RPE65 from Cone-dominant Chicken Is a More Efficient Isomerohydrolase Compared with That from Rod-dominant Species*

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Cones recover their photosensitivity faster than rods after bleaching. It has been suggested that a higher rate regeneration of 11-cis-retinal, the chromophore for visual pigments, is required for cones to continuously function under bright light conditions. RPE65 is the isomerohydrolase catalyzing a key step in regeneration of 11-cis-retinal. The present study investigated whether RPE65 in a cone-dominant species is more efficient in its enzymatic activity than that from rod-dominant species. In vitro isomerohydrolase activity assay showed that isomerohydrolase activity in the chicken retinal pigment epithelium (RPE) was 11.7-fold higher than in the bovine RPE, after normalization by RPE65 protein levels. Similar to that of human and bovine, the isomerohydrolase activity in chicken RPE was blocked by two specific inhibitors of lecithin retinal acyltransferase, indicating that chicken RPE65 also uses all-trans-retinyl ester as the direct substrate. To exclude the possibility that the higher isomerohydrolase activity in the chicken RPE could arise from another unknown isomerohydrolase, we expressed chicken and human RPE65 using the adenovirus system in a stable cell line expressing lecithin retinal acyltransferase. Under the same conditions, isomerohydrolase activity of recombinant chicken RPE65 was 7.7-fold higher than that of recombinant human RPE65, after normalization by RPE65 levels. This study demonstrates that RPE65 from the cone-dominant chicken RPE possesses significantly higher specific retinol isomerohydrolase activity, when compared with RPE65 from rod-dominant species, consistent with the faster regeneration rates of visual pigments in cone-dominant retinas.

Visual pigments of rods and cones consist of protein opsins and the chromophore, 11-cis-retinal covalently bound to opsins via a Schiff base (1). Upon light absorption, the 11-cis-retinal is photoisomerized to all-trans-retinal, which subsequently activates opsin and triggers the phototransduction cascade (1, 2). The 11-cis-retinal chromophore regenerates through a series of reactions of the retinoid visual cycle (3, 4). The visual cycle has been intensively studied in rod-dominant species such as bovine and mouse. The two-cell (photoreceptor and RPE)2 system of 11-cis-retinal recycling has been proposed for the visual cycle. After reduction of all-trans-retinal by retinol dehydrogenase, the generated all-trans-retinol is transported from the photoreceptor to the RPE and esterified to all-trans-retinyl esters by lecithin retinol acyltransferase (LRAT). The resulting all-trans-retinyl ester can either be stored in the RPE or directly converted to 11-cis-retinol by the isomerohydrolase, which was recently identified as the microsomal protein RPE65 (5–7). RPE65 has been shown to be an essential enzyme in the retinoid visual cycle for 11-cis-retinal regeneration (8). After oxidation of 11-cis-retinol by retinol dehydrogenase-5, the generated 11-cis-retinal is transported back to the photoreceptors to regenerate the visual pigments.

It is known that cones have lower sensitivities but faster responses to light, when compared with rods (9, 10). There is some evidence suggesting that visual cycle reactions also proceed faster in cones than in rods (11, 12). The rate of conversion of all-trans-retinal to all-trans-retinol is 10–40 times faster in isolated salamander cones than that in rods (11). These observations suggest that pigment regeneration is faster in cones than in rods under light conditions. This faster pigment regeneration may require a more efficient visual cycle in cone-dominant species.

Because isomerohydrolase RPE65 is a key enzyme in the visual cycle for the regeneration of 11-cis-retinal, the present study investigated whether RPE65 in the RPE of cone-dominant chicken is a more efficient isomerohydrolase than in the rod-dominant bovine and human.

