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Identification of the Key Residues Determining the Product Specificity of Isomerohydrolase

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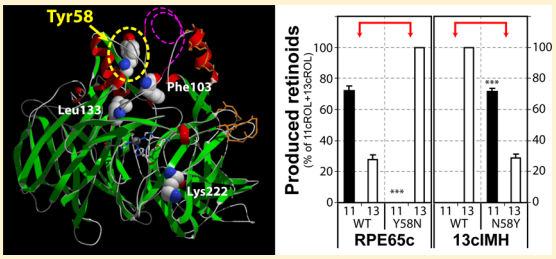
ABSTRACT: The efficient recycling of the chromophore of visual pigments, 11-*cis*-retinal, through the retinoid visual cycle is an essential process for maintaining normal vision. RPE65 is the isomerohydrolase in retinal pigment epithelium and generates predominantly 11-*cis*-retinol (11cROL) and a minor amount of 13-*cis*-retinol (13cROL), from all-*trans*-retinyl ester (atRE). We recently identified and characterized novel homologues of RPE65, RPE65c, and 13-*cis*-isomerohydrolase (13cIMH), which are expressed in the zebrafish inner retina and brain, respectively. Although these two homologues have 97% identical amino acid sequences, they exhibit distinct product specificities. Under the same assay conditions, RPE65c generated predominantly 11cROL, similar to RPE65, while 13cIMH generated exclusively 13cROL from atRE substrate. To study the impacts of the key residues determining the isomerization product specificity of RPE65, we replaced candidate residues by site-directed mutagenesis in RPE65c and 13cIMH. Point mutations at residues Tyr58, Phe103, and Leu133 in RPE65c resulted in significantly altered isomerization product specificities. In particular, our results showed that residue 58 is a primary determinant of isomerization specificity, because the Y58N mutation in RPE65c and its reciprocal N58Y mutation in 13cIMH completely reversed the respective enzyme isomerization product specificities. These findings will contribute to the elucidation of molecular mechanisms underlying the isomerization reaction catalyzed by RPE65.



To maintain normal vision, an efficient recycling of the chromophore [11-*cis*-retinal (11cRAL)] of visual pigments is essential. Chromophore recycling, termed the retinoid visual cycle, involves multiple enzymes and retinoid-binding proteins in the photoreceptors and retinal pigment epithelium (RPE).[1,2] The key step of the retinoid visual cycle is the conversion of all-*trans*-retinyl ester (atRE) into 11-*cis*-retinol (11cROL), which is catalyzed by a single enzyme, isomerohydrolase, in the RPE.[3−5] We and other groups independently showed that an RPE-specific protein with apparent molecular mass of 65 kDa (RPE65) is the isomerohydrolase in the RPE and catalyzes the conversion of atRE to 11cROL, which is subsequently oxidized to 11cRAL, the chromophore of visual pigments.[6−8] Furthermore, we successfully purified the active form of RPE65 and demonstrated that purified RPE65 has robust isomerohydrolase activity.[9] This study provided solid evidence that RPE65 is the isomerohydrolase in the retinoid visual cycle. Finally, Kiser et al. recently reported the crystal structure of RPE65,[10] which confirmed our previous findings regarding the key residues for the enzymatic activity of RPE65 using structure modeling and site-directed mutagenesis.[11−13]

On the other hand, the molecular mechanisms underlying the isomerization from all-*trans* to 11-*cis* retinoids and its isomerization specificity are not well understood. It was reported that an apocarotenoid-15, 15′-oxygenase (ACO), which belongs to the same enzyme family as RPE65, possesses a bent tunnel from the nonpolar patch to the active center, and that the actual isomerization may occur when the substrate passes through the bent tunnel to the catalytic domain.[14] Moreover, Redmond et al. showed that RPE65 produces both 11cROL and 13-*cis*-retinol (13cROL) from atRE.[15] It was shown that several amino acid residues in the potential "substrate cleft" contribute to the isomerization specificity of RPE65. Specifically, the F103L mutation in canine RPE65 significantly increased the level of 13cROL production, whereas the T147S mutation decreased the level of 13cROL production, compared to wild-type RPE65.[15] These reports suggested that the structure of the "bent tunnel" and "substrate cleft" may contribute to its product specificity. However, these two mutations did not completely reverse the product specificity from dominant 11cROL to 13cROL, suggesting that there are other residues contributing to the product specificity of RPE65.

We recently identified and characterized a novel homologue of RPE65, 13-*cis*-specific isomerohydrolase (13cIMH), from the zebrafish brain.[16] Although 13cIMH belongs to the same isomerohydrolase family as the RPE-specific RPE65 (RPE65a in zebrafish[17]), the gene encoding 13cIMH is located in a different chromosome than that encoding RPE65a, and it generates exclusively 13cROL without any detectable 11cROL.[16] It is worth mentioning that 13cIMH was previously named "RPE65b" on the basis of its sequence homology to RPE65.[17] Later, we named it 13cIMH, because it generates

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Table 1. Primer Sets for Site-Directed Mutagenesis in This Study

