The 11-cis-Retinol Dehydrogenase Activity of RDH10 and Its Interaction with Visual Cycle Proteins

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PURPOSE. The final step in the retinoid visual cycle is catalyzed by 11-cis-retinol dehydrogenases (11-cis-RDHs) that oxidize 11-cis-retinol (11cROL) to 11-cis-retinaldehyde (11cRAL). Genetic studies in mice indicate that the full repertoire of 11-cis-RDH enzymes remains to be identified. This study was conducted to characterize the 11-cis-RDH activity of RDH10 in vitro and specifically to determine whether RDH10 can functionally and physically interact with visual cycle proteins.

METHODS. Human RDH10 was expressed in COS1 cells to measure its 11-cis-RDH activity in the presence or absence of purified recombinant cellular retinaldehyde-binding protein (CRALBP). The RPE visual cycle was reconstituted in HEK-293A cells by co-expressing RDH10, CRALBP, RPE-specific 65-kDa protein (RPE65) and lecithin retinol acyltransferase (LRAT). The cells were subsequently treated with all-trans-retinol (atROL), and retinoid profiles were quantified by HPLC. Immunocytochemical and co-immunoprecipitation analyses were performed to determine whether RDH10 physically interacts with other visual cycle proteins.

RESULTS. RDH10 oxidized 11cROL to generate 11cRAL in vitro in the presence of CRALBP. RDH10 can use both NAD⁺ and NADP⁺ as cofactors for 11-cis-RDH activity, although NAD⁺ cofactor confers more robust activity. In a cell culture model co-expressing RDH10 with RPE65, LRAT and CRALBP, the visual chromatophore 11cRAL was generated from atROL. Immunohistochemistry showed that RDH10 co-localizes with RPE65 and CRALBP in vivo in primary bovine RPE cells. Immunoprecipitation analysis demonstrated that RDH10 physically interacts with CRALBP and RPE65.

CONCLUSIONS. RDH10 may function in the RPE retinoid visual cycle as an 11-cis-RDH, and thereby partially compensate for the loss of RDH5 function in patients with fundus albinus punctatus. (Invest Ophthalmol Vis Sci. 2009;50:5089–5097) DOI:10.1167/iovs.09-3797

Retinal photoreceptor cells contain photosensitive visual pigments that are composed of opsin proteins bound by the visual chromophore, 11-cis-retinaldehyde (11cRAL). Upon absorption of light, the bound 11cRAL is isomerized to the all-trans form, causing the opsin molecule to undergo a conformational change to initiate the phototransduction signaling cascade, with eventual disassociation of all-trans-retinaldehyde (atRAL). In order for photoreceptors to sustain sensitivity to light stimuli, 11cRAL must be regenerated in a process termed the retinoid visual cycle, which involves the concerted action of several specialized enzymes and retinoid-binding proteins that serve to metabolize and traffic retinoids between the photoreceptors and the retinal pigment epithelium (RPE).¹² Initially, atRAL is reduced to all-trans-retinol (atROL) by all-trans-retinol dehydrogenases (atRDHs) in the photoreceptors, and then atROL is transported to the RPE, where lecithin retinol acyltransferase (LRAT) converts atROL into all-trans-retinyl esters (atRE), which are stored in retinosomes.³ RPE-specific 65-kDa protein (RPE65) isomerohydrolyzes atRE into 11-cis-retinol (11cROL),⁺⁻ which is then oxidized by 11-cis-retinol dehydrogenases (11-cis-RDHs) to form 11cRAL. Finally, 11cRAL is transported back to photoreceptor outer segments, where it combines with rod and cone opsin proteins to regenerate visual pigments (for review, see Refs. 1, 2).

