Identification of a Novel Palmitylation Site Essential for Membrane Association and Isomerohydrolase Activity of RPE65*‡§

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RPE65 is a membrane-associated protein abundantly expressed in the retinal pigment epithelium, which directs all-trans-retinyl ester to cis-retinol, a key step in the retinoid visual cycle. Although three cysteine residues (Cys-231, Cys-329, and Cys-330) were identified to be palmitylated in RPE65, recent studies showed that a triple mutant, with all three Cys residues replaced by an alanine residue, was still palmitylated and remained membrane-associated, suggesting that there are other yet to be identified palmitylated Cys residues in RPE65. Here we mapped the entire RPE65 using mass spectrometry analysis and demonstrated that a trypsin-digested RPE65 fragment (residues 98–118), which contains two cysteine residues (Cys-106 and Cys-112), was singly palmitylated in both native bovine and recombinant human RPE65. To determine whether Cys-106 or Cys-112 is the palmitoylation site, these Cys were separately replaced by alanine. Mass spectrometry analysis of purified wild-type RPE65 and C106A and C112A mutants showed that mutation of Cys-106 did not affect the palmitoylation status of the fragment 98–118, whereas mutation of Cys-112 abolished palmitoylation in this fragment. Subcellular fractionation and immunocytochemistry analyses both showed that mutation of Cys-112 dissociated RPE65 from the membrane, whereas the C106A mutant remained associated with the membrane. In vitro isomerohydrolase activity assay showed that C106A has an intact enzymatic activity similar to that of wtRPE65, whereas C112A lost its enzymatic activity. These results indicate that the newly identified Cys-112 palmitoylation site is essential for the membrane association and activity of RPE65.

Both rod and cone visual pigments in vertebrates require 11-cis-retinal as the chromophore. Isomerization of 11-cis-retinal to all-trans-retinal by a photon triggers the phototransduction cascade and initiates vision (1, 2). Recycling of 11-cis-retinol through the retinoid visual cycle is an essential process for the regeneration of visual pigments and for normal vision (3, 4). The key step in the visual cycle is to isomerize all-trans-retinyl ester to 11-cis-retinol in retinal pigment epithelium (RPE) (5, 6). This isomerization process is known to be catalyzed by an isomerohydrolase in the RPE. Several recent lines of evidence suggest that RPE65 is the isomerohydrolase in the visual cycle (7–9).

RPE65 is a microsomal protein, abundantly expressed in the RPE (10–12). RPE65 knock-out (Rpe65−/−) mice showed a lack of 11-cis-retinoids, overaccumulation of all-trans-retinyl ester, impaired visual function, and early degeneration of cone photoreceptors (7–9). RPE65 is an iron(II)-dependent enzyme, in which an iron is coordinated by four conserved histidine (His) residues (His-180, -241, -313, and -527) based on molecular modeling using a crystal structure of apocarotenoid monooxygenase as a template (8, 13–15). RPE65 lacks any predicted transmembrane helix and is associated with the microsomal membrane (11). Previous studies have shown that membrane association of RPE65 is essential for its isomerohydrolase activity (7). However, the structural basis for its membrane association has not been revealed. An earlier study showed that three cysteine residues (Cys-231, 329 and 330) in RPE65 were palmitylated, which were suggested to be responsible for its membrane association (16). However, triple mutations of all the three cysteine residues did not completely dissociate RPE65 from the membrane (17, 18). Moreover, the triple Cys mutant remains palmitoylated (17). These results suggested that either the site of palmitoylation responsible for the membrane association of RPE65 had not yet been identified or other mechanisms, such as hydrophobic interactions, anchor the protein to cellular membranes (17, 19).

In this study, we used the combination of mass spectrometric analysis and site-directed mutagenesis to identify the palmitoylated site in RPE65. Moreover, we determined the role of this site in the membrane association and enzymatic activity of RPE65.

