Aberrant Metabolites in Mouse Models of Congenital Blinding Diseases: Formation and Storage of Retinyl Esters†

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Received November 21, 2005; Revised Manuscript Received January 31, 2006

ABSTRACT: Regeneration of the visual chromophore, 11-*cis*-retinal, is a critical step in restoring photoreceptors to their dark-adapted conditions. This regeneration process, called the retinoid cycle, takes place in the photoreceptor outer segments and the retinal pigment epithelium (RPE). Disabling mutations in nearly all of the retinoid cycle genes are linked to human conditions that cause congenital or progressive defects in vision. Several mouse models with disrupted genes related to this cycle contain abnormal fatty acid retinyl ester levels in the RPE. To investigate the mechanisms of retinyl ester accumulation, we generated single or double knockout mice lacking retinoid cycle genes. All-*trans*-retinyl esters accumulated in mice lacking RPE65, but they are reduced in double knockout mice also lacking opsin, suggesting a connection between visual pigment regeneration and the retinoid cycle. Only *Rdh5*-deficient mice accumulate *cis*-retinyl esters, regardless of the simultaneous disruption of RPE65, opsin, and prRDH. 13-*cis*-Retinoids are produced at higher levels when the flow of retinoid through the cycle was increased, and these esters are stored in specific structures called retinosomes. Most importantly, retinylamine, a specific and effective inhibitor of the 11-*cis*-retinol formation, also inhibits the production of 13-*cis*-retinyl esters. The data presented here support the idea that 13-*cis*-retinyl esters are formed through an aberrant enzymatic isomerization process.

Vitamin A transformations in the eye are essential for vision. Absorption of light by the vitamin A-derived chromophore of visual pigments, 11-*cis*-retinal, leads to its photoisomerization to all-*trans*-retinal and the initiation of the signal transduction cascade (*1*−*4*). Through a chain of reactions, all-*trans*-retinal is recycled enzymatically back to 11-*cis*-retinal (*5*−*8*). These retinoid cycle reactions (Figure 1) take place in the outer segments of photoreceptor cells and in the adjacent retinal pigment epithelium (RPE).[1] Characterization of the knockout mice lacking genes involved in the retinoid cycle provides important insight into the flow of retinoids in the eye.

11-*cis*-Retinol dehydrogenase (RDH), also known as RDH5, catalyzes the final oxidation reaction of 11-*cis*-retinol in the RPE (*9*, *10*). Although RDH5 is responsible for the majority of 11-*cis*-RDH activity, RDH11 and other RDHs also have a measurable role in regenerating the visual pigment by complementing RDH5 in RPE cells (*11*). Disruption of the gene encoding RDH5 causes fundus albipunctatus in humans (*12*, *13*). Fundus albipunctatus is an autosomal recessive form of congenital stationary night blindness characterized by the appearance of numerous small white dots located in the RPE, a delayed course of dark adaptation, and occasionally progressive cone dystrophy (*14*). Deletion of 11-*cis*-RDHs in mice, *Rdh5*−/− and *Rdh11*−/−, causes delayed dark adaptation and delayed 11-*cis*-retinal regeneration (*15*−*18*).

Lipid droplet-like organelles known as retinosomes were characterized as specific sites of all-*trans*-retinyl ester accumulation in the RPE (*19*, *20*). All-*trans*-retinyl esters accumulate in the RPE of wild-type mice as they are trapped from circulation and also during the recycling of all-*trans* chromophore from visual pigments (Figure 1). The vast majority of retinyl esters in wild-type mice maintain an all-*trans* configuration. 11-*cis*-Retinal appears to be produced on demand when high numbers of visual pigments are uncoupled from their chromophore (*21*). Deletion of specific genes of the retinoid cycle, however, leads to severe abnormalities in ester levels. For example, the levels of all-*trans*-retinyl esters are highly elevated in *Rpe65*−/− mice (*22*), while in *Rdh5*−/− mice, 13-*cis*-retinyl ester levels are surprisingly high (*15*). RPE65 has been proposed to be the isomerase responsible for transformation of all-*trans*-retinyl esters to 11-*cis*-retinol (*23*−*26*) (Figure 1). However, in a purified form, it has been reported that RPE65 does not possess the isomerase activity (*27*).

In this study we examine the formation, accumulation, and utilization of retinyl esters in the retinoid cycle, employing knockout mice lacking genes that encode proteins of the phototransduction and retinoid cycle. Our results point to relaxed stereospecificity of the isomerization complex as the cause of abnormal all-*trans*- to 13-*cis*-retinoid isomerization. 13-*cis*-Retinyl esters are stored in retinosomes. We show also a connection/communication between rod photoreceptors and RPE in the eye.

† This research was supported by NIH Grant EY09339.

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[1] Abbreviations: CRALBP, cellular retinaldehyde-binding protein; CRBP, cellular retinoid-binding protein; LRAT, lecithin:retinol acyltransferase; RDH, retinol dehydrogenase; RPE, retinal pigment epithelium; retinosomes, retinyl ester storage particles; Ret-NH_2, retinylamine; RPE65, an RPE-specific 65 kDa protein.

