The Roles of Three Palmitoylation Sites of RPE65 in Its Membrane Association and Isomerohydrolase Activity

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PURPOSE. RPE65, a membrane-associated protein predominantly expressed in the retinal pigment epithelium, is the isomerohydrolase in the retinoid visual cycle. Three cysteine (Cys) residues, Cys231, Cys329, and Cys330, in RPE65 have been shown to be palmitoylated and have been suggested to be responsible for its membrane association. The purpose of this study was to define the role of these Cys residues in palmitoylation, membrane association, and isomerohydrolase activity of RPE65.

METHODS. The three Cys residues in RPE65 were replaced by Alanine (Ala) with site-directed mutagenesis. The mutant protein levels and subcellular localizations were determined by Western blot analysis and subcellular fractionation, respectively. Their enzymatic activities were evaluated with the in vitro isomerohydrolase activity assay. Palmitoylation of the mutants was examined by labeling of the protein with [3H]-labeled palmitic acid.

RESULTS. Mutation of any single residue of these three Cys significantly reduced protein levels of RPE65. Similar to wild-type RPE65, however, all three single Cys mutants were predominantly present in the membrane fraction. Mutations of any one or two of these Cys substantially weakened the isomerohydrolase activity of RPE65, whereas mutations of all three Cys (triple mutant) completely abolished the enzymatic activity. However, this triple Cys mutant was still palmitoylated and associated with the membrane, although at a reduced level.

CONCLUSIONS. There are additional yet to be identified palmitoylation sites in RPE65. The structural distortions induced by the Cys mutations may be responsible for the mislocalization and decreased isomerohydrolase activities of RPE65. (Invest Ophthalmol Vis Sci. 2006;47:5191–5196) DOI:10.1167/iovs.06-06141

Isomerization of 11-cis retinal, the chromophore in vertebrate visual pigments, by a photon initiates activation of visual pigments and the phototransduction cascade.1,2 To maintain vision, the photosomizerized chromophore, all-trans retinal, must be efficiently recycled via multistep enzymatic reactions, termed the retinoid visual cycle.3,4 It has been suggested that the rate-limiting step of the visual cycle is the reduction of all-trans retinal5 or delivery of 11-cis retinal to opsin.6 The key step of the visual cycle is the isomerization of all-trans retinyl ester to 11-cis retinol by an enzyme, referred to as isomerohydrolase, which is associated with the RPE microsomal membrane.7 Recently, we and the other groups reported that RPE65 is the isomerohydrolase in the retinoid visual cycle.8–10 RPE65 is abundantly expressed in the retinal pigment epithelium (RPE) and has been shown to bind stereospecifically to all-trans retinyl ester with a high affinity.11–13 It has been known that several RPE65 gene mutations cause inherited retinal diseases such as retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA).14–17 RPE65 gene knockout in the mouse abolishes the isomerohydrolase activity and interrupts the regeneration of 11-cis retinol.18–20 Moreover, gene delivery of RPE65 restores the isomerohydrolase activity in the RPE and prevents early cone degeneration in the RPE65 gene knockout mouse.19 Recently, we have shown that RPE65 is an iron-dependent enzyme.20 It has been reported that four histidine residues, perfectly conserved in the β-carotene oxygenase (BCO) family, which includes RPE65 and carotenoid monooxygenase, are essential for the isomerohydrolase activity of RPE65.9,21–23 However, the functional parameters of this enzyme, such as the structural elements responsible for membrane association and detailed mechanism of isomerization, have not been elucidated.

It has been demonstrated that three cysteine (Cys) residues, Cys231, Cys329, and Cys330, in RPE65 are palmitoylated, and the palmitoylation has been suggested to be responsible for the membrane association of RPE65.24 Our previously reported evidence suggests that the membrane association of RPE65 is essential for its enzymatic activity, as it is essential for it to obtain highly hydrophobic all-trans retinyl ester from L-α-β-RAT in the same membrane.25 In the present study, we investigated the roles of the three Cys residues in palmitoylation and the membrane association and isomerohydrolase activity of RPE65, by using site-directed mutagenesis.

METHODS

Construction of the RPE65 Expression Vector and Site-Directed Mutagenesis

Human RPE65 cDNA was subcloned into a vector (pBluescript SK(+); Stratagene, La Jolla, CA). Single, double, and triple mutants of three Cys residues, C231A, C329A, C330A, C231A+C329A, C231A+C330A, C329A+C330A, and C231A+C329A+C330A (Cys×3), were generated with a site-directed mutagenesis kit (QuickChange; Stratagene) according to the protocol recommended by the manufacturer. The full-length cDNA sequences of the mutants were confirmed from both directions with a DNA sequencer (model 3730; Applied Biosystems, Inc., Foster City, CA) and subcloned into a transfer vector (pShuttle-CMV; Stratagene). The recombinant adenoviruses expressing the mutants were generated, verified, amplified, and titered, as described previously.22 Furthermore, the gene expression induced by adenovirus vectors was confirmed by Western blot analysis with an anti-RPE65 antibody and normalized by respective β-actin levels.

