Immunocytochemical Localization of
Two Retinoid-binding Proteins in Vertebrate Retina

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ABSTRACT   The recent discovery and characterization of several proteins that purify with endogenous, bound retinoid have given rise to the suggestion that these proteins, which are abundant in retina, perform a role in transport and function of vitamin A. Immunocytochemical techniques were used to localize two retinoid-binding proteins in the retina of four species. Antisera to cellular retinal-binding protein (CRALBP) and an interphotoreceptor retinoid-binding protein (IRBP) were obtained from rabbits immunized with antigens purified from bovine retina. Antibodies from each antiserum reacted with a single component in retinal homogenates and supernatants which corresponded to the molecular weight and charge of the respective antigen (non-SDS and SDS PAGE, electrophoretic transfer to nitrocellulose, immunochemical staining). Immunocytochemistry controls were antibodies from nonimmune serum and antibodies absorbed with purified antigen. Antigens were localized on frozen-sectioned bovine, rat, monkey, and human retina using immunofluorescence and the peroxidase-antiperoxidase technique. Specific staining with anti-IRBP was found in the space that surrounds photoreceptor outer segments, with heaviest labeling in a line corresponding to the retinal pigment epithelium (RPE) apical surface. Cone outer segments were positive. Staining with anti-CRALBP was found in two cell types in all species: the RPE and the Müller glial cell. Within the RPE, labeling filled the cytoplasm and was heaviest apically, with negative nuclei. Labeling of Müller cells produced Golgi-like silhouettes with intense staining of all cytoplasmic compartments. Staining of the external limiting membrane was heavy, with labeled microvilli projecting into the interphotoreceptor space. Localization of IRBP to this space bordered by three cell types (RPE, photoreceptor, and Müller) is consistent with its proposed role in transport of retinoids among cells. Localization of CRALBP in RPE corroborates previous biochemical studies; its presence in the Müller cell suggests that this glial cell may play a hitherto unsuspected role in vitamin A metabolism in retina.

The low solubility of retinol leads to its nonspecific interaction with surfaces, lipids, and apolar regions of proteins. Transport of vitamin A to target cells is accomplished by serum retinol-binding protein which serves to solubilize and enhance the stability of the vitamin and to prevent its nonspecific absorption (22). Vitamin A appears to be delivered to only cells with surface receptors for serum retinol-binding protein (3, 18, 33). The problems associated with insolubility, as well as toxicity of free vitamin A, do not end with its delivery to a target cell by serum retinol-binding protein. In the eye, for example, the vitamin must be transported repeatedly between the retinal pigment epithelium (RPE[1]) and photoreceptors, depending on the amount of visual pigment bleached (4, 10, 48). In

addition, vitamin A must undergo enzymatic processing, including oxidation/reduction, esterification, de-esterification, and geometrical isomerization (5, 9). The recent discovery and characterization of several retinoid-binding proteins from retina suggest that they may be involved in these processes, perhaps as transport proteins or enzyme substrate carrier proteins (5, 9, 36, 38).

In addition to the photopigment rhodopsin, three cellular proteins have been purified from bovine retina and shown to carry endogenous retinoid: cellular retinol-binding protein, cellular retinoic acid-binding protein, and cellular retinal-binding protein (CRALBP) (37). Recently several laboratories have described the existence of an extracellular protein capa-

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ble of binding retinoids as well as other hydrophobic substances (1, 2, 5, 9, 24, 28, 29). This protein has been termed interstitial retinol-binding protein (5), interphotoreceptor retinol-binding protein (9), 7S protein (9), and 290,000-dalton retinol-binding protein (1). We have independently isolated a protein that appears to be identical to those described by the other laboratories. The term we employ for this protein, interphotoreceptor retinoid-binding protein (IRBP), refers to its extracellular localization in the retina and its ability to bind a number of vitamin A derivatives (retinoids). Cellular retinol-binding protein and cellular retinoic acid-binding protein have been isolated from non-ocular tissues as well as retina and may be involved in systemic functions of vitamin A (31, 32, 34). In contrast, CRALBP and IRBP have been found only in extracts of retina and/or RPE (15). This restricted localization of CRALBP and IRBP to the retina-RPE complex suggests that they may subserve functions of vitamin A specific to its role in vision.

Localization of these proteins to specific cell types within the retina would aid considerably in understanding their roles in metabolism and function of vitamin A. Some information has been obtained from biochemical studies: CRALBP has been purified from both RPE and retina and found to carry different endogenous retinoids in these two tissues (37); it is absent in isolated outer segments. IRBP was initially obtained from buffer washes of intact retinas, suggesting that this protein might be found in an extracellular compartment of the retina, probably the interphotoreceptor matrix (1, 2, 5, 28, 29). In this report we describe the immunocytochemical localization of these two proteins, CRALBP and IRBP, in the retina of four vertebrates.

MATERIALS AND METHODS

Preparation of Binding Proteins: CRALBP from bovine retina and RPE was purified as described previously (35). An additional high performance liquid chromatography (HPLC)-anion exchange step was included in some preparations. Fractions containing CRALBP from a hydroxylapatite column (35) were pooled, concentrated, dialyzed against 20 mM Tris, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol and applied to a Synchropak AX-300 anion exchange column (0.3 × 25 cm; SynChrom, Inc., Linden, IN) equilibrated in the same buffer. After washing the column with equilibration buffer for 2-3 column volumes (flow rate 1 ml/min), CRALBP was eluted from the column by linearly increasing the concentration of NaCl from 0 to 1 M over 30 min. Fractions containing CRALBP were collected, pooled, concentrated, and stored at −80°C. IRBP was purified from bovine retinal washes according to the procedure of Adler and Klucznik (2) or from retinal homogenates by a combination of gel filtration, ion exchange, and affinity chromatography.

Purification of CRALBP by Reverse-phase HPLC: Samples of purified CRALBP were analyzed by reverse-phase HPLC on a Vydac C₄ column (The Separations Group, Hesperia, CA) (5-μm particle size, 0.3 × 25 cm). The column was equilibrated in 0.1% trifluoroacetic acid in H₂O with a flow rate of 1 ml/min. Samples of CRALBP (90 μg in 250 μl) in 20 mM Tris, pH 7.5, were injected onto the column which was developed with a series of linear gradients of acetonitrile—0.01% trifluoroacetic acid, 0–45% in 1 min, 45–75% in 30 min, and 75–100% in 5 min (Fig. 1). In preparative experiments, fractions corresponding to CRALBP were collected and dialyzed immediately against PBS. The resulting suspension of precipitated CRALBP was added to samples of anti-CRALBP in control experiments to give a concentration of 25 μg/ml. Following an incubation of 3–6 h the absorbed antibody was used for immunocytochemistry.