Primer names	*Sequences*
RPE65c Y58N Fwd	5′-GAGATGAACCATTTAATCATCTTTTTGATGGCC-3′
RPE65c Y58N Rev	5′-GGCCATCAAAAAGATGATTAAATGGTTCATCTC-3′
RPE65c F103L Fwd	5′-GTTGTGATCACAGAGCTTGGCACCACTGCATATC-3′
RPE65c F103L Rev	5′-GATATGCAGTGGTGCCAAGCTCTGTGATCACAAC-3′
RPE65c L133S Fwd	5′-GTGACAGACAATTGCTCTGTAAACATTTACCC-3′
RPE65c L133S Rev	5′-GGGTAAATGTTTACAGAGCAATTGTCTGTCAC-3′
RPE65c K222M Fwd	5′-CCGATCAGTTTGAGATGTCAAAGATTTTGG-3′
RPE65c K222M Rev	5′-CCAAAATCTTTGACATCTCAAACTGATCGG-3′
13cIMH N58Y Fwd	5′-GAGATGAACCATTTTACCATCTTTTTGATGGCC-3′
13cIMH N58Y Rev	5′-GGCCATCAAAAAGATGGTAAAATGGTTCATCTC-3′
13cIMH L103F Fwd	5′-GTTGTGATTACAGAATTCGGCACCGCTGCATATC-3′
13cIMH L103F Rev	5′-GATATGCAGCGGTGCCGAATTCTGTAATCACAAC-3′
13cIMH S133L Fwd	5′-GTTACAGACAACTGTCTTGTAAACATTTACCC-3′
13cIMH S133L Rev	5′-GGGTAAATGTTTACAAGACAGTTGTCTGTAAC-3′
13cIMH M222K Fwd	5′-CTGATCCACTTGCGAAGTCAAAGGTTTTGG-3′
13cIMH M222K Rev	5′-CCAAAACCTTTGACTTCGCAAGTGGATCAG-3′

exclusive 13cROL from atRE substrate in our enzymatic assay.[16] In addition to 13cIMH, we have identified another homologue of RPE65, RPE65c, which is expressed in the inner retina of zebrafish, likely in retinal Müller cells and not in the RPE.[18] RPE65c generates predominantly 11cROL (72.2 ± 3.0%) and a minor amount of 13cROL (27.8 ± 3.0%) from the atRE substrate in our in vitro assay, similar to the RPE-specific RPE65. Zebrafish is a cone-dominant species with 79% cones and 21% rods based on immunohistochemistry analysis at 7 dpf.[19] RPE65c expressed in the inner retina may serve as an alternative isomerohydrolase in the inner retinal visual cycle to meet the high demand for recycling of the chromophore in the cone-dominant retina.

It is known that a number of genes were duplicated because of fish-specific whole-genome duplication.[20,21] It is likely that the two novel homologues of RPE65 (13cIMH and RPE65c) are generated through gene duplication after the separation of fish RPE65 from the ancestral RPE65, because they exhibited an extremely high level of sequence identity (97%) and are located in the same chromosome 8.[18] However, they are encoded by distinct genes, show different tissue distributions, and generate distinct isomerization products.[16,18] A high degree of sequence identity and distinct product specificities of zebrafish RPE65c and 13cIMH make them ideal molecular models for identifying the key residues determining the product specificity of isomerohydrolase.

In this study, we identified the key residues that determine the product specificity of these isomerohydrolases using site-directed mutagenesis and the in vitro isomerohydrolase assays.

MATERIALS AND METHODS

Construction of Expression Vectors and Site-Directed Mutagenesis. The wild-type (wt) zebrafish 13cIMH, RPE65c, and chicken RPE65 were subcloned into cloning and expression vectors as described previously (Table 1).[16,18,22] It is noteworthy that the enzymatic activity of RPE65a in zebrafish has not been studied or characterized. At present, we know neither its enzymatic kinetics nor its product specificity. In contrast, chicken is another cone-dominant species; chicken RPE65 has been cloned and expressed, and its enzymatic activity and product specificity have been well characterized.[9,22] Therefore, we used chicken RPE65 as a control for product specificity analysis in this study. The site-directed mutations of 13cIMH and RPE65c were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the protocol recommended by the manufacturer. The introduced mutations were confirmed by sequencing from both strands using ab ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) and subcloned into an expression vector, pcDNA3.1(−) (Invitrogen, Carlsbad, CA). Following the sequence confirmation, the expression constructs were purified with a QIAfilter Maxi Prep kit (Qiagen, Valencia, CA).

Plasmid Transfection. The expression plasmids of RPE65 homologues and their mutants were transfected into 293A cells using the Fugene 6 transfection reagent (Roche, Indianapolis, IN) following manufacturer's instructions. Forty-eight hours following the transfection, the cells were harvested with a cell scraper and rinsed twice with ice-cold PBS. The protein expression and its enzymatic activity were measured by Western blot analyses and in vitro activity assays.