10.1021/bi052382x CCC: $33.50
Published on Web 03/14/2006

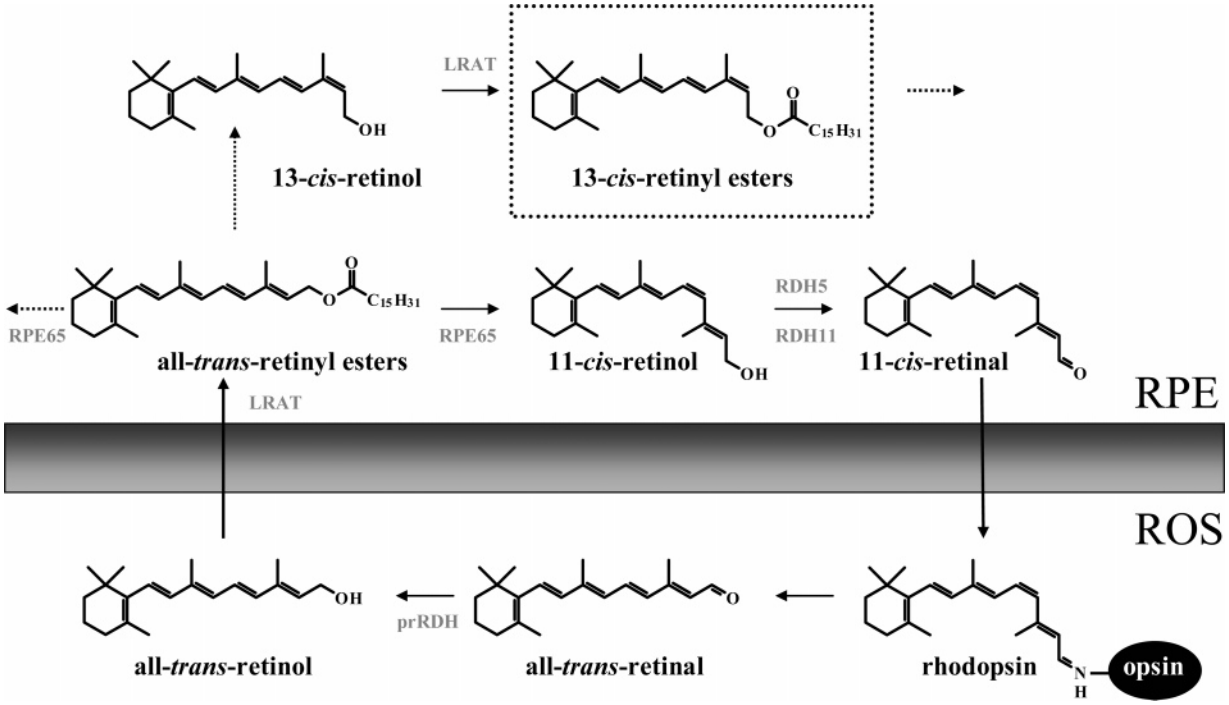

FIGURE 1: Chemistry of the retinoid cycle reactions in the vertebrate retina. The retinoid cycle reactions were reviewed recently (*6*). In the rod outer segments (ROS), light causes the isomerization of the rhodopsin chromophore, 11-*cis*-retinylidene, to all-*trans*-retinylidene, which is hydrolyzed and released from opsin. All-*trans*-retinal is then reduced in a reaction catalyzed by all-*trans*-retinal-specific RDH(s) including prRDH. All-*trans*-retinol diffuses to retinal pigment epithelium (RPE) where it is esterified by LRAT to fatty acid all-*trans*-retinyl esters. All-*trans*-retinyl esters or its derivative is isomerized to 11-*cis*-retinol in a reaction that involves an abundant RPE protein, termed RPE65. 11-*cis*-Retinol is then oxidized by 11-*cis*-RDH (RDH5, RDH11) and other dehydrogenases to 11-*cis*-retinal, completing the cycle. 11-*cis*-Retinal diffuses across the extracellular space, is taken up by the ROS, and recombines with opsin to regenerate rhodopsin. In aberrant reactions, all-*trans*-retinyl esters or its derivative is isomerized to 13-*cis*-retinol. 13-*cis*-Retinol can also be esterified by LRAT to form 13-*cis*-retinyl esters, stored in retinosomes.

EXPERIMENTAL PROCEDURES

Animals. All animal experiments employed procedures approved by the Case Western Reserve University, and initially by University of Washington, Animal Care Committee, and conformed to the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the recommendations of the Association of Research for Vision and Ophthalmology. Animals were maintained in complete darkness or on a 12 h light/12 h dark cycle, and all manipulations were done under dim red light employing a Kodak No. 1 safelight filter (transmittance >560 nm). Typically, 2−3-month-old mice were used in all of the experiments. *Rdh11*-deficient mice were generated by Dr. P. Nelson (Fred Hutchinson Cancer Research Center, Seattle, WA), and their genotyping and characterization were described previously (*11*). *Rdh5*-deficient mice were previously generated and characterized by Drs. C. Driessen and J. J. Janssen (*15*). Genotypes of *Rdh5*−/− mice were determined by PCR (*15*). *Opsin*−/− mice were obtained from Dr. J. Lem (Tufts University, Boston, MA). These mice were genotyped as described previously (*28*). *Rpe65*-deficient mice were obtained from Dr. M. Redmond (NEI, National Institutes of Health) and genotyped as described previously (*22*). *Prrdh*- and *Lrat*-deficient mice were generated and characterized in our laboratory in collaboration with Dr. W. Baehr (University of Utah) as described previously (*29*, *30*). Double knockout mice were generated by cross-breeding single knockout mice to genetic homogeneity. All mice were outbred by standard procedures into the pigmented C57BL/6J strain (Jackson, Bar Harbor, ME).

Immunoblotting. The immunoblotting was carried out according to standard protocols using Immobilon-P to adsorb proteins [poly(vinylidene difluoride); Millipore Corp.]. Monoclonal anti-rhodopsin antibody was provided by Dr. R. Molday, rabbit polyclonal anti-RPE65 was a gift from Dr. J. Saari, and monoclonal anti-LRAT antibody was generated in our laboratory (*29*). Alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Promega) was used as a secondary antibody. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color development substrate (Promega).

Retinoids: *Analyses, Synthesis, and Treatments.* All of the experimental procedures related to the analysis of dissected mouse eyes, derivatization, and separation of retinoids have been described previously (*31*). Retinoic acids and their derivatives were measured as reported previously (*32*). Typically, two mouse eyes were used per assay, and the assays were repeated three to six times. Retinylamine (Ret-NH_2) was synthesized by the method described previously (*33*). Oral gavage was carried out as described previously (*31*, *34*).

Isomerization Assay. Isomerization assay and preparation of proteins were carried out as described in prior publications (*35*, *36*).

Intraocular Injection. Mice were anesthetized by intraperitoneal injection using 20 μL/g body weight of 6 mg/mL ketamine and 0.44 mg/mL xylazine diluted with 10 mM sodium phosphate, pH 7.2, containing 100 mM NaCl. Anesthetized mice were injected with 1 μL of DMSO solution containing all-*trans*-retinol in the vitreous cavity of

Table 1: Single Gene Knockout Mice Used in the Experiments Described in This Report

genotype	role of the gene	phenotype	ref[a]
opsin−/−	photoreceptor of rod cells, G protein-coupled receptor	lack of ROS formation, lack of rod-mediated vision	*49*
Rpe65−/−	abundant RPE protein involved in the isomerization process of all-*trans*-retinyl esters to 11-*cis*-retinol (isomerase)	lack of 11-*cis*-retinal production and residual visual functions, accumulation of retinyl esters in large inclusion droplets	*22*
Rdh5−/−	*cis*-retinol dehydrogenase	delayed dark adaptation, accumulation of 13-*cis*- and 11-*cis*-retinyl esters	*15*
Lrat−/−	lecithin:retinol acyltransferase, a major enzyme involved in retinal esterification in many tissues	lack of 11-*cis*-retinal production and residual visual functions, lack of significant levels of retinyl esters in the eye and liver	*29*
Rdh11−/−	dual specificity (*cis/trans*) retinol dehydrogenase	delayed dark adaptation	*11*
Prrdh−/−	photoreceptor-specific all-*trans*-retinol dehydrogenase	delayed dark adaptation	*30*

[a] Reference to original report on mouse genotype.

the eye. The injection was performed with a 30 gauge Hamilton microneedle syringe through the sclera at a point 1 mm from the limbus to avoid puncture through the lens.

