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Retinoid processing proteins in the ocular ciliary epithelium

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Purpose: To identify retinoids and retinoid processing proteins in the ocular ciliary epithelium (CE), and to compare in cultured ciliary epithelial cell lines promoter activities of the cellular retinaldehyde binding protein (CRALBP) and interphotoreceptor retinoid binding protein (IRBP).

Methods: Retinoid processing proteins were detected by RT-PCR, western analysis and immunocytochemistry. PCR products were verified by DNA sequence analysis. Retinoids were measured by normal phase HPLC and UV visible spectroscopy. Reporter product from CRALBP and IRBP promoter fragments was measured following transient transfection in bovine pigmented and nonpigmented CE cells.

Results: CRALBP, IRBP, cellular retinol binding protein (CRBP), 11-cis-retinol dehydrogenase (11-cis-RDH), lecithin:retinol acyltransferase (LRAT), and ATP binding cassette receptor (ABCR) were detected in human CE tissue by RT-PCR. Retinal pigment epithelium specific protein 65 kDa (RPE65) mRNA and protein were also detected. CRALBP and IRBP were detected by western analysis in tissue extracts from bovine CE and were localized to the PE and NPE cell layers, respectively, by immunocytochemistry. IRBP immunoreactivity was also detected in aqueous humor. Retinoids identified in the bovine CE include retinyl esters (7.4±3.5 pMol/mg of protein) and all-trans-retinol (14.9±1.1 pMol/mg of protein). Carotene was also tentatively identified. 11-cis-Retinoids were not detected. In CE cell cultures, the CRALBP p2.1-kb promoter construct exhibited reporter activity 15-30 fold above basal level, with 2 fold more activity in pigmented than nonpigmented CE cells. IRBP promoter constructs exhibited low level reporter activities in vitro in both CE cell layers.

Conclusions: The ocular CE expresses genes encoding components of the rod visual cycle. The differential localization of CRALBP and IRBP along the bilayer of the CE suggests a potential role in retinoid transport and/or retinoid metabolism. However, the absence of 11-cis-retinoids suggests that the function of retinoid processing proteins in the CE differs from that of the retina.

Photoisomerization of rhodopsin bound 11-cis-retinal to all-trans-retinal initiates the phototransduction signal cascade within rod photoreceptor cells, with subsequent regeneration of the 11-cis-retinoid occurring within the adjacent retinal pigment epithelium (RPE) [1]. The epithelial cell layers of the iris and ciliary epithelium (CE) are differentiated cell layers related to both the RPE and retina, sharing with these tissues a common embryological origin [2]. Thus, the pigmented (PE) cell layer of the CE is continuous with the RPE, and the nonpigmented (NPE) cell layer is related to the multiple cell layers of the retina. The bilayer CE is a secretory neuroepithelium that controls the production and protein composition of the aqueous humor and regulates (in conjunction with the trabecular meshwork and Schlemm’s canal) intraocular pressure (IOP). Earlier studies have raised the possibility that the iris/CE might display an intrinsic non-visual phototransduction.

Because expression of rod specific and cone specific genes have been detected in these tissues [3,4].

Although there is no evidence that the mammalian CE is intrinsically photosensitive, several functions ascribed to this tissue follow a 24 h light-dark circadian cycle, including aqueous humor secretion and regulation of IOP. In experimental animals entrained in a 24 h light-dark cycle, the circadian rhythm of IOP persists in constant dark [5]. The signals responsible for maintaining the circadian rhythm are believed to derive from the central nervous system (i.e., suprachiasmatic nuclei) and to arrive at the superior cervical ganglia by way of preganglionic fibers of the sympathetic system and travel to the eye through postganglionic adrenergic nerves [6]. Superior cervical ganglionectomy or preganglionic sectioning of the cervical sympathetic trunk in rabbits does not abolish the circadian IOP [7]. It is difficult to explain the continued light induced decrease of IOP in sympathectomized animals without considering an additional light mediated response mechanism.

In mice lacking rods and cones, for example, melatonin synthesis and the circadian cycle remains unaltered in the pineal gland, suggesting that there are additional photoreceptor cells in the eye capable of integrating a light response [8,9]. Recently, it has been found that a subset of ganglion cells in the inner retina are sensitive to light. These retinal ganglion...
cells contained the opsin based molecule melanopsin, and they are believed to transduce ambient light signals to areas of the brain involved in tasks including circadian entrainment, pupillary light reflexes, and melatonin synthesis [10-12]. However, the light induced transduction mechanism linked to the activation of the photopigment in these cells is, at present, unknown.

