Promotion of the Release of 11-*cis*-Retinal from Cultured Retinal Pigment Epithelium by Interphotoreceptor Retinoid-Binding Protein†

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ABSTRACT: This study investigates whether the interphotoreceptor retinoid-binding protein (IRBP) is necessary for the release of 11-*cis*-retinaldehyde (RAL) or if the retinoid is constitutively released from the retinal pigment epithelium (RPE) following synthesis. The strategic location of IRBP in the interphotoreceptor matrix (IPM) and its retinoid-binding ability make it a candidate for a role in 11-*cis*-RAL release. Fetal bovine RPE cells were grown in permeable chambers, and their apical surfaces were incubated with medium containing either apo-IRBP, the apo form of cellular retinaldehyde-binding protein (CRALBP), the apo form of serum retinol-binding protein (RBP), or bovine serum albumin (BSA) or with medium devoid of binding proteins. [^{3}H]-*all-trans*-Retinol (ROL) was delivered to the basal surface of the cells by RBP. High-performance liquid chromatography demonstrated that [^{3}H]-11-*cis*-RAL was optimally released into the apical medium when apo-IRBP was present. The most surprising result was the diminished level of [^{3}H]-11-*cis*-RAL when apo-CRALBP was in the apical medium. Circular dichroism demonstrated that CRALBP had not been denatured by the photobleaching required for endogenous ligand removal. Therefore, apo-CRALBP should have been able to bind [^{3}H]-11-*cis*-RAL if it was constitutively released into the apical medium. In addition, when proteins other than apo-IRBP were present, or if the cells were incubated with medium alone, the observed decrease in apical [^{3}H]-11-*cis*-RAL was concomitant with a buildup of intracellular [^{3}H]-*all-trans*-retinyl palmitate and [^{3}H]-*all-trans*-ROL in the basal culture medium. Together these results suggest that IRBP is actively involved in the apical release of [^{3}H]-11-*cis*-RAL, perhaps via a receptor in the RPE apical membrane.

Rhodopsin must be regenerated following illumination of the retina; therefore *all-trans*-retinol (ROL)[1] produced during bleaching must be reconverted into the visual pigment chromophore 11-*cis*-retinaldehyde (RAL). Since the retinal pigment epithelium (RPE) is the site of synthesis of 11-*cis*-RAL (Bernstein et al., 1987; Okajima et al., 1990; Flannery et al., 1990), *all-trans*-ROL needs to reach this layer of cells by traveling from the photoreceptors through the interphotoreceptor matrix (IPM) and, therefore, the extracellular space separating the RPE and the photoreceptors. Once *all-trans* ROL is converted into 11-*cis*-RAL, it is released from the RPE and then it too must traverse the IPM before reaching the photoreceptors. Since retinoids are hydrophobic and susceptible to oxidative degradation in an aqueous environment (Futterman & Heller, 1972; Ho et al., 1989), one would expect a retinoid-binding protein to exist in the IPM, which would assist in retinoid transport. Interphotoreceptor retinoid-binding protein (IRBP) is a candidate for this role.

The exact role that IRBP plays in the visual cycle is unresolved, but there are a number of theories regarding its function. IRBP can bind a variety of retinoids endogenously (Adler et al., 1985; Liou et al., 1982; Fong et al., 1984; Saari et al., 1985), and in intact eyes, it is associated with 11-*cis*-RAL or *all-trans*-ROL as they appear in the IPM in conjunction with fluctuations in the dark–light cycle (Saari et al., 1985; Alder & Evans, 1985; Lin et al., 1989). One theory, therefore, is that IRBP facilitates the intercellular movement of retinoids and may actually shuttle them between the photoreceptors and the RPE (Adler et al., 1985; Liou et al., 1982; Lai et al., 1982). However, the high concentration of IRBP in the IPM (30–100 μM), its large size (133 kDa), and its relatively weak affinity for retinoids, as compared to other retinoid-binding proteins (Adler et al., 1985; Liou et al., 1982; Adler & Klucznik, 1982), make it difficult to believe that IRBP actively transports retinoids through the IPM. 11-*cis*-RAL and *all-trans*-ROL may, instead, be rapidly exchanged among IRBP molecules as they travel through the IPM along concentration gradients (Ho et al., 1989; Lin et al., 1989; Chader, 1989). IRBP, in this second model, may have the additional role of protecting retinoids from oxidative degradation as well as protecting cell membranes from retinoid damage (Bangham, 1964). Others believe that IRBP does not facilitate retinoid transport since retinoids can be rapidly transferred across lipid membranes as well as through the aqueous phase between liposomes. In fact, retinoid transport was retarded by IRBP in these studies (Ho et al., 1989).

In the present study we used cultured fetal bovine RPE cells grown on porous supports in order to study the role of IRBP in the apical release of 11-*cis*-RAL from the RPE. Because of its strategic location in the IPM and its ability to bind retinoids, it is of interest to determine whether IRBP is specifically required for the release of 11-*cis*-RAL or whether the retinoid is constitutively released from the cell following its synthesis. If the latter is true, 11-*cis*-RAL will be detected

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[1] Abbreviations: RAL, retinal; RPE, retinal pigment epithelium; IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; NGM, normal growth medium; RBP, serum retinol-binding protein; TTR, transthyretin; CRALBP, cellular retinaldehyde-binding protein; BSA, bovine serum albumin; CD, circular dichroism; NBP, no binding protein; CRBP, cellular retinol-binding protein.

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in the apical medium as long as any protein capable of binding and protecting it is present.