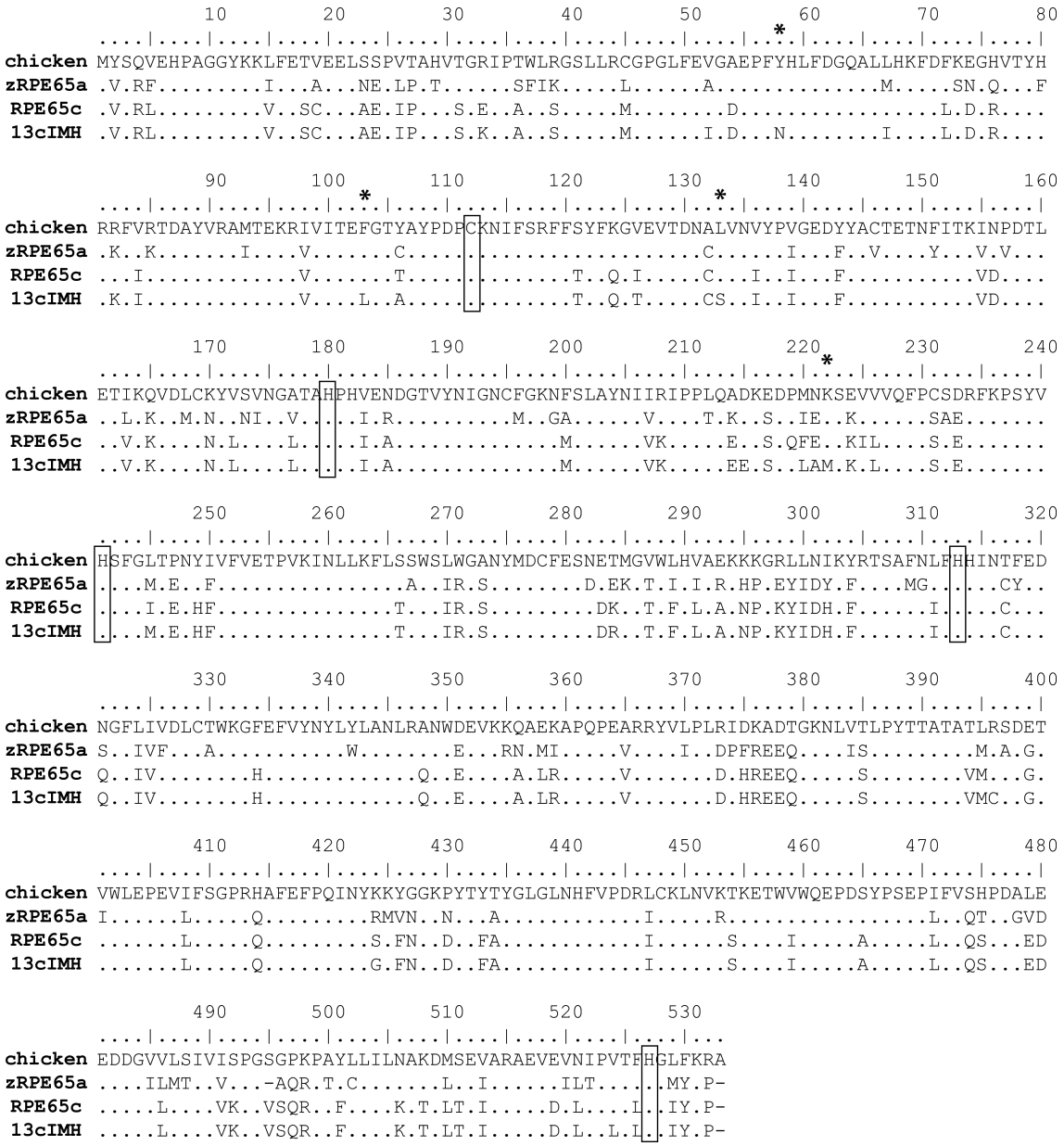

Figure 1. Amino acid comparison of RPE65 and its homologues. Amino acid sequences of chicken RPE65, zebrafish RPE65 (zRPE65a), RPE65c, and 13cIMH were aligned. Amino acid residues identical to those of chicken RPE65 are denoted with periods, while only the different residues are shown. The known key residues (four His residues forming the iron binding site and a palmitylated Cys residue[7,11,27]) are boxed. The four amino acid residues in 13cIMH (at positions 58, 103, 133, and 222) that may contribute to exclusive 13cROL production of 13cIMH are denoted with asterisks.

Western Blot Analysis. Total cellular protein concentrations were measured by the Bradford assay.[23] Equal amounts of protein (25 μg) were resolved by electrophoresis through an 8% Tris-glycine SDS−polyacrylamide gel and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 30 min and subsequently incubated overnight at 4 °C with a 1:1000 dilution of an anti-RPE65 polyclonal antibody.[24] After three washes with TBST, the membrane was incubated for 1.5 h with 1:6700 dilutions of an HRP-conjugated anti-rabbit IgG antibody (Millipore, Billerica, MA) in TBST containing 1% nonfat dry milk. After four washes with TBST, the bands were detected using Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL) or a homemade ECL solution.[25] As needed, the membrane was stripped in the stripping buffer (Pierce) and reblotted with an antibody specific for β-actin (Sigma-Aldrich, St. Louis, MO) for a loading control. The bands (intensity × area) were semiquantified by densitometry using Fluorochem-Q software (AlphaInnotech, San Leandro, CA), averaged over at least three independent experiments.