Although many of the enzymes responsible for key steps in the visual cycle pathway have been identified and partially characterized, identifying the full repertoire of retinol dehydrogenases (RDHs) involved in the visual cycle remains challenging. This is because RDH and RDH-like enzymes are represented by two large classes, the microsomal short-chain dehydrogenase/reductases (SDRs) and the cytosolic medium-chain alcohol dehydrogenases (ADHs), and these enzymes have overlapping expression patterns and redundant enzymatic activities.⁷⁻⁹ Hereditary null mutations in one 11-cis-RDH, RDH5, have been linked to fundus albinus punctatus (FA), a disease characterized primarily by congenital night blindness due to severely delayed dark adaptation.¹⁰ RDH5 is highly expressed in the RPE, and analysis of Rdb5⁻/⁻ mice has shown that RDH5 accounts for most of the 11-cis-RDH activity in the RPE.¹¹,¹² However, both patients with null mutations in RDH5 and Rdb5⁻/⁻ mice are able to regenerate 11cRAL, which indicates other 11-cis-RDHs contribute to the visual cycle in the RPE.¹⁰⁻¹² RDH11 was identified from the RPE and found to have dual specificity for both cis- and trans-retinoid substrates, prompting speculation that RDH11 could compensate for loss of RDH5 activity.¹⁵ However, double gene knockout Rdb5⁻/⁻ Rdb11⁻/⁻ mice are still able to regenerate 11cRAL indicating that one or more 11-cis-RDH(s) of the visual cycle remain to be identified.¹⁴ Recently, RDH10 was found to have 11-cis-RDH activity in vitro, suggesting that it may function in the visual cycle to generate the visual chromophore.¹⁵

We originally cloned RDH10 from the RPE, and found that it associates with microsal membranes and primarily acts as an atRDH in vitro.¹⁶¹⁷ RDH10 has since been found to be essential for all-trans-retinoic acid synthesis during embryonic development, as mice harboring a null mutation in RDH10 die by day 13 of gestation, unless the maternal diet is supplemented with retinoic acid.¹⁸ Therefore, the requirement of RDH10 for viability may have precluded the discovery of any role for RDH10 in the adult RPE visual cycle by means of an
RDH10-knockout model. In adult animals, RDH10 is expressed specifically in the RPE and retinal Müller cells,17 which suggests it could function in the retinoid visual cycle. However, the function of RDH10 in the RPE has not been established.

In the present study, we characterized the 11-cis-RDH activity of RDH10 in vitro and in a cell culture model under conditions that mimic the unique environment of the RPE, to determine whether RDH10 displays the enzymatic properties that are necessary for 11-cis-RDH activity in the RPE visual cycle. Further, we examined the physical interactions of RDH10 with other visual cycle proteins in the RPE.

METHODS

Construction of Vectors

A bacterial vector expressing histidine-tagged CRALBP, pET19b-CRALBP-His (gift from John Crab, Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, OH), was used to express CRALBP-His in Escherichia coli. CRALBP-His was subsequently purified by nickel affinity chromatography, as described elsewhere for use in in vitro RHDI activity assays that included purified CRALBP. A CRALBP mammalian expression vector was constructed with PCR amplification from pET19b-CRALBP-His vector with the following primers: forward, 5'-GAATTCTGTCGACGGGCGAGCGCTTCG-3' containing an EcoRI site (italic) and reverse, 5'-CCGCGGCGCATGCAAGACGTCGTCG-3' containing a NotI site (italic). The PCR product was TA-cloned into the pGEM-T easy vector (Promega, Madison, WI), subsequently digested with EcoRI and NotI and subcloned into the pcDNA6/V5/His mammalian expression vector (Invitrogen, Carlsbad, CA). A stop codon was inserted upstream of the V5-His coding region to produce untagged CRALBP.

A RDH5 mammalian expression vector was constructed with PCR from a commercially available RDH5 cDNA clone (Clone ID 4984311; Open Biosystems, Huntsville, AL) with the following primers: forward, 5'-GAATTCTGTCGACGGGCGAGCGCTTCG-3' containing an EcoRI site (italic) and reverse, 5'-CCGCGGCGCATGCAAGACGTCGTCG-3' containing a NotI site (italic). The PCR product was subcloned into pcDNA6/V5/His mammalian expression vector (Invitrogen, Carlsbad, CA). A stop codon was inserted upstream of the V5-His coding region to produce untagged CRALBP.