Our results demonstrate that [^{3}H]-11-*cis*-RAL is optimally released when apo-IRBP is present. When binding proteins other than IRBP are available, the level of [^{3}H]-11-*cis*-RAL released into the apical medium is dramatically reduced, and as a result, [^{3}H]retinoids build up at various points in the visual cycle. IRBP, therefore, must be interacting with the RPE apical membrane, which suggests that it may have more than a passive role in the visual cycle.

MATERIALS AND METHODS

Isolation and Culture of Fetal Bovine RPE Cells. Cultures of fetal bovine RPE cells were established by following the method of Pfeffer et al. (1986) as modified by William O'Day in our laboratory. The RPE cells used to initiate the cultures were derived from fetal bovine eyes obtained from a local abattoir. The posterior half of each eye was incubated in Dispase solution for 2–3 h to release sheets of RPE. The cultures were maintained on plastic dishes at 37 °C in an atmosphere of 5% CO_2–95% air with a low-Ca^{2+} growth medium (CEM 2000 without $CaCl_2$; Scott Laboratories, Fiskeville, RI), containing supplements and 10% heat-inactivated calf serum. At confluence the cells were trypsinized, concentrated by centrifugation, and then either frozen in medium with cryoprotectant or subcultured directly. The cells were seeded at a density of 4×10^5 cells/cm^2 onto 12-mm Millicell-HA culture wells with 0.45-μm pores (Millipore Corp., Bedford MA) which had been coated with human extracellular matrix (Collaborative Research Inc., Bedford, MA). The cultures were fed twice weekly with normal growth medium (NGM) containing 10% heat-inactivated calf serum and were maintained 2–6 months prior to use. Transepithelial resistance measurements were made with an epithelial voltohmmeter (World Precision Instruments Inc., New Haven, CT).

Retinoids. [11,12-^{3}H]-*all-trans*-ROL (41.9 Ci/mmol, 1 mCi/mL) was purchased from Du Pont-New England Nuclear (Boston, MA). 11-cis-RAL was a gift from Hoffman La-Roche, and 11-*cis*-ROL was produced by reduction of the RAL congener with $NaBH_4$. 9-*cis*-RAL, 13-*cis*-RAL, *all-trans*-RAL, and *all-trans*-ROL were purchased from Sigma Chemical Co. (St. Louis, MO). A mixture of nonradioactive retinoid standards was prepared in the following way. *all-trans*-RAL was photoisomerized for 2 h in methanol (4 °C) 6 in. away from two 15-W fluorescent bulbs. 9-*cis*-, 11-*cis*-, 13-*cis*-, and *all-trans*-RAL were subsequently reduced with $NaBH_4$ in order to produce the corresponding ROL isomers. Retinyl palmitate isomers were prepared from the ROLs by standard methods (Bridges & Alvarez, 1982) using palmitoyl chloride (Sigma Chemical Co.). All experiments utilizing retinoids were performed in the dark in the presence of Sylvania (Minneapolis, MN) gold fluorescent F40/GO lamps (Landers & Olson, 1986), unless stated otherwise.

Preparation of Retinoid-Binding Proteins. Serum retinol-binding protein (RBP) was purified by transthyretin (TTR) affinity chromatography by following the method of Vahlquist et al. (1971) as modified by Shingleton et al. (1989). Cellular retinaldehyde-binding protein (CRALBP) and IRBP were purified from frozen, dark-adapted, adult bovine retinas (J. Lawson, Lincoln, NB) following the method of Saari and Bredberg (1988). The purity of each of the binding protein preparations was determined by SDS–PAGE using the Laemmli (1970) buffer system and by western blot analysis (Towbin et al., 1979).

Apo-CRALBP was produced by modification of a method by Saari et al. (1984). Purified CRALBP was bleached for 10 min at 4 °C, 1 in. from a fiberoptic light source, and then the sample was passed over a 200-uL bovine serum albumin (BSA)–agarose column (Sigma Chemical Co.) in order to remove the free retinoid. Apo-RBP was produced by bleaching the holoprotein at 328 nm in a Farrand Mark 1 spectrofluorometer (Valhalla, NY) for 3 h at 25 °C. IRBP was stripped of its ligand during Con A chromatography (Adler & Evans, 1985). The conversion of each of the holoproteins to the corresponding apoprotein was confirmed spectrophotometrically by the reduction of the absorbance ratios 425:280 nm, 328:280 nm, and 330:280 nm for CRALBP, RBP and IRBP, respectively. BSA was purchased fatty acid-free from Sigma Chemical Co.

Circular Dichroism (CD). CD spectra between 250 and 600 nm were recorded from a J-600 circular dichroism spectropolarimeter (Jasco Inc., Easton, MD) interfaced with a PC computer. Spectra were recorded at 25 °C, using a 1-cm quartz cuvette with a scan speed of 100 nm/min, a sensitivity of 5 mdeg, a time constant of 0.5 s, and a spectral band width of 1.0 nm. A baseline spectrum was obtained with Tris buffer (25 mM Tris acetate, pH 7.5–0.1 mM dithiothreitol) to which the spectra were compared. 11-*cis*-RAL (2 μL) in ethanol (2.8 mM) was added to 1 mL of CRALBP (2.8 μM, Tris buffer) in order to ensure saturation of the protein with the retinoid. Saturation was determined by the inability to detect an increase in the CD signal with continued addition of the retinoid. Holo-CRALBP was then bleached, as described above, producing the apoprotein. 11-*cis*-RAL was added to apo-CRALBP in order to regenerate the holoprotein.