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Coexpression of LRAT with wild-type (wtRPE65) or Its Cysteine Mutants

The 293A-LRAT cell line, a QBI-293A cell line stably expressing human LRAT were generated and cultured as described previously.22 The 293A-LRAT cells were infected by Ad-wtRPE65 or the Cys mutants separately at a multiplicity of infection (MOI) of 100 or 400, and cultured for 24 hours after infection. The cells were harvested by cell scraper, washed twice with ice-cold PBS (phosphate-buffered saline) and immediately stored at −80°C. The expression of RPE65 and LRAT was confirmed by Western blot analyses.

Isomerohydrolase Activity Assay

The 293A-LRAT cells were separately infected by adenoviruses expressing wtRPE65 or the Cys mutants. Noninfected 293A-LRAT cells were used as the negative control. Cell lysates were sonicated on ice for 20 seconds in a reaction buffer (10 mM BTP [pH 8.0] (1,3-bis[tris(hydroxymethyl)methylamino]propane) and 100 mM NaCl), quantified by the Bradford assay,25 and the same amount of total cell lysate was used for activity assays. All-trans [11,12-3H]-retinol in ethanol (1 mcg/mL, 52 Ci/mmol) Perkin Elmer, Boston, MA) was dried under argon and resuspended in the same volume of dimethyl formamide (DMF). For each reaction, 2 μL of the nondenatured all-trans [11,12-3H]-retinol in DMF and 250 μg of total cell lysate were added into 200 μL of the reaction buffer containing 0.5% BSA and 25 μL recombinant human CRALBP (cellular retinaldehyde-binding protein) expressed in Escherichia coli. The CRALBP expression vector was kindly provided by John Crabb (Cleveland Clinic Foundation), and recombinant CRALBP was purified to homogeneity by Ni2+-His tag affinity chromatography, as described by Crabb et al.26 After 2 hours’ incubation in the dark at 37°C, the retinoids generated were extracted by the addition of 300 μL cold methanol and 300 μL hexane. The upper organic phase was collected and analyzed by normal-phase HPLC, as described.27 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 11-cis retinol peak with commercial software (Radiomatic 610TR, Perkin Elmer, Boston, MA), with synthetic 11-cis [3H]-retinol used as a standard.

Subcellular Fractionation of Cultured Cells

The 293A cells expressing RPE65 or the Cys mutants were harvested and washed twice with ice-cold PBS. Subsequently, cells were separated into cytosolic, membrane, nuclear, and cytoskeletal–inclusion body fractions (FracPrep, BioVision, Mountain View, CA), according to the manufacturer’s protocol. The same amount of proteins (10 μg) of each fraction was resolved by 8% SDS-PAGE and analyzed by Western blot analysis with a purified RPE65 antibody,12 to confirm subcellular localization.

Palmitoylation Assay

QBI-293A cells were separately infected by Ad-wtRPE65 at MOI 100 and the triple Cys mutant (Ad-Cys×3) at MOI 400. The infected cells were incubated for 2 hours, followed by the addition of 3 mcg [9,10-3H] palmitic acid (American Radiolabeled Chemical, Inc., St. Louis, MO), and incubated for another 16 hours. The cells were harvested by cell scraper, washed twice with ice-cold PBS, and lysed by sonication. Expressed wtRPE65 and the Cys triple mutant were purified by immuno precipitation. Briefly, the cell lysates were dissolved by PBS containing 0.5% CHAPS (3-[3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate), 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 100 μM phenylmethylsulfonyl fluoride (PMSF), for 1.5 hours on ice. The fraction solubilized by CHAPS was separated by centrifugation, and gently mixed with cyanogen bromide–activated Sepharose 4B (GE Healthcare, Piscataway, NJ) conjugated with the antibody for human RPE6522 in a cold room overnight. The Sepharose was washed five times with PBS containing 0.5% CHAPS and the proteinase inhibitors described earlier. The final elution was performed by the addition of SDS-PAGE sample buffer. Purified wtRPE65 and the triple Cys mutant were separated in an 8% SDS-PAGE gel, analyzed by Western blot, and semiquantified by densitometry. The same volumes of purified wtRPE65 and Cys×3 were separated on an 8% SDS-PAGE gel. The gel was treated with amplification reagent (Amplify; GE Healthcare), according to the manufacturer’s protocol and dried with a gel dryer. The dried gel was exposed to intensifying screens (Biomax Ms, Eastman Kodak, Rochester, NY) in an autoradiography cassette at −80°C for 8 days, and the film was then developed.