Intracellular RDH Activity Assays

HEK-293A-LRAT cells (described elsewhere)25 were transfected with pcDNA6-CRALBP alone or in combination with individual RHDI expression vectors: pcDNA6-RDH10, pcDNA6-RDH5-His, or pTarget-Flag-RDH8. Six hours after transfection, the cells were infected with adenovirus expressing chicken RPE65 at a multiplicity of infection (MOI) of 100.23 Fifteen hours after infection, the cells were treated with 2 μM all-trans-retinol in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were harvested after 8 hours of treatment by scraping with a rubber policeman and were washed with PBS to remove the culture medium. During the PBS wash steps, 20% of the cells were removed for immunoblot analysis. The remaining cells were pelleted and stored at −80°C. The cell pellets were thawed on ice and lysed by sonication (three pulses of 20 seconds each with a 1-minute pause between each pulse) in 300 μL of extraction buffer containing 50% ethanol and 50 mM MOPS (pH 6.5). The retinoids were immediately extracted with 300 μL of hexane and analyzed by HPLC, as described earlier.

Immunoblot Analysis

For in vitro activity assays, protein extracts were prepared in RDH activity buffer and 10 μg of proteins per sample were analyzed by immunoblot analysis. For in-cell activity assays, cells in 15-cm culture plates that were 90% confluent were washed and resuspended in PBS. Twenty percent of total cells was removed and directly resuspended in Laemmli buffer, and ultimately 8% of cells from each sample were analyzed by immunoblot analysis.

Protein concentration was determined by Bradford analysis,24 and proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane for subsequent immunoblot analysis. The membranes were blocked in 5% milk/Tris-buffered saline Tween-20 (TBST) for 30 minutes at room temperature (RT), and primary antibodies were applied in 5% milk/TBST for either 1 hour at RT or overnight at 4°C. Unbound primary antibodies were removed by four washes in TBST for 5 minutes/wash. Horseradish peroxidase–conjugated secondary antibodies were applied at 1:5000 in 5% milk/TBST for 1 hour at RT and subsequently removed by four washes in TBST for 5 minutes. Antibody-binding was detected using enhanced chemiluminescence reagent (Pierce, Rockford, IL) and an imaging station (GeneTools; SynGene, Frederick, MD). As needed, stripping buffer (Pierce) was used to repeat immunoblot analysis with different antibodies. Primary antibodies and dilutions were rabbit anti-RDH10 (1:1000; polyclonal raised against peptide QRKQATNNEAKNG),15 mouse anti-histidine (1:2000; Millipore, Billerica, MA), mouse anti-CRALBP (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-RPE65 (1:5000; Millipore), rabbit anti-LRAT,25 mouse anti-Flag (1:1000; Sigma-Aldrich, St. Louis, MO), and mouse anti-β-actin (1:5000; AbCam, Cambridge, MA).

Immunocytochemistry

Bovine eyes were obtained within 1 hour postmortem from a local abattoir, transported on ice, and dissected within 1 hour. The anterior segment, vitreous, and retina were removed, and 1 mL of DMEM with
10% FBS was added to each eye cup. RPE cells were collected by gentle brushing, and 20 to 40 μL of RPE cell suspension was directly applied to positively charged microscope slides and dried at 37°C for 2 hours. RPE cell specimens were fixed in 4% paraformaldehyde/PBS for 15 minutes, and permeabilized in 0.1% Triton X-100/PBS for 10 minutes. The specimens were blocked in 3% BSA/PBS for 30 minutes, and primary antibodies were incubated in 3% BSA/PBS for 1 hour. After the specimens were washed with PBS, secondary antibodies were incubated in 3% BSA/PBS in the dark for 1 hour, and the unbound antibodies were washed away with PBS. Mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Prolong Gold; Invitrogen) was applied, and the slide was coverslipped. Primary antibodies and dilutions were: rabbit anti-RDH10 (1:50), mouse anti-RPE65 (1:100; Millipore), and mouse anti-CRALBP (1:100; Santa Cruz Biotechnology Inc.). Secondary antibodies were goat anti-rabbit fluorescent dye (Alexa 488, 1:500; Invitrogen) and goat anti-mouse fluorescent dye (Alexa 568, 1:500; Invitrogen). Images were acquired with an upright laser scanning confocal microscope (SP2 M2; Leica, Wetzlar, Germany) with a 63× plan APO objective, and image analysis was performed (LCS Lite software; Leica, Wetzlar, Germany).