The stability of apo-CRALBP following 16 h at 37 °C was examined as follows. The CD spectrum of a 1-mL sample of holo-CRALBP (9 μM in 20 mM Tris acetate, pH 7.5) was obtained and the sample was bleached as described above. Apo-CRALBP was divided into two Centricon-10 microconcentrators (Amicon, Danvers, MA) and dialyzed against Eagle's minimal essential medium (with Earle's salts and without L-glutamine or phenol red; Gibco Laboratories, Grand Island, NY). Following dialysis, the samples were concentrated in the Centricons to the original 1-mL volume. Two RPE monolayers each received 500 μL of apo-CRALBP on their apical surface and an equivalent volume of 0.2 μM holo-RBP on their basal surface, and the cells were then incubated in the dark for 16 h at 37 °C. Postincubation, the apical medium from each chamber was dialyzed in separate Centricons against 20 mM Tris acetate, pH 7.5, and these were concentrated to a final total volume of 1 mL. CD spectra of this sample were obtained before and after two additions of 1 μL of 11-*cis*-RAL in ethanol (4 mM).

Fetal Bovine RPE Incubations. (*a*) *Preparation of the [^{3}H]Holo-RBP Complex.* [11,12-^{3}H]-*all-trans*-ROL (200 μCi) in ethanol (4.8×10^{-3} μmol) was dried under a stream of nitrogen and resuspended in 50 μL of ethanol. [^{3}H]Holo-RBP was then regenerated by adding the [^{3}H]-*all-trans*-ROL to 2 mL of apo-RBP (5.2×10^{-2} μmol) which had been dialyzed against phosphate-buffered saline. [^{3}H]Holo-RBP was separated from apo-RBP by TTR affinity chromatography, as described above. The holoprotein was identified by detecting the eluted peak of radioactivity with a Packard scintillation counter. These fractions were pooled and stored at −20 °C until needed.

(*b*) *Incubation Conditions.* The Millicell chambers selected for the experiments were confluent and had high transepithelial resistances (300–600 Ω cm^2). The apical and basal

surfaces of the cells were washed three times with NGM in the absence of added calf serum. [^{3}H]Holo-RBP (0.2 μM) in NGM without calf serum (300 μL) was then added to the basal compartment of each chamber. This compartment, therefore, approximated the vascular supply in vivo. The apical compartment, which represented the IPM, contained 200 μL of either 3.0 μM apo-IRBP; 0.3, 1.0, or 3.0 μM apo-CRALBP; 3.0 μM apo-RBP; or 90 μM BSA (Okajima et al., 1989) in NGM without calf serum or 200 μL of medium in the absence of binding proteins. In other experiments, the basal medium contained 0.2 μM [^{3}H]holo-RBP–TTR while the apical medium contained 3 μM apo-IRBP. The cells were incubated in the dark for 16 h at 37 °C in an atmosphere of 5% CO_2–95% air. Triplicate dishes were analyzed for each of the binding proteins studied.

(*c*) *Extraction and Analysis of* [^{3}*H*]*Retinoids*. Postincubation, the apical and basal media were collected, the monolayer was washed twice with phosphate-buffered saline, and the cells were lysed by osmotic shock with 3 × 300 μL of H_2O. The retinoids in the media and the cell lysates were extracted three times with organic solvents by following the Suzuki (1986) method with the following modifications: the samples were mixed with (1) an equal volume of 6 M formaldehyde (0.1 M phosphate buffer, pH 7.5) instead of 100 mL and (2) 50 μL of a nonradioactive retinoid mixture (prepared as described above), which served as an internal standard. The extracts of each sample were maintained at 4 °C, then pooled, dried under a stream of nitrogen, and redissolved in 3 mL of hexane. These samples were then dehydrated with sodium sulfate, filtered through an Acrodisc LC13 PVDF filter (Gelman Sciences, Ann Arbor, MI), dried under nitrogen, and finally redissolved in 250 μL of hexane and 0.1 mg/mL butylated hydroxytoluene.

The media and cell lysate retinoid extracts were analyzed with a Dynamax-60A column (8-μm silica, 25-cm bed; Rainin Instrument Co., Inc., Emeryville, CA) by normal-phase HPLC using a Vista 5500 HPLC (Varian, Walnut Creek, CA) linked to a β-RAM 1B (IN/US Systems Inc.; Fairfield, NJ) in-line liquid scintillation counter. The retinoids were separated by isocratic elution at 1 mL/min using the following mixtures: 4% dioxane in *n*-hexane (RALs), 8% dioxane in *n*-hexane (ROLs), and 0.5% ethyl ether in *n*-hexane (retinyl esters). The identity of each [^{3}H]retinoid peak was verified by comparing its retention time with that of the identical nonradioactive retinoid standard which was eluted simultaneously and detected at 326 nm with an in-line Varian UV200 UV detector. A Hyundai Super 286E computer (San Jose, CA), using β–RAM software, quantitated the [^{3}H]retinoid peaks by integrating the counts per minute (cpm) recorded for each peak.

Statistics. All statistics of the data were performed using StatView II software (Abacus Concepts, Inc., Berkeley, CA) for the Macintosh computer. Results were statistically significant when unpaired, two-tailed *t*-tests demonstrated that $P < 0.05$.

RESULTS

Cultured Fetal Bovine RPE. The fetal bovine RPE grown on the millicell porous supports were well differentiated as demonstrated by a number of morphological characteristics. The electron micrograph in Figure 1 shows a cultured fetal bovine RPE cell with its basal surface adjacent to the millicell membrane upon which it has deposited extracellular matrix proteins. The cell displays characteristic basal infoldings, basally polarized mitochondria, apically polarized premelanosomes and melanosomes, and short apical processes. The transepithelial resistances of the confluent monolayers were between 300 and 600 Ω cm^2.