Preparation, Detection, and Purification of Antibodies to Retinoid-binding Proteins: Rabbits (New Zealand white females ∼10 wk old) were immunized in groups of three following the general procedure of Hurn and Chantler (21). Each animal received 80–100 μg of purified binding protein in PBS emulsified with Freund's complete adjuvant fortified with 5 mg/ml *Mycobacterium tuberculii* (1 ml of emulsion, distributed intramuscularly in the hind limbs). After 4 wk a second injection of antigen in Freund's complete adjuvant was given. Samples of blood were obtained 10 d after this boost. Antibodies were detected using an enzyme-linked immunosorbent assay
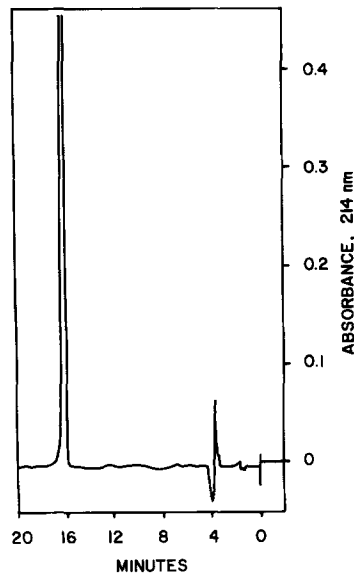

FIGURE 1   Reverse-phase HPLC of cellular retinal-binding protein. A sample of purified binding protein (250 μl, 90 μg, in 10 mM Tris, pH 7.5) was injected onto a reverse-phase C₄ column. Conditions of elution are described in the text.

procedure based on that given by Engvall (11). The following solutions were added to the wells of 96-well microtiter plates and incubated for the indicated times (all steps at room temperature except where indicated): antigen, 200 μl, 5 μg/ml in 0.1 M NaHCO₃, 3 h, 37°C; wash solution three times (PBS, 0.05% Tween-20); serial dilutions of antiserum, 200 μl, 3 h; wash solution three times; anti-rabbit IgG-alkaline phosphatase conjugate (Cappell Laboratories, Cochranville, PA) (1:500 dilution) 50 μl, 3 h; wash solution three times; 200 μl 1 mg/ml *p*-nitrophenylphosphate in 1 M diethanolamine, pH 8.9, 15 min; 50 μl 2 M NaOH, 0.75 ml H₂O. Color was determined spectrophotometrically at 405 nm. Antisera giving >50% maximum color response at >5,000-fold dilution were used as sources of the IgG fractions employed in this study. The immunoglobulin fraction was precipitated from serum with ammonium sulfate and an IgG fraction was isolated using Protein A Sepharose (19), concentrated to A_{280} ∼2, and stored frozen at −20°C.

Electrophoresis and Electrotransfer to Nitrocellulose: PAGE systems employed were those of Fairbanks et al. (12), with or without SDS, Laemmli (23) and Weber and Osborn (46). CRALBP was charged with 11-*cis*-retinal as previously described (36), and analyzed by non-SDS PAGE under deep red illumination (ruby red bulbs, Westinghouse Electric Corp., Pittsburg, PA) to prevent photoisomerization of bound chromophore. Conditions for transfer of proteins from gels to nitrocellulose sheets were those described by Towbin et al. (44) as modified by Burnette (8). Transferred antigen was demonstrated using the peroxidase-antiperoxidase technique (PAP) (W. Stahl, University of Washington, personal communication). The nitrocellulose sheets were soaked in the following solutions at room temperature for the indicated times: 0.015 M Tris, pH 7.4, 0.15 M NaCl, 1% BSA, 1 h; specific IgG fraction diluted 1:200, 1 h; wash (see below); goat anti-rabbit IgG (Cappell Laboratories), 1:1000, 1 h; wash; rabbit PAP (Cappell Laboratories) 1:500, 30 min; wash; 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO), 0.5 mg/ml in Tris NaCl, 0.025% H₂O₂ 3–5 min. The latter reagent was made fresh at 3 mg/ml in methanol and diluted with 5 vol of Tris-NaCl. The wash procedure consisted of Tris-NaCl, 10 min; Tris-NaCl-0.05% NP-40, 20 min, two times; Tris-NaCl, 10 min. Antibodies and PAP were diluted in 0.015 M Tris, pH 7.4, 0.15 M NaCl, 1% BSA. Transfer of binding proteins to nitrocellulose was quantitative in 1 h at 0.8–1.2 A as judged by staining of the gel with Coomassie Blue after transfer.

Fixation of Tissue: Bovine and monkey (*Macaca fascicularis*) retinas were obtained immediately after death; human retinas were obtained from surgically enucleated eyes; albino rat (Sprague-Dawley) eyes were enucleated after intracardiac perfusion of fixative. Only the tapetal region of the bovine retina was used for these studies so that melanin granules in the RPE would not interfere with the PAP staining. Eye cups were fixed for 2–12 h at room temperature in one of the following solutions: 4% methanol-free formaldehyde (prepared from paraformaldehyde) in 0.13 M phosphate buffer (pH 7.4), 4% formaldehyde and 0.1% glutaraldehyde in 0.13 M phosphate buffer (pH 7.4), 2% formaldehyde in 0.01 M periodate and 0.75 M lysine in 0.073 M phosphate buffer (pH 6.2) (30) or 2% formaldehyde and 0.2% picric acid in 0.13 M phosphate buffer (pH 7.4; 4 h fixation only; [41]). Retinas were dissected, sunk overnight in 30% sucrose in 0.13 M phosphate buffer, frozen-sectioned at 20- or 40-μm thickness and collected in 10% sucrose in 0.13 M phosphate buffer.

Immunofluorescence: 20-μm sections were mounted on chrome alum-gelatin coated slides and air-dried overnight at room temperature. Plastic rings (0.75-cm diam) were mounted around the sections using fingernail polish

to form incubation wells. The sections were treated for 10 min at room temperature with 1% goat serum and 4% BSA in PBS, followed by overnight incubation at 4°C in primary antibody (1:200 in PBS containing 0.3% Triton X-100). Control sections were treated identically with either an IgG fraction from nonimmune or preimmune rabbit serum or specific antibody absorbed with purified antigen (concentration 10–25 μg/ml IRBP or CRALBP). Sections were washed twice (15 min each) with PBS at room temperature and incubated for 30 min at room temperature in the dark in goat anti-rabbit IgG-fluorescein isothiocyanate (Cappell Laboratories), diluted 1:50 in PBS with 0.3% Triton X-100. After two 15 min washes in phosphate buffer, the plastic rings were removed and the sections were coverslipped with 80% glycerol in 0.13 M phosphate buffer containing 5% n-propyl gallate (17). The sections were examined with a Zeiss microscope equipped for epifluorescence.

PAP: 40-μm free floating sections or 20-μm sections mounted on slides as above were incubated overnight at room temperature in primary antibody diluted 1:200 in PBS with 0.3% Triton X-100 and 1% normal sheep serum. Controls consisted of an IgG fraction from nonimmune or preimmune serum and specific antibody absorbed with purified antigen. The sections were washed three times, 1 h each, with 0.2 M Tris buffer (pH 7.4) at room temperature and then incubated in sheep anti-rabbit IgG (Cappell Laboratories) diluted 1:10 in PBS for 30 min at 37°C. The sections were washed three times, 1 h each at room temperature with Tris buffer and then incubated in rabbit PAP (42) diluted 1:50 in PBS for 30 min at 37°C. Following three washes, 1 h each at room temperature with Tris buffer, the sections were reacted with diaminobenzidine and H_2O_2 in Tris buffer, pH 7.4 for 30 min at room temperature, washed 30 min with Tris buffer at room temperature, and some sections were mounted on glass slides in glycerol with 1% formaldehyde. Other sections were postfixed in 1% OsO_4 in 0.13 M phosphate buffer and processed in EPON. Sections were examined by light and electron microscopy with no counterstain. The cytochemical control was treatment of all samples (sections treated initially with immune, preimmune, or antigen-absorbed antibody), with diaminobenzidine solution lacking H_2O_2.