In Vitro Isomerohydrolase Activity Assay. The plasmids expressing red fluorescence protein (Rfp, as a negative control), wt chicken RPE65 (as a positive control of cone-dominant species), zebrafish 13cIMH, RPE65c, and their mutants were separately transfected into 293A cells. The liposome-based isomerohydrolase activity assay was conducted as described previously.[9,16] The peak of each retinoid isomer was identified by high-performance liquid chromatography (HPLC) based on its characteristic retention time and the absorption spectrum of

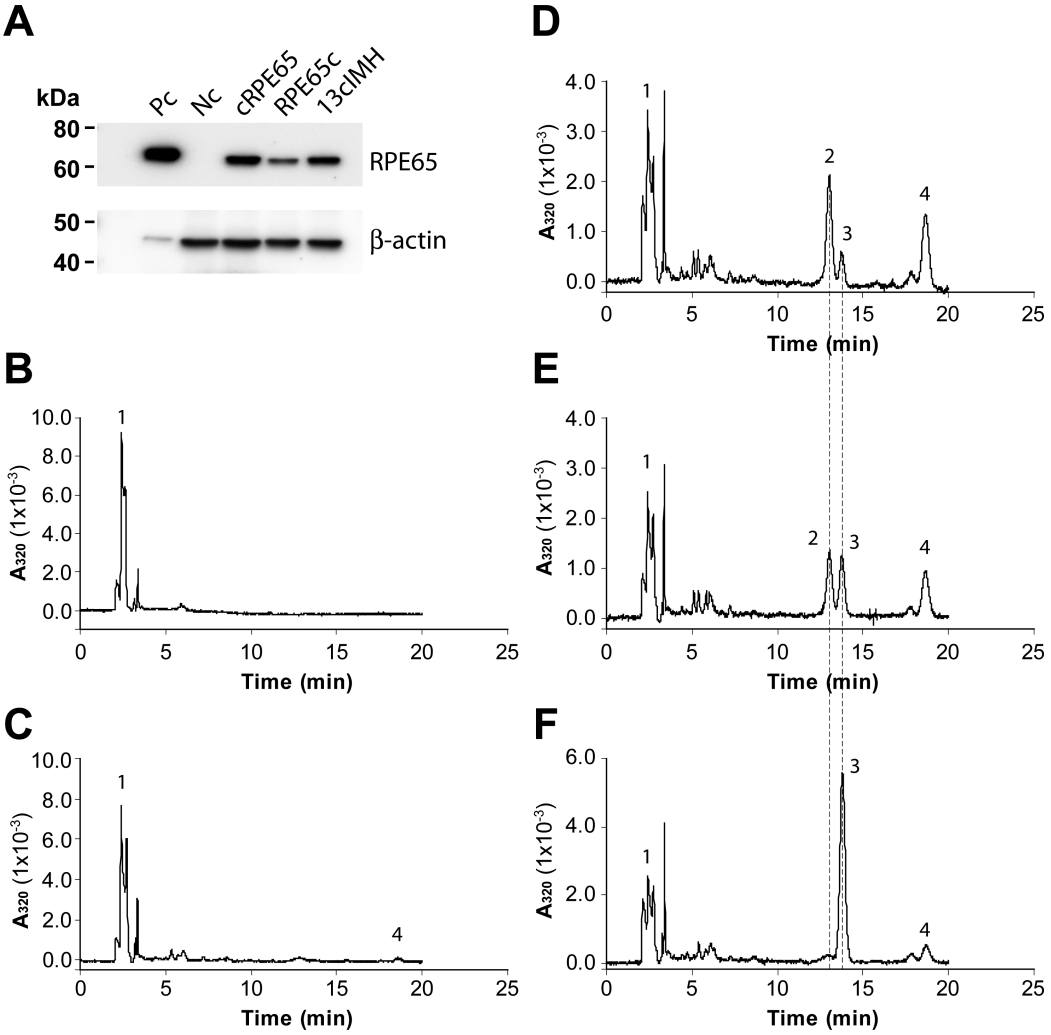

Figure 2. HPLC profiles showing products from the isomerohydrolase assay of RPE65 and its homologues. The 293A cells were separately transfected with the plasmids expressing wt chicken RPE65, zebrafish RPE65c, and 13cIMH and cultured for 48 h. The cells were harvested, and the cell lysate was incubated with liposomes containing atRE (250 μM lipids and 3.3 μM atRE) for 1 h at 37 °C, and the generated retinoids were analyzed by HPLC. Protein expression was confirmed by Western blot analysis. (A) Pc, positive control (2.5 μg of bovine microsomal fraction); Nc, negative control (25 μg of total cellular protein of 293A cells expressing Rfp); cRPE65, chicken RPE65. (B–F) HPLC profiles: (B) without cell lysate (only atRE incorporated in the liposome), (C) negative control cell lysate (atRE liposome and cell lysate expressing Rfp), (D) wt chicken RPE65, (E) zebrafish RPE65c, and (F) 13cIMH. The peaks were identified as follows: **1**, retinyl esters; **2**, 11cROL; **3**, 13cROL; **4**, atROL.