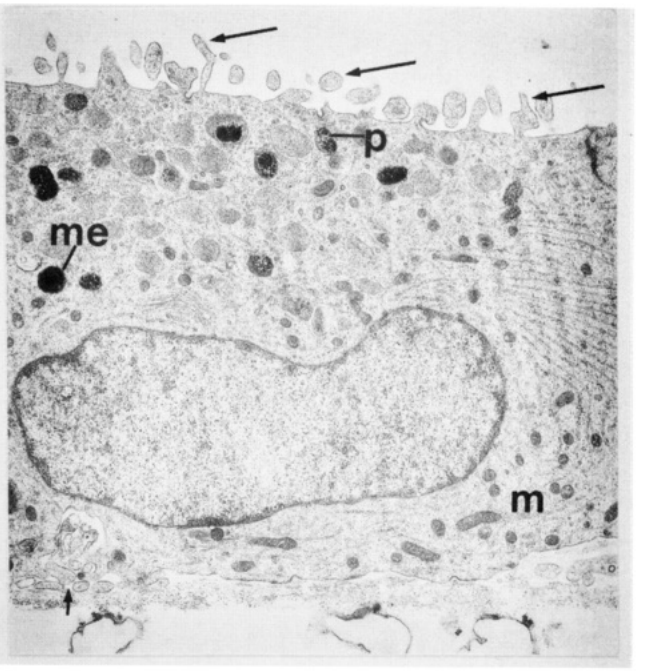

FIGURE 1: Electron micrograph of a cultured fetal bovine RPE cell grown on a porous support film. The normal morphology of the cell is characterized by apically polarized premelanosomes (p) and melanosomes (me), basally polarized mitochondria (m), short apical processes (long arrows), and basal infoldings (short arrow). Pores in the support film can be observed at the bottom of the figure. Magnification, 7200×.

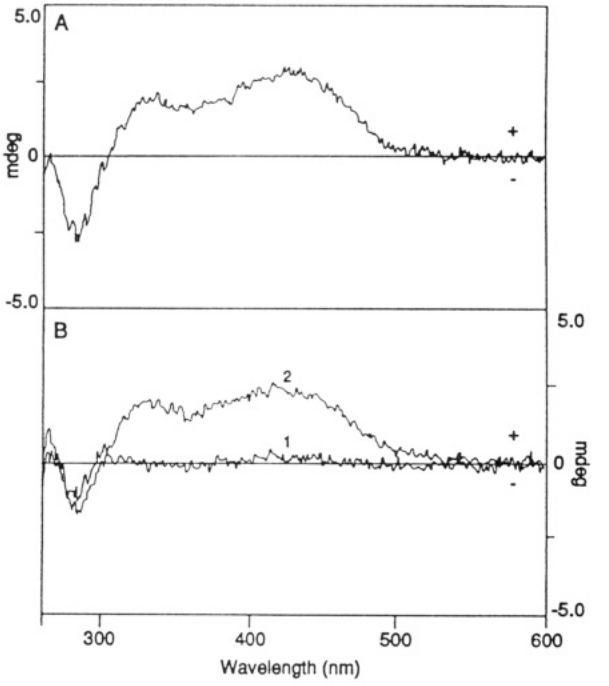

FIGURE 2: CD spectra of apo- and holo-CRALBP: (A) CRALBP saturated with 11-*cis*-RAL. (B) Bleaching holo-CRALBP produces the apoprotein (1), which does not emit a CD signal. Addition of 11-*cis*-RAL to the apoprotein regenerates holo-CRALBP (2).

Circular Dichroism of CRALBP. 11-*cis*-RAL bound to CRALBP displayed a bimodal spectrum with peaks of optical activity at 325 and 425 nm (Figure 2A). CRALBP purified from the frozen, dark-adapted bovine retinas was determined to be essentially saturated with 11-*cis*-RAL by CD spectral

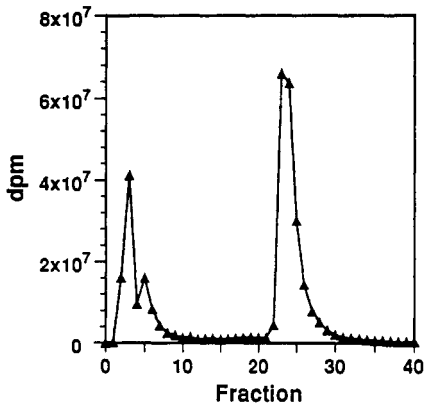

FIGURE 3: TTR affinity column purification of [^{3}H]holo-RBP. [^{3}H]-holo-RBP (peak at right) was eluted from the TTR affinity column with H_2O. The peak at the left represents [^{3}H]-*all-trans*-ROL or [^{3}H]holo-RBP which was unable to bind TTR and therefore passed through the column uninhibited. See Materials and Methods for experimental details.

analysis since the addition of 11-*cis*-RAL in ethanol resulted in only a slight increase of the CD signal (not shown). Illumination of the sample to produce the apoprotein resulted in a baseline CD signal (Figure 2B, spectrum 1).

In order to determine that CRALBP was not damaged by the bleaching procedure, the holoprotein was reconstituted by the addition of 11-*cis*-RAL to the apo-CRALBP preparation. This was verified by the return of a bimodal spectrum which displayed only a slight decrease in the CD signal (Figure 2B, spectrum 2) as compared to that obtained prior to bleaching (Figure 2A).