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The abbreviations used are: RPE, retinal pigment epithelium; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; L RAT, lecithin-retinol acyltransferase; MMTS, methyl-methanethiosulfonate; MS, mass spectrometry; Bistris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propane; HPLC, high pressure liquid chromatography; Ni-NTA, nickel-nitrilotriacetic acid.
Palmitylation and Membrane Association of RPE65

EXPERIMENTAL PROCEDURES

Peptide Generation for MS—Purified native bovine RPE65 (14) as well as recombinant human wild-type RPE65 with a histidine tag (wtRPE65-His), and the mutants with Cys-106 and Cys-112 replaced by alanine fused with a histidine tag, C106A-His and C112A-His, were prepared for MS analysis in the original buffers. Up to 100 μg samples were reduced with 2 μl of 5 mM tris(2-carboxyethyl)phosphine hydrochloride (Applied Biosystems, Foster City, CA) for 60 min at 60 °C and then alkylated with 1 μl of 10 mM methyl-methanethiosulfonate (MTS; Applied Biosystems, Foster City, CA) for 10 min. The protein was then precipitated with 10% trichloroacetic acid (Fisher) at -20 °C for 10 min followed by two washes with water (5 min of centrifugation at 100,000 × g, removal of supernatant) and was then brought up in 5–25 μl of 6 M urea. After solubilization, the protein was diluted with 7 × volume of 10 mM Tris (pH 7.4) and 10% volume of acetonitrile (Sigma). Thereafter, 10 μl of sequencing grade modified trypsin (Promega, Madison, WI, 1 μg/μl) was added and incubated with the sample at 37 °C overnight to cleave the protein.

HPLC/MS/MS—Peptide separation from the trypsin cleavage mixture of native bovine RPE65 was performed using a binary HPLC pump system (Agilent Technologies, Santa Clara, CA). These samples were analyzed in normal flow mode (200 μl/min initial flow with a 10:1 split in front of the instrument source) as with a narrowbore Brownlee Aquapore C18 reverse phase column (OD-300, 100 mm × 2.1 mm, Bodman Industries, Aston, PA) (for injection, the initial sample was diluted to 3 ml with water). Solvent A was 0.05% trifluoroacetic acid (Fisher) in HPLC-grade water (Fisher), and solvent B was 0.05% trifluoroacetic acid in 2:1 isopropl alcohol/acetonitrile (both from Fisher). The column gradient was from 2% B to 98% B over 90 min. The separation of human recombinant RPE65 peptides was performed on an Ultimate 3000 nanoflow HPLC system (LC Packings, Sunnyvale, CA) at a flow rate of 180 nl/min with a nanobore Vydac C18 reverse phase column (2385V.5.057515; 150 mm × 75 μm, Bodman) (10 μl of the final sample was injected). Solvent A was 0.2% formic acid (Fisher) in HPLC-grade water, and solvent B was 0.2% formic acid in acetonitrile. The column gradient was from 2% B to 98% B over 120 min. The effluent was directed into the ion Max source (normal flow) or nanospray source (nanoflow) of an LTQ XL ETD linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Mass spectra and five data-dependent tandem mass spectra were measured by the Thermo Scientific Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) in HPLC-grade water. The column was a 2.1 mm × 100 mm C18 column (YMC, Kyoto, Japan) and the mobile phase was a linear gradient from 0 to 100% B over 8 min. The column was maintained at 80 °C, and the flow rate was 0.3 ml/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 275 °C and a heater temperature of 360 °C. The spray voltage was set at 2.5 kV, and the capillary temperature was 280 °C. The ion trap was operated in the extended dynamic exclusion mode with a exclusion duration of 45 s and a list length of 200. The mass spectrometer was controlled by Xcalibur version 1.4SR1 software. For MS/MS analysis, dynamic exclusion was used. Bioworks 3.3.1 Software (Thermo Fisher Scientific, Waltham, MA) was used for data analysis.