Two-Photon Vitamin A Imaging. Two-photon excitation microscopy was performed using a confocal/two-photon laser scanning microscope (LSM 510 MP-NLO; Carl Zeiss, Inc., Thornwood, NY) with LSM510 software, version 3.0 (*19*, *20*). Briefly, 76 MHz, 100 fs pulses of 730 nm light from a mode-locked Ti:Sapphire laser (Mira-900; Coherent, Mountain View, CA) were focused on the sample by a Plan-Neofluar 40×/1.3 NA objective lens (Carl Zeiss). The intensity of the laser was measured at the back aperture of the objective lens and kept at ∼3 mW. Autofluorescence from the sample (390−545 nm) was collected by the objective, separated from the excitation light by a dichroic mirror, filtered to remove scattered excitation light, and directed to a photomultiplier tube detector. The objective lens was heated to 37 °C by an air stream incubator. A temperature-controlled microscopic stage was installed on the microscope to maintain the reaction at 37 °C. Fluorescent intensities, expressed in pixel values, were calculated by off-line analysis of the collected raw images (SCION image; Scion Co., Frederick, MD). Fluorescent intensity was measured for the tangential sections of the RPE cells and averaged per pixel for randomly chosen areas (mean ± SD, $n = 3$) enclosing 200 × 200 pixels (∼32 × 32 μm^2).

Mice were light adapted under room light for 1 h, and isolated eyecups were located at the center of a glass-bottomed 35 mm dish and perfused with the oxygenized (95% O_2, 5% CO_2) Ames medium (Sigma) at 37 °C. In case of a slight movement of the eye, the same area of the retina was traced by using the unique texture of the RPE cell layer formed by randomly arranged single and double nucleated RPE cells.

Electron Microscopy. For transmission electron microscopy, mouse eyecups were analyzed as described previously (*30*).

RESULTS

To investigate the mechanisms of retinyl ester accumulation, we used single or double knockout mice with disrupted genes of the retinoid cycle (Figure 1). The roles of these gene products are described in Table 1.

Lack of Rdh5 Results in cis-Retinyl Ester Accumulation. *cis*-Retinyl esters, mostly in the form of 13-*cis*-retinyl esters, accumulated to significant levels only in mice lacking the functional *Rdh5* gene (Figure 2) (Table 2). The highest levels of 13-*cis*-retinyl esters were observed in the eyes of *Rdh5*−/− *Rdh11*−/− mice. Interestingly, high levels of *cis*-retinyl esters were observed not only in *opsin*−/−*Rdh5*−/− mice (Table 2) but also in heterozygote *opsin*+/−*Rdh*5−/− mice (Figure 3). Disruption of the *Rpe65* and *Prrdh* genes did not considerably affect the level of *cis*-retinyl esters. The ablation of *Rdh5* and *Rdh11* genes did not appreciably affect the expression levels of LRAT or RPE65 (Figure 4).

All-*trans*-retinyl esters accumulated to significant levels only in mice lacking RPE65 (Figure 2A) (Table 2). Additional disruption of the *opsin* gene significantly lowered the level of all-*trans*-retinyl esters in *Rpe65*-deficient animals and increased levels of *cis* esters in *Rdh5*-deficient animals. In *opsin*−/− animals, 11-*cis*-retinal was present at a low level (Figure 2A) (Table 2), of which a majority most likely bound to CRALBP, as the cone pigments are sparse in mice (*37*). Simultaneous disruption of *Rdh5* and *Rpe65* genes led to lower levels of all-*trans*-retinyl esters than did single knockouts, while these and other retinoids were not detected in *Lrat*−/−*Rpe65*−/− mice.

Employing electron microscopy, we analyzed the retinas for morphological changes in double knockout *Lrat*−/− *Rpe65*−/− mice and compared them with *Rpe65*−/− mice (*19*, *22*). The outer segments of *Lrat*−/−*Rpe65*−/− mice were shortened, loaded with opsin instead of rhodopsin as indicated by retinoid analysis, and displayed an underdeveloped synaptic inner plexiform layer (IPL) (Figure 5A−E), which was likely due to overstimulation by free opsin in the rod photoreceptor cells. No lipid droplets were observed in *Lrat*−/−*Rpe65*−/− mice. Similarly, the lack of opsin in *opsin*−/−*Rpe65*−/− mice reduced the formation of these ester droplets (Figure 5F−H), while they remained prominent, although reduced in size, in *Rdh5*−/−*Rpe65*−/− mice, which had fewer total esters than *Rpe65*−/− mice (Figure 5I−K). Reducing levels of retinyl esters by rescuing visual pigments with 9-*cis*-retinal also led to the shrinkage of these droplets in *Rpe65*−/− mice (*34*).

13-cis-Retinoids Are Produced at Higher Levels in Light/Dark-Reared Mice. 13-*cis*-Retinoids were produced at higher levels in light/dark-reared *Rdh5*−/−*Rdh11*−/− mice than in mice never exposed to light (Figure 6A), while light exposure did not lead to accumulation of these esters in wild-type mice. Exposure of dark-adapted *Rdh5*−/−*Rdh11*−/− mice to intense illumination enhanced production of 13-*cis*-retinyl esters. After exposure to light, transiently elevated all-*trans*-retinyl esters decayed to normal dark-adapted levels concurrently with the regeneration of rhodopsin, while 13-*cis*-retinyl

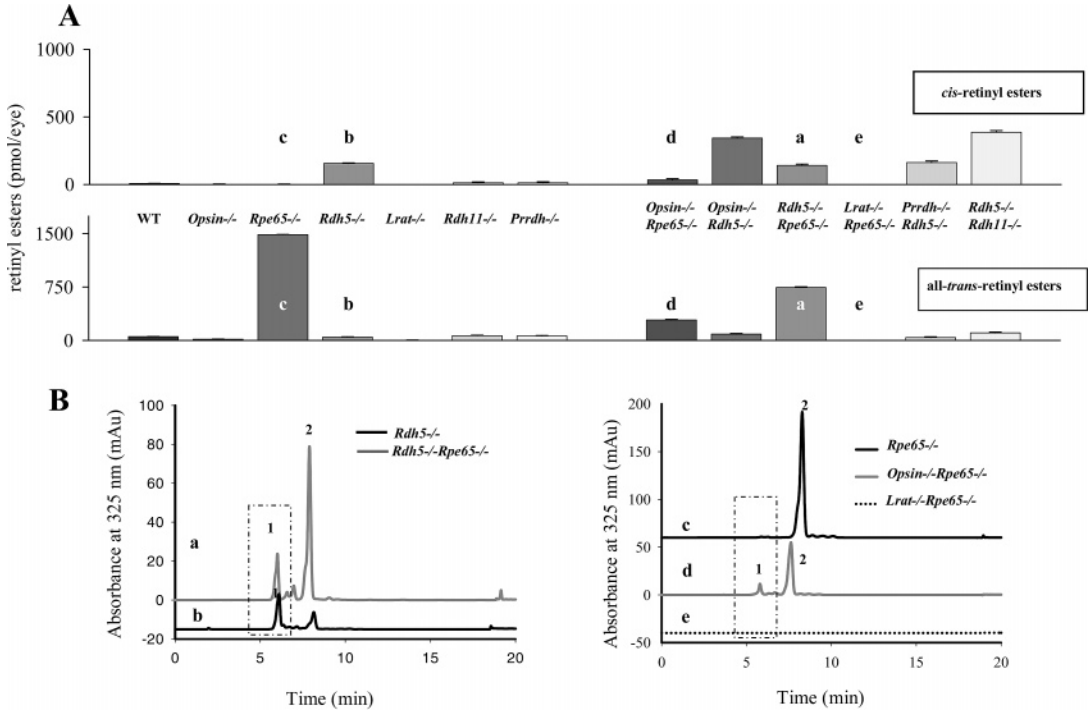

FIGURE 2: Retinyl esters in the eyes of mice of different genetic backgrounds. (A) All-*trans*- and *cis*-retinyl esters in mice of different genetic backgrounds. Retinoids were extracted from 6-week-old eyes and separated on normal-phase HPLC as described in Experimental Procedures. (B) Chromatographic separation of all-*trans*- and *cis*-retinyl esters from (a) *Rpe65*−/−, (b) *opsin*−/−*Rdh65*−/−, (c) *Lrat*−/−*Rpe65*−/−, (d) *Rdh5*−/−*Rpe65*−/−, and (e) *Rdh5*−/− mice. Retinoids were extracted from 6-week-old eyes and separated on normal-phase HPLC. The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters) also indicated as peak 1. Peak 2 represents all-*trans*-retinyl esters. The mice were reared under dim red light.