We have hypothesized that the expression of functional components of rod phototransduction in the iris-CE may be linked to neuroendocrine signals underlying the regulation of the circadian rhythms of aqueous humor secretion and IOP [13]. In the present work we set out to determine whether functional components of the rod visual cycle critical for the regeneration of photopigment are also expressed in the CE. So far, the only component of the retinoid cycle previously described in the iris-CE is the cellular retinaldehyde binding protein (CRALBP) [14]. CRALBP in the visual cycle serves as an 11-cis-retinol acceptor for the enzymatic isomerization of all-trans-retinyl esters to 11-cis-retinol and as a substrate carrier for 11-cis-retinol dehydrogenase (RDH5) [15]. We do not know, at present, the function of CRALBP in the CE.

We screened for relevant mRNA, retinoid processing proteins in human CE tissues and cells in vitro and report evidence supporting the presence of visual cycle proteins in the CE. We also report evidence of transcriptional regulation in the CE of genes encoding the cellular retinaldehyde binding protein (CRALBP), and the interphotoreceptor retinoid binding protein (IRBP). Finally, we analyzed by normal phase HPLC retinoids extracted from bovine ciliary processes.

**METHODS**

**Source of eye tissues and ciliary epithelial cell lines:** Human eyes were obtained from cadavers within 24 h after enucleation through the National Disease Research Interchange (Philadelphia, PA). Under a dissecting microscope the ciliary body and retina were dissected and stored in liquid nitrogen until further analysis. A human nonpigmented ciliary epithelial cell line, ODM-C4, was used in this study and cultured as previously described [16]. Investigations with human subjects, which involved only residual material from autopsy, was approved by the Human Subjects Committee of Yale University and, followed the tenets of the Declaration of Helsinki.

Bovine eyes from 1- to 12-week-old animals were obtained from a local slaughterhouse and dissected within 2 h of enucleation. To eliminate cross contamination between the CE and the retina, eyes were opened by a circumferential incision approximately 2-3 mm away from the corneoscleral limbus and 2 mm inside of the ciliary body from the ora serrata. The ciliary processes from the pars plicata region of the CE were then dissected from the lens or cornea. The retina and RPE were also dissected. Two bovine ciliary epithelial cell lines established from the pigmented (PE) and nonpigmented (NPE) CE were also used in the present study and cultured as previously described [17].

**Reverse transcription (RT-) polymerase chain reaction (PCR) assays:** Total RNA was isolated from human retinas, ciliary processes, and cultured human ODM-C4 cells using TRIzol reagent (GIBCO BRL, Gaithersburg, MD), according to the manufacturer’s protocol and quantitated by spectrophotometry. Primer sets for the listed genes were selected, as indicated in Methods, from their corresponding published cDNAs (GenBank accession number). The predicted length for each of the PCR DNA products in base pairs (bp), annealing temperature, and location of the forward and reverse sequence for each primer pair are also listed.