**EXPERIMENTAL PROCEDURES**

Cloning of the Chicken RPE65 cDNA and Construction of the Expression Vector—The human RPE65 cDNA was cloned as described previously (13). For cloning of chicken RPE65, ani-

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s were purchased from a local vendor. The chicken eyes were enucleated, and the RPE carefully dissected. The total RNA was isolated from the RPE using TRIzol reagent (Invitrogen) and further purified with an RNeasy column (Qiagen, Valencia, CA). Reverse transcription was performed with the TaqMan reverse transcriptase system (Applied Biosystems Inc., Foster City, CA) using an oligo(dT) primer. To clone the full-length chicken RPE65 cDNA, the gene-specific primers (forward primer: 5'GCGCGGCCCACCATGTACGCCAG-GTGGAGC-3' containing a NotI site and the Kozak sequence (14) and reverse primer: 5'-AAGGTTCTAGCTTTTGAAGATCCATGG-3'; containing a HindIII site) were used for PCR. PCR was performed with Pfu-Turbo (Stratagene, La Jolla, CA), a high fidelity enzyme, at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min. The size of the PCR product was confirmed by 0.8% agarose gel electrophoresis, and the DNA band with expected size was isolated from the gel using the QIAquick gel extraction kit (Qiagen). The extracted RPE65 cDNA was treated with TaqDNA polymerase (Roche Applied Science) at 72 °C for 10 min.

The extracted RPE65 cDNA was treated with TaqDNA polymerase (Roche Applied Science) at 72 °C for 10 min. Finally, the full-length chicken RPE65 cDNA was cloned into the pGEM-T easy vector (Promega, Madison, WI) and transformed into Escherichia coli. The positive clones were sequenced by an ABI-3770 automated DNA sequencer (Applied Biosystems Inc.) from both directions to exclude any mutations. Confirmed human and chicken RPE65 cDNAs were cloned between the NotI and HindIII sites of the pShuttle-CMV vector (Qbiogene, Montreal, Canada) for construction of adenoviruses. Preparations, amplification, and titration of the recombinant adenoviruses were performed as described previously (5).

**Purification of Native Bovine and Recombinant Chicken RPE65**—Native bovine RPE65 was purified from bovine RPE microsomes as described previously (15). Recombinant chicken RPE65 was purified from 293A cells infected by the adenovirus-expressing chicken RPE65 (Ad-chRPE65) at multiplicity of infection of 100 for 24 h. Microsomes were prepared from the infected 293A cells as described previously (16). The microsomal proteins were solubilized in 1% CHAPS and centrifuged at 100,000 × g for 1 h. The supernatant was applied onto a DEAE-Sepharose column (1 × 20 cm). RPE65 in the collected fractions was analyzed by Western blotting. The fractions with the highest amounts of RPE65 were combined and concentrated using a Centricon with 10,000-Da cut-off (Millipore, Billerica, MA). The purity of RPE65 was analyzed by SDS-PAGE with Coomassie Brilliant Blue R-250 staining as described previously (15).

**Western Blot Analysis and Quantification of RPE65 in Microsomes**—Total protein concentrations in cell lysates and microsomal fractions were measured using the Bradford method (17). Purified chicken and bovine RPE65 standards were quantified by both the Bradford method (17) and the method of Gill and von Hippel (18). Both methods produced similar protein concentration values. Serial dilutions of total microsomal proteins (10–40 μg) from chicken and bovine RPE were loaded onto the gel and blotted with an antibody for RPE65 (13). The antibody that was used in this manuscript was raised against the epitope VSHPDALEEDGV, which is 100% conserved in human, bovine, and chicken RPE65. Increasing amounts (0.1–2 μg) of purified chicken and bovine RPE65 were used as standards in the gel. The image of the blot was captured by the imager Chemi Genius™ (Syngene, Frederick, MD). The protein level of RPE65 was quantified by densitometry using the program GENETOOLS (Syngene). The intensity of the purified RPE65 protein standard was plotted as the function of amounts of purified proteins loaded. The amounts of RPE65 in the microsomes used were calculated based on the standard curves.