Table 2: Retinoid Levels in Genetically Varied Mice Lacking Proteins of the Retinoid Cycle[a]

genotype	13-*cis*-/11-*cis*-retinyl esters (pmol/eye)	all-*trans*-retinyl esters (pmol/eye)	11-*cis*-retinal (pmol/eye)	11-*cis*-retinol (pmol/eye)	all-*trans*-retinal (pmol/eye)	all-*trans*-retinol (pmol/eye)
single knockout						
WT	6.6 ± 0.8	52.6 ± 4	575 ± 30	18 ± 4	45 ± 6.5	4.6 ± 0.6
opsin−/−	2.0 ± 0.5	15.8 ± 4.6	13.7 ± 1.2	7.1 ± 1.9	trace	4.8 ± 1.3
Rpe65−/−	trace	1488 ± 65	none	none	3 ± 0.1	7.4 ± 0.7
Rdh5−/−	155 ± 40	47 ± 9.8	508 ± 24	9.2 ± 3.5	22.3 ± 2.8	4.8 ± 1.3
Lrat−/−	trace	trace	trace	trace	trace	7.9 ± 1.3
Rdh11−/−	12 ± 1.4	67 ± 13.4	510 ± 24	13.1 ± 1.1	17 ± 0.1	0.8 ± 1.5
Prrdh−/−	12 ± 0.8	62 ± 4.2	514 ± 30	20 ± 7	40.9 ± 9.4	7.9 ± 1.0
double knockout						
opsin−/−*Rpe65*−/−	34 ± 7.5	288 ± 107	none	trace	trace	89 ± 3.2
opsin−/−*Rdh5*−/−	343 ± 91	88.8 ± 29	10.1 ± 3.9	trace	trace	trace
Rdh5−/−*Rpe65*−/−	140 ± 24	744 ± 98	none	trace	trace	96 ± 21
Lrat−/−*Rpe65*−/−	trace	trace	none	trace	trace	8 ± 2.1.4
Prrdh−/−*Rdh5*−/−	162 ± 17	39 ± 7.9	536 ± 39	19.7 ± 2.5	9.2 ± 6.5	3.5 ± 1.0
Rdh5−/−*Rdh11*−/−	186 ± 34	104 ± 6	480 ± 18	trace	29.5±2.0	trace

[a] Mice were genotyped, and retinoid analysis was performed as described in Experimental Procedures. *cis*-Retinyl esters were integrated as 13-*cis*-retinyl esters (>90% *cis*-retinyl esters).

esters once formed remained stable for at least 1 week (data not shown). As investigated by two-photon microscopy (*19*), 13-*cis*-retinyl esters likely accumulated in the retinosomes (Figure 6B), because these structures became inflated and displayed higher RPE fluorescence compared with those of control wild-type and *Rdh5*+/− *Rdh11*+/− mice (Figure 6C).

13-cis-Retinol Is Formed through an Enzymatic Isomerization Process. Biochemical data described below supported the idea that 13-*cis*-retinyl esters are formed through an isomerization process. When all-*trans*-retinol was injected into the eyes of *Rdh5*−/−*Rdh11*−/− mice, the amount of 13-*cis*-retinyl esters formed in darkness increased from 83.1 ± 9.3 pmol (in control mice treated only with DMSO) to 220.4 ± 33.2 pmol in 30 min and to 498.5 ± 49 pmol in 2 days (Figure 7). Thus, 13-*cis*-retinyl esters are formed independently of light. Second, when *Rdh5*−/−*Rdh11*−/− mice were gavaged with a potent inhibitor of 11-*cis*-retinal production, all-*trans*-Ret-NH_2 (*38*), and then exposed 24 h later to intense light for 3 min at 500 cd·m^{-2}, only a modest increase in 13-*cis*-retinyl ester production was observed, whereas untreated control mice showed a severalfold increase in 13-*cis*-retinyl esters. No formation of these *cis* esters was observed in wild-type treated or untreated mice (Figure 8A). Finally, we examined the effects of Ret-NH_2 on 13-*cis*-retinyl ester production after intraocular injection of all-*trans*-retinol. The inhibitor completely stopped isomerization to 13-*cis*-retinyl esters in *Rdh5*−/−*Rdh11*−/− mice (Figure 8B).