**Table 1. Oligonucleotide primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
<th>GenBank accession number</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCR</td>
<td>5’-GTGCTCCGGCTACTTTGTTT-3’ 5’-TACTCCTCAGCTGTTTCA-3’</td>
<td>824</td>
<td>60</td>
<td>NM_000350</td>
<td>4132–4155</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4932–4955</td>
</tr>
<tr>
<td>11-cis-RDH</td>
<td>5’-ACCCCTGGTCGTCTCGTGC-3’ 5’-TGATCCCCGTTGGGTTGTCG-3’</td>
<td>415</td>
<td>63</td>
<td>U43559</td>
<td>430–451</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>823–844</td>
</tr>
<tr>
<td>CRBP</td>
<td>5’-CGCAAATCGCCAACCTT-3’ 5’-GACCCGCTCTCCACTTCAT-3’</td>
<td>288</td>
<td>56</td>
<td>M11433</td>
<td>216–232</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>485–503</td>
</tr>
<tr>
<td>LRAT</td>
<td>5’-GCTCCGGACCCCACTGACC-3’ 5’-GACGCCATCCCAAAGCTGCTGA-3’</td>
<td>456</td>
<td>61</td>
<td>AF071510</td>
<td>159–177</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>591–614</td>
</tr>
<tr>
<td>IRBP</td>
<td>5’-CCCCAGGCTTCCCAACAAG-3’ 5’-GGCCGCTCCCCAGCTCC-3’</td>
<td>505</td>
<td>62</td>
<td>M22453</td>
<td>400–419</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>904–888</td>
</tr>
<tr>
<td>CRALBP</td>
<td>5’-TGGGGGAGGAGGTGCAAGAGAAGG-3’ 5’-TTTAAACGGGCTGGGAAGGAATC-3’</td>
<td>458</td>
<td>65</td>
<td>L34219</td>
<td>251–274</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>708–685</td>
</tr>
<tr>
<td>RPE65</td>
<td>5’-GTGCGCTCTCCTATACACT-3’ 5’-CAGCCGGCACTTCACT-3’</td>
<td>400</td>
<td>55</td>
<td>AH006489</td>
<td>1150–1170</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1549–1531</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-TCGGGTGACATTTAGAAGAGAAGG-3’ 5’-CTAGAAGACTTTGCCGTTGACGAT-3’</td>
<td>506</td>
<td>60</td>
<td>BC013835</td>
<td>695–718</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1200–1177</td>
</tr>
</tbody>
</table>

Primer sets for the listed genes were selected, as indicated in Methods, from their corresponding published cDNAs (GenBank accession number). The predicted length for each of the PCR DNA products in base pairs (bp), annealing temperature, and location of the forward and reverse sequence for each primer pair are also listed.
Polyadenylated RNA (poly(A)+ RNA) was purified from total RNA using an oligoT RNA mini kit from Qiagen Inc., Valencia, CA following the manufacturer’s protocol. Oligonucleotide primers (Table 1) specific for the following human retinoid processing proteins were designed with the aid of DNASTAR software (DNASTAR Inc. Madison, WI) and GenBank: ATP binding cassette receptor (ABCR), 11-cis-retinol dehydrogenase (11-cis-RDH), cellular retinol binding protein (CRBP), lecithin:retinol acyltransferase (LRAT), interphotoreceptor retinoid binding protein (IRBP), cellular retinaldehyde binding protein (CRALBP), and retinal pigment epithelium specific protein 65 kDa (RPE65). Primers were annealed to cDNA templates synthesized in vitro from 100 to 500 ng of poly(A)+ mRNA.

To eliminate possible genomic DNA contamination in the RNA preparation, samples were pretreated with RNase free DNase (Boehringer Mannheim, Indianapolis, IN). RT-PCR reactions were performed using a RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Each PCR cycle consisted of a denaturation step at 94 °C for 1 min, 1 min of annealing at the optimal temperature indicated in Table 1, and 2 min of polymerization at 72 °C. This cycle was repeated 35 times for each of the set of primers used in this work. The final polymerization step was extended an additional 5 min.

Indirect immunofluorescence and immunoblotting: For indirect immunofluorescence, two antibodies were used; a polyclonal anti-peptide (K25Q) antibody to CRALBP raised in rabbits [18] and a monoclonal antibody mAb F7 against the bovine IRBP (personal communication, J. Huang and J. C. Saari). Cryostat sections (1-2 µm thick) of the bovine ciliary processes were prepared as described [19]. Sections were incubated with the anti-K25Q antibody at a dilution 1:100, or with mAb F7 at a dilution 1:50 in 10% normal horse serum containing 1% BSA in PBS for 2 h at 37 °C in a humidified atmosphere. After washing with 1% BSA in PBS, the sections were incubated for 1 h at 37 °C with the secondary antibody (rhodamine conjugate anti-rabbit or anti-mouse immunoglobulin, respectively) followed by three 10-min washes in PBS. The sections were mounted in a solution of glycerol pH 7.0 and analyzed in a Zeiss microscope equipped with epifluorescent microscopy.