**Isomerohydrolase Activity Assay**—The 293A-LRAT cells, a stable cell line expressing LRAT established previously (19), were separately infected with Ad-chRPE65 and the adenovirus expressing human RPE65 (Ad-hRPE65) with a multiplicity of infection of 100. Un-infected 293A-LRAT cells were used as a negative control. Cell lysates were sonicated on ice for 20 s in a reaction buffer (10 mM BTP, pH 8.0, 100 mM NaCl), and microsomal fractions were prepared as described previously (16). Briefly, sucrose was added to the cell homogenate to the concentration of 0.32 M. The homogenate was centrifuged (20 min, 20,000 × g) to sediment unbroken cells, nuclei, and mitochondria. The supernatant was recrystallized (1 h, 100,000 × g), and the microsomal pellet was resuspended in reaction buffer, washed, and stored at −80 °C. Similarly, chicken and bovine RPE microsomes were prepared. All-trans-[1,12-3H]retinol (1 mCi/ml, 45.5 Ci/mol, American Radiolabeled Chemical, Inc., St. Louis, MO), dried under argon and resuspended in the same volume of N,N-dimethyl formamide, was used as the substrate for the isomerohydrolase assay. For each reaction, indicated amounts of microsomal protein were added into 200 μl of reaction buffer (10 mM BTP, pH 8.0, 100 mM NaCl) containing 0.2 μM of all-trans-retinol, 1% bovine serum albumin, and 25 μM of cellular retinaldehyde-binding protein. The reaction was stopped, and retinoids were extracted with 300 μl of cold methanol and 300 μl of hexane and centrifuged at 10,000 × g for 5 min. The generated retinoids were analyzed by normal phase HPLC as described (16). The peak of each retinoid isomer was identified based on the retention time of retinoid standards. The isomerohydrolase activity was calculated from the area of the 11-cis-retinol peak using Radiochromatic 610TR software (PerkinElmer Life Sciences) with synthetic 11-cis-[3H]retinol as a standard.

**RESULTS**

**Cloning of Chicken RPE65**—The chicken RPE65 cDNA was cloned as described under “Experimental Procedures.” The chicken RPE65 cDNA contains an open reading frame encoding a protein of 533 amino acids with a predicted molecular mass of 60,927.40 Da. The derived amino acid sequence shares 90% identity with human RPE65 (Fig. 1). All of the key amino acid residues required for enzymatic activity, such as the His in the catalytic center (7, 19), and the reported palmitoylated Cys residues, Cys-231 and Cys-329 (20), are identical between the chicken and human RPE65 except that the chicken RPE65 lacks the Cys-330, which was previously (5).
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| Residue | Human | Bovine | Mouse | Chicken |
|---------|-------|--------|-------|---------|
| 10      | I     | I      | I     | I       |
| 20      | I     | I      | I     | I       |
| 30      | I     | I      | I     | I       |
| 40      | I     | I      | I     | I       |
| 50      | I     | I      | I     | I       |
| 60      | I     | I      | I     | I       |
| 70      | I     | I      | I     | I       |

The identical residues are indicated by dots. The four conserved histidine residues in the catalytic center are marked by arrows. The potential palmitoylation site Cys-330 conserved in mammalian RPE65 is boxed.

Figure 1. Alignment of the deduced amino acid sequences of human, bovine, mouse, and chicken RPE65. The identical residues are indicated by dots. The four conserved histidine residues in the catalytic center are marked by arrows. The potential palmitoylation site Cys-330 conserved in mammalian RPE65 is boxed.

human, bovine, and mouse RPE65, but is replaced by Thr in chicken RPE65 (Fig. 1).

Lower Abundance of RPE65 in the Chicken RPE than in the Bovine RPE—Chicken RPE65, expressed in 293A cells, was purified by chromatography as described under “Experimental Procedures.” The purified chicken RPE65 was electrophoretically homogeneous as shown by SDS-PAGE (Fig. 2). The identity of the purified RPE65 was confirmed by Western blot analysis as described previously (16).