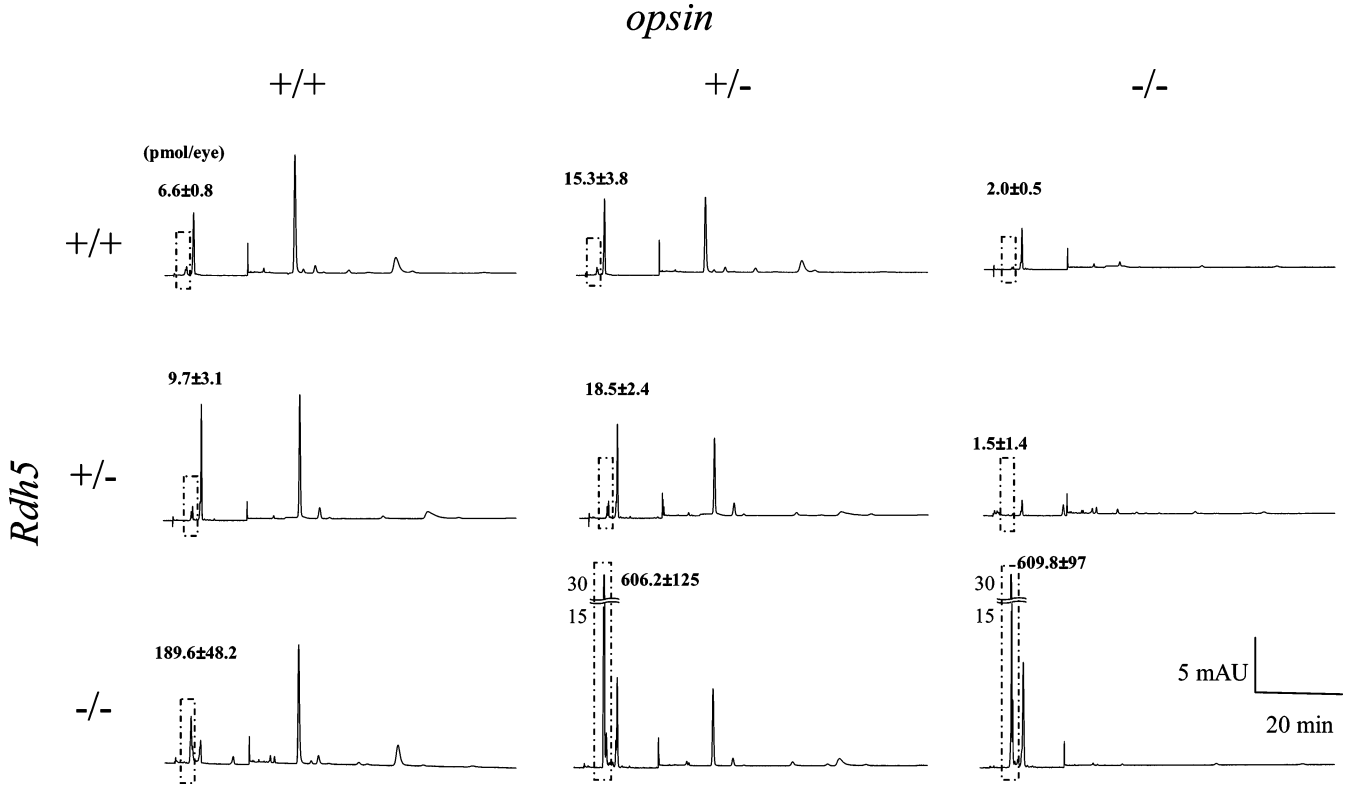

FIGURE 3: Chromatographic separation of nonpolar retinoids from *opsin*−/−*Rdh5*−/− mice. Retinoids were extracted from 6-week-old eyes and separated on normal-phase HPLC. The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD).

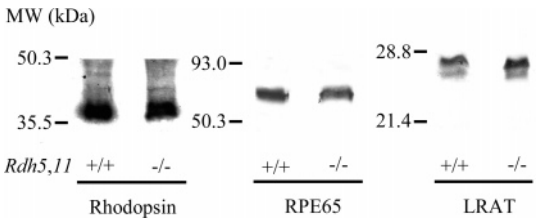

FIGURE 4: Immunoblotting of eyecup extracts from wild-type and *Rdh5*−/−*Rdh11*−/− mice probed with anti-rhodopsin (1D4), anti-RPE65, or anti-LRAT antibodies. The eyecup extracts were prepared from mice by homogenizing with the sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer. The proteins (30 μg) were analyzed by 12.5% SDS−PAGE. The eyecup extract from *Rdh5*−/−*Rdh11*−/− mice showed no significant difference in the levels of rhodopsin, RPE65, or LRAT compared with wild-type mice.

To prove that the inhibitor blocks this aberrant isomerization, we demonstrated that Ret-NH_2 stops production of 13-*cis*-retinol in vitro in the presence of bovine serum albumin (BSA) (Figure 9). These experiments are an extension of the observations made by McBee and coworkers establishing that isomerization depends on the specificity of the acceptor protein used in the isomerization reaction (*36*). Production of both 11-*cis*- and 13-*cis*-retinol in the presence of Ret-NH_2 was inhibited in the presence of appropriate retinoid-binding proteins (Figure 9). Injection of 13-*cis*-retinol into the eyes of *Rdh5*−/−*Rdh11*−/− and wild-type mice did not lead to differences in production of retinoic acid or its oxidation products (data not shown), indicating that the accumulation of 13-*cis*-retinyl esters in these knockout mice is not the result of a defective clearing process in the oxidation pathway (*32*).

DISCUSSION

The retinoid cycle is a two-cell biochemical pathway that requires the concerted action of several enzymes. It has been recently reported that RPE65 possesses retinol isomerase activity (*23*−*26*), while RDH5 is one of the enzymes catalyzing the last step of the retinoid cycle, oxidation of 11-*cis*-retinol to 11-*cis*-retinal (*11*, *12*, *17*, *39*). In mice, disruption of the *Rdh5* gene results in the accumulation of *cis*-retinyl esters (*15*). Interestingly, 13-*cis*-retinyl esters are observed in *Rdh5*−/−*Rpe65*−/− mice, which we believe are formed enzymatically through a molecular mechanism similar to that for 11-*cis*-retinol (*36*). To gain insight into the mechanism of 13-*cis*-retinyl ester formation, we produced animals with simultaneous disruption of *Rdh5* and other genes of the retinoid cycle as well as opsin. Disruption of the *Rdh5* gene concomitantly with the *Rpe65* gene results in a different phenotype than the disruption of the *Rpe65* gene alone. For example, *Rpe65*−/− mice accumulate all-*trans*-retinyl esters; however, *Rdh5*−/−*Rpe65*−/− double knockouts have far fewer all-*trans*-retinyl esters than the *Rpe65*−/− mice, but these mice still accumulated 13-*cis*-retinyl esters. This suggests that the process of hydrolysis−isomerization−oxidation of all-*trans*-retinyl esters to 11-*cis*-retinal is aberrant in a unique way when one or more components of the retinoid cycle genes are eliminated. Hence, the retinoid analysis of mice with a combination of different retinoid cycle genes provides the opportunities to uncover interconnections between these gene products and understanding in more detail their involvements in the cycle.

Accumulation of 13-cis-Retinyl Esters in the RPE in Rdh5−/− *Mice.* Accumulation of 13-*cis*-retinyl esters is a result of isomerization of all-*trans*-retinoids to 13-*cis*-retinol