CD analysis of apo-CRALBP recovered following its incubation with an RPE monolayer at 37 °C for 16 h demonstrated a normal CD signal with peak maxima at 325 and 425 nm. Although the signal intensity was 20% of the original holo-CRALBP CD spectrum, there was a comparable decrease in the amount of CRALBP in the sample, as verified by SDS–PAGE, which was a result of the extensive manipulation of the sample, including multiple dialyses and concentrations (not shown).

[^{3}H]Holo RBP. Figure 3 demonstrates the [^{3}H]holo-RBP which was purified from apo-RBP by TTR affinity chromatography. The effluent contained either free [^{3}H]-*all-trans*-ROL or [^{3}H]holo-RBP. The latter may represent RBP which was damaged during bleaching such that it retained the ability to bind the retinoid but was unable to bind TTR. The eluted peak contained the [^{3}H]holo-RBP, which was used in the subsequent incubation experiments.

HPLC Analysis of [^{3}H]Retinoids in the Media and Intracellularly. (a) Effect of 11-cis-RAL-Binding Proteins in the Apical Medium. Following the 16-h incubation, the [^{3}H]retinoids in the media and in the cell lysates were extracted and analyzed by HPLC as described above. From these analyses it was determined that on average 62 ± 7% of the [^{3}H]-*all-trans*-ROL remained in the basal medium, whereas the RPE contained 17 ± 8% of the recovered radioactivity. The amount of radioactivity recovered in the apical medium varied widely, depending on the binding protein present in the apical medium during the incubation (21 ± 11%).

The HPLC traces in Figure 4 represent the [^{3}H]retinoids found in the apical medium after the cells had been incubated for 16 h with 0.2 μM [^{3}H]holo-RBP basally and either 3 μM apo-IRBP, 3 μM apo-CRALBP, 3 μM apo-RBP or 90 μM BSA apically or no binding protein (NBP) in the apical medium. In the presence of IRBP, the cells synthesized and

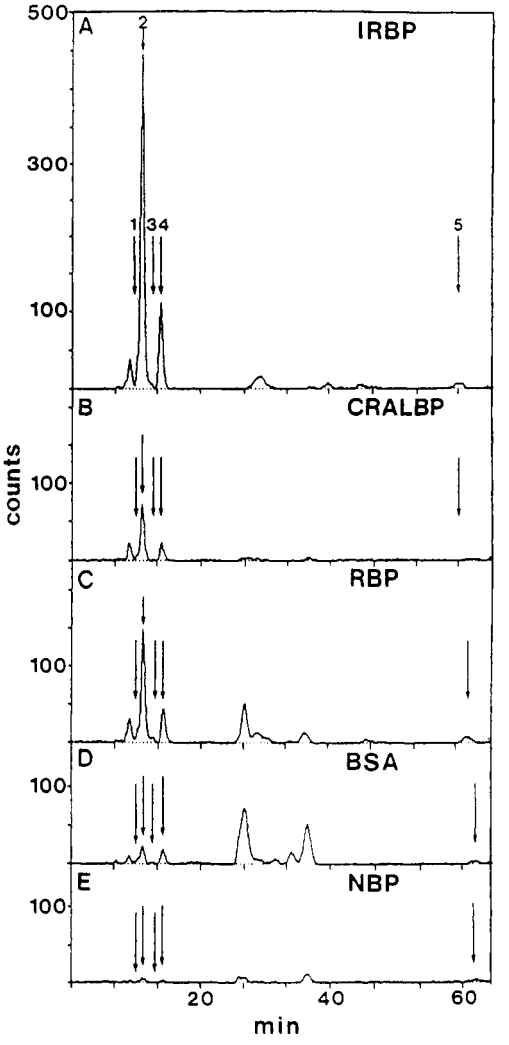

FIGURE 4: Normal-phase HPLC separation of [^{3}H]retinoids released from fetal bovine RPE into the apical medium. The basal surface of the fetal bovine RPE was incubated in the dark, at 37 °C, for 16 h in the presence of 0.2 μM [^{3}H]holo-RBP. Throughout the incubation period the apical medium contained either (A) 3 μM apo-IRBP, (B) 3 μM apo-CRALBP (C) 3 μM apo-RBP, (D) 90 μM BSA, or (E) NBP. [^{3}H]Retinoids in the apical medium were extracted and analyzed by HPLC. [^{3}H]Retinoids were eluted isocratically with 4% dioxane in *n*-hexane at 1 mL/min. Standard elution of the retinoids was the following: 1, 13-*cis*-RAL; 2, 11-*cis*-RAL; 3, 9-*cis*-RAL; 4, *all-trans*-RAL; 5, 9-*cis*- and *all-trans*-ROL.

released the most [^{3}H]-11-*cis*-RAL (22 ± 1% of the total recovered cpm, Figures 4A and 5A). On the other hand, when the cells were incubated overnight with apo-CRALBP, apo-RBP, or BSA or when they were incubated in the absence of binding proteins (Figure 4B–E, respectively), the level of [^{3}H]-11-*cis*-RAL found in the apical medium was significantly reduced to 5 ± 1%, 9 ± 1%, 2 ± 1%, and 0%, respectively (Figure 5A, $P < 0.05$). The apical medium in each case also contained a small amount of [^{3}H]-*all-trans* RAL which was significantly higher in the presence of IRBP (Figure 4A–E and Figure 5A, 6 ± 1%, 2 ± 1%, 3 ± 1%, 1 ± 0% and 0%, respectively, $P < 0.05$). Occasionally, a minor amount of [^{3}H]-*all-trans*-ROL was detectable in the apical medium (Figure 4).