Construction of wtRPE65 Expression Vector and Site-directed Mutagenesis—The human RPE65 cDNA with the Kozak sequence (gccacc (20)) upstream of the translational start codon was subcloned into the EcoRI and HindIII sites of pBluescript SK (+) vector. Cys-106 and Cys-112 were replaced by Ala residue using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the protocol recommended by the manufacturer. To process efficient purification of wtRPE65 and its mutants for mass spectral analysis, a histidine hexamer tag (His6 tag) was inserted in-frame into the N-terminal region of RPE65 by PCR. The mutants were confirmed by sequencing from both strands using ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) and subcloned into an expression vector, pCDNA3.1 (+) (Invitrogen). Following the sequence confirmations, the expression constructs were purified by QIAfilter maxi prep kit (Qiagen, Valencia, CA).

Expression and Purification of Recombinant wtRPE65 and Its Mutants—For mass spectral analysis, the expression plasmids of wtRPE65-His, C106A-His, and C112A-His were transfected into 293A-LRAT cells, a stable cell line expressing LRAT, which was established previously (13), and harvested at 48 h post-transfection. Cells were lysed by sonication in a buffer containing 6 M guanidine hydrochloride, 20 mM Tris HCl (pH 8.0), 500 mM NaCl, 0.1% CHAPS, 1 mM imidazole, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μM phenylmethanesulfonyl fluoride. The cell lysate was applied onto Ni-NTA His-Bind Resin™ (Novagen, Madison, WI) and washed with 25 ml of washing buffer containing 6 M urea, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% CHAPS, 10 mM imidazole, and protease inhibitors as described above. wtRPE65 and the Cys mutants containing the His6 tag were eluted with the washing buffer containing 400 μM imidazole. When necessary, the purified wtRPE65 and the Cys mutants were concentrated using Microcon YM-10 (Millipore, Billerica, MA) and/or by dialysis using the Slide-A-Lyzer mini dialysis unit (Pierce).

Western Blot Analysis—Total cellular protein concentrations were measured by the Bradford assay (21). Equal amounts of protein (25 μg) were resolved by electrophoresis through 8% Tris-glycine SDS-polyacrylamide gel and electrophoresed 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 1:1000 dilution of an anti-RPE65 polyclonal antibody (10). After three washes with TBST, the membrane was incubated for 1.5 h with 1:6700 dilutions of an horseradish peroxidase-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) or Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). As needed, the membrane was stripped in the stripping buffer (Pierce) and re-blotted with an antibody specific for β-actin (Abcam, Cambridge, MA) for loading control. The bands (intensity × area) were semi-quantified by densitometry using GeneTools (SynGene, Frederick, MD), averaged from at least three independent experiments.

Subcellular Fractionation of Cultured Cells—The 293A-LRAT cells expressing wtRPE65, C106A, or C112A mutants were harvested and washed twice with ice-cold phosphate-buffered saline. Subsequently, cells were fractionated into cytosolic, membrane, nuclear, and cytoskeletal (containing inclusion body) fractions using FractionPrep™ (BioVision, Mountain View, CA) following the manufacturer’s protocol. Equal amounts of protein (25 μg of total protein and 5 μg of each fraction) from each fraction were resolved by 8% SDS-PAGE.
and analyzed by Western blot analyses using a purified antibody for RPE65 to identify the subcellular localization.

**Immunohistochemistry**—The 293A-LRAT cells were cultured in glass chamber slides and transfected separately with wtRPE65, C106A, C112A expression vectors and the empty pcDNA3.1 (mock transfection). At 24 h post-transfection, the cells on the chamber slides were rinsed with phosphate-buffered saline and fixed in 4% paraformaldehyde. Following blocking with 3% bovine serum albumin and 10% goat normal serum, the slides were incubated with 1:1000 dilution of an anti-RPE65 monoclonal antibody (Chemicon, Temecula, CA) and 1:300 dilution of a rabbit polyclonal antibody specific for an endoplasmic reticulum (ER) marker calnexin (Abcam, Cambridge, MA). After three washes, the slides were incubated with 1:200 dilution of Cy3-labeled anti-mouse IgG and fluorescein isothiocyanate-labeled anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Following three washes, the slides were treated with mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, San Diego). The fluorescent signals were observed using a Zeiss LSM-510META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