The purified chicken RPE65 was used as a standard to quantify RPE65 in chicken RPE by Western blot analysis. RPE65 purified from the bovine RPE was used as a standard for quantifying bovine RPE65. Total protein concentration was 4.8 mg/ml in chicken RPE microsomes used for the assay, and the concentration of chicken RPE65 was 0.19 mg/ml, as determined by Western blot analysis. Total protein concentration was 2.9 mg/ml in bovine RPE microsomes, and the concentration of bovine RPE65 was 0.54 mg/ml. Therefore, the calculated abundance of RPE65 was 39 ± 3 μg/mg of total microsomal proteins in chicken RPE (mean ± S.D.). The abundance of RPE65 in chicken RPE was significantly lower than that of bovine RPE65, which was 186 ± 20 μg/mg of total microsomal proteins.

Chicken Isomeryohydrolase Activity Is Dependent upon the Formation of All-trans-retinyl Ester—We investigated whether the chicken isomeryohydrolase utilizes all-trans-retinyl ester as substrate, similar to that in rod-dominant species such as the bovine and human RPE65. We measured the dependence of chicken isomeryohydrolase activity upon the generation of all-trans-retinyl ester using two specific LRAT inhibitors, apo-cellular retinol-binding protein Type 1 (apo-CRBP) and 10-N-acetamido-dodecyl chloromethyl ketone (AcDCMK), which have been characterized previously (16, 21). In the absence of the inhibitors, a significant amount of 11-cis-retinol was generated from all-trans-retinol after 1.5-h incubation with bovine or chicken RPE microsomes (Fig. 3, A and D). The addition of 13 μM of apo-CRBP, a potent competitive inhibitor of retinyl ester formation, to the RPE microsomes almost completely blocked the formation of both retinyl esters and 11-cis-retinol (Fig. 3, B and E).

Previously, we have shown that AcDCMK is also a potent and specific inhibitor of retinyl ester synthesis (16). To block all-trans-retinyl ester formation from all-trans-retinol, bovine and chicken RPE microsomes were separately preincubated with 20 μM of AcDCMK for 15 min prior to the addition of all-trans[^1H]retinol. Similar to apo-CRBP, AcDCMK also completely inhibited the generation of retinyl esters and 11-cis-retinol in both the chicken and bovine RPE microsomes (Fig. 3, C and F). These results suggest that the chicken isomeryohydrolase also uses retinyl esters as its substrate for the generation of 11-cis-retinol.
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Comparison of the Isomerohydrolase Activities of RPE65 in Chicken and Bovine RPE Microsomes—Most of the previous measurements of RPE65 isomerohydrolase activity were performed using bovine RPE microsomes (22, 23). However, it is not known if the specific activity of RPE65 is the same in cone-dominant species. We have prepared bovine and chicken RPE microsomes using the same method as described previously (16). Although all-trans-retinyl ester has been shown to be the substrate of the isomerohydrolase (16, 24), the poor solubility of the highly hydrophobic retinyl ester limits its use in this assay. Therefore all-trans-[3H]retinol was used to generate retinyl esters by LRAT, and these esters were then isomerized/hydrolyzed to 11-cis-[3H]retinol by RPE65.

To compare the RPE65 isomerohydrolase activity, we plotted the time course of 11-cis-[3H]retinol formation following incubation of all-trans-[3H]retinol with 14.5 μg of chicken RPE microsomes and 29 μg of bovine RPE microsomes. The isomerization reaction was stopped at various time points, and the generated 11-cis-[3H]retinol was quantified using HPLC and by comparing with 11-cis-retinol standard. The time course of 11-cis-retinol generation was linear at its initial reaction period (Fig. 4, A and B). The dependence of isomerohydrolase activities on the enzyme concentration was also linear both for bovine and chicken enzymes.

From the slope of the time curves, we calculated the specific isomerohydrolase activity in bovine RPE microsomes to be 1.97 pmol of 11-cis-retinol per mg of total microsomal protein per min (pmol/mg/min). The time course of 11-cis-retinol generation in chicken RPE microsomes showed a specific activity of isomerohydrolase 4.62 pmol/mg/min.