The binding protein present during the incubation period affected not only the level of the [^{3}H]-11-*cis*-RAL apically but the level of other extracted [^{3}H]retinoids as well. More [^{3}H]-*all-trans*-ROL was retained in the basal medium when the cells were incubated with apo-CRALBP (61 ± 4%), apo-RBP (57 ± 2%), BSA (57 ± 3%) or medium alone (61 ± 4%)

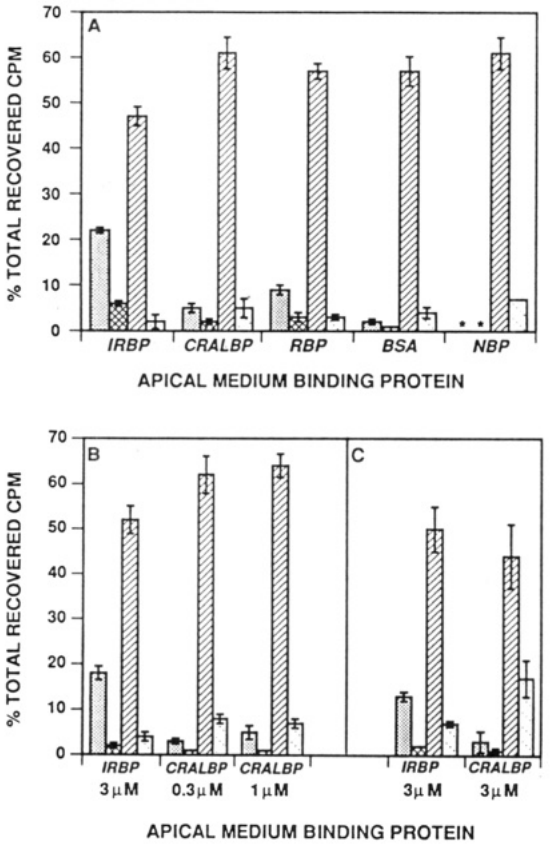

FIGURE 5: Distribution of [^{3}H]retinoids. HPLC analysis determined the identity of [^{3}H]retinoids present intracellularly and in the media, following incubation of the RPE with the following apical medium binding proteins: (A) 3 μM apo-IRBP, 3 μM apo-CRALBP, 3 μM apo-RBP, 90 μM BSA, and NBP. (B) 3 μM apo-IRBP and 0.3 and 1 μM apo-CRALBP. (C) 3 μm apo-IRBP and 3 μM apo-CRALBP. A–C are the results of three separate experiments. The error bars represent the standard deviation for $n = 3$. (▦) apical [^{3}H]-11-*cis*-RAL, (▩) apical [^{3}H]-*all-trans*-RAL, (▨) basal [^{3}H]-*all-trans*-ROL, (▧) intracellular [^{3}H]-*all-trans*-retinyl palmitate. Asterisk indicates that data not detectable.

than when they were incubated with IRBP (47 ± 2%, $P < 0.05$; Figure 5A). In addition, the RPE incubated under these conditions had elevated levels of intracellular [^{3}H]-*all-trans*-retinyl palmitate (5 ± 2%, 3 ± 1%, 4 ± 1%, and 7 ± 0% versus 2 ± 2% for IRBP; Figure 5A). Other experiments compared apo-IRBP with apo-CRALBP only. These results demonstrated elevated levels of basal [^{3}H]-*all-trans*-ROL (64 ± 3% and 62 ± 4% versus 52 ± 3%) and intracellular [^{3}H]-*all-trans*-retinyl palmitate (7 ± 1% and 8 ± 1% versus 4 ± 1%, $P < 0.05$; Figure 5B) or that only the level of [^{3}H]-*all-trans*-retinyl palmitate was elevated (17 ± 4% versus 7 ± 1%, $P < 0.05$, Figure 5C) in the presence of apo-CRALBP. The decrease in basal [^{3}H]-*all-trans*-ROL was not statistically significant. Varying the concentration of apo-CRALBP in the apical medium did not affect the results (Figure 5B and C). On the other hand, the levels of [^{3}H]-13-*cis*-ROL in the basal medium as well as apical [^{3}H]-*all-trans*-ROL and intracellular [^{3}H]-11-*cis*-RAL, [^{3}H]-*all-trans*-ROL, [^{3}H]-*all-trans*-RAL, and [^{3}H]-13-*cis*-retinyl palmitate did not vary among the incubation conditions (not shown).

(*b*) *Effect of the [^{3}H]-all-trans-ROL Vehicle.* [^{3}H]-*all-trans*-ROL was also delivered to the basal surface of the RPE by the RBP–TTR complex, instead of RBP. In this experiment, apo-IRBP was again in the apical medium in order to bind the [^{3}H]-11-*cis*-RAL released by the cells. HPLC analysis demonstrated that the distribution of [^{3}H]retinoids in the media and intracellularly was the same when RBP and

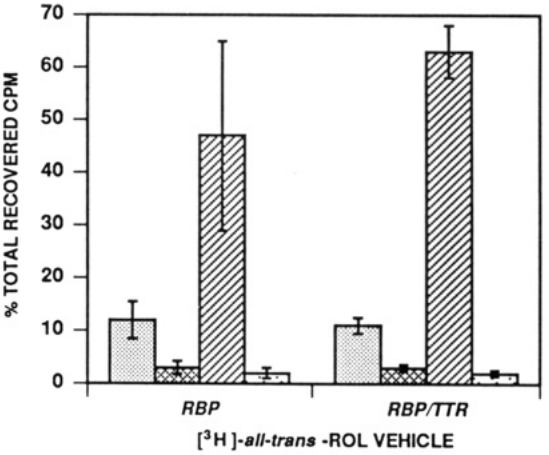

FIGURE 6: Effect of [^{3}H]-*all-trans*-ROL vehicle on [^{3}H]retinoid distribution. The RPE was incubated with 3 μM apo-IRBP apically and either 0.2 μM [^{3}H]holo-RBP or 0.2 μM [^{3}H]holo-RBP–TTR basally. (▦) apical [^{3}H]-11-*cis*-RAL, (▩) apical [^{3}H]-*all-trans*-RAL, (▨) basal [^{3}H]-*all-trans*-ROL, (▧) intracellular [^{3}H]-*all-trans*-retinyl palmitate.