RESULTS

Characterization of Antibodies

CRALBP and IRBP were examined with several SDS- and non-SDS PAGE systems before use as antigens. No evidence was found for heterogeneity. Retinal homogenates and supernatants were prepared and analyzed in duplicate sets by SDS PAGE and non-SDS PAGE. The separated proteins from one half of a gel were electrophoretically transferred to nitrocellulose and analyzed by the PAP technique with specific antibody. The other half of the gel was stained with Coomassie Blue. Fig. 2 illustrates the results obtained with antibody to CRALBP and retinal supernatants. One band was produced by staining with the specific antibody whether the retinal supernatant proteins were separated by size (SDS PAGE, Fig. 2A) or charge and size (non-SDS PAGE, Fig. 2B). An identical result was obtained when retinal homogenates were analyzed by SDS PAGE. This result indicates that anti-CRALBP recognizes a single component in retinal homogenates and supernatants which corresponds to the size and charge of purified CRALBP. A similar result was obtained when anti-IRBP was used for the analysis (Fig. 3, A and B) indicating that this antibody recognizes a single component in retinal homogenates and supernatants which corresponds in size and charge to purified IRBP.

Immunofluorescence

The best preservation of retinal morphology was achieved with the formaldehyde-picric acid fixative but this yielded virtually no specific labeling. The formaldehyde-glutaraldehyde mixture yielded acceptable morphology but weak labeling. Formaldehyde alone gave relatively poor preservation, with prominent ballooning of photoreceptor inner segments, but slightly better specific immunofluorescence with the anti-CRALBP and the strongest reactivity with the anti-IRBP. Finally, the formaldehyde-lysine-periodate fixative produced

the strongest specific labeling with anti-CRALBP but usually gave relatively poor preservation of morphology, with frequent detachment of the retina from the RPE-choroid com-
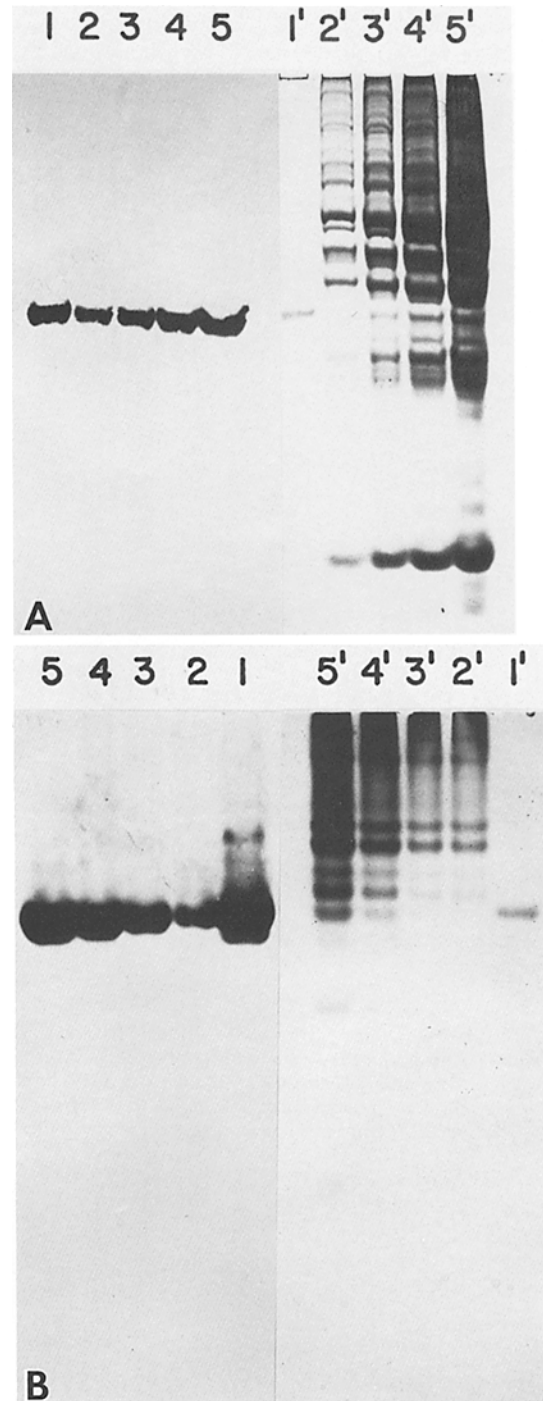

FIGURE 2   Characterization of anti-CRALBP. The figure shows results obtained when purified CRALBP (lane *1, 1'*; 5 μg) and retinal supernatants (lanes *2–5, 2'–5'*; 16, 32, 82, and 164 μg total protein, respectively) were analyzed by (*A*) SDS PAGE or (*B*) non-SDS PAGE, and stained with Coomassie Blue (*1'–5'*) or transferred to nitrocellulose and stained by immunochemistry with anti-CRALBP (*1–5*). A single polypeptide was recognized by anti-CRALBP in retinal homogenates which corresponds to the molecular weight (*A*) and charge (*B*) of purified CRALBP. Lane *1, B* indicates the presence of a minor charge variant in addition to CRALBP in this preparation. The variant is not present in fresh retinal homogenates (lanes *2–5, B*) and appears to form after purification of CRALBP. The gels were 10% in acrylamide (12).
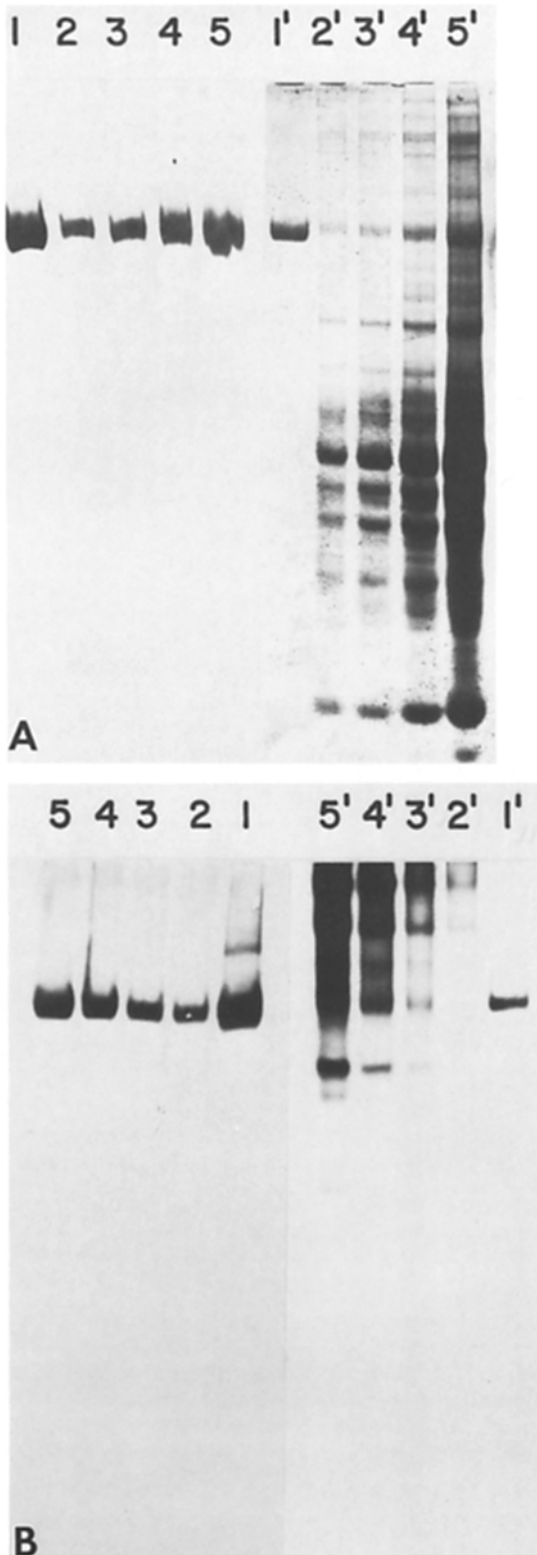
Figure 3   Characterization of anti-IRBP. The figure shows results obtained when purified IRBP (lane 1, 1'; 5 µg) and bovine supernatants (lanes 2–5, 2'–5'; 16, 32, 82, and 164 µg total protein, respectively) were analyzed by (A) SDS PAGE or (B) non-SDS PAGE, and stained with Coomassie Blue (1'–5') or transferred to nitrocellulose and stained by immunochemistry with anti-IRBP (1–5). A single polypeptide was recognized by anti-IRBP which corresponds in size (A) and charge (B) to purified IRBP. Purified IRBP contains a minor charge variant (lane 1, B) which is not present in freshly prepared retinal supernatants. The gels were 5% in acrylamide (12).