**Nickel Affinity Chromatography**

**Pull-Down Assays**

Bovine eyes were obtained and dissected as described earlier, except that bovine RPE cells were collected by gentle brushing into RDH activity buffer (100 mM sodium phosphate; pH 7.4). Cells were pelleted and resuspended in the RDH activity buffer with 0.25 M sucrose for cell lysis by sonication. Lysates were centrifuged at 10,000 g for 10 minutes to remove cell debris. The supernatant was then centrifuged at 100,000 g for 1 hour to separate cytosolic and microsomal fractions. The pellet (microsomes) was resuspended in RDH activity buffer and frozen at ~80°C.

Bovine RPE microsomes were thawed and resuspended in histidine-binding buffer (300 mM NaCl, 50 mM Tris, pH 8.0) supplemented with 0.1% CHAPS and solubilized by brief sonication. For pull-down assays, 200 μg of solubilized bovine RPE microsomes was incubated with 10 μg histag-crABLP or histag-KBP (a kallikrein-binding protein, SERPINA3K) in the presence of 50 μM 11-cis-retinal for 1.5 hours at 4°C. Nickel beads (GE Healthcare, Amersham, UK) were preblocked in 1% BSA, and then 10 μL of beads was incubated with each sample for 2 hours at 4°C in the dark. After adsorption, unbound proteins were removed, and the beads were washed with 80 mM imidazole. Bound proteins were eluted with 500 mM imidazole, and diluted with 4× Laemml buffer. Immunoblot analysis was performed with 25% bound proteins, 2% unbound proteins, and 5% bovine RPE microsomal input proteins.

**Co-immunoprecipitation Assays**

Bovine RPE microsomes (200 μg) were resuspended in the histidine-binding buffer supplemented with 0.1% CHAPS and solubilized by brief sonication. Microsomal lysates were incubated with mouse anti-RPE65 or mouse IgG (15 μg) for 2 hours at 4°C, and then 50 μL of protein A beads (Santa Cruz Biotechnology, Inc.) were added to each sample and incubated for an additional 2 hours at 4°C. After adsorption, unbound proteins were removed, and beads were washed eight times with 500 μL of the histidine binding buffer. Immunoprecipitated proteins were eluted with Laemml Buffer. Immunoblot analysis was performed using 56% of immunoprecipitates, 5% of unbound proteins, and 5% of input microsomes.

**RESULTS**

**RDH10 Activity with 11-cis-Retinoids**

Previous efforts to purify RDH10 have resulted in loss of enzymatic activity, as detergents are required to solubilize the protein. Therefore, membrane fractions were prepared from RDH10-transfected COS1 cells (Supplementary Fig. S1, http://www.iovs.org/cgi/content/full/50/11/5089/DC1) to measure the kinetics of RDH10 activity with 11-cis-retinoids. To determine cofactor preference, membrane fractions (62 μg protein) were mixed with 1 mM NAD+/NADH or NADP+/NADPH cofactor in RDH activity buffer containing 1% BSA, and the reaction was initiated by addition of 11cROL or 11cRAL substrates. The kinetic constants of RDH10 for 11-cis-retinoids were determined by assaying RDH10 activity with increasing amounts of 11cROL or 11cRAL substrates. The Lineweaver-Burke analyses. These assays demonstrated that RDH10 oxidizes 11cROL to generate the visual chromophore 11cRAL with either NAD+ or NADP+ cofactor with K_m of 2.0 and 0.69 μM, respectively (Figs. 1A, 1B, peak 1). The specific activity of the RDH10-containing membrane fractions with