Table 2. Product Isomerization Specificities of RPE65 and Mutants Tested in This Study

	11cROL (%)	13cROL (%)	11cROL (pmol/h)	13cROL (pmol/h)	% of substrate isomerization[a]
wt chicken	87.3 ± 3.9	12.7 ± 3.9	43.1 ± 13.2	6.1 ± 1.9	7.5
wt RPE65c	72.2 ± 3.0	27.8 ± 3.0	24.8 ± 8.8	9.3 ± 2.6	5.2
Y58N	0	100	nd[b]	51.6 ± 2.9	7.8
F103L	4.3 ± 1.1	95.7 ± 1.1	1.6 ± 0.3	35.1 ± 2.0	5.6
L133S	28.7 ± 1.1	71.3 ± 1.1	7.3 ± 0.5	18.2 ± 1.4	3.9
K222M	44.9 ± 1.2	55.1 ± 1.2	13.9 ± 1.0	17.1 ± 0.4	4.7
Y58N/F103L	74.1 ± 0.5	25.9 ± 0.5	30.2 ± 1.3	10.6 ± 0.3	6.2
wt 13cIMH	0	100	nd[b]	48.4 ± 7.1	7.3
N58Y	71.3 ± 2.4	28.7 ± 2.4	11.2 ± 3.2	4.4 ± 0.9	2.4
L103F	37.5 ± 2.6	62.5 ± 2.6	8.1 ± 1.3	13.5 ± 1.0	3.3
S133L	5.6 ± 1.4	94.4 ± 1.4	1.2 ± 0.3	20.4 ± 1.1	3.3
M222K	0	100	nd[b]	45.9 ± 6.7	7.0
N58Y/L103F	0	100	nd[b]	41.1 ± 0.7	6.2

[a]Each reaction mixture (200 μL) contained 660 pmol of atRE (3.3 μM atRE), and the percent of substrate isomerization in 1 h was the sum of 11cROL and 13cROL production divided by the amount of atRE substrate. [b]Nondetectable.

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each retinoid standard. The isomerohydrolase activity was calculated from the area of the 11cROL and 13cROL peaks. The ratio of the generated 11cROL to the sum of generated 11cROL and 13cROL was expressed as the mean ± the standard deviation (SD) from three independent measurements.

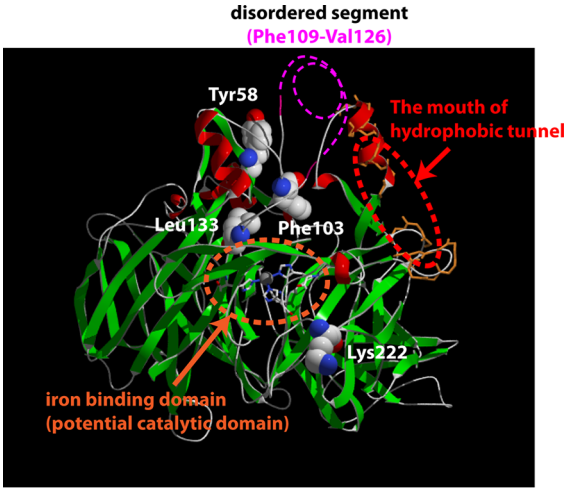
Statistical Analysis. The product generated by RPE65 and its mutants were presented as means ± SD from three independent measurements and evaluated with a Student's *t* test. It is considered "statistically significant" at a *p* value of <0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), but a *p* value of >0.05 is "nonsignificant" (n.s, $p > 0.05$).

RESULTS

Prediction of Key Residues Contributing to the Product Specificity of Isomerohydrolases. To predict the candidate residues that determine the product specificity of the isomerohydrolase, amino acid sequences of chicken RPE65, zebrafish RPE65a, RPE65c, and 13cIMH were aligned using Clustal-W in BioEdit (Ibis Therapeutics, Carlsbad, CA). We selected four candidate residues (Asn58, Leu103, Ser133, and Met222) that are specific in 13cIMH and were substituted by their counterparts in RPE65c and chicken RPE65 (Figure 1).

Isomerohydrolase Activities of RPE65 and Its Homologues. HEK-293A cells were separately transfected with plasmids expressing chicken RPE65, zebrafish RPE65c, and 13cIMH and cultured for 48 h. Protein expression was confirmed by Western blot analysis (Figure 2A), and the same batches of total cellular proteins were used for the in vitro isomerohydrolase activity assay. To avoid experimental variations of retention time in the HPLC profile, we performed the isomerohydrolase assay for all of the enzymes at the same time. The HPLC profiles were presented side by side to compare the retinoid profiles produced by the three isomerohydrolases (Figure 2D–F). Chicken RPE65 and zebrafish RPE65c generated predominantly 11cROL with a minor amount of 13cROL (actual values will be shown in Figures 4 and 5 and listed in Table 2), whereas 13cIMH generated exclusively 13cROL. This result is consistent with our previous studies.[16,18]

Structural Model of RPE65 and Location of Candidate Residues. The structure of bovine RPE65 [Protein Data Bank (PDB) entry 3FSN] was analyzed with SwissPDB Viewer version 4.01 (http://www.expasy.org/spdbv/),[26] and the results were displayed with POV-Ray version 3.61 (http://www.povray.org/) (Figure 3). The candidate residues Tyr58, Phe103, and Leu133 are located in the random coils, which may be close to the "hydrophobic tunnel" of the substrate or product,[10] the "substrate cleft" that determines the isomerization product specificity of RPE65,[15] and Lys222 is located in α-helix 4. The disordered segment (Phe109–Val126;[10] pink dotted line in Figure 3) may be responsible for its membrane association, because this segment contains a palmitylated Cys112 residue.[10,27] The straight-line distances from the four residues to an iron in the predicted catalytic domain are 19.15 Å for Tyr58, 14.56 Å for Phe103, 10.79 Å for Leu133, and 25.91 Å for Lys222, suggesting that these four residues are likely too far from the catalytic center to directly impact the hydrolysis and/or isomerization of the substrate. These residues, however, can directly or indirectly alter the structure of the hydrophobic tunnel and substrate cleft to affect its product specificity.