For western blotting, protein concentration was measured by colorimetry [20]. Microsomal fractions from ocular tissues were prepared as previously described [19]. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (BioRad, Hercules, CA) by semidy electroblotting, as previously described [4]. Blots containing proteins from whole tissue extracts were probed with anti-CRALBP (at a dilution of 1:1000), and anti-IRBP (at a dilution of 1:500). Blots containing proteins from the microsomal fractions of RPE and ciliary process were probed with a rabbit polyclonal antibody raised against a conserved human/bovine peptide of RPE65 (a gift from Dr. Michael Redmond, National Eye Institute, NIH, Bethesda) [21,22] at a dilution 1:250. Secondary antibodies were peroxidase conjugated goat-anti-rabbit or goat-

![Figure 1. Detection of components of the retinoid cycle in human ciliary processes and in a human ciliary epithelial cell line. Sets of oligonucleotide primers were selected to amplify by RT-PCR coding regions from the following genes; ATP binding cassette, retin a (ABCR), 11-cis-retinol dehydrogenase (RDH), cellular retinol binding protein (CRBP), lecithin:retinol acyltransferase (LRAT), interphotoreceptor retinoid binding protein (IRBP), and cellular retinaldehyde binding protein (CRALBP). DNA products amplified by PCR were resolved on 1.2% agarose gels, stained with ethidium bromide, and photographed. Lane 1 represents retina, lane 2 represents ciliary processes, and lane 3 represents the ODM-C4 cell line. To the left of each gel is a 1 Kb DNA ladder marker (M).](http://www.molvis.org/molvis/v11/a42)
anti-mouse immunoglobulin G (Amersham, Arlington, IL) at a dilution of 1:5000, and antigen-antibody complexes detected using the ECL chemiluminescent substrate (Amersham, Arlington, IL). As a control, pre-immune mouse serum or culture medium was used. In both cases the signal was negative. To verify differences in protein loading in each lane separated by electrophoresis, blots were probing also with an anti-β-actin antibody (Miles Scientific, Naperville, IL) at a 1:500 dilution.

Retinoid extraction and analysis: Bovine eyes were adapted to the dark for at least 2-3 h after enucleation and before dissection of ciliary processes under dim red light to avoid photoisomerization. Retinoids were extracted from pooled ciliary processes from six eyes of one-week-old calves, and from six eyes of 6-12-week-old cows and analyzed in a normal phase HPLC column, as previously described [23]. Spectra of selected components were obtained at least 4 times. Ethyl all-trans-9-(methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (etretinate) was used as an internal standard. Carotenoid elution was marked by a negative deflection because the reference beam of the HPLC instrument was set at a wavelength close to the maximal absorbance of the compounds (450 nm).

CRALBP luciferase and IRBP chloramphenicol acetyl transferase (CAT) reporter-gene assays: Two previously characterized CRALBP-luciferase (Luc) reporter gene constructs were used in this study and contained CRALBP promoter regions designated as p2.1-Luc (-2089 to +80 bp) and p0.2-Luc (-200 to +80 bp) [24]. Three previously characterized IRBP-CAT reporter gene constructs were also used and contained promoter regions of the mouse IRBP gene designated as p156 (-156 to +101), p70 (-70 to +101), and p45 (-45 to +101) [25].

Transient transfections of two bovine ciliary epithelial cell lines, PE and NPE [17], with either the CRALBP-Luc constructs or the IRBP-CAT, were carried out following conditions previously described in detail [24,25]. For comparative purposes, cultured HeLa cells (a cell line of non-ocular origin) were also used as control. The different constructs were transfected in the presence of pSV β-galactosidase (Promega, Madison, WI), using lipofectamine-2000 (Invitrogen, Carlsbad, CA). Cell extracts were assayed for luciferase, chloramphenicol acetyl transferase (CAT), or β-galactosidase activity as previously described [26]. To correct for variation in transfection efficiency, luciferase levels and CAT activity were normalized to protein concentration and β-galactosidase, using commercially available assay systems (Promega). All values are expressed as the mean ± standard deviation of 3-6 separate determinations.

Nucleotide sequencing: PCR DNA products were sequenced on both strands in an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer), using the rhodamine terminator cycle sequencing ready reaction from a DNA sequencing kit (PE Applied Biosystems, Foster City, CA).