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** RDH10 oxidized 11-cis-retinol in vitro. The membrane fraction (62 μg) of RDH10-expressing or untransfected (UNTF) COS1 cells was incubated with 11-cis-retinol substrate (2.6 μM) in the RDH activity buffer containing 1% BSA in the presence of 1 mM NAD+/NADH (A, C) or NADP+/NADPH (B, D) cofactor for 30 minutes at 37°C. The retinoids were extracted and analyzed by HPLC. Pure retinoid standards were used to confirm peak identities: (1) 11-cis-retinal; (2) all-trans-retinal; and (3) 11-cis-retinol.
TABLE 1. Kinetic Constants of Membrane-Associated RDH10 for 11-cis-Retinoids

<table>
<thead>
<tr>
<th>Substrate (Cofactor)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ (pmol/mg membrane protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-cis retinol (NAD$^+$)</td>
<td>2.00</td>
<td>69.44</td>
</tr>
<tr>
<td>11-cis retinol (NADPH)</td>
<td>3.85</td>
<td>289.10</td>
</tr>
<tr>
<td>11-cis retinal (NADH)</td>
<td>4.32</td>
<td>340.25</td>
</tr>
</tbody>
</table>

The $K_m$ and $V_{max}$ for each substrate were determined at fixed saturating concentrations (1 mM) of the listed cofactors using the same preparation of membranes containing RDH10.

Effect of CRALBP on RDH10 Activity

CRALBP is a water-soluble, retinoid-binding protein that has high affinity and selectivity for 11-cis-retinoids and is expressed in the RPE and Müller cells. CRALBP-binding of 11-cis-retinoids is known to be important for directing the flow of retinoids through the visual cycle, and in vitro studies have confirmed that the well-characterized 11-cis-RDH, RDH5, can use CRALBP-bound 11cROL (holo-CRALBP) as a substrate to generate the visual chromophore. Therefore, it has been proposed that any physiologically relevant 11-cis-RDH must be able to use holo-CRALBP as a substrate.

To determine whether RDH10 can oxidize 11cROL bound in holo-CRALBP, we assayed RDH10 activity with 11cROL substrate (2.6 $\mu$M) in the presence of increasing concentrations of purified recombinant CRALBP. Increasing CRALBP concentrations caused a decrease in the production of atRAL (Figs. 2A–C; peak 2), indicating that CRALBP binds to 11cROL in the reaction mixture, preventing thermal isomerization of 11cROL to atROL and effectively increasing the 11cROL substrate concentrations in the reaction. At a 1:1 molar ratio of CRALBP to 11cROL, the rate of oxidation was approximately double the rate without CRALBP (Figs. 2A, 2B, 2D). At ratios of 2:1 and 5:1, the reaction rate was maintained at approximately 100% and 50%, respectively (Figs. 2C, 2D). The decreased activity observed at higher molar ratios of CRALBP:11cROL may reflect inhibition due to apo-CRALBP competing with holo-CRALBP for binding to RDH10, as we demonstrated that RDH10 physically associates with CRALBP (see Figs. 5A, 5B).