**Isomerohydrolase Activity Assay**—The 293A-LRAT cells were separately transfected with plasmids expressing wtRPE65, C106A, and C112A. Un-transfected 293A-LRAT cells were used as a negative control. The cells were lysed by sonication in a reaction buffer (10 mM Bis Tris propane (pH 8.0), 100 mM NaCl). All-trans-[11,12-3H]retinol (1 mCi/ml, 45.5 Ci/mmol, American Radiolabeled Chemical, Inc., St. Louis, MO) in N,N-dimethylformamide was used as the substrate for the isomerohydrolase assay. For each reaction, microsomal proteins from the cells were added into 200 μl of reaction buffer (10 mM Bis Tris propane (pH 8.0), 100 mM NaCl) containing 0.2 μM all-trans-retinol, 1% bovine serum albumin, and 25 μM cellular retinaldehyde-binding protein. The reaction was stopped and retinoids extracted with 300 μl of cold hexane and centrifuged at 10,000 × g for 5 min. The generated retinoids were analyzed by normal phase HPLC as described. The peak of each retinoid isomer was identified based on its characteristic retention time of retinoid standards. The isomerohydrolase activity was calculated from the area of the 11-cis-retinol peak using Radiomatic 610TR software (PerkinElmer Life Sciences) with synthetic 11-cis-[3H]retinol as a standard.

**RESULTS**

**Identification of the Peptide Fragment Containing the Palmitylated Cys Residue**—Purified native bovine RPE65 was digested with trypsin, and the digested peptide fragment mixture was analyzed with HPLC/MS-MS following a methodology developed previously and adapted in our laboratory (22). This methodology allows the observation of the entire protein sequence map in a single experiment. RPE65 is a 61-kDa protein with 533 amino acids, producing 52 individual peptide fragments, as well as various incomplete cleavage products after digestion with trypsin. In consequence of the high number of fragments, the individual peptides were identified with the utilization of the TurboSequest search engine. The search engine provides a score for how reliable the identification is (XCorr). The higher XCorr, the more reliable is the identification. This analysis resulted in complete sequence mapping with a minimal score limit of 1.4 XCorr.

Palmitylation was identified only in a peptide fragment (amino acids 98–118) and its incomplete cleavage products. The identity of the palmitylated peptide identified by the search engine was also confirmed by de novo sequencing. Moreover, this peptide was not identified as unpalmitylated. Other peptides were not palmitylated above XCorr values of 1.0, below which the identification is not accepted to be reliable and could not be confirmed with de novo sequencing. The cysteine residues, which have previously been reported to be palmitylated (Cys-231, Cys-329, and Cys-330), were positively identified as unpalmitylated with their individual peptides showing XCorr values above 2.0.

The sequence for the identified palmitylated peptide (amino acids 98–118, IVITEFGTCAFPDPCKNIFSR) is shown in Fig. 1A. The peptide identified by the search engine was a +2 charge state ion, m/z = 1323.9, with single palmitylation and MMTS label, which corresponds to a molecular mass of 2643.7 Da. The identified mass lines in Fig. 1 are indicated by red and blue coloring, but some of the most abundant ions are also labeled. The observed sequences and the expected b and y fragment ions are also shown with the identified ions underlined in Fig. 1, indicating single palmitylation on the peptide fragment. However, this peptide contains two Cys residues, Cys-106 and Cys-112, which can potentially be palmitylated (Table 1). Therefore, it was important to determine the abundance of this peptide versus its unpalmitylated (molecular mass of 2451.5 Da with two MMTS labels) and doubly palmitylated (molecular mass of 2835.9 Da with two S-palmityl cysteines) counterparts. Fig. 1B shows normalized selected ion chromatograms for the corresponding +2 charge state ions. The unpalmitylated peptide (Fig. 1B, top panel) is shown with a low abundance at retention time of 39.5 min, whereas the peptide with single palmitylation formed a distinct peak (Fig. 1B, middle panel). In contrast, the peptide that contained two palmitylated Cys residues (Cys-106 and Cys-112) was not detected above background (Fig. 1B, bottom panel). The position of the peak for the singly palmitylated peptide corresponded to the retention time where the sequence shown in Fig. 1A was identified.