It has been shown that Rpe65−/− mice lack any detectable isomerase activity in their RPE or retina (8), suggesting that RPE65 is the only retinol isomerase in the mouse eye. As chicken RPE has lower abundance of RPE65 than bovine RPE, we quantified RPE65 both in bovine and chicken RPE microsomes used for the enzymatic activity assays by Western blot analysis using purified bovine and chicken RPE65 as standards and normalized the isomerohydrolase activity by the abundance of RPE65. The specific isomerohydrolase activities, as calculated based on the initial slope of the normalized time course of 11-cis-retinol generation, was 115.1 ± 9.5 pmol of 11-cis-retinol per mg of RPE65 per min (pmol/mg/min) for the chicken RPE65. Under the same condition,
the bovine RPE65 showed a specific activity of 9.8 ± 2.6 pmol/mg/min, ~11.7-fold lower than that of chicken RPE65 (Fig. 4).

Comparison of the Isomerohydrolase Activity of Recombinant Human and Chicken RPE65—As there is a possibility that the higher specific isomerase activity in the chicken RPE microsomes observed may be due to the existence of another unknown isomerase in addition to RPE65, chicken RPE65 was expressed using the adenovirus expression system in the 293A-LRAT cell line as described previously (19). This cell line has been shown to express LRAT and to lack endogenous isomerase activity assay as described under “Experimental Procedures.” The reaction was stopped at various time points from 0 to 90 min of the reaction time. The amount of generated 11-cis-retinol was analyzed and quantified using HPLC (Fig. 5, A and B). The initial rate of the 11-cis-retinol generation was determined from the slope of the linear part of the kinetic curves.

The initial rate of the isomerization was calculated to be 8.75 × 10⁻³ pmol/min in the cells expressing human RPE65 and 0.17 pmol/min in those expressing chicken RPE65, when the same amount of total microsomal proteins was used. After normalization of the enzymatic activity by the RPE65 levels in the microsomes, the specific isomerohydrolase activity of chicken RPE65 was 36.2 ± 4.5 pmol/mg/min, ~8-fold higher than that of human RPE65 activity, which was 4.7 ± 1.3 pmol/mg/min, suggesting that recombinant chicken RPE65 has a higher specific enzymatic activity than human RPE65 under the same conditions.

To ensure that the LRAT activity is not rate-limiting in the production of 11-cis-retinol from all-trans-retinol, we measured LRAT activities in both chicken and bovine RPE microsomes under the same conditions as that for the isomerohydrolase assay (Fig. 6) and normalized it by the total microsomal protein level. These values were compared with isomerohydrolase activities in the same microsomal preparations and normalized by the total microsomal protein level. The specific activity of bovine LRAT was 13.0 ± 1.2 pmol/mg/min, which was significantly higher than that of the isomerohydrolase activity of 2.0 ± 0.3 pmol/mg/min in the same samples.

Similarly, chicken LRAT and isomerohydrolase activities were 18.6 ± 1.1 pmol/mg/min and 4.6 ± 0.5 pmol/mg/min, respectively. This experiment demonstrates that LRAT activity was severalfold higher than the isomerohydrolase activity and is not rate-limiting in the assay.

DISCUSSION

The chicken retina contains ~60% cones, whereas human and bovine retinas have ~5% cones (12). Physiological measurements have shown that cones have much faster photoreceptor kinetics than rods (9, 10). The sensitivity of cones after bleaching recovers ~10 times faster than that of rods (9, 10). The faster photoreceptor in cones requires a faster regeneration of 11-cis-retinal after its bleaching. Thus, we set out to determine if animals with cone-dominant retinas have a more efficient isomerohydrolase in their RPE. RPE65 (isomerohydrolase) is the key enzyme of the visual cycle, which regenerates 11-cis-retinal, the chromophore for rod and cone visual pig-
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In chicken RPE arises from higher levels of the isomerohydrolase protein or a higher activity of the enzyme, we have measured the abundance of RPE65, the isomerohydrolase and compared the specific enzymatic activity of chicken RPE65 with those of human and bovine RPE65. To avoid possible difference in affinity of the antibody to RPE65 from different species, purified chicken and bovine RPE65 were used separately as protein standards for quantification of chicken and bovine RPE65 in the RPE, respectively. Moreover, the antibody used for quantifying RPE65 recognizes an epitope that is 100% conserved in human, bovine, and chicken RPE65, to avoid possible difference caused by different antibody-antigen affinity. The semi-quantitative measurement showed that the RPE65 abundance in chicken RPE microsomes is lower than that in the bovine RPE. In contrast, our isomerohydrolase activity assay showed that the specific isomerohydrolase activity measured in chicken RPE microsomes is 11.7 times higher than that in bovine RPE microsomes, after normalization by RPE65 levels in the RPE microsomes. These observations suggest that the higher rate of