RDH10 Activity in Reconstituted RPE Visual Cycle in Cultured Cells

To determine whether RDH10 can function as an 11-cis-RDH in an intracellular environment, we developed a unique cell culture model. HEK293A cells stably expressing LRAT were co-transfected with the CRALBP and RDH5 expression vectors and then infected with adenovirus expressing RPE65 to reconstitute expression of these key players of the RPE visual cycle (Fig. 3A). The cells were incubated with 2 $\mu$M atROL for 8 hours, and the retinoids were extracted and analyzed by HPLC. As a negative control, the cells were transfected with all-trans-retinoid-specific RDH8 in place of RDH5. Cells transfected with RDH5 generated a detectable and significant amount of 11cRAL from atROL (Fig. 3B, peak 2), demonstrating that the cell culture model sufficiently reiterates the RPE visual cycle (Fig. 3). RDH8 transfection did not generate any detectable amount of 11cRAL (Fig. 3B). When RDH10 was substituted for RDH5, a significant amount of 11cRAL was generated (Fig. 3B, peak 2), confirming that RDH10 can functionally cooperate with other visual cycle proteins to complete the RPE visual cycle (Fig. 3).

Subcellular Co-localization of RDH10 with CRALBP and RPE65

We have previously shown that RDH10, like RDH5 and RPE65, is located in the microsomal membrane. To determine whether RDH10 is co-localized with RPE65 in RPE cells, im-
munocytochemistry with both anti-RDH10 and anti-RPE65 antibodies was performed on primary bovine RPE cells. Confocal microscopy revealed that RDH10 co-localized with RPE65 in the intracellular space outside the nucleus (Figs. 4A–H). We also double stained the RPE cells using the anti-RDH10 and anti-CRALBP antibodies. Although CRALBP is a soluble cytosolic protein, we detected significant co-localization of CRALBP with RDH10 (Figs. 4I–P). To confirm the specificity of immunocytochemistry results, double immunostaining showed that a non-visual cycle protein, β-catenin, was not co-localized with RPE65 (Supplementary Fig. S2, http://www.iovs.org/cgi/content/full/50/11/5089/DC1).

Association of RDH10 with CRALBP and RPE65

Since we demonstrated that RDH10 can functionally interact with CRALBP and RPE65 in vitro, we performed pull-down and immunoprecipitation assays to determine whether RDH10 physically associates with CRALBP and RPE65. His-tagged CRALBP (10 μg) was added to solubilized bovine RPE microsomes in the presence or absence of 50 μM 11cRAL. Nickel affinity chromatography was performed to pull down CRALBP, and immunoblot analysis showed that RDH10 co-purified specifically with CRALBP, demonstrating that RDH10 binds to CRALBP in vitro (Fig. 5A). Furthermore, this binding was enhanced in the presence of 11cRAL (Fig. 5A), suggesting that retinoid-binding may bridge this interaction. To further confirm the specificity of this interaction, we added an unrelated histagged protein, KBP, to RPE microsomes and also pulled it down by Ni-resin. Under the same conditions used for the pull-down of CRALBP, RDH10 did not co-precipitate with KBP (Fig. 5B). Immunoblot analysis with an anti-clathrin antibody demonstrates that CRALBP binding to RDH10 is not a result of CRALBP nonspecifically binding to membrane proteins (Fig. 5B).

To determine whether RDH10 could also bind to RPE65, the anti-RPE65 antibody was added to solubilized bovine microsomes and subsequently immunoprecipitated using protein A affinity chromatography. We found that native RDH10 specifically co-immunoprecipitated with native RPE65, demonstrating that RPE65 binds to RDH10 in vivo (Fig. 5C). However, it is unlikely that 100% of RPE65 molecules are in complex with RDH10, as a small amount of RPE65 binds nonspecifically to mouse IgG but does not result in pull-down of any detectable amount of RDH10 (Fig. 5C). Last, we sought to determine whether the binding of RDH10 to CRALBP and RPE65 was direct or whether it required the presence of RPE-expressed adaptor proteins. RDH10, CRALBP, and RPE65 were co-expressed in COS1 cells, and cell extracts were subjected to pull-down assays as described for the RPE pull-down assays. However, no co-precipitation was observed (data not shown).