**Expression and Purification of wtRPE65 and the Cys Mutants without or with a His6 Tag**—The obtained MS/MS data were not sufficient to unequivocally distinguish between the two Cys residues in the peptide, as the recorded sequence may be a mixture of the two different singly palmitylated peptides. Therefore, to identify whether Cys-106 or Cys-112 is palmitylated and to establish the functional significance of the palmitylation, Cys-106 and Cys-112 were separately replaced by Ala. Human wtRPE65 and the mutants C106A and C112A were expressed in 293A-LRAT cells. To purify wtRPE65 and the mutants efficiently, the His6 tag was inserted at the N terminus of wtRPE65 and the Cys mutants. Expressed wtRPE65 and the Cys mutants fused with the His6 tag were purified through a Ni-NTA column (Fig. 2A). Approximately 66, 120, and 22 μg of wtRPE65-His, C106A-His, and C112A-His were purified from
the 5 mg of total cellular protein, respectively, and confirmed by Western blot analysis (Fig. 2B).

Palmitylation of Cys-106 and Cys-112 in the Recombinant Proteins—Purified wtRPE65, C106A, and C112A were digested with trypsin and used for MS analysis. Although the abundance of these recombinant proteins after purification was less than that of the native bovine RPE65, with the utilization of the nanoflow HPLC system, all of the three recombinant proteins were successfully analyzed by MS and provided complete peptide fragment maps with XCorr values above 1.0.

Fig. 3A shows the MS/MS spectrum for the +2 charge state of the 98–118 fragment ion from recombinant human wtRPE65. The data provide evidence of complete sequence coverage for the 98–118 peptide. Cys-106 was not labeled with either iodoacetamide or MMTS, which probably indicates a buried localization of the residue. Palmitylation on Cys-112 is evidenced by the observed sequence ladder and, in particular, by mass lines \( m/z 574 \) and \( m/z 826 \). The analysis of the peptide (residues 98–118) from the mutants reinforced the observations in native bovine and recombinant human wtRPE65 that this peptide was singly palmitylated. The peptide from the C106A mutant also showed palmitylation on Cys-112 (Fig. 3B) as evidenced by mass lines \( m/z 770 \), 941, 593, and 423, although the peptide from the C112A mutant was not palmitylated (Fig. 3C). The Ala substitutions were confirmed in both mutants in the obtained sequences. Additionally, the sequence ladders demonstrated that Lys-113 was acetylated and Thr-101, Thr-105, and Ser-117 were phosphorylated in human wtRPE65 (Fig. 3). These post-translational modifications were not detected in native bovine RPE65. Similar to the native bovine protein, no other cysteine residues in the recombinant RPE65 were found to be palmitylated using the minimal XCorr limit (1.0), and no other sites could be confirmed to be palmitylated with de novo sequencing.

Subcellular Localization of wtRPE65 and the Cys Mutants—To evaluate the significance of Cys-106 and Cys-112 residues on membrane association of RPE65, the cells expressing wtRPE65 and the Cys mutants were fractionated and analyzed by Western blot analysis. wtRPE65 predominantly existed in the membrane fraction (Fig. 4A), consistent with our previous reports (17). Similarly, the C106A mutant was present predominantly
TABLE 1
Alignment of Cys residues in the RPE65 from different species

| Species                  | Cys-231 | Cys-329 | Cys-330 |
|--------------------------|---------|---------|---------|
| Human                    | C       | C       | C       |
| African green monkey     | C       | C       | C       |
| Bovine                   | C       | C       | C       |
| Dog                      | C       | C       | -       |
| Rat                      | C       | C       | -       |
| Mouse                    | C       | C       | -       |
| Chicken                  | Y       | -       | -       |
| Tiger salamander         | F       | -       | -       |
| Japanese Fireberry newt  | F       | -       | -       |
| African clawed frog      | F       | -       | -       |
| Zebrafish                | L       | V       | S       |