RESULTS

Retinoid processing proteins in the human CE processes: The predicted PCR DNA products for ABCR, 11-cis-RDH, CRBP,
LRAT, IRBP, and cellular CRALBP were amplified from poly(A)+ mRNA prepared from the retina and ciliary processes of a human eye donor (cadaver), and from an established human cell line (ODM-C4) derived from CE and analyzed by agarose gel electrophoresis (Figure 1). As expected, mRNA for each of these genes of the retinoid visual cycle was detected in retina (Figure 1, lane 1). Surprisingly, each was also detected in ciliary processes (Figure 1, lane 2) and in the ODM-C4 cell line (Figure 1, lane 3). The DNA products from PCR amplification were gel purified and their nucleotide sequences were found to exhibit 100% identity with the corresponding published cDNA sequences.

Immunolabeling of CRALBP and IRBP in bovine CE: In earlier studies we demonstrated immunodetection of CRALBP along the pigmented CE in bovine eyes [14]. In the present study, for comparative reasons, we have analyzed the cell-restricted immunolocalization of CRALBP along the CE and its pattern of expression with that of IRBP. Using a different, well characterized anti-peptide CRALBP antibody designated anti-K25Q [18], we confirmed the restricted immunolocalization of CRALBP along the PE cell layer of the bovine CE (Figure 2A,B). From western analysis, the anti-K25Q CRALBP antibody crossreacted with a single band of approximately 36 kDa in whole tissue extracts prepared from ciliary processes (Figure 3A, lane 1), RPE cells (Figure 3A, lane 2), and retina (Figure 3A, lane 3), but not in the aqueous humor (Figure 3A, lane 4). In contrasting immunocytochemical analyses for IRBP, the mAb F7 against IRBP labeled the NPE cell layer of the bovine CE, but not the PE (Figure 2C,D). From western analyses, the mAb to IRBP crossreacted with a doublet band of approximately 138 to 140 kDa in whole tissue extracts from ciliary processes (Figure 3C, lane 1), RPE (Figure 3C, lane 2), retina (Figure 3C, lane 3), and aqueous humor (Figure 3C, lane 4). To verify that approximately similar amounts of protein from tissue extracts were loaded in each line (30 µg), blots shown in Figure 3 were reprobed with a β-actin antibody (Miles Scientific, Naperville, IL) at a dilution of 1:500.

These results support the cytosolic nature of CRALBP and the secreted nature of IRBP, and the distinct PE and NPE specific cellular distribution of these retinoid processing proteins in the CE.

Expression of RPE65 mRNA and protein in the bovine ciliary epithelium: It is generally accepted that RPE65 is a key component in the processing of all-trans-retinol into 11-cis-retinol [27,28]. To verify whether RPE65 mRNA and protein were expressed in the ciliary process, we carried out RT-PCR and western blot analysis, respectively, and used RPE tissue extract as a positive control. By RT-PCR, a set of oligonucleotide primers based on the human RPE65 cDNA sequence were selected (see Table 1), and annealed to cDNA templates prepared from bovine RNA from RPE and ciliary processes. A 400 bp DNA product was amplified from both RPE and ciliary process (Figure 4A, lanes 1 and 2). The nucleotide sequence of the gel purified PCR DNA product confirmed its homology with bovine RPE65. By western blot analysis, an anti-peptide antibody to RPE65 [21,22] recognized an abundant protein of 65 kDa of molecular mass in the microsomal fraction from bovine RPE cells (Figure 4B, lane 1). To verify whether RPE65 mRNA and protein were expressed in the ciliary process, we carried out RT-PCR and western blot analysis, respectively, and used RPE tissue extract as a positive control. By RT-PCR, a set of oligonucleotide primers based on the human RPE65 cDNA sequence were selected (see Table 1), and annealed to cDNA templates prepared from bovine RNA from RPE and ciliary processes. A 400 bp DNA product was amplified from both RPE and ciliary process (Figure 4A, lanes 1 and 2). The nucleotide sequence of the gel purified PCR DNA product confirmed its homology with bovine RPE65. By western blot analysis, an anti-peptide antibody to RPE65 [21,22] recognized an abundant protein of 65 kDa of molecular mass in the microsomal fraction from bovine RPE cells (Figure 4B, lane 1). In contrast, the predicted 65 kDa protein corresponding to RPE65 was much less abundant in extracts of ciliary body (Figure 4B, lane 2). To determine the abundance of RPE65 signal in the RPE relative to the ciliary process, we reprobed the blot membrane shown in Figure 4B with a β-actin antibody. After
normalizing the RPE65 signal to that of the β-actin signal, we estimated that the RPE65 signal in the RPE was approximately 20 fold higher than in ciliary process.