Residue	Distance (Å) carbon-α to iron-II
Tyr58	19.15
Phe103	14.56
Leu133	10.79
Lys222	25.91

Figure 3. Locations of unique residues in the three-dimensional structure of RPE65. The locations of the four residues are shown in the model of the crystal structure of bovine RPE65 (PDB entry 3FSN). The iron-binding site that may be a part of the catalytic domain is denoted with an orange dotted circle. Tyr58, Phe103, and Leu133, which had an impact on isomerization specificity, are located in the random coils, which may be a part of the substrate cleft or hydrophobic tunnel contributing to the isomerization specificity of RPE65.[15] The disordered segment (Phe109–Val126), which contains a palmitylated Cys residue (Cys112) that may be responsible for its membrane association, is shown as a pink dotted line.[10,27] The potential mouth of substrate entry and product exit of the hydrophobic tunnel[10] is represented by a red dotted line. The Lys222 residue that had a minor effect on isomerization specificity is located in α-helix 4.

Impacts of the Candidate Residues on the Product Specificity of RPE65c. To analyze the impacts of the candidate residues on the product specificity of the isomerohydrolase, Tyr58, Phe103, Leu133, and Lys222 residues in zebrafish RPE65c were substituted with their counterparts in 13cIMH (Asn58, Leu103, Ser133, and Met222, respectively) using site-directed mutagenesis. wt RPE65c and its mutants (Y58N, F103L, L133S, and K222M) were expressed in 293A cells by plasmid transfection. The protein expression and its enzymatic activities were confirmed by Western blot analysis and an in vitro isomerohydrolase activity assay. The isomerization product specificity and efficiency of isomerization (picmoles per hour) of all the tested wt enzymes and its mutants are summarized in Table 2. As a positive control, wt chicken RPE65 generated 87.3 ± 3.9% of 11cROL and 12.7 ± 3.9% of 13cROL. Similarly, zebrafish RPE65c generated 72.2 ± 3.0% of 11cROL and 27.8 ± 3.0% of 13cROL. As shown by the in vitro isomerohydrolase assay, the Y58N mutant of RPE65c generated exclusively 13cROL, the same as wt 13cIMH. The F103L and L133S mutations substantially increased the level of production of 13cROL (F103L, 95.7 ± 1.1%; L133S, 71.3 ± 1.1%), whereas the K222M mutation had a weaker effect on

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increasing the level of 13cROL generation (55.1 ± 1.2%), compared to the F103L and L133S mutations. Interestingly, the double mutant Y58N/F103L of RPE65c generated predominantly (74.1 ± 0.5%) 11cROL, which is similar to the cases for wt RPE65 and RPE65c (Figure 4B and Table 2).

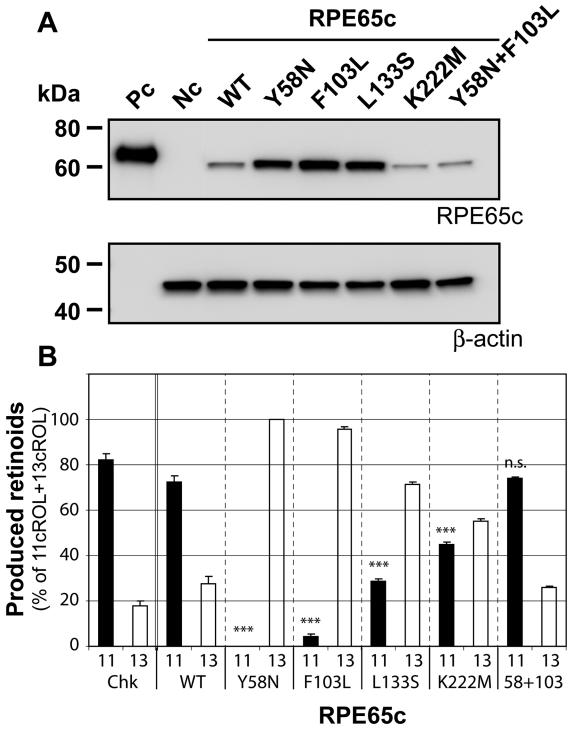

Figure 4. Site-directed mutagenesis of zebrafish RPE65c and the impacts on its isomerization specificities. Four candidate residues in RPE65c were replaced with their counterparts in 13cIMH. (A) The expression plasmids were separately transfected into 293A cells, and the protein expression was confirmed by Western blot analysis. Pc, positive control (2.5 μg of bovine microsomal fraction); Nc, negative control (25 μg of total cellular protein of 293A cells expressing Rfp); WT, wild-type RPE65c; Y58N, Tyr58Asn mutant of RPE65c; F103L, L133S, and K222M, single mutants; Y58N+F103L, Y58N/F103L double mutant of RPE65c. Equal amounts of total cellular proteins (125 μg) from the cells expressing zebrafish RPE65c and its mutants were incubated with liposomes containing atRE (250 μM lipids and 3.3 μM atRE) for 1 h at 37 °C, and the generated retinoids were analyzed by HPLC. The produced 11cROL and 13cROL were separately quantified from the areas of the 11cROL and 13cROL peaks, respectively (mean ± SD; n = 3). (B) Retinoids produced by wt chicken RPE65 (Chk), RPE65c and its mutants were expressed as the percent of produced 11cROL and 13cROL (*p < 0.05; **p < 0.01; ***p < 0.001; n.s, p > 0.05).