plex. The formaldehyde-lysine-periodate fixative gave rather weak labeling with anti-IRBP, possibly because this antigen is a glycoprotein (1, 5) and the sugar moieties, which are altered by the formaldehyde-lysine-periodate (30), may be antigenic determinants.

Control sections treated with antibodies from preimmune serum or antibodies absorbed with purified antigen showed only the autofluorescence of retina (Figs. 4A and 5B). This was strongest with the paraformaldehyde-picric acid fixative and consisted of a weak green fluorescence of the neural retina, particularly in the zones rich in mitochondria (inner and outer plexiform layers and photoreceptor ellipsoids). Lipofuscin granules in the RPE showed a bright yellow-orange autofluorescence.

Specific labeling was found only in sections that had been treated with anti-CRALBP or anti-IRBP in all retinas examined. The fluorescence after anti-CRALBP was confined to two cell types, the RPE and the Müller cells of retina (Fig. 4, B and C). Within the RPE, labeling filled the cytoplasm and was concentrated in the apical portion of the cells; RPE nuclei were negative. In occasional sections where the retina was partially detached from the RPE, the labeled RPE apical processes adhered to the photoreceptor outer segment tips (Fig. 5A).

Labeling of the Müller cells produced a Golgi-like silhouette, with intense fluorescence of the basal end feet, triangular somata in the inner nuclear layer, and fine cytoplasmic processes that surround neuronal elements in the plexiform and nuclear layers. Müller cell nuclei were negative. The presence of CRALBP in astrocytes could not be excluded due to the heaviness of labeling of Müller end feet. In monkey, the fluorescence was intense at the external limiting membrane, with tufts of labeled microvilli projecting into the interphotoreceptor space (Figs. 5C and 7D). Labeled microvilli of rat Müller cells were inconspicuous, in agreement with previous anatomical descriptions (45). Labeling by anti-CRALBP of the following retinal elements was never observed: outer or inner segments of rods and cones, photoreceptor or other neuronal somata, or the interphotoreceptor space located beyond the Müller apical microvilli.

Specific immunofluorescence with anti-IRBP was restricted to the interphotoreceptor space that surrounds the photoreceptor outer segments (Fig. 6, A and B). Heaviest labeling occurred in a line corresponding to the RPE apical surface, which was accentuated in the PAP sections (Fig. 6C). No labeling of any intracellular compartment was found, although cone outer segments were positive (see Fig. 11 below).

Immunoperoxidase

Control sections that had been treated with preimmune or antigen-absorbed antibodies showed very light tan staining of the plexiform layers, photoreceptor ellipsoids, and Müller end feet against the internal limiting membrane; erythrocytes were stained dark brown owing to endogenous peroxidase activity (Figs. 7A and 8A). Cytochemical controls that had been treated with anti-CRALBP and reacted with diaminobenzidine but no H_2O_2 showed no tan or brown coloration, with the exception of melanin in the RPE and choroid of human, monkey, and bovine, and an occasional brown premelanosome in the otherwise melanin-free bovine RPE (Fig. 7B).

Specific peroxidase activity was found only in those retinas that had been incubated with anti-CRALBP or anti-IRBP. As found by immunofluorescence, specific PAP staining due to
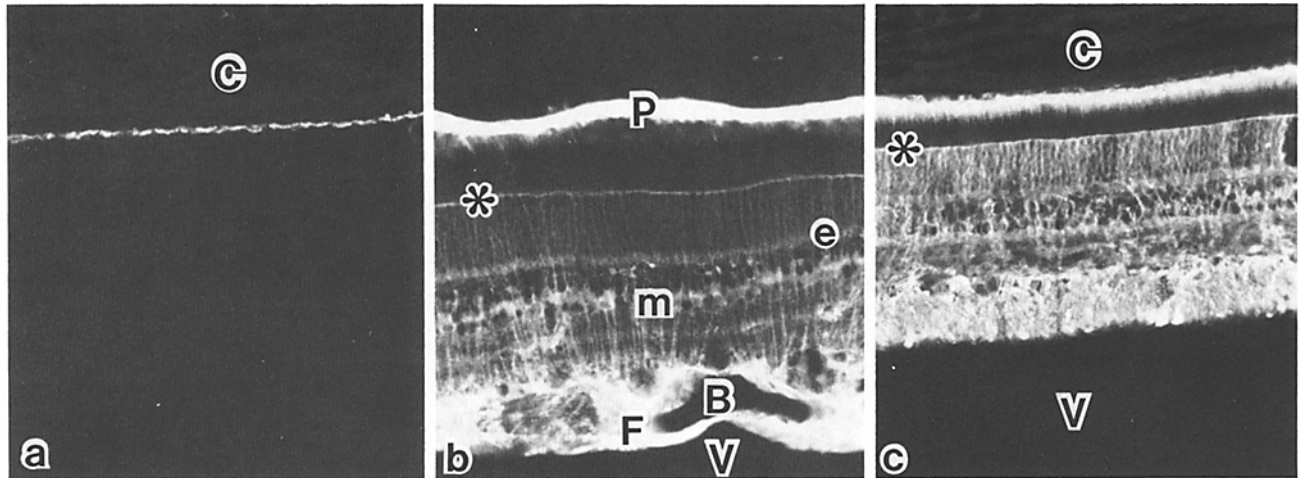
Figure 4 Frozen-sectioned retinas examined by fluorescence microscopy; formaldehyde-lysine-periodate fixation. (a) Control bovine retina treated with CRALBP absorbed antibodies, demonstrating autofluorescent lipofuscin granules in retinal pigment epithelium. C, choroid. (b) Specific fluorescence of bovine retina treated with antibodies to CRALBP. Note heavy staining of retinal pigment epithelium, p. Müller cells are brightly fluorescent and span the retina from the external limiting membrane (*) to the internal limiting membrane. Neuronal components of the retina appear as black negative images. e, external plexiform layer; m, layer of Müller somata in inner nuclear layer; B, blood vessel; F, Müller foot processes against the inner limiting membrane and blood vessel. V, vitreous humor. (c) Monkey retina treated with anti-CRALBP showing staining of retinal pigment epithelium and Müller cells. *, external limiting membrane. C, choroid; V, vitreous. × 65.
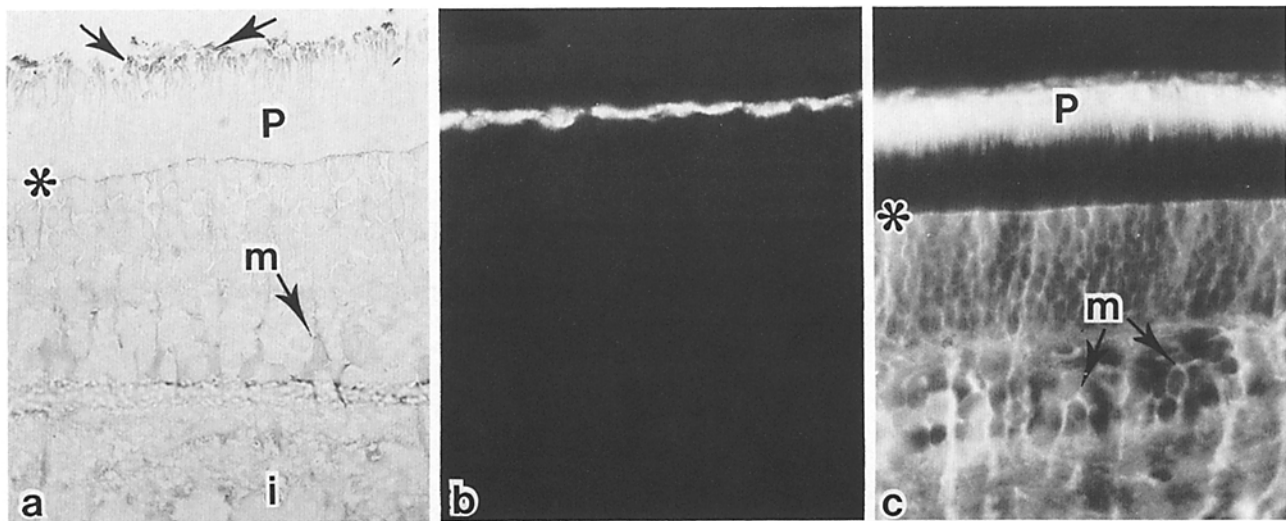