**CRALBP and IRBP promoter activity in cultured ciliary epithelial PE and NPE cells:** To further examine the cell specific expression of CRALBP and IRBP in the CE, transient transfection assays were performed to compare promoter activities of CRALBP and IRBP in vitro in bovine CE cultures established with either the PE or NPE cell layers. Reporter activities from two human CRALBP promoter constructs (p2.1 and p0.2-Luc) [24,26] and three mouse IRBP promoter constructs (p156, p70, and p45) [25,29] are shown in Figure 5. The CRALBP p2.1-Luc construct elicited about 15-30 fold more reporter activity than the p0.2-Luc construct (or promoterless vector) in both cell lines and exhibited approximately 2 fold greater reporter activity in PE cells than in NPE cells (Figure 5A). The IRBP-CAT p156 and p70 constructs elicited somewhat higher reporter activities than the p45 or promoterless controls in both NPE and PE cells (about 0.5-2 fold), however no significant differences were apparent between NPE and PE cells. HeLa cells transfected with these IRBP constructs did not show any CAT reporter activity (data not shown).

**Retinoids in bovine ciliary processes:** Retinoids were extracted from bovine ciliary processes from calves (n=6 eyes) and older cows (n=6 eyes) and analyzed by normal phase HPLC, with components identified based on their relative elution times and characteristic absorbance spectra. A representative HPLC result is shown in Figure 6, with peaks 1 and 2 (Figure 6B) corresponding to all-trans retinyl esters, most likely stearate and palmitate, respectively, peak 4 corresponding to all-trans-retinol and peak 3 to an internal standard. The negative deflection at 2.5 min corresponds to a carotenoid, possible β-carotene and the second negative deflection at 25 min may be another carotenoid. Interestingly, the negative deflection at 2.5 min was absent in one-week-old calves (see Figure 6A). Overall, we estimate from these results that bovine ciliary processes from older cows contain about 7.40±3.53

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**Figure 4.** Expression of RPE65 mRNA and protein in the ciliary epithelium. **A:** The expected DNA product sizes amplified by PCR for RPE65 (400 bp) and β-actin (506 bp) were amplified from cDNA templates, synthesized in vitro from poly (A)+ mRNA prepared from bovine RPE (lane 1) and ciliary process (lane 2). DNA products were resolved on 1% agarose gels, stained with ethidium bromide, and their identity confirmed by direct sequencing. **B:** A representative western blot analysis of the microsomal fractions from bovine RPE (lane 1) and ciliary process (lane 2), with polyclonal anti-peptide antibody to RPE65. A β-actin antibody was used to verify loading of protein in each lane. Arrow in **B** indicates the migration of RPE65.

**Figure 5.** Reporter activity of CRALBP and IRBP promoter constructs in bovine PE and NPE cells. **A:** CRALBP-luciferase (Luc) reporter constructs (p2.1-Luc [-2089 to +80 bp] and p0.2-Luc [-200 to +80 bp]). **B:** IRBP chloramphenicol acetyl transferase (CAT) reporter constructs (p156 [-156 to +101], p70 [-70 to +101] and p45 [-45 to +101]). The transcriptional activity generated by the CRALBP-Luc and IRBP-CAT constructs were normalized to β-galactosidase and expressed as relative light units (RLU) and CAT activity (fold pvoCAT), respectively. Basic (pGL2) and pvoCAT indicate two control vectors used in the transfection assay lacking promoter regions for CRALBP and IRBP genes, respectively. Bars represent the mean and standard error of the mean for at least three independent experiments. Asterisks indicate statistical significance calculated using Student’s t-test (p<0.05).
pmoles/mg of protein all-trans-retinyl palmitate (7 analyses) and about 14.86±1.13 pmoles/mg of protein all-trans-retinol (7 analyses). No evidence was detected for 11-cis-retinal or all-trans-retinal in any of the bovine CE samples. Additional experiments employing hydroxylamine during retinoid extraction also did not provide evidence for 11-cis-retinal oxime (results not shown).