African clawed frog

Bovine

Human

Japanese Fireberry newt

Tiger salamander

African clawed frog

Zebrafish

FIGURE 2. Purification of recombinant RPE65 for MS analyses. A, 293A-LRAT cells were transfected separately with the plasmids expressing wtRPE65-His, C106A-His, or C112A-His mutants and then cultured for 48 h. The His-tagged RPE65 was expressed and purified through a Ni-NTA column. M, molecular weight marker. Total, total cell lysate; FT, flow-through; E1, E2, and E3, the elution fractions from the column. B, purified wtRPE65-His (0.1 μl of 80 μl), C106A-His (0.1 μl of 105 μl), C112A-His (0.1 μl of 90 μl), and purified native RPE65 (10–250 ng) from bovine RPE were used for Western blot analysis. The same samples were used for in vitro isomerohydrolase activity assay. wtRPE65-His and C106A-His exhibited robust isomerohydrolase activities, whereas C112A-His showed no detectable enzymatic activity (Fig. 6B, C–E).

DISCUSSION

RPE65 is the retinoid isomerohydrolase in the visual cycle, a key enzyme for regenerating 11-cis-retinal, the chromophore of visual pigment (7–9). It is associated with the microsomal membrane in the RPE (11, 12). The structural basis responsible for membrane association of RPE65 has not been revealed. Previously, it was reported that three Cys residues (Cys-231, -329, and -330) were palmitylated, which was suggested to be responsible for membrane association of RPE65 (16). However, we reported previously that a triple Cys mutant, in which all of the three Cys were mutated, is present in the membrane fraction, but C112A mutant dissociated from the membrane.

Impacts of the Cys Mutations on RPE65 Protein Levels and Isomerohydrolase Activities with and without the His6 Tag—wtRPE65 and the mutants C106A and C112A were expressed in 293A-LRAT cells. The protein levels of these mutants were compared with wtRPE65 by Western blot analysis. Both C106A and C112A mutants showed RPE65 levels similar to that of wtRPE65 (Fig. 6, A and B), suggesting that these mutations did not affect protein expression or stability. However, wtRPE65 and C106A showed robust isomerohydrolase activities as they both generated 11-cis-retinol from all-trans-retinol in the 293A-LRAT cells that stably express LRAT, whereas in the same cells the C112A mutant did not show any detectable enzymatic activity (Fig. 6, C–E).

Furthermore, to exclude the possibility that the fusion with the His6 tag could affect the isomerohydrolase activity of RPE65, wtRPE65-His, C106A-His, and C112A-His were separately expressed in 293A-LRAT cells. As shown by Western blot analysis, His-tagged wtRPE65 and the mutants had expression levels similar to that without the His tag (Fig. 6G). The same samples were used for in vitro isomerohydrolase activity assay. wtRPE65-His and C106A-His exhibited robust isomerohydrolase activities, whereas C112A-His showed no detectable enzymatic activity (Fig. 6H), consistent with the results in the proteins without the His tag (see Fig. 6, E and F).
brane association of RPE65. This study identified the novel palmitylation site, Cys-112, in RPE65 through MS analysis and site-directed mutagenesis. Furthermore, our data demonstrated that Cys-112 is essential for membrane association of RPE65 and its enzymatic activity.

In search for novel palmitylation sites in RPE65, we utilized a new MS analysis strategy. This strategy is based on observing the sequence of the protein in its entirety. This MS analysis allows us not only to see which peptide is palmitylated but also to confirm at the same time that the peptide is not present in unpalmitylated form and that other peptides are not palmitylated either. Our analyses showed that one of the trypsin-digested fragments (residues 98–118), containing two Cys residues (Cys-106 and -112), was singly palmitylated in both native bovine RPE65 and recombinant human RPE65 (Fig. 1 and Fig. 3). Considering the close locations of these two Cys sites and the relatively low (<2.0) XCorr values, we used site-directed mutagenesis to replace Cys-106 and Cys-112 individually by Ala, to determine whether Cys-106 or Cys-112 is the palmitylation site. MS analysis of the trypsin fragment (residues 98–118) from the C106A and C112A mutants demonstrated that the peptide from the C106A mutant was singly palmitylated, the same as wtRPE65. In contrast, when Cys-112 was replaced by Ala, the palmitylation in this peptide was lost as shown by MS. These results together support the conclusion that Cys-112 is the palmitylation site in the peptide 98–118.