RBP–TTR were the ROL vehicles ($P > 0.05$; Figure 6). As in the experiments described in (a), the amounts of apical [^{3}H]-*all-trans*-ROL, intracellular [^{3}H]-11-*cis*-RAL, [^{3}H]-*all-trans*-RAL, [^{3}H]-*all-trans*-ROL, [^{3}H]-13-*cis*-retinyl palmitate, and basal [^{3}H]-13-*cis*-ROL did not vary when either RBP or RBP–TTR was the vehicle.

DISCUSSION

The cultured fetal bovine RPE cells of the type used for this study were highly differentiated by virtue of their morphology and pigmentation, polarized water flow (formation of domes on plastic), distribution of membrane proteins (Mircheff et al., 1990), and polarized viral budding (Bok et al., 1992). For the current study these cells were incubated with [^{3}H]holo-RBP basally and apo-IRBP apically in order to mimic the in vivo situation. Postincubation, the apical medium was found to contain [^{3}H]-11-*cis*-RAL, as well as significantly lower amounts of [^{3}H]-*all-trans*-RAL and [^{3}H]-*all-trans*-ROL. It had been demonstrated previously, by our laboratory, that fetal human RPE cells grown on plastic dishes synthesize 11-*cis*-RAL and release it into the culture medium (Flannery et al., 1990). In the current study, the presence of [^{3}H]-11-*cis*-RAL in the apical medium, but not in the basal medium, demonstrated that the cells synthesized [^{3}H]-11-*cis*-RAL and released it in a polarized fashion. These results, together with the morphology of the RPE cells and their high resistances, provide further evidence that our cultured RPE cells are both highly differentiated and polarized.

The small level of [^{3}H]-*all-trans*-RAL in the apical medium probably resulted from the thermal isomerization of the [^{3}H]-11-*cis*-RAL whereas the [^{3}H]-*all-trans*-ROL, bound by RBP, may have been translocated by RPE transcytosis from the basal medium (unpublished results). It is unlikely that the presence of [^{3}H]-*all-trans*-ROL in the apical medium is the result of leakage between the cells comprising the monolayer, since we would have expected every major [^{3}H]retinoid in the apical and basal media to equilibrate across the monolayer during the 16-h incubation. In addition, the transepithelial resistance of each of the chambers was too high to suggest that there was a flaw in the integrity of the monolayer.

Since the RPE released [^{3}H]-11-*cis*-RAL into the apical medium in the presence of IRBP, we questioned whether this event required IRBP specifically or whether the retinoid was

constitutively released from the cell as soon as it was produced. In order to answer this question, we incubated the RPE with apical medium which was devoid of binding proteins or which contained one of a number of apoproteins, other than apo-IRBP. These proteins included apo-CRALBP, apo-RBP, and fatty acid-free BSA, none of which are native to the IPM but can all bind retinoids, including 11-*cis*-RAL, to varying degrees (Futterman et al., 1977; Horwitz & Heller, 1973). By HPLC analysis it was determined that if the RPE was incubated with a protein other than apo-IRBP, or if the medium was devoid of binding proteins, the level of [^{3}H]-11-*cis*-RAL detected apically was reduced by 3–11-fold.

The absence of [^{3}H]-11-*cis*-RAL when the RPE was incubated with medium alone was not surprising, even in the event of constitutive 11-*cis*-RAL release, due to the high probability of retinoid oxidation in an aqueous environment (Futterman & Heller, 1972; Ho et al., 1989). Nor was the diminished level of [^{3}H]-11-*cis*-RAL in the presence of apo-RBP (~3-fold) surprising since it could be explained by a low affinity of apo-RBP for 11-*cis*-RAL. Okajima et al. (1989) estimated that 30-fold more BSA (90 μM), relative to IRBP (3 μM), was necessary in order to obtain comparable levels of [^{3}H]retinol associated with each protein. Surprisingly, a diminished level of [^{3}H]-11-*cis*-RAL (11-fold) was detected in the apical medium, despite the presence of 90 μM BSA. The greatest surprise was the reduced level of [^{3}H]-11-*cis*-RAL when the cells were incubated with apo-CRALBP overnight (~6 fold). CRALBP is the natural binding protein for 11-*cis*-RAL in the RPE (Futterman et al., 1977), and it has a high affinity for this retinoid (Livrea et al., 1987). In addition, it is highly stereoselective for 11-*cis*-RAL and protects it well in solution (Saari & Bredberg, 1987).

Saari and Bredberg (1988) have speculated that bleaching holo-CRALBP isomerizes 11-*cis*-RAL into *all-trans*-RAL, the latter having a deleterious effect on the protein. We were concerned, therefore, that CRALBP was denatured during the bleaching procedure, resulting in its inability to bind the [^{3}H]-11-*cis*-RAL released by the cell. In order to verify that this was not an issue, the ability of apo-CRALBP to be reconstituted into the holo form was monitored by CD.