Figure 5 Localization of CRALBP; formaldehyde-lysine-periodate fixation. (a) 1-μm EPON section of bovine retina demonstrating localization of CRALBP by the peroxidase-antiperoxidase technique. The pigment epithelium has detached leaving stained apical processes adherent to outer segment tips (arrows). P, photoreceptors; *, external limiting membrane; m, Müller soma; i, inner plexiform layer. No counterstain. (b) Fluorescence micrograph of monkey retina after treatment with anti-CRALBP absorbed with purified CRALBP. Note intense autofluorescence of lipofuscin granules in retinal pigment epithelium. (c) Monkey retina after treatment with anti-CRALBP. Note labeled microvilli of pigment epithelium (P) and finer Müller cell microvilli extending into the interphotoreceptor space from the external limiting membrane (*) which appears beaded due to alternating intercellular junctions. Note labeled Müller somata (m) with negative nuclei. × 150.

anti-CRALBP was restricted to two cell types, RPE and Müller cells of the retina (Figs. 7 C and 8 B). Reaction product filled the cytoplasm but was absent from the nuclei of both cell types. The increased sensitivity of the PAP technique enabled resolution of labeled Müller cell microvilli extending beyond the external limiting membrane into the interphotoreceptor space (Figs. 7 D and 9) as well as RPE apical microvilli which interdigitate with photoreceptor outer segments (Fig. 10).

As found by immunofluorescence, reaction product due to anti-IRBP was localized in the interphotoreceptor space that surrounds rod and cone outer segments and the apical surfaces

of inner segments (Fig. 6 C). By electron microscopy, peroxidase label surrounded rod and cone inner segments and filled the intralamellar spaces of cone outer segments (Fig. 11). This extracellular staining stopped abruptly at the external limiting membrane and the surfaces of Müller microvilli were heavily stained. Staining was pronounced in a band along the RPE apical margin; by electron microscopy, this labeled band was resolved as heavily labeled IRBP coating the RPE microvilli.

DISCUSSION

Previous biochemical studies from this laboratory have provided information concerning the cellular localization of
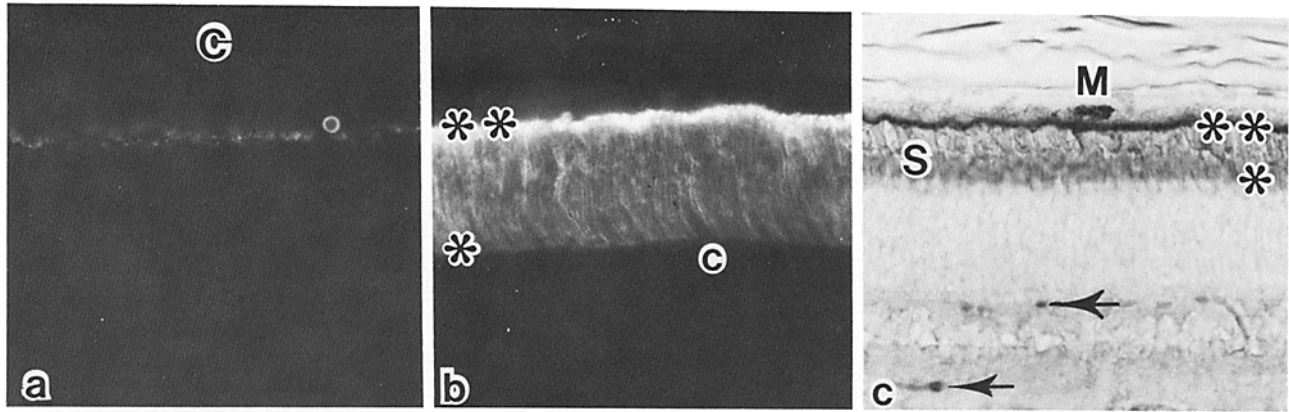
FIGURE 6 Localization of IRBP; formaldehyde-glutaraldehyde fixation. (a) Bovine retina after treatment with anti-IRBP which had been absorbed with purified IRBP. Only autofluorescent lipofuchsin granules of the retinal pigment epithelium are apparent. C, choroid. (b) Bovine retina following treatment with anti-IRBP. The space delineated by the apex of the retinal pigment epithelium (**) and the external limiting membrane (*) is brightly fluorescent. Note negative image of cone inner segment (above c) and vertical striations in the interphotoreceptor space, corresponding to staining of external surfaces of rod outer segments. (c) Bovine retina reacted with the immunoperoxidase technique to demonstrate localization of IRBP in the interphotoreceptor space (S). Staining is most intense in a line (**) just apical to the retinal pigment epithelium; *, external limiting membrane; M, melanin in a solitary cell of the pigment epithelium; arrows, erythrocytes. × 380 (a and b); × 100 (c).
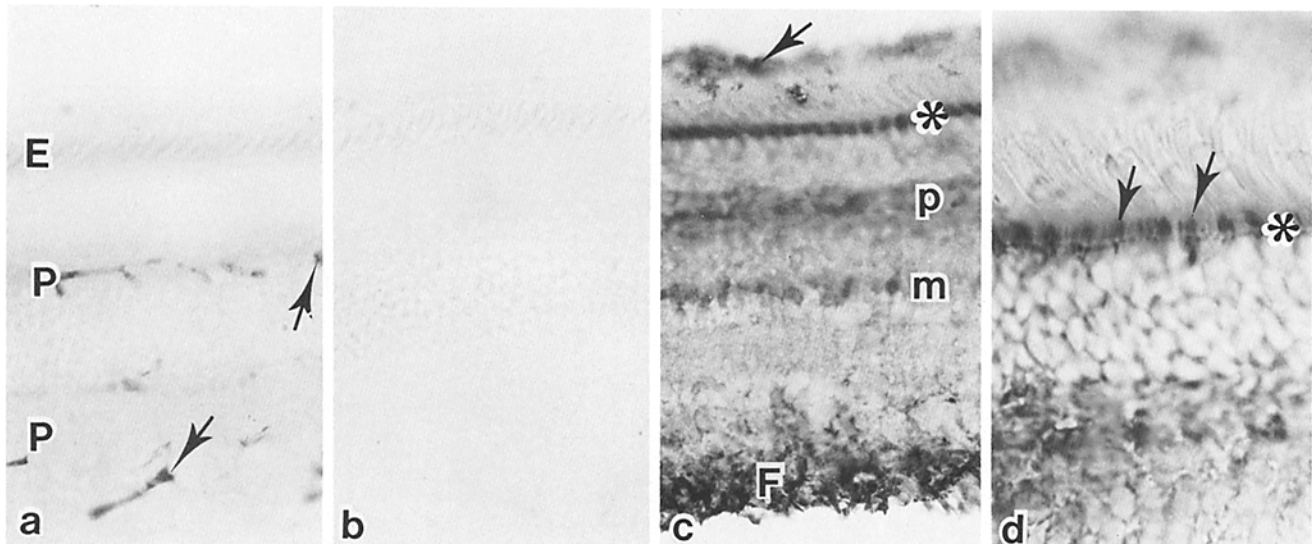