FIGURE 3. RDH10 generated the visual chromophore, 11-cis-retinal, in a cell culture model that reconstituted the RPE visual cycle. HEK293A-LRAT cells (stably expressing LRAT) were co-transfected with CRALBP and Histagged RDH5, untagged RDH10, Flag-tagged RDH8, or no RDH. The cells were infected with adenovirus that expressed RPE65 at an MOI of 100 and treated with 2 μM all-trans-retinol for 8 hours. Intracellular retinoids were extracted and analyzed by HPLC. (A) Immunoblot analysis was performed to confirm expression of visual cycle proteins. All lanes contain samples from cells expressing LRAT, RPE65, and CRALBP as well as one specific RDH. Lane 1: nothing additional; lane 2: RDH10; lane 3: His-RDH5; and lane 4: Flag-RDH8. (B) Representative HPLC chromatograms are shown for each sample. Pure retinoid standards were used to confirm peak identities: (1) retinyl esters and (2) 11-cis-retinol. (C) Quantification of 11-cis-retinal generated in each sample. The graph represents the mean and SD of samples treated and measured in triplicate and is representative of results in three independent experiments.
suggesting the association of RDH10 with CRALBP and RPE65 requires RPE-specific scaffolding proteins.

**DISCUSSION**

In the present study, RDH10 had 11-cis-RDH activity and oxidized 11cROL from holo-CRALBP, which is the endogenous form of 11cROL found in the RPE. Furthermore, RDH10 functionally interacted with visual cycle proteins to generate 11cRAL and reconstitute the RPE visual cycle in a cell culture model that co-expressed recombinant LRAT, RPE65, CRALBP, and RDH10. Pull-down assays demonstrated that RDH10 physically interacted with CRALBP and RPE65, and immunocytochemical analyses revealed that RDH10 co-localized with CRALBP and RPE65 in vivo. These data suggest that RDH10 may serve as an 11-cis-RDH in the RPE visual cycle in vivo, and thereby partially compensate for the loss of RDH5 function in rdh5−/− mice and in patients with FA.

RDH5 is believed to account for most of the 11-cis-RDH activity in the mouse retina. However, Rdb5−/− mice are able to regenerate 11cRAL and only show delayed dark adaptation after intense bleaching. The residual RDH5-independent 11-cis-RDH activity has been characterized with RPE microsomal and soluble fractions from Rdb5−/− mice. The most efficient RDH5-independent 11-cis-RDH activity was characterized as membrane-associated, primarily NADP+-dependent with a ∼5-fold lower efficiency than RDH5.12 Characterization of RDH11 activity in vitro indicated that it could compensate for the loss of RDH5. However, studies later found that Rdb5−/−Rdb11−/− mice can still regenerate 11cRAL and have only slightly more delayed dark adaptation than Rdb5−/− mice, indicating that another 11-cis-RDH can compensate for the loss of both enzymes. This remaining 11-cis-RDH may have NAD+ or NADP+ preference and is far less efficient than RDH5.14

In the present study, RDH10 had 11-cis-RDH activity in vitro and had a preference for NAD+ cofactor over NADP+, as the NAD+ cofactor yields a sevenfold higher specific activity for 11cROL oxidation. However, in contrast to a previous report, we found that RDH10 can use NADP(H) cofactors for oxidation/reduction of 11-cis-retinoids. Likewise, we have previously shown that RDH10 prefers NADP+ cofactor for atROL oxidation in vitro. Although the amino acid sequence of the cofactor binding motif in RDH10 predicts that RDH10 should have a preference for NAD(H) over NADP(H), RDH10
RDH10 physically associated with CRALBP and RPE65. (A, B) Purified CRALBP-His was incubated with bovine RPE microsomes, and pulled down by nickel affinity chromatography. Nickel bead-bound and unbound proteins were analyzed by immunoblot to detect RDH10 and His-tagged proteins. (A) Addition of 11cRAL enhanced the binding of RDH10 and CRALBP-His (lane 3 compared with lane 2). Therefore, 11cRAL was added to all samples for subsequent assays. (B) Binding of RDH10 and CRALBP-His was shown to be specific, as RDH10 did not co-precipitate with His-tagged KBP. Furthermore, the anti-clathrin immunoblot indicates that CRALBP did not bind to membrane proteins nonspecifically. (C) Anti-RPE65 or mouse IgG was added to bovine RPE microsomes and subsequently immunoprecipitated with protein A affinity chromatography. Immunoblot analysis was performed on immunoprecipitated (bound) and unbound samples. Native RDH10 co-immunoprecipitated with RPE65 (lane 2).