**DISCUSSION**

In spite of the embryological relationship between the bilayer CE with the RPE and sensory retina, there is no evidence that the ocular CE is intrinsically photosensitive. However, indirect evidence raises the possibility that the mammalian CE may be photosensitive [13,30]. This hypothesis is based on the findings that the human and bovine CE and iris express many genes encoding proteins involved in phototransduction [3,4] and the retinoid cycle (this work). Further suggestive evidence comes from studies in transgenic mice carrying tandem copies of the rhodopsin promoter region fused to the lacZ reporter gene, which have shown β-galactosidase activity in photoreceptor cells, in the brain, and in the ocular CE [31].

The absorption of light by rhodopsin in the photoreceptor cells in the retina causes a cis to trans photoisomerization of its bound chromophore, 11-cis-retinal, initiating the phototransduction signaling cascade. Complementary to this cascade are the reactions of the retinoid visual cycle in RPE, which provide the necessary enzymatic machinery for regeneration of visual pigment. We have hypothesized that within the ocular CE (by analogy with the retina and the RPE) there is also compartmentalization of the genes expressed in phototransduction, and the retinoid cycle. For example, gene expression of opsin, rhodopsin kinase, and visual arrestin have been shown in the NPE cells [3], and of CRALBP in the PE (this work and [14]).

We also detected IRBP in the ocular CE, distributed among the NPE cells, and in the aqueous humor. IRBP is a poorly understood, large (140 kDa) glycoprotein synthesized in photoreceptors and secreted into the interphotoreceptor matrix (IPM) [32]. However, in zebrafish eyes IRBP has been shown to be synthesized in both photoreceptor cells and RPE cells [33]. It has been suggested that IRBP plays a role in the trafficking of 11-cis retinal and all-trans retinol between photoreceptors and the RPE [32]. Although IRBP can not freely diffuse out of the IPM [34,35], it appears to be internalized also into the RPE [36,37]. The protein binds several hydrophobic ligands, including 11-cis-retinal, all-trans-retinol, other retinoids, several fatty acids and vitamin E [38,39]. IRBP knockout mice do not exhibit retarded rhodopsin regenera-

![Figure 6. Chromatographic separation of retinoids from bovine ciliary processes. Retinoids were extracted from pooled ciliary processes of eyes of one-week-old calves and six- to eight-week-old cows, and analyzed on normal phase HPLC as previously described [21]. The plots show the chromatographic separation of retinoids from one-week-old (A) and from older cows (B). The peaks from older cows (B) are numbered for identification: 1 and 2 correspond to all-trans-retinyl esters (stereate and palmitate, respectively); 3 corresponds to control (etretinate); and 4 corresponds to all-trans-retinol. The negative deflection at 3 min corresponds to elution of a carotenoid, likely β-carotene (only present in older animals). The absorption spectra of components 1, 2, 4, and the carotenoid, are shown in insets (B). The elution profile of retinoids was obtained at 325 nm.](http://www.molvis.org/molvis/v11/a42)
tion [40]. The function of IRBP remains to be determined but it has been proposed to serve a protective role for visual cycle retinoids [32,41]. Perhaps IRBP protects ocular tissues by scavenging, trapping, and facilitating the removal of oxidized retinoids, lipids, and fragments thereof. To the best of our knowledge, this is the first report describing the detection of IRBP mRNA and protein in the NPE cells of the CE and in aqueous humor. The relative strong immunodetection of the 138 to 140 kDa IRBP protein in the bovine aqueous humor suggested its accumulation in this fluid in large amounts. The presence of IRBP in this fluid also suggests that it could provide binding sites for retinoids (or other ligands) and allow vectorial diffusion of the ligand through the aqueous phase through a concentration gradient. Further studies are necessary to confirm de novo synthesis of IRBP in the ciliary epithelium, for example, by immunoprecipitation with IRBP antibodies from proteins of extracts taken from metabolic labeling of dissected ciliary processes in vitro, with isotopes such as 35S-Methionine.

Although CE cell expression of all the proteins involved in the retinoid visual cycle has yet to be demonstrated, we have provided evidence of the distinct cell specific distribution of CRALBP and IRBP in the bilayer CE. RPE65, a unique RPE specific protein required for the conversion of all-trans retinyl esters to 11-cis retinol has been suggested to function as a retinyl ester binding protein [27,28]. Using RPE65 antibodies, we were able to detect very low levels of this protein in a microsomal fraction of bovine ciliary process, when compared (by western blot) to a similar fraction prepared from RPE. However, by indirect immunofluorescence, RPE65 antibodies fail to label the CE (data not shown).