Reciprocal Mutations in 13cIMH and Their Impacts on Its Isomerization Specificity. To study the impacts of the selected residues on the product specificity of RPE65, the reciprocal mutants of 13cIMH (N58Y, L103F, S133L, and M222K) were generated. The expression plasmids of wt 13cIMH and these mutants were separately transfected into 293A cells. Their protein expression and enzymatic activities were measured by Western blot analysis (Figure 5A) and the in vitro isomerohydrolase activity assay, respectively. As shown in Figures 2F and 5B, wt 13cIMH exclusively generated 13cROL, whereas point mutations of 13cIMH, N58Y and L103F, dramatically increased the level of 11cROL (N58Y, 71.3 ± 2.4%; L103F, 37.5 ± 2.6%) while decreasing the level of

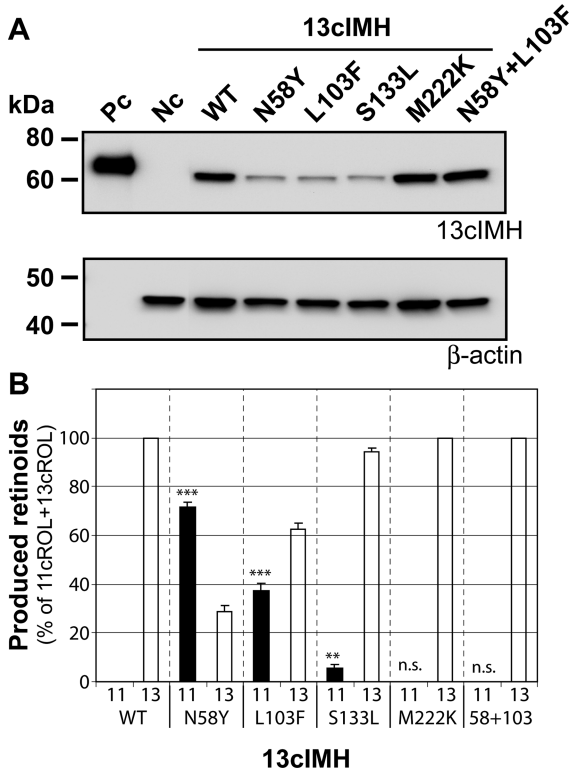

Figure 5. Reciprocal mutants of zebrafish 13cIMH and the impacts on its isomerization specificities. Similar to the RPE65c mutants, the reciprocal mutants of 13cIMH for the four candidate residues were generated and separately expressed in 293A cells. (A) The protein expression was confirmed by Western blot analysis. Pc, positive control (BMF); Nc, negative control (Rfp); WT, wild-type 13cIMH; N58Y +F103L, double mutant of 13cIMH containing N58Y and L103F mutations. Equal amounts of total cellular proteins (125 μg) from the cells expressing zebrafish 13cIMH and its mutants were incubated with liposomes containing atRE for 1 h at 37 °C, and the generated retinoids were analyzed by HPLC. The produced 11cROL and 13cROL were separately quantified from the area of the 11cROL and 13cROL peaks, respectively (mean ± SD; n = 3). (B) Retinoids produced by 13cIMH and its mutants were expressed as the percent of the produced 11cROL and 13cROL (*p < 0.05; **p < 0.01; ***p < 0.001; n.s, p > 0.05).

13cROL generation. The S133L mutant showed a slight increase (5.6 ± 1.4%) in the level of 11cROL generation (Figure 5B and Table 2). In particular, the N58Y single mutation in 13cIMH completely reversed its product from exclusively 13cROL to a mixture of 11cROL and 13cROL (71.3:28.7 11cROL:13cROL ratio), at an 11cROL:13cROL ratio similar to that in wt chicken RPE65 (87.3:12.7 11cROL:13cROL ratio) and RPE65c (72.2:27.8 11cROL:13-cROL ratio). On the other hand, the M222K single mutation in 13cIMH, which is far from the catalytic center in the RPE65 structural model, had no effect on product specificity, which generated exclusively 13cROL. Interestingly, an N58Y/L103F double mutant showed a product specificity identical to that of wt 13cIMH (Figure 5B and Table 2), although each of the single mutations, N58Y and L103F, in 13cIMH substantially increased the level of 11cROL production.

■ DISCUSSION

RPE65 in the RPE was previously known as the only enzyme that generates 11cROL from all-*trans* retinoids.[28] Recently, we

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have identified a homologue of RPE65, RPE65c, in the inner retina of zebrafish and showed that RPE65c is another isomerohydrolase that converts atRE to predominantly 11cROL with a minor amount of 13cROL, a product profile similar to that of the RPE-specific RPE65.[16] This is the first isomerohydrolase in the retina that generates 11cROL in vertebrates and, thus, is thought to participate in the generation of additional 11-*cis* retinoids to meet the high demand of the chromophore in the cone-dominant retina in zebrafish. Interestingly, we have identified another isomerohydrolase, 13cIMH, which is encoded by a distinct gene in the same chromosome as that of RPE65c in zebrafish. Although the sequence of 13cIMH is 97% identical with that of RPE65c, it generates exclusive 13cROL from the same substrate, atRE.[16,18] The features of these RPE65 homologues make them ideal models for studying the structural basis for the product specificity of isomerohydrolase. Using site-directed mutagenesis and the isomerohydrolase assay, this study for the first time identified key residues responsible for the different isomerization products generated by these highly homologous enzymes.