11-cis-retinal regeneration in chicken RPE microsomes cannot be ascribed to the higher RPE65 levels.

It has been reported that a unique characteristic of the isomerase in cone-dominant species is that it directly utilizes all-trans-retinol as the substrate and converts it directly to 11-cis-retinol without formation of the intermediate retinyl esters (12, 26). On the contrary, the rod-dominant retinol isomerases requires the formation of retinyl esters that are a direct substrate for the isomerohydrolase (16, 24). To investigate the nature of the substrate in the isomerization reaction catalyzed by chicken RPE microsomes, we used two specific inhibitors of LRAT, a major retinyl ester-synthesizing enzyme in the eye. Both specific inhibitors of LRAT, AcDCMK and apo-CRBP, completely blocked the isomerization reaction, indicating that retinyl ester is a direct substrate of chicken RPE isomerase, similar to that in rod-dominant species. Our result is in line with the previous observations by Rando’s group who demonstrated using the pulse-chase method that 11-cis-retinoid biosynthesis in cone-dominant species follows the same pathway, including the formation of retinyl esters as that in rod-dominant species (27).

It was also reported that chicken eyecups contain lower levels of all-trans-retinyl ester but higher levels of 11-cis-retinyl esters.
than that in mammalian eyes (12, 26). The higher isomerohydrolase activity in the chicken RPE could also be responsible for the lower level of all-trans-retinyl esters in the chicken RPE, because all-trans-retinyl esters are more efficiently converted to 11-cis-retinol, which is consequently esterified to 11-cis-retinyl esters for storage (12, 26).

It has been shown that Rpe65−/− mice lack any 11-cis-retinoids in the retina or RPE (8), suggesting that RPE65 is the only isomerase in the RPE, at least in mammals. It has been proposed that some cone-dominant species, including chicken and zebrafish larvae may have an additional retinol isomerase in their eyes (12, 26, 28). However, this enzyme has not been identified at the molecular level. As there is a possibility that higher isomerohydrolase activity in chicken RPE may arise from another unidentified isomerase, we cloned the chicken RPE65 and expressed it in 293A-LRAT cells, which express LRAT but lack any endogenous isomerase activity (19). The isomerohydrolase assay using isolated microsomes showed that the recombinant chicken RPE65 has an isomerohydrolase activity 7.7-fold higher than that in human RPE65 after normalization by the recombinant RPE65 levels, suggesting that recombinant chicken RPE65 itself is a more efficient enzyme than that in humans. This finding suggests that high catalytic activity of chicken RPE65 may contribute to a faster regeneration rate of 11-cis-retinol in cone-dominant species.

Chicken and human RPE65 proteins share 90% sequence identity at the amino acid level. It remains to be investigated which of the 10% amino acid differences are responsible for the higher retinol isomerase activity in RPE65. It is also possible that different post-translational modifications could contribute to the higher enzymatic activity of chicken RPE65. Elucidation of the structural basis responsible for the higher isomerase activity of chicken RPE65 could have significant impacts on understanding of the mechanism for the isomerohydrolase and could also contribute to the development of a more efficient RPE65 gene therapy for retinal dystrophies caused by RPE65 mutations.

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