when incubated with a plasma membrane fraction from testis (another ob-
ligate target organ for retinol), retains its affinity for the receptor longer than when it is incubated with whole cells. One plausible explanation is that the complete transfer of retinol from RBP to the cell can not occur without the participation, on the cytosol side of the plasmalemma, of a cellular retinol binding protein. We recently have obtained evidence by radioimmunoassay that human RPE cells maintain high levels of CRBP both in primary and first passage culture for as long as 3 months (Pfeffer, Ong, and Bok, in preparation). It is intriguing that all-trans retinol bound to CRBP can serve as a substrate for RPE retinyl ester synthase. If the currently accepted scheme for intracellular transport and storage of retinol is correct, all the necessary macromolecular components are found in the cultured human RPE employed in this study.

Despite the fact that the RPE cells were cultivated in medium containing retinyl acetate, this exogenously supplied retinyl ester made no contribution to endogenous ester stores. Likewise, the low levels of retinoids in serum and retinal extract in the growth medium had a negligible effect on the accumulation of intracellular retinoids. It is not yet known whether the levels of RBP receptors expressed in target cells are modulated by circulating retinoids or hormones. There is also no information on whether the levels of RBP to which the cells are exposed, or the amount of intracellular retinol, plays any part in regulating the activity or synthesis of the receptor. These are questions that we plan to address in the future. Thus, functionally differentiated RPE in culture as utilized in the current study has high potential as a model system for studying such effects, as well as for further elucidation of the role of the RPE in the visual cycle.

**Key words:** retinal pigment epithelium, receptors, vitamin A, epithelial cell culture, retinol binding protein

**Acknowledgments**

The authors gratefully acknowledge the assistance of William O'Day, coordinator, UCLA Eye Bank. Radiolabeled retinal was a generous gift of Wayne Hubbell. Joseph Horwitz provided helpful comments. Some of the initial purifications of RBP and TTR were performed by Lin-lin Ding, as well as by Paul FitzGerald. Alice Van Dyke and Marcia Lloyd provided technical assistance with photography and microtomy, respectively.

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