In this study, we predicted the key residues contributing to different product specificities of isomerization by amino acid sequence comparisons among these enzymes (see Figure 1) and examined if the substitutions of the four predicted residues of RPE65c result in altered products. All four single mutations in RPE65c (Y58N, F103L, L133S, and K222M) significantly increased the level of 13cROL production while decreasing the level of 11cROL production (see Figure 4B and Table 2), suggesting that these residues exert significant impacts on determining the isomerization specificity of products.

Among the four residues identified, our results showed that residue 58 plays a crucial role in determining the isomerization at the 11 or 13 bond of atRE. Substitution of Tyr58 in RPE65c with Asn, the counterpart of 13cIMH, completely reversed the isomerization product from predominantly 11cROL to exclusively 13cROL, a profile identical to that of wt 13cIMH. On the other hand, the reciprocal mutation in 13cIMH, N58Y, also completely reversed the product to predominantly 11cROL, similar to that of RPE65c. These results indicate that the amino acid residue at position 58 is the primary determinant of its isomerization specificity for generating either 11-*cis*- or 13-*cis*-retinol, at least in the zebrafish RPE65 homologues. Interestingly, an additional mutation at position 103 in the Y58N mutant of RPE65c and in the N58Y mutant of 13cIMH (double mutants, Y58N/F103L in RPE65c and N58Y/L103F in 13cIMH) reversed these phenotypic changes induced by the single mutations at residue 58 to that of wt enzymes (Figures 4 and 5). These results suggest that the additional mutation may reverse the tunnel structure in the mutant to that of the wt.

Recently, Redmond et al. experimentally showed that a replacement of Phe103 with Leu (F103L mutant) in canine RPE65 resulted in an approximately 2-fold increase in its level of 13cROL production compared to that of wt RPE65.[15] Actually, Phe103 is conserved in all known RPE-specific RPE65 forms from different species and is substituted with a Leu residue in zebrafish 13cIMH, suggesting that Leu103 in 13cIMH may contribute to its exclusive 13cROL production. Here, we examined the impact of residue 103, and the results showed that the F103L mutation in RPE65c greatly increased its level of 13cROL production compared to that of wt RPE65c (see Figure 4B), consistent with the observation by Redmond's group.[15] Likewise, a reciprocal mutant of 13cIMH at the same position, L103F, showed a significantly increased level of 11cROL production, compared to that of wt 13cIMH (see Figure 5B). Unlike the N58Y mutant of 13cIMH, the L103F mutant did not completely change the products of 13cIMH to those of RPE65c and RPE65 (Figure 5B). This result suggests that the residue at position 103 has significant impacts on isomerization specificity, but it is not the primary residue determining isomerization specificity. Similarly, our results showed that residues at positions 133 and 222 play minor roles in determining isomerization specificity, because L133S and K222M mutants of RPE65c both showed significant increases in the level of 13cROL production, whereas the reciprocal mutants of 13cIMH at the same positions, S133L and M222K, had minor or no effects on 11cROL production.

On the basis of the three-dimensional structural model of RPE65, all four residues identified here are not in the proximity of the catalytic domain or the iron-binding site. Instead, they are located relatively close to the bent hydrophobic tunnel and substrate cleft, through which the substrate reaches the catalytic center. Our results suggest that the structures of the bent tunnel and substrate cleft are crucial for determining its product specificity. It is likely that isomerization of the substrate (atRE) in RPE65 occurs when the substrate reaches the catalytic domain, similar to that in apocarotenoid-15,15′-oxygenase (ACO).[14]

An efficient visual cycle for regenerating the chromophore (11cRAL) of visual pigments is crucial for maintaining normal vision. It has been shown that depletion of 11cRAL in the retina causes vision loss as well as cone opsin mislocalization and accelerates photoreceptor death, whereas supplementation of 11cRAL restores vision and normalizes opsin localization.[29,30] RPE65 is a key enzyme in the visual cycle and is essential for regenerating 11cRAL. Numbers of nonsense and missense mutations in RPE65 have been reported to impair vision by disrupting the visual cycle.[31−33] Despite the significance of the efficient regeneration of 11cRAL, the molecular mechanism by which RPE65 generates 11cROL from atRE has not been well understood. This study has identified the key residues contributing to the generation of 11cROL, a precursor for 11cRAL. These findings will contribute to the understanding of the structure and function of RPE65. Furthermore, identification of key residues determining the product specificity will also contribute to the elucidation of the mechanism for isomerization of atRE to 11cROL catalyzed by RPE65.

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Notes

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■ ABBREVIATIONS

11cRAL, 11-*cis*-retinal; RPE, retinal pigment epithelium; atRE, all-*trans*-retinyl ester; 11cROL, 11-*cis*-retinol; RPE65, RPE-specific 65 kDa protein; 13cROL, 13-*cis*-retinol; ACO, apocarotenoid-15,15′-oxygenase; 13cIMH, 13-*cis*-isomerohydrolase; wt, wild-type; RFP, red fluorescent protein; HPLC, high-performance liquid chromatography.

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