In the present work, we compared reporter gene activity with CRALBP and IRBP promoter constructs in two cell lines representative of the two cell layers of the bovine CE. We found enhanced reporter activity with the p2.1 CRALBP construct in the PE cells compared to NPE. This result is consistent with the cell restricted localization of CRALBP in vivo in the PE cells [14]. Interestingly, in an earlier study, a human CE derived cell line had also the in vitro capability to drive reporter activity from the p2.1 CRALBP promoter construct similar to RPE cells but differently than in retinal Müller cells [26]. In contrast to CRALBP, the reporter gene activity with IRBP promoter constructs was less marked between both NPE and PE cells, even though IRBP appears to be restricted to the NPE cell layer in vivo. Earlier studies have shown that the p70 and p156 IRBP constructs contain photoreceptor specific consensus sequences that confer partial tissue specificity to IRBP gene expression [25,29]. The relatively low level of reporter activity observed for the IRBP constructs in CE may be due to species differences, and/or the lack of essential cis or trans factors.

Finally, we identified retinoids present in the ciliary body of bovine eyes. The absorption spectra and chromatographic characteristics of the main retinoids resolved by HPLC were consistent with the presence in this tissue of all-trans retinyl esters (palmitate and stearate) and all-trans-retinol. Evidence for a carotenoid was also obtained in hexane extracts of ciliary bodies, which is likely to be β-carotene based on its early elution and absorption spectrum. However, additional experiments are necessary to confirm this assignment. All-trans-retinyl esters and all-trans-retinol are common constituents in many cells and their presence in ciliary body may reflect systemic functions of the vitamin A, or perhaps contamination from retinal. The presence of β-carotene in ciliary bodies from adult cows and its absence in calf tissues may reflect a dietary contribution of carotenoid. The accumulation of carotenoids in the ciliary body is also consistent with the anti-oxidant functions of the CE. This tissue expresses abundantly anti-oxidant enzymes involved in detoxification and protection from oxidative damage induced in part by light [42,43].

All-trans-Retinyl esters and all-trans retinol can be rapidly interconverted by the action of the enzymes lecithin retinol acyl transferase (LRAT) and retinyl ester hydrolase (REH) [44,45]. The detection of the substrate (all-trans retinol) for LRAT indicates that the bilayered CE is involved in retinoid metabolism. However, the lack of detection of 11-cis-retinol or 11-cis-retinal among the retinoids extracted from the bovine ciliary processes, contrasted with the expression (mRNA) of 11-cis-retinol dehydrogenase (11-cis-RDH). This dehydrogenase is involved in the conversion of 11-cis-retinol into 11-cis-retinal [46]. Possible explanations for the lack of a chromophore in the ciliary processes are: (a) it is not generated; (b) it is unstable upon retinoid extraction; or (c) it is below the limits of HPLC detection. Overall, the levels of retinoid in the ciliary processes are very low, compared to those in the retina. Interestingly, HEK293S cells express in culture many of the phototransduction and retinoid cycle proteins described in the present study. However, it is not known whether they can elicit light responses [47]. Recent studies have also documented the capability of the NPE cells to differentiate in vivo into neurons in response to mitogens [48], which may be underlying the “neural” characteristic and perhaps the stem-progenitor cell capacity of the CE [49].

A recent study documented the lack of detection of 11-cis chromophore in the pineal gland of the golden hamster, in spite of vestigial levels of components of phototransduction [50]. We do not believe that the levels of many of the components of phototransduction and the retinoid visual cycle in bovine and human CE are present at the vestigial level or that they are due to tissue cross contamination with the retina. Several of the key components of phototransduction and of the retinoid cycle have been immunolocalized along the CE in a cell specific manner (Figure 2) [3]. However, because of the limitations in retinoid detection, the present study does not rule out the possibility of non-visual photosignaling based on retinal chromophore. Furthermore, the potential role of retinoid processing proteins in retinoid metabolism in the CE, underlying circadian tasks such as aqueous humor secretion and intraocular pressure, requires further study.

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