Palmitylation is a common posttranslational modification responsible for anchoring proteins to the membrane (23–26). However, mutations on the previously reported palmitylation sites, Cys-231, Cys-329, and Cys-330 did not affect membrane association of RPE65, suggesting that Cys-231, Cys-329, and Cys-330 are not essential for membrane association. The previous observation, which reported that Cys-231, -329, and -330 were not palmitylated (18), is now independently confirmed by our experiments. To determine the role of the palmitylation at Cys-112 in the membrane association of RPE65, we have expressed wtRPE65 and the C106A and C112A mutants and determined their subcellular localizations using fractionation and immunocytochemistry analyses. wtRPE65
Palmitylation and Membrane Association of RPE65

FIGURE 4. Subcellular fractionation of wtRPE65 and its Cys mutants. The 293A-LRAT cells were transfected separately with the plasmids expressing wtRPE65, C106A, and C112A mutants and cultured for 48 h after the transfection. A, cells were harvested and fractionated using FractionPREPTM (BiorVision, Mountain View, CA). Equal amounts of total cellular protein from each cell lysate (25 μg) and subcellular fraction (5 μg/fraction) were used for Western blot analysis using the anti-RPE65 antibody. Note that the cytoskeletal fraction contains the inclusion bodies. B, relative RPE65 levels in each fraction were semi-quantified by densitometry, averaged from three independent experiments, and expressed as percent of total RPE65 levels (mean ± S.E., n = 3). WT, wild type.

and the C106A mutant showed similar subcellular distributions, predominantly present in the membrane fraction and co-localized with an ER membrane marker as shown by double immunostaining. This subcellular localization is similar to that of mutants C231A, C329A, and C330A reported previously (17). Consistent with the MS results, the C112A mutant almost completely lost its membrane association. This observation is supported by our immunocytochemistry results that showed that C112A is not localized on the ER membrane. These results suggest that palmitylation at Cys-112 is important for membrane association of RPE65.

A residue with functionally significant palmitylation should meet two major criteria. First, it should be well conserved among different species. RPE65 has high amino acid sequence homologies among different vertebrates (human versus bovine, 98%; mouse, 94%; chicken, 90%). Key residues essential for its enzymatic activity, such as four His residues forming the iron binding pocket, are perfectly conserved in all of the known RPE65 proteins (13). Likewise, in all of the RPE65 sequences analyzed, Cys-112 is perfectly conserved, whereas Cys-106 is not (see Table 1). Second, a palmitylation site responsible for membrane association should be exposed to the surface of proteins. Although the three-dimensional structure of RPE65 has not been revealed, molecular modeling based on the three-dimensional structure of apocarotenoid monooxygenase (15) showed that Cys-112 is located on the surface of RPE65 (see supplemental Fig. 1). These structural features support the role of Cys-112 in membrane association.

Although the C106A and C112A mutants showed expression levels similar to that of wtRPE65, the C112A mutant lost its enzymatic activity, whereas C106A retained the intact isomerohydrolase activity of RPE65. Our previous studies have shown that RPE65 utilizes highly hydrophobic all-trans-retinyl esters as its direct substrate (27). Membrane association has been considered an important feature of RPE65, which may be essential for RPE65 to access its hydrophobic substrate from LRAT through the membrane (7). Dissociation of the C112A mutant from the membrane may contribute to the loss of the enzymatic activity. As shown in cell fractionation analysis, the C112A mutant is predominantly present in the fraction of the cytoskeletal and inclusion body. The mislocalization of this mutant