FIGURE 7 Monkey retina reacted by the immunoperoxidase technique for demonstration of CRALBP; formaldehyde-lysine-periodate fixation. (a) Control section treated with anti-CRALBP absorbed with CRALBP, followed by all steps in the PAP technique. Observe light tan staining of photoreceptor ellipsoids (E) and plexiform layers (P) presumably due to mitochondrial cytochrome activity. Erythrocytes (arrows) are stained dark brown due to endogenous peroxidase activity. The retinal pigment epithelium has artifactually detached. (b) Control section that had been incubated with anti-CRALBP and processed by the PAP method with the omission of H_2O_2 in the final cytochemical step. There is no endogenous coloration of the retina. (c) Specific staining with anti-CRALBP results in dark brown reaction product in Müller end feet (F), the row of Müller somata (m), the line of Müller processes which ramify in the external plexiform layer (p) and the external limiting membrane (*). The retinal pigment epithelium has detached, but remnants of its heavily stained microvilli (arrow) adhere to the tips of unstained outer segments (O). (d) Higher magnification of c to demonstrate tufts of labeled Müller microvilli (arrows) extending into the interphotoreceptor space from the beaded external limiting membrane (*). × 230 (a–c); × 570 (d).

CRALBP. The protein was purified from RPE in substantial amounts (~0.7 nmol per bovine retina) and found to carry endogenous 11-cis-retinal (37). The present study corroborated this finding with localization of CRALBP to RPE cytoplasm by immunocytochemistry. Its presence in RPE apical processes is particularly interesting in view of the known exchange of retinoid between RPE and photoreceptor cells (4, 10, 48). CRALBP has also been purified from retina in substantial amounts (~1 nmol per bovine retina). Moreover, the endogenous retinoid content of CRALBP from retina (11-

cis-retinal and 11-cis-retinol) differs from that of CRALBP purified from RPE (11-cis-retinal only; [37]) suggesting that two distinct populations of CRALBP may be localized in different cell types. In view of the known unique involvement of 11-cis-retinoids in the visual cycle, it seemed reasonable to assume that CRALBP would be found in the photoreceptor cell. However, most surprisingly, photoreceptors did not react with the antibody and the Müller cell stained for the binding protein. To our knowledge, this is the first evidence that this glial cell might be involved in vitamin A metabolism in the
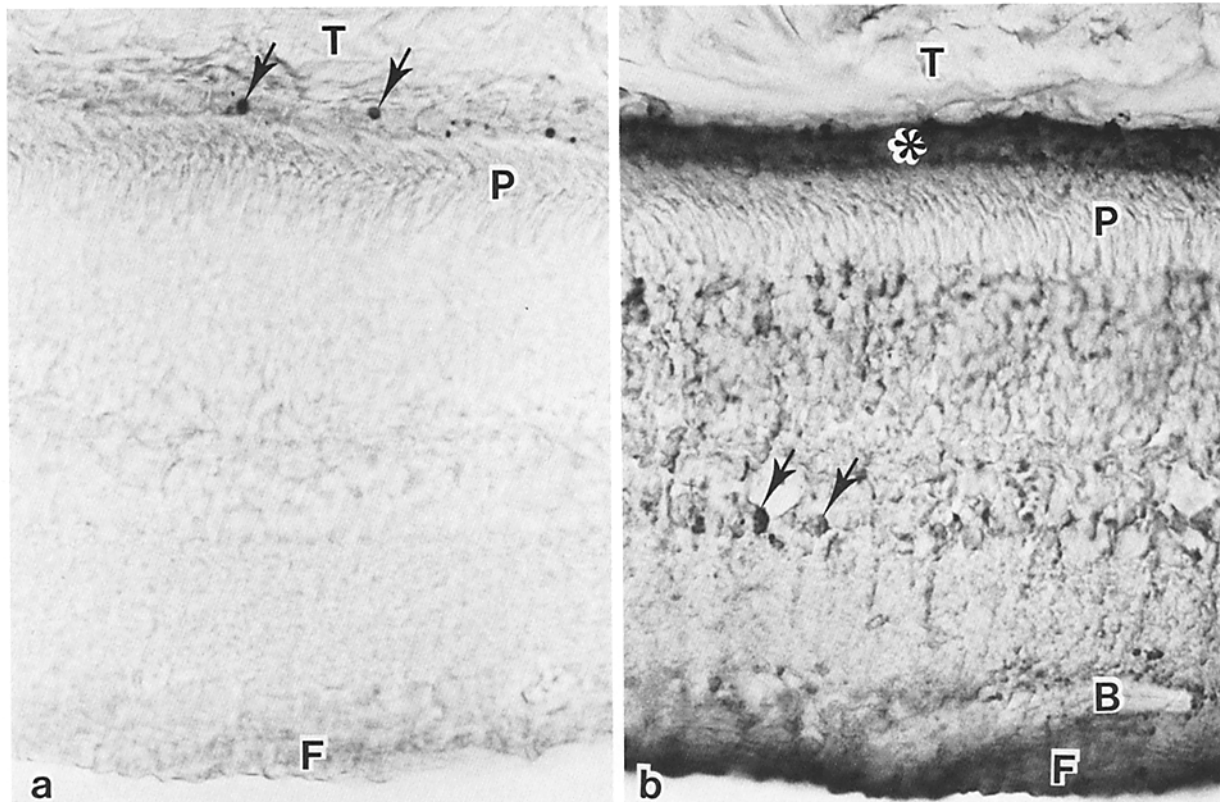
FIGURE 8 Bovine retina reacted by the PAP technique for the localization of CRALBP; formaldehyde-lysine-periodate fixation. (a) Control section treated with CRALBP-absorbed antibodies. There is light tan staining of Müller end feet (F) and the photoreceptor layer (P). Endogenous coloration is due to premelanosomes (arrows) in the retinal pigment epithelium. T, unstained tapetum. (b) Bovine retina as in a following incubation with anti-CRALBP. Note intense staining of retinal pigment epithelium (∗) and Müller cells, including foot processes (F) and somata (arrows) in the inner nuclear layer. Photoreceptor inner and outer segments (P) as well as retinal blood vessels (B) and the tapetum (T) are unstained. × 170.

retina.