We prepared apo-CRALBP by loading the protein onto a BSA–agarose column immediately after bleaching. This step effectively removed the free retinoid without contaminating the apo-CRALBP preparation with BSA. The addition of 11-*cis*-RAL to apo-CRALBP produced a CD signal characteristic of the 11-*cis*-RAL–CRALBP complex with peak maxima at 425 and 325 nm, representing holo-CRALBP and the cis absorption band, respectively. This indicated not only that the protein was capable of binding the retinoid but also that bleaching, followed by BSA–agarose chromatography, did not result in a large loss of functional protein. CD analysis also demonstrated that apo-CRALBP survived the conditions of the 16-h incubation. Apo-CRALBP should, therefore, be able to bind [^{3}H]-11-*cis*-RAL released into the apical medium as well as or better than apo-IRBP, if the retinoid was indeed being constitutively released. Since [^{3}H]-11-*cis*-RAL was detected in greatest amounts when IRBP was present, retinoid release was therefore dependent upon the presence of IRBP. However, the low but detectable levels of [^{3}H]-11-*cis*-RAL in the apical medium when IRBP was not present is indicative that there may be a constitutive component of retinoid release.

Since the level of [^{3}H]-11-*cis*-RAL detected in the apical medium was markedly reduced when IRBP was not present, we predicted that there should be a buildup of [^{3}H]retinoid at one or more places in the visual cycle under these conditions. Indeed, when the RPE was incubated with apo-CRALBP, apo-RBP, BSA, or medium alone, the level of [^{3}H]-*all-trans*-ROL retained in the basal medium and/or the level of intracellular [^{3}H]-*all-trans*-retinyl palmitate was greater than when the cells were incubated with IRBP. These increases are not surprising since (1) retinyl palmitate is an intracellular retinoid storage form and (2) the RPE may limit the amount of *all-trans*-ROL it takes up if it is unable to process it to completion and release the end product. Therefore, the observed increases in [^{3}H]retinyl palmitate and [^{3}H]-*all-trans*-ROL, concomitant with the decrease in apical [^{3}H]-11-*cis*-RAL, provide convincing evidence that IRBP is actively involved in the apical release of [^{3}H]-11-*cis*-RAL.

The intracellular levels of [^{3}H]-11-*cis*-RAL and [^{3}H]-*all-trans*-ROL did not vary with the change in apical binding protein. These levels may not change if they represent the amount of [^{3}H]retinoid required to fully saturate CRALBP and CRBP, respectively. In addition, neither [^{3}H]-11-*cis*-ROL nor [^{3}H]-11-*cis*-retinyl palmitate was detected intracellularly. It has been shown that 11-*cis*-ROL, normally bound by CRALBP in the neural retina, exists only in trace amounts in the RPE (Saari et al., 1982). This discrepancy may result from 11-*cis*-ROL's position at a branch point in the visual cycle such that it may either be converted into 11-*cis*-RAL or be stored as 11-*cis*-retinyl palmitate (Saari, 1990). It is possible that [^{3}H]-11-*cis*-retinyl palmitate was present in the RPE early in the incubation but was then metabolized into another retinoid form. Perhaps HPLC analysis of the RPE, extracted at an earlier time point, will show that [^{3}H]-11-*cis*-retinyl palmitate is also an intermediate of the visual cycle whose level is affected by the presence or absence of IRBP.

Since a significant amount of [^{3}H]-*all-trans*-ROL was present in the basal medium postincubation, we questioned whether the [^{3}H]retinoid was effectively delivered to the basal surface of the RPE by RBP. Since the RBP–TTR complex is presumably the physiological *all-trans*-ROL vehicle, we delivered [^{3}H]-*all-trans*-ROL to the basal surface of the RPE via the RBP–TTR complex. The distribution of [^{3}H]retinoids under these conditions was the same as that observed when [^{3}H]-*all-trans*-ROL was delivered by RBP alone. This supports Heller's finding (1975) that TTR is not necessary for the binding of *all-trans*-ROL–[^{125}I]RBP to the cell membranes of intact, isolated RPE.

The most interesting finding of the study is that IRBP seems to induce the RPE to release the greatest amount of [^{3}H]-11-*cis*-RAL. Even when the cells were incubated with CRALBP, the level of [^{3}H]-11-*cis*-RAL detected in the apical medium was much reduced. The ability of IRBP to enhance the extraction of 11-*cis*-RAL from the RPE is logical given its strategic location in the IPM and its putative role in retinoid movement across the IPM. This is in agreement with previous work which demonstrated that IRBP was not only better than BSA, RBP, or buffer at delivering [^{3}H]-*all-trans*-ROL to frog RPE eyecups but that IRBP was again the preferred vehicle at extracting [^{3}H]-11-*cis*-RAL from the RPE eyecups and delivering it to isolated photoreceptors (Okajima et al., 1989; 1990).

The results presented in this study shed some light on the role of IRBP in the visual cycle. It has been shown that IRBP is necessary for obtaining the greatest release of [^{3}H]-11-*cis*-RAL from cultured fetal bovine RPE, and it must, therefore, have a special relationship with the RPE apical membrane. Although there is currently no evidence to prove that an IRBP membrane receptor exists on either the RPE apical surface or in the photoreceptor plasma membrane, it

is conceivable that IRBP needs to interact with some kind of intrinsic membrane protein, perhaps a receptor, in order to communicate to the cell that it is available to bind 11-*cis*-RAL. In addition, it can be imagined that a membrane protein might be necessary to coordinate the delivery of 11-*cis*-RAL to the apical membrane by CRALBP and its uptake extracellularly by IRBP. A similar mechanism can be surmised for the delivery and uptake of *all-trans*-ROL at the RPE apical surface by IRBP and CRBP, respectively.

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