RESULTS

Impact of the Cys Mutations on Protein Levels of RPE65

QBI-293A cells were infected with adenovirus expressing wtRPE65; the three single Cys mutants, Ad-C231A, Ad-C329A and Ad-C330A; the three double mutants, Ad-C231A+C329A, Ad-C231A+C330A, Ad-C329A+C330A; and the triple Cys mutant Ad-Cys×3 (C231A+C329A+C330A), separately at the same titer as the virus (MOI 100). At 24 hours after infection, the RPE65 levels were measured by Western blot analysis with the same amount of total cellular proteins (50 μg), semiquantified by densitometry, and normalized by the respective β-actin levels. The results were averaged among five independent experiments. All the Cys mutants showed significantly reduced protein levels of RPE65, compared with wtRPE65 in the same culture condition (Fig. 1A). The single mutants C231A, C329A, and C330A showed protein levels of 54%, 18%, and 60%, respectively, of the wtRPE65 level. The double mutants C231A+C329A, C231A+C330A, and C329A+C330A showed RPE65 levels further decreased to 8%, 27%, and 16% of the wtRPE65, respectively. These results suggest that C329 is more important than the other two Cys for the stability of the RPE65 protein. The triple Cys mutant showed a protein level of 5% of wtRPE65 at the same MOI (Figs. 1A, 1B).

For the in vitro isomerohydrolase activity assay, a 293A cell line stably expressing LRAT was infected by adenoviruses expressing the mutants at a higher MOI (MOI 400), to achieve protein levels of the mutants comparable to that of wtRPE65 (MOI 100). Protein levels of wtRPE65 and the Cys mutants were examined by Western blot analysis (Fig. 1C), normalized by β-actin levels, and semiquantified by densitometry (Fig. 1D). The values were averaged from independent experiments and expressed as percentages of wtRPE65. The result showed that a higher MOI (400) substantially increased the expression of the mutants to levels 40% to 100% of the wtRPE65 level when expressed at MOI 100 (Fig. 1D).

Isomerohydrolase Activities of the Cys Mutants

An in vitro isomerohydrolase activity assay with the same lysates as for the Western blot analysis showed that the three single Cys mutants C231A, C329A, and C330A exhibited robust
isomerohydrolase activity, approximately 49%, 35%, and 61% of that in wtRPE65, after normalization by the relative RPE65 protein level measured by Western blot analysis (Figs. 2A–D, 2I). The three double mutants showed further reduced but detectable isomerohydrolase activities (Figs. 2E–G). The triple Cys mutant, however, showed no detectable enzymatic activity.
The decreased or abolished isomerohydrolase activities of these mutants, even after normalization by their protein levels of RPE65, indicate that the decrease in the enzymatic activity cannot be ascribed only to the reduced protein levels of the mutants (Fig. 2I). Instead, the reduced enzymatic activities in these mutants may be explained by structural distortions introduced by these point mutations.

Subcellular Localization of the Cys Mutants

We sought to determine whether replacement of these palmitoylated Cys residues by alanine would alter the membrane association, by using subcellular fractionation and Western blot analysis. Similar to wtRPE65, all the single Cys mutants were still predominantly present in the membrane fraction, suggesting that the replacement of any of the three Cys is not sufficient to dissociate RPE65 from the membrane (Fig. 3A, left; 3B). The membrane-associated RPE65 of the double mutants was significantly reduced, whereas the RPE65 mutants in the cytosolic and cytoskeletal-inclusion body fractions were increased substantially (Fig. 3B). The substitution of all the three Cys residues caused a great reduction of RPE65 in the membrane fraction. A substantial amount of the triple Cys mutant was present in the inclusion body, suggesting that possible misfolding of the triple mutant may indirectly affect the membrane association (Fig. 3A).

Palmitoylation Status of The Triple Cys Mutant

We performed a palmitoylation assay with [9,10-3H] palmitic acid in the cells expressing the triple Cys mutant and wtRPE65 control, to determine whether the substitution of all three known palmitoylation sites abolishes the palmitoylation of the mutant protein. Western blot and autoradiography analyses were performed with the same samples. The triple Cys mutant exhibited lower protein levels of RPE65, compared with that of wtRPE65, but it was clearly labeled by [9,10-3H] palmitic acid, although at reduced levels, compared with that in wtRPE65 (Fig. 4). This result is consistent with our subcellular fractionation assay showing that a fraction of the triple Cys mutant remains to be associated with the membrane (Fig. 3A) and indicates that there are other palmitoylation sites in addition to...
these three Cys sites, or there may be other mechanisms for the protein to associate with the membrane.28

**DISCUSSION**

RPE65 is a membrane-associated protein with isomerohydrolase activity in the RPE.8–10 It has been reported that Cys231, Cys329, and Cys330 in RPE65 are palmitoylated, and the palmitoylation at these sites has been suggested to be responsible for the membrane association of RPE65.24 With site-directed mutagenesis, we show that replacement of these Cys residues with Ala reduced protein levels and impaired the isomerohydrolase activity. However, all the mutant proteins remained to be palmitoylated and membrane associated, even after all three Cys residues were mutated, suggesting that there are other palmitoylation sites in the protein.