FIGURE 5. RDH10 physically associated with CRALBP and RPE65. (A, B) Purified CRALBP-His was incubated with bovine RPE microsomes, and pulled down by nickel affinity chromatography. Nickel bead-bound and unbound proteins were analyzed by immunoblot to detect RDH10 and His-tagged proteins. (A) Addition of 11cRAL enhanced the binding of RDH10 and CRALBP-His (lane 3 compared with lane 2). Therefore, 11cRAL was added to all samples for subsequent assays. (B) Binding of RDH10 and CRALBP-His was shown to be specific, as RDH10 did not co-precipitate with His-tagged KBP. Furthermore, the anti-clathrin immunoblot indicates that CRALBP did not bind to membrane proteins nonspecifically. (C) Anti-RPE65 or mouse IgG was added to bovine RPE microsomes and subsequently immunoprecipitated with protein A affinity chromatography. Immunoblot analysis was performed on immunoprecipitated (bound) and unbound samples. Native RDH10 co-immunoprecipitated with RPE65 (lane 2).
long-standing hypothesis that CRALBP mediates diffusion of retinoids from the apical processes to the visual cycle enzymes in the somata. There is also abundant evidence that CRALBP drives substrate flow through the retinoid visual cycle by transferring retinoids between visual cycle enzymes. Therefore, the co-localization and binding interaction of RDH10 with both RPE65 and CRALBP in bovine RPE indicate that RDH10 could function within a retinoid-processing complex.

The data presented herein suggest that RDH10 is likely to function in vivo as an 11-cis-RDH in the retinoid visual cycle. Until now, RDH10 has been most often overlooked as a potentially significant RDH in the retinoid visual cycle, because no human retinal dystrophies have been linked to deficiencies in RDH10; however, it was recently shown that RDH10 is essential for development in mice. RDH10 is the only RDH enzyme that has been found to be essential for development, presumably because RDH10 is necessary to oxidize aTROL for retinoic acid synthesis, despite the fact that numerous other enzymes with inherent stability would be incompatible with survival. This would explain why no retinal dystrophies have been linked to deficiencies in RDH10. The present study demonstrates that RDH10 has a uniquely robust activity in vivo. Therefore, despite previous studies in mice that demonstrate significant genetic redundancy in the RDH activity of the visual cycle, RDH10 cannot be disregarded as a potentially significant RDH in the visual cycle. Furthermore, since RDH10 is essential for development, any mutation in RDH10 that would be deleterious to its oxidoreductase activity or inherent stability would be incompatible with survival. This would explain why no retinal dystrophies have been linked to deficiencies in RDH10. The present study demonstrates that RDH10 could account for the residual 11-cis-RDH activity found in Rdb5−/−/Rdh11−/− mice and that RDH10 may partially compensate for the loss of RDH5 function in human patients with FA. RPE and Müller cell-specific conditional knockouts of Rdh5 have been found to be essential for development, presumably because RDH10 is necessary to oxidize aTROL for retinoic acid synthesis. Furthermore, since RDH10 is necessary to oxidize aTROL for retinoic acid synthesis, it would be deleterious to its oxidoreductase activity or inherent stability.

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References

5. Moiseyev G, Crouch RK, Goletz P, Oatis J Jr, Redmond TM, Ma JX. Retinyl esters are the substrate for isomerohydrolase. Biochimbi-


25. Ruiz A, Winston A, Lim VH, Gilbert BA, Rando RR, Bok D. Molecular and biochemical characterization of lecithin retinol acyltrans-


35. Bliss AF. The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. Arch Biochem. 1951;31:197–204.