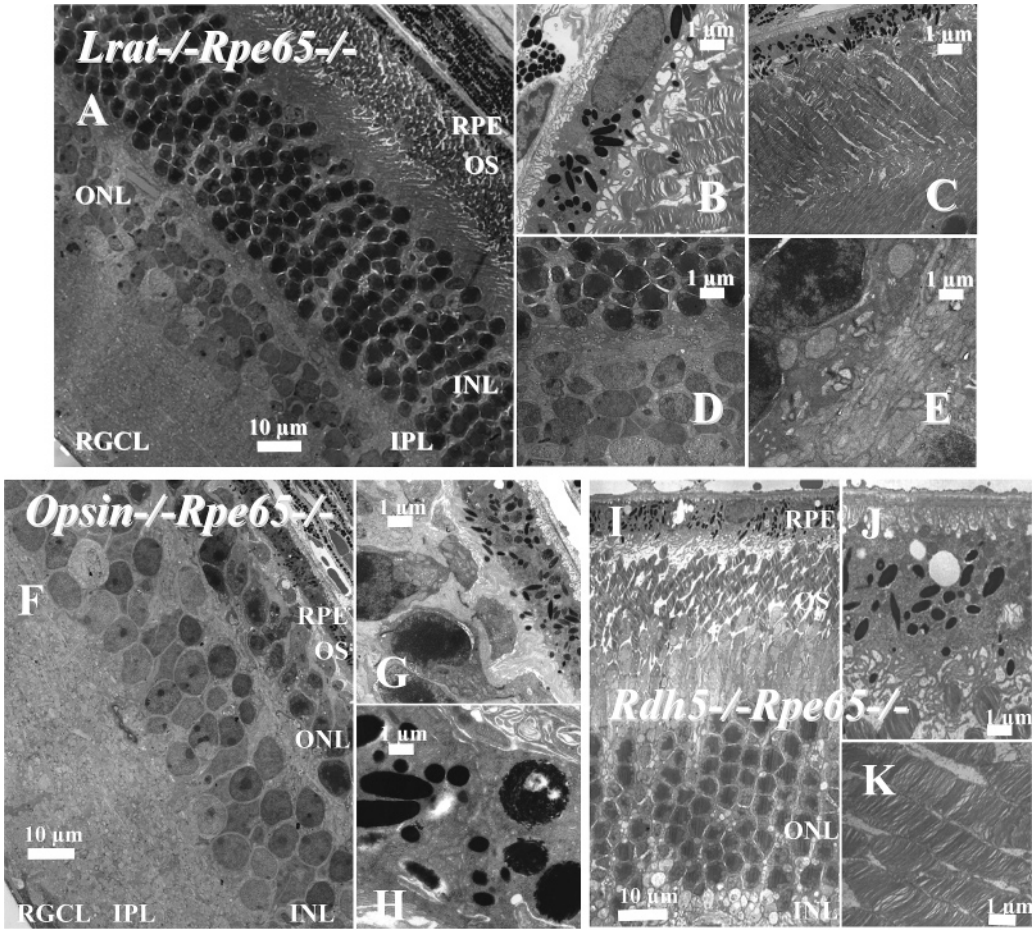

FIGURE 5: Montage of cross sections of the retinas of 2-month-old *Lrat*−/−*Rpe65*−/− mice analyzed by transmission electron microscopy. Panel A shows the cross section of the RPE and the photoreceptor cells. Panels B and C show higher magnification sections of the RPE and ROS (B, C) and the IPL (D, E). (F−H) Montage of cross sections of the retinas of 2-month-old *opsin*−/−*Rpe65*−/− mice analyzed by transmission electron microscopy. Panel F shows the cross section of the RPE and the photoreceptor cells. Panels G and H show higher magnification sections of the RPE and ROS, repectively. (I−K) Montage of cross sections of the retinas of 2-month-old *Rdh5*−/−*Rpe65*−/− mice analyzed by transmission electron microscopy. Panel I shows the cross section of the RPE and photoreceptor cells. Panels J and K show a higher magnification of the RPE and ROS. The sections were prepared as described in Experimental Procedures. The scale bar represents 1 or 10 μm as indicated.

and esterification of the product by LRAT to 13-*cis*-retinyl esters (Figures 1 and 2). Even *Rdh5*−/−*Rdh11*−/− mice kept in normal room light showed very high levels of 13-*cis*-retinyl esters, but after exposure to high-intensity light these mice further accumulated 13-*cis*-retinyl esters. These esters were not observed in wild-type mice. The retinosomes of the RPE are the storage site of 13-*cis*-retinyl and all-*trans*-retinyl esters. The storage amount was enlarged to 400−600 pmol/eye, and accumulated 13-*cis*-retinyl esters remained for more than 1 week in the RPE.

What can account for this mechanism? For one, the lack of RDH5 may perturb the highly compartmentalized retinoid cycle (see, for example, ref *20*), resulting in the production of *cis*-retinol according to the thermodynamics of the isomerization reaction and distribution of the hydration product of the putative carbocation intermediate (*40*). This aberrant isomer is produced not only from all-*trans*-retinal liberated from opsin during a high demand for 11-*cis*-retinal but also in *Rdh5*−/−*Rpe65*−/− mice lacking rhodopsin. The product of this isomerization is not utilized but is stored in the retinosomes. Perhaps this is due to the lack of 13-*cis*-retinol to 13-*cis*-retinal oxidation activity in *Rdh5*−/− mice (*16*) and further oxidation reactions producing inactive oxidized retinoid, which could eliminate this aldehyde.

Alternatively, the isomerization complex lacking RDH5 may have relaxed specificity, producing both 11-*cis*- and 13-*cis*-retinoids. The fact that there is a substantial 13-*cis*-retinyl ester formation in the *Rdh5*−/−*Rpe65*−/− mice supports the notion that RPE65 is not required for 13-*cis*-retinoid formation, but these animals have only trace amounts of 11-*cis*-retinoids, consistent with RPE65 being essential for the 11-*cis*-retinal production. Hence, RPE65 is not responsible for enzymatic conversion of all-*trans*-retinoids to their corresponding 13-*cis* isomers but essential for 11-*cis*-retinal production (*22*).

In wild-type mice, light can convert a small amount of all-*trans*-retinoids to 13-*cis*-retinoids, and potentially RPE65 is capable of interconverting all-*trans*-, 11-*cis*-, and 13-*cis*-retinol, but in normal conditions the reaction is driven by RDH5 and the retinoid-binding proteins selectively removing the 11-*cis* product. However, our *Rdh5*−/− mice maintained under dim red light to avoid photoisomerization still accumulated 13-*cis*-retinyl esters, diminishing the probability of this mechanism. Moreover, *Rdh5*−/−*Rpe65*−/− mice lacking visible light absorbing rhodopsin still produce 13-