Our previous studies have suggested that the membrane association of RPE65 is essential for RPE65 to obtain the hydrophobic substrate, all-trans retinyl ester, generated by LRAT in the membrane.8 RPE65 lacks any hydrophobic transmembrane helix, based on its amino acid sequence.11 As palmitoylation is a common mechanism for membrane association of proteins, Rando’s group proposed that the three palmitoylated Cys residues may be responsible for the membrane association of RPE65.24 Based on these previous findings, we have explored the role of these Cys in isomerohydrolase activity and membrane association of RPE65. All the single Cys mutants decreased RPE65 protein levels and isomerohydrolase activities. The lower enzymatic activities of the mutants cannot be explained by their decreased protein levels, as when the mutants were overexpressed to levels comparable to that of wtRPE65, the mutants still show significantly lower enzymatic activities than wtRPE65. This finding suggests that the structure distortion induced by mutations at these Cys residues may be responsible for the impaired isomerohydrolase activities of RPE65. Of interest, these Cys mutants are still associated with the membrane. This result suggests that substitution of one of these Cys is not sufficient for dissociating the protein from the membrane. Therefore, we further mutated two of the three and all three Cys in this study. The double and triple mutants showed significantly reduced protein levels in the membrane fraction and greatly increased mutant protein levels in cytosolic and cytoskeletal–inclusion body fractions. The altered levels of the double and triple mutants in the membrane fraction are probably due to disturbance of the protein conformation by two and three of the Cys mutations.

After all the three Cys residues were replaced by alanine, levels of the triple Cys mutant were significantly decreased. Although a higher titer of the adenovirus infection (MOI 400) achieved higher protein levels of this mutant (approximately 40% of wild type at MOI 100), the high levels of mutant protein did not show any detectable enzymatic activity. This result suggests that the lack of enzymatic activity in the triple mutant cannot be ascribed to the reduced protein level of the mutant protein. However, this triple Cys mutant still exhibited a lower level of membrane association, suggesting that at least, the palmitoylation at Cys231, Cys329, and Cys330 is not the only mechanism responsible for the association of RPE65 with the membrane. Moreover, palmitoylation assay demonstrated that the triple Cys mutant is still labeled by [3H]-palmitic acid. A possible explanation is that in addition to the three Cys residues, there are other yet to be identified palmitoylation sites in RPE65 protein, which may be responsible for the membrane association of the triple Cys mutant. It is known that most of the palmitoyl modification occurs in Cys residues, referred to as S-palmitoylation, and a very few in Leu residues, referred to as O-palmitoylation.29–32 The additional palmitoylation sites in RPE65 protein and the exact mechanism for the protein to associate with the membrane remain to be investigated.

Human RPE65 possesses 13 Cys residues in its amino acid sequences. Sequence alignment of RPE65 from 11 vertebrates has shown that Cys329 is absolutely conserved across the species, but Cys231 and Cys330 are not (data not shown), which suggests that Cys329 may play more important roles in the structure and function of RPE65 than the other two palmitoylated Cys residues. Indeed, we showed that the mutation Cys329Ala caused more significant reduction of RPE65 level and enzymatic activity than the other two Cys mutations. This observation, in consistency with the assumption based on the sequence alignment, suggests that Cys329 is essential for the structure and function of RPE65, such as protein folding and stability.

It is also possible that RPE65 is associated with the membrane through other mechanisms than palmitoylation. A recent report showed that two fragments of RPE65 (fragments 1 and 2, 1-125 and 126-250, respectively, of RPE65) bind to the lipid monolayer (dioleoylphosphatidylcholine; [DOPC]).
larly, fragment 2 of RPE65 can penetrate the lipid monolayer.24
This fragment is postulated to contain a hydrophobic patch of seven-bladed β-propeller, based on sequence and structure comparison with apocarotenoid oxygenase.25 The water-soluble apocarotenoid oxygenase uses this hydrophobic patch to dip into the membrane and extract its nonpolar substrate from the membrane.25 Consistent with this hypothesis, we showed that substitution of Cys231 did not affect its membrane association (Fig. 3). It is possible that fragment 2 of RPE65 can bind to the membrane independent of palmitoylation of Cys231. This report, together with our Cys mutant results, suggests that hydrophobic domains of RPE65 may be responsible for its membrane association.

References