The validity of this study depends on the purity of the antigen, CRALBP. The presence of a Müller cell contaminant in our preparations could be responsible for the result we have obtained. However, several lines of evidence and reasoning suggest that the antibodies are directed solely against CRALBP: (a) Antisera prepared against three different preparations of CRALBP (each injected into three rabbits for a total of nine rabbits) produced staining of both RPE and Müller cells. (b) Staining of both cell types was eliminated by adding CRALBP (purified by both our normal procedures and by an additional reverse-phase HPLC step (Fig. 1)) to antiserum before immunocytochemistry. (c) Antibodies used in this study were prepared against CRALBP from retina, yet staining of both RPE and Müller cells was abolished when CRALBP purified from RPE was added to the antibodies before immunocytochemistry. It is unlikely that CRALBP from these two sources would contain the same contaminants. (d) Immunostaining of nitrocellulose electroblots prepared after both SDS PAGE and non-SDS PAGE of retinal supernatants showed a single band, indicating that CRALBP was the only antigen recognized. (e) The presence of CRALBP in a cell type of the retina was not unexpected since it had been purified from this tissue (35). Therefore the presence of a second antigen in addition to CRALBP in our preparation is unlikely; such a second antigen would have to co-purify with CRALBP and have charge and size properties identical to CRALBP in all gel systems and reverse-phase HPLC systems

we have employed. Our interpretation of the data is that CRALBP is present in the cytoplasm of both Müller and RPE cells.

Localization of CRALBP in the Müller cell suggests that this glial cell may play a hitherto unsuspected role in vitamin A metabolism in the retina. Other suggested functions for the Müller cell include secretion and/or maintenance of the internal limiting membrane and vitreous humor, ion exchange and participation in neurotransmitter metabolism, and structural support of the various layers of the retina (14, 20, 45). The Müller apical processes terminate as numerous fine microvilli (39) which "may serve to exchange metabolites with the pigment epithelium as well as to maintain a homeostatic environment in the region of the photoreceptor outer segments" (40), that is, the interphotoreceptor space.

IRBP was localized to this interphotoreceptor space in all species examined, corroborating previous biochemical evidence for its extracellular location, since it was isolated from retinae by washing techniques developed initially to extract interphotoreceptor matrix (1, 2, 5, 28, 29). Other investigators have proposed that IRBP is involved in transport of retinol between RPE and retina based on its intercellular localization, ability to bind retinoids and content of endogenous retinoid (1, 9, 24, 28). Our findings strengthen this suggestion since we have demonstrated here that IRBP is confined to the interphotoreceptor space across which such transport occurs. Use of the PAP technique at the fine structural level enabled resolution of the structural correlate of the heavily labeled
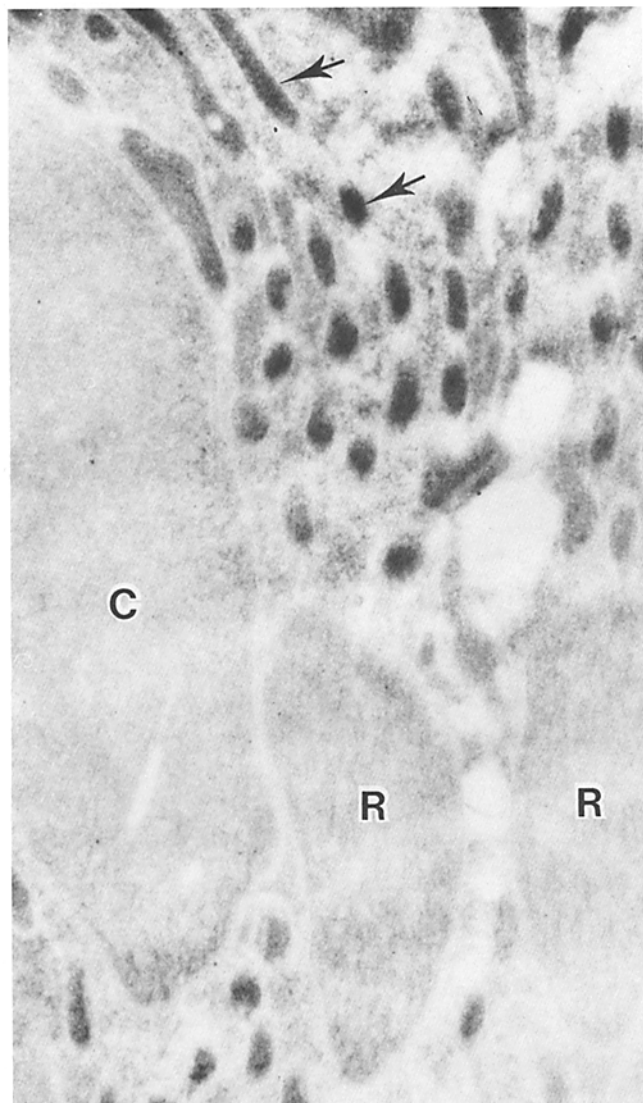
FIGURE 9   Electron micrograph of monkey retina just sclerad to the external limiting membrane showing localization of CRALBP by the PAP technique. The Müller microvilli (arrows) are positive whereas the cone (C) and rod (R) inner segments are negative. Formaldehyde-lysine-periodate fixation. × 19,000.
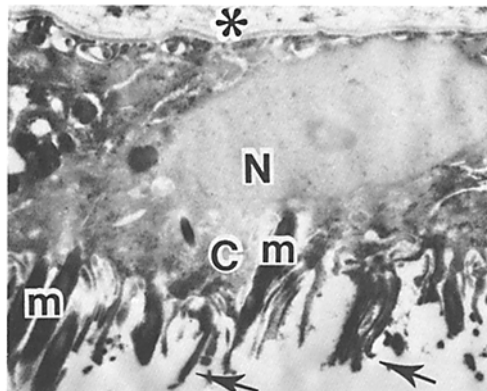

FIGURE 10   Electron micrograph of monkey retinal pigment epithelial cell demonstrating CRALBP localization by the PAP technique; formaldehyde-lysine-periodate fixation. Note positive staining of the cytoplasm (C) and intense staining of apical microvilli (arrows). Melanin granules (m) are intrinsically electron dense and Bruch's membrane (*) and the nucleus (N) are unstained. Retina has artifactually detached. × 5,000.

band at the outer segment-RPE interface as IRBP coating the RPE microvilli. Other investigators have noted the presence of a similar intensely positive band at the outer segment-RPE interface after staining for acid mucopolysaccharides (13, 27, 47). This band may play a role in phagocytosis of outer segments since it appears in the developing rat and mouse retina (27, 47) at the time outer segment shedding and phagocytosis commence (26, 43) and is incompletely formed in the retina of Royal College of Surgeons rats whose RPE shows defective phagocytosis of shed outer segment tips (27).

Staining of the intralamellar spaces in cone outer segments with anti-IRBP is similar to that observed with markers such as Procion yellow and horseradish peroxidase and may reflect the patency of these spaces to the extracellular compartment (6, 7, 25). Labeling was restricted to external surfaces of rod outer segments; the question remains open as to why IRBP was not localized in rod intradisk spaces since they, too, are open to the extracellular space at the time of initial development at the base of the outer segment (25). If IRBP is

entrapped in forming disks, perhaps its antigenicity is lost during disk maturation and distal migration, accounting for the apparent lack of staining of rod intradisk spaces.