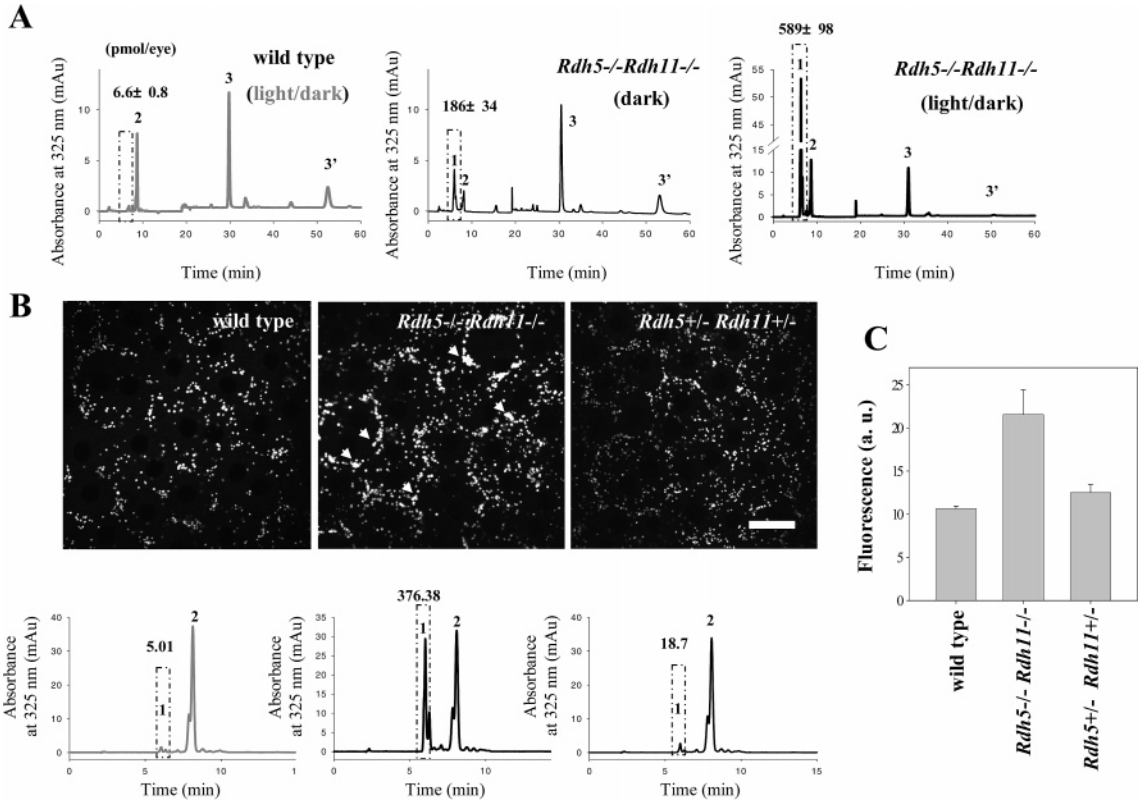

FIGURE 6: Light-dependent formation and storage of retinyl esters in eyes from *Rdh5*−/−*Rdh11*−/− mice. (A) Effect of dark or light rearing on the accumulation of 13-*cis*-retinyl esters in eyes from *Rdh5*−/−*Rdh11*−/− mice and wild-type controls. The dark-reared mice were exposed to no other light than dim red illumination. Peaks 1, 2, and 3/3′ represent *cis*-retinyl esters, all-*trans*-retinyl esters, and 11-*cis*-retinal oximes, respectively. The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD). (B) Imaging of retinyl esters by two-photon microscopy (top row) and quantification of retinyl esters by HPLC (bottom row). Left column: Wild-type mice contained all-*trans*-retinyl esters in retinosomes (RESTs). Middle column: *Rdh5*−/−*Rdh11*−/− mice stored all-*trans*- and 13-*cis*-retinyl esters in retinosomes (white arrow). Retinosome fluorescence in *Rdh5*−/−*Rdh11*−/− mice is more intense than in wild-type mice (arrowheads). Right column: Distribution and quantity of all-*trans*-retinyl esters in eyes from *Rdh5*+/−*Rdh*+/− mice were similar to those of wild type. The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD). (C) Quantification of fluorescence intensity measured by two-photon microscopy. Fluorescence intensity is higher in *Rdh5*−/−*Rdh11*−/− compared to wild-type and *Rdh5*+/−*Rdh*+/− mice ($n = 3$). The mean ± SD was indicated.

cis-retinoids. Alternatively, not light, but thermal isomerization of retinoids could be the responsible factor. To test this possibility, we employed a very potent inhibitor of isomerization, Ret-NH_2, which does not bind to most of the RPE65 pool present in the RPE (*38*, *41*). This inhibitor prevents the isomerization of all-*trans*- to both the 11-*cis*- and the 13-*cis*-retinol configuration, providing evidence that this reaction is enzymatic. This conclusion is also supported by the earlier chemical evidence of a similar mechanism of enzymatic isomerization of *trans* isomers to 11-*cis*- and 13-*cis*-retinol through the alkyl cleavage (*36*). Observed differences between *Rpe65*-null (no production of 11-*cis*-retinal) and Ret-NH_2-administrated *Rdh5*−/−*Rdh11*−/− mice (inhibition of 11-*cis*- or 13-*cis*-retinoid production) thus suggest the existence of an enzyme which requires the other protein components such as RPE65 and RDH5 for the production of 13-*cis* isomer or an activity which is suppressed by the presence of RPE65 and RDH5 in wild-type mice. This observation is puzzling when it is considered that RPE65 has been recently proposed to be an isomerohydrolase, the enzyme responsible for isomerization and hydrolysis of all-*trans*-retinyl esters to 11-*cis*-retinol (*23*−*26*). It should be noted that purified RPE65 has no isomerase activity (*27*). Further studies are required to understand the protein complex responsible for the retinoid isomerization in the RPE cells.

This aberrant isomerization process to 13-*cis*-retinoids occurs to a significant degree when RDH5 is eliminated, and it is further enhanced when opsin is deleted (Figures 2 and 3). High levels of *cis*-retinyl esters were observed not only in *opsin*−/−*Rdh5*−/− mice but also in heterozygote *opsin*+/−*Rdh*5−/− mice (Figure 3), suggesting that the lack of 11-*cis*-retinal, the final product of the retinoid cycle, or the lack of the full complement of rhodopsin in rod outer segments of *opsin*+/− mice (*42*) enhances this aberrant isomerization. Opsin, or the physical interaction of the rod outer segment structure with the RPE processes, can influence the retinoid cycle through an uncovered yet signaling pathway. This hypothesis is supported by accumulation of all-*trans*-retinyl esters in mice of different genetic backgrounds and production of 11-*cis*-retinal on demand.

Accumulation of All-trans-Retinyl Esters in the RPE. If esterification and hydrolysis depended only on mass action, a steady level of retinyl esters would be present in the eye. However, this is not what is observed in knockout mice deprived of key proteins of the retinoid cycle. In addition,

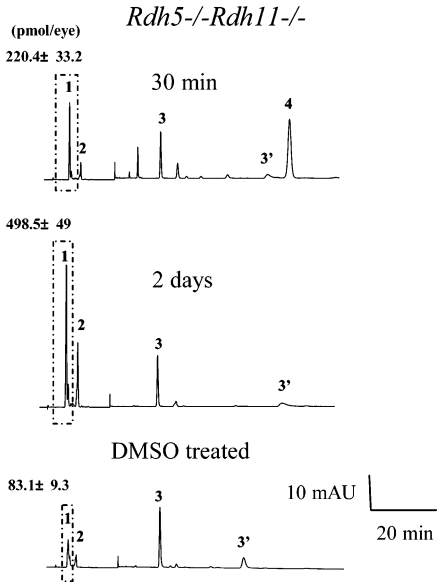

FIGURE 7: Enzymatic production of 13-*cis*-retinyl esters by *Rdh5*−/−*Rdh11*−/− mice. Mice were dark adapted for 48 h, followed by intravenous injection of 40 nmol of all-*trans*-retinol in 1 μL of DMSO with a 30 gauge needle under anesthesia. Retinoid analysis was performed both 30 min and 2 days after injection. All experiments were carried out in the dark. A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD). Retinoids were extracted from 6-week-old eyes and separated on normal-phase HPLC. The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). Peaks 1, 2, 3/3′, and 4 represent *cis*-retinyl esters, all-*trans*-retinyl esters, 11-*cis*-retinal oximes, and all-*trans*-retinol, respectively.