FIGURE 3. Sequence spectra of human recombinant RPE65 fragment (residues 98–118). A, sequence of the human wtRPE65 peptide shows single palmitylation on residue Cys-112. Cys-106 was not labeled during the workup procedure. Together with this modification, in this peptide Lys-113 was acetylated, and Thr-101 and Thr-105 were phosphorylated. B, sequence of the recombinant C106A mutant peptide also shows palmitylation on the single Cys site, Cys-112. Thr-105 and Ser-117 were also phosphorylated. The identified b ions are shown in red, and the y ions are shown in blue. The most abundant ions identified are labeled with their molecular weights. The observed sequences and expected +2 charge state fragmentation ions are indicated. The identified ions and amino acids are underlined, showing complete sequence coverage for both the recombinant human RPE65 peptides. The Cys-106 and Cys-112 are indicated by orange color, ‘c’ is the S-palmitylated Cys residue; S*, phosphoserine; T*, phosphothreonine; K*, acetylated lysine.

FIGURE 5. Double immunostaining of RPE65 and an ER membrane marker. The 293A-LRAT cells in the culture slides were separately transfected with plasmids expressing wtRPE65, C106A, and C112A and then cultured for 24 h. The cells were double stained with an anti-RPE65 antibody (red) and an antibody for calnexin (ER membrane marker, green). Only the ER marker was detected in the control with mock transfection (A). Both wtRPE65 (B) and the C106A mutant (C) but not the C112A mutant (D) showed similar subcellular localization of RPE65 with the calnexin. Scale bars, 20 μm.
Our previous studies have shown that most of the point mutations of RPE65 destabilized the protein and resulted in a short half-life and, consequently, a lower protein level of RPE65 (13, 28, 29). However, C112A showed unchanged protein levels, although it had a disturbed subcellular localization and lacked any enzymatic activity. Therefore, the Cys-112 residue seems not important for RPE65 protein stability. The structural significance of Cys-112 remains to be further analyzed.

FIGURE 6. Impacts of the Cys mutations on RPE65 protein levels and isomerohydrolase activities. wtRPE65 and mutants C106A and C112A were expressed in 293A-LRAT cells, and their protein levels and isomerohydrolase activities were analyzed by Western blotting and the in vitro isomerohydrolase assay, respectively. A, equal amount of total cellular protein (25 μg) was applied for Western blot analysis using an antibody specific for RPE65. The membrane was stripped and re-blotted with an anti-β-actin antibody as loading control using untransfected cells; WT, wild type. B, relative RPE65 levels were semi-quantified by densitometry and normalized by β-actin levels on the same membrane. The protein levels were averaged in three independent experiments and expressed as percent of wtRPE65 (mean ± S.E., n = 3). C–E, in vitro isomerohydrolase activities in 293A-LRAT cell lysates expressing wtRPE65 (C), C106A (D), and C112A (E). wtRPE65 and C106A showed similar isomerohydrolase activity, whereas C112A completely lost its enzymatic activity. Peak 1, all-trans-retinyl esters; peak 2, 11-cis-retinol. F, comparison of isomerohydrolase activity of wtRPE65 and the Cys mutants. The isomerohydrolase activities were quantified by measuring the generated 11-[3H]cis-retinol and normalized by the relative RPE65 protein level of each mutant based on Western blot analysis. The amount of 11-cis-retinol was calculated based on standards (picomole) (mean ± S.E., n = 3). G, equal amount of total cellular protein (25 μg) was applied for Western blot analysis using the anti-RPE65 antibody. The membrane was stripped and re-blotted with an anti-β-actin antibody as loading control. H, comparison of isomerohydrolase activities of wtRPE65 and the Cys mutants with the His6 tag. The isomerohydrolase activities were quantified by amounts of the generated 11-[3H]cis-retinol and normalized by their RPE65 protein levels. The amount of 11-cis-retinol was calculated based on standards (picomole) (mean ± S.E., n = 3).

could be ascribed to the loss of membrane association of RPE65, as RPE65 has limited solubility and stability in the cytosol in the absence of detergents.

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