Differences in the properties of IRBP and CRALBP must be kept in mind when considering the functional roles of the two proteins. CRALBP displays a high degree of specificity; of the compounds tested so far, only retinoids bind to the protein, and among the retinoids, only retinols and retinals of the 11-cis- configuration are bound (16). Direct binding of other cis-isomers has not yet been investigated. In addition, CRALBP purifies with its binding site saturated with 11-cis-retinoid (37), a relatively strong indication that the protein is involved in vitamin A metabolism. IRBP, on the other hand, is nonspecific in its binding properties. Not only does the protein bind all retinoids tested to date (retinoic acid, retinal, retinol, retinyl acetate, retinyl palmitate) but also binds exogenous vitamin E and palmitate (J. C. Saari, unpublished observations and references 5, 28, and 29). Thus the physiologically important ligand (if any) carried by IRBP has not yet been established.

A fortuitous observation in this study prompts cautious interpretation of results from biochemical experiments purporting to isolate proteins from RPE or retina. In sections where the retina had detached from the RPE, labeled RPE apical processes adhered to outer segments (Fig. 5A). If similar incomplete cleavage occurs during dissection of retinas for biochemical studies, some CRALBP in retinal homogenates may originate from RPE. It is possible that this is the source of 11-cis-retinal in CRALBP isolated from retina and that CRALBP derived solely from retina contains only 11-cis-retinol. Further experiments are required to resolve this question.

In summary, two separate binding proteins with different specificities for vitamin A have been localized in the retina-RPE complex of four species of vertebrates (Fig. 12). Neither has been found in photoreceptors, where vitamin A forms an integral component of rod and cone photopigments. Rather, the two binding proteins have been found in the three compartments which surround photoreceptors, the RPE (CRALBP), the interphotoreceptor matrix (IRBP), and the Müller cell (CRALBP). These two cell types have not been considered functionally similar, although certain anatomic
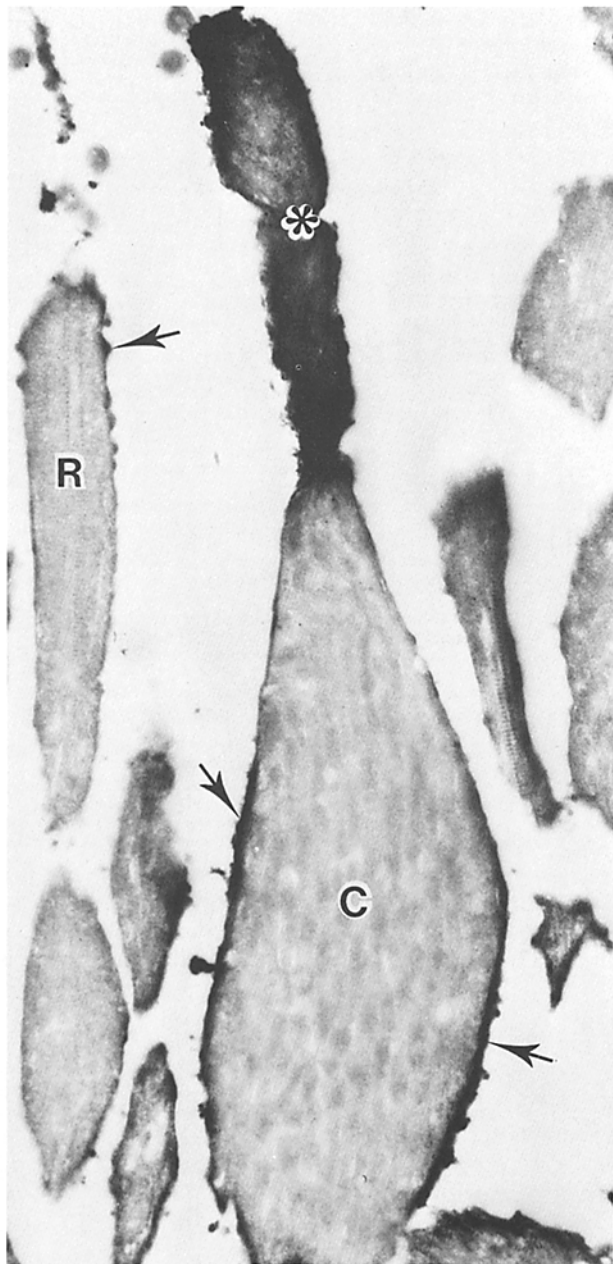
FIGURE 11   Electron micrograph of IRBP localization in monkey retina by the PAP technique; formaldehyde-glutaraldehyde fixation. Note electron dense reaction product (arrows) coating plasma membranes of the unstained cone (C) and rod (R) inner segments. The obliquely sectioned cone outer segment (*) is diffusely filled with reaction product. × 7,500.
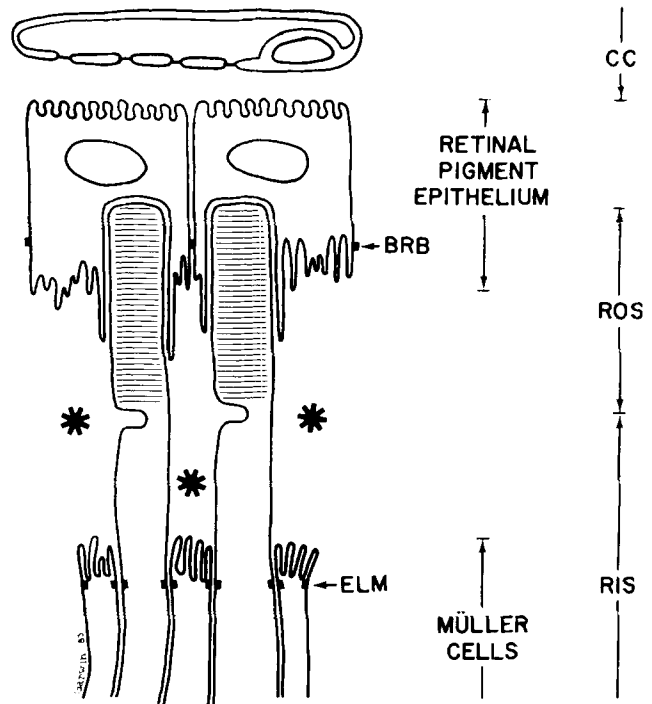

FIGURE 12   Schematic drawing of the cells of the outer retina and pigment epithelium. The present study has localized CRALBP to Müller and pigment epithelial cells and IRBP in the interphotoreceptor space (*). No retinoid-binding proteins have been localized in the photoreceptors where vitamin A is an integral component of photopigments in the outer segments. ELM, external limiting membrane; RIS, photoreceptor inner segments; ROS, photoreceptor outer segments; BRB, choroidal blood retinal barrier; CC, choriocapillaris.

similarities have been noted between the two (14, 20), including an abundance of smooth endoplasmic reticulum in the cytoplasm (45) (as found in other cell types specialized for lipid synthesis); intimate association with the two sources of blood supply to the retina, the choriocapillaris (RPE) and retinal capillaries (Müller cells); and apical microvilli which extend into the interphotoreceptor matrix and surround the distal ends of outer segments (RPE) and inner segments (Müller cells). Since microvilli function to increase surface membrane area of cells for absorption and/or secretion, their extension from both RPE and Müller cells into the interphotoreceptor compartment, which is filled with a second binding protein (IRBP), is consistent with a physiologic transfer, me-

diated by this protein, of different forms of vitamin A among RPE, Müller, and photoreceptor cells.

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