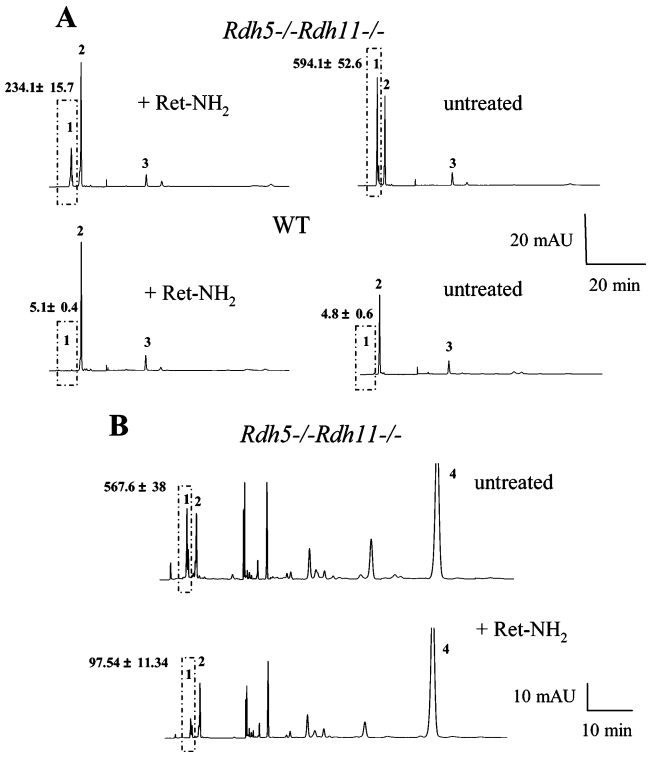

FIGURE 8: Effects of Ret-NH_2 on 13-*cis*-retinyl ester production. (A) Effects of Ret-NH_2 on 13-*cis*-retinyl ester production induced by light. Mice were dark adapted for 48 h, gavaged with 1 mg of Ret-NH_2, and then exposed to intense light for 3 min at 500 cd·m^{-2}, 24 h postgavage. A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD). The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). (B) Effects of Ret-NH_2 on 13-*cis*-retinyl ester production from injected all-*trans*-retinol. Mice were dark adapted for 48 h and gavaged with 1 mg of Ret-NH_2. After 24 h, 400 nmol of all-*trans*-retinol in 1 μL of DMSO was intravenously injected. A representative chromatogram is shown, and the average data from three mice are indicated with standard deviation above the ester peaks (mean ± SD). The box represents *cis*-retinyl esters (>90% 13-*cis*-retinyl esters). Peaks 1, 2, 3/3′, and 4 represent *cis*-retinyl esters, all-*trans*-retinyl esters, 11-*cis*-retinal oximes, and all-*trans*-retinol, respectively.

excessive all-*trans*-retinyl esters normally accumulate in the eyes of wild-type mice with age (*34*).

Redmond et al. first observed that *Rpe65*−/− mice do not possess an 11-*cis*-retinal chromophore and also tend to overaccumulate retinyl esters in lipid droplets (*22*). The esters accumulated with age (*34*) by means of the budding of naturally occurring retinyl ester storage particles, termed retinosomes (*19*, *20*). Two hypotheses can be postulated that are not exclusive of each other. First, the RPE may recognize a signal from photoreceptor cells by an undefined chemical sensory means that opsin is unliganded and more 11-*cis*-retinal needs to be produced. In such a scenario, the RPE would first accumulate retinyl esters to provide a substrate for further transformations leading to the production of 11-*cis*-retinal. A genetic defect or a lack of RPE65 would stall the isomerization process, and consequently the chromophore would not be produced, while the esters would continue to accumulate. Recent identification of RPE65 as the isomerase is consistent with this view (*23*−*26*). When the *opsin* gene is deleted from *Rpe65*−/− mice, a 3−4-fold decrease in the ester content is observed (Figure 2), which also supports this hypothesis. This effect of opsin on the level of all-*trans*-retinyl ester levels revealed a very important connection between phototransduction and the retinoid cycle. This hypothesis is also supported by the observation that the retinyl ester accumulation defect can be bypassed via oral gavage of 9-*cis*-retinal very early in life, slowing the accumulation of esters (*34*). It appears that unliganded opsin sends a signal to RPE, which responds to increased absorption of all-*trans*-retinol from circulation.

A second possibility is that RPE65 is also involved in the mobilization of stored retinyl esters (*43*). The accumulation of retinyl esters is clearly dependent on LRAT activity (Figure 2), and these esters accumulate in *Rpe65*−/− mice. RPE65 was also proposed to be a retinyl ester-binding protein (*27*, *44*, *45*), hinting of its possible role in this process. Most insightful was the observation made by Pepperberg et al. that, in normal RPE, there is a substantial exchange of all-*trans*-retinol with the blood circulation, whereas in *Rpe65*−/− mice, despite the presence of abnormally high molar levels of RPE retinyl esters, the outward movement is inhibited (*43*).

There are other circumstances in which retinyl esters accumulate in knockout mice, as in *Rgr*−/− (*46*), *Cralbp*−/− (*47*), and *Crbp*−/− mice (*48*) after bleach. However, this accumulation is due to the dynamic process during the retinoid cycle, a different process than discussed in this study. Here, we demonstrated that 13-*cis*-retinyl esters are formed through an aberrant enzymatic isomerization process involving an enzyme that is inhibited by Ret-NH_2 as is the isomerase that normally produces 11-*cis*-retinoids.

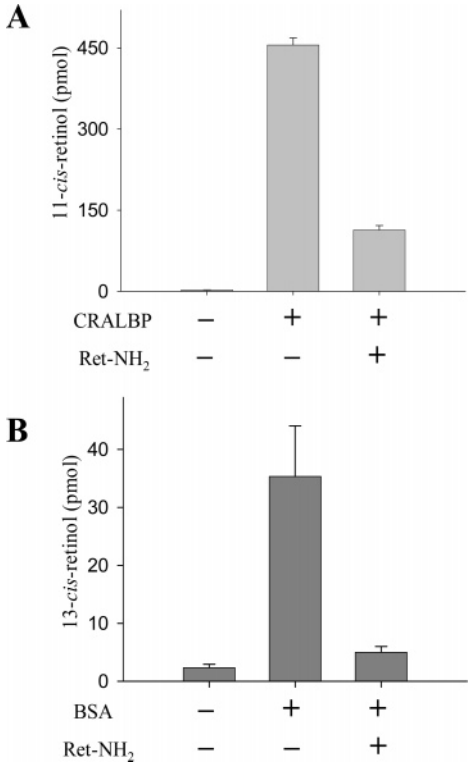

FIGURE 9: Influence of retinoid-binding proteins and Ret-NH_2 on the formation of 11-*cis*-retinol and 13-*cis*-retinol. The isomerization reaction was performed in 10 mM BTP buffer, pH 7.5, containing 1 mM ATP and 10% BSA. UV-treated bovine RPE microsomes were used as a source of enzyme (150 μg of protein). For inhibition assays, all-*trans*-Ret-NH_2 was used at a concentration of 3 mM. The reaction was initiated by adding all-*trans*-retinol in DMF and a solution of CRALBP or BSA to the final concentration of 10 mM or 10%, respectively. The reaction mixture was incubated in 37 °C for 1 h.

ACKNOWLEDGMENT

We thank Daniel Possin for electron microscopic analysis, Amy Look for technical help during the course of this study, and Rebecca Birdsong for help with manuscript preparation.

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BI052382X