Expression, Purification, and MALDI Analysis of RPE65

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PURPOSE. RPE65 is preferentially expressed in the retinal pigment epithelium (RPE) and is essential for retinal function. The purpose of the study was to develop methods for the expression of the protein, determine the accurate molecular weight of this expressed protein, and quantitate the amount of RPE65 in the bovine RPE.

METHODS. Human RPE65 was expressed in S99 cells using the baculovirus system. The subcellular localization was determined by Western blot analysis and immunocytochemistry. An ELISA was developed for RPE65 and used to measure levels in bovine RPE. Recombinant and native RPE65 were purified by affinity chromatography. Molecular mass was determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

RESULTS. Recombinant human (rH)RPE65 was expressed as a major protein associated with cell membrane in S99 cells. The recombinant protein was purified to apparent homogeneity from both the membrane and nonmembrane fractions. The identity of the purified protein was confirmed by Western blot analysis and by partial peptide sequencing. rHRPE65 from the nonmembrane fraction has a mass of 64,867 ± 80 which is close to the calculated molecular weight from the amino acid sequence including the His-tag (64,663), whereas the membrane-associated rHRPE65 has a molecular mass of 65,380 ± 150, which is significantly higher than that of the non-membrane-associated form and the calculated molecular weight, suggesting posttranslational modifications. Similarly, native RPE65 was detected in the cytosolic and microsomal fractions of the bovine RPE, with an average level of 3.8 ± 1.3 and 7.2 ± 0.4 µg RPE65 per eye, respectively. The cytosolic form had a molecular mass of 61,161 ± 60, which is close to the calculated value (60,944), whereas that of the microsomal form was 61,961 ± 170.

CONCLUSIONS. RPE65 is expressed in two forms, one of which is membrane associated and contains significant posttranslational modifications, similar to the native membrane-associated form. (Invest Ophthalmol Vis Sci. 2001;42:1429–1435)

RPE65 is a protein predominantly expressed in the retinal pigment epithelium (RPE).1,2 Bovine RPE65 consists of 533 amino acids and has a calculated molecular mass of 60,944. The RPE65 sequence is highly conserved across species from human to salamander.3-6 RPE65 has been reported to associate with the membranes in the RPE, preferentially in the microsomal fraction.2,6 However, it is not an integral membrane protein, because it is without both hydrophobic transmembrane domains and a signal peptide.5,7

Mutations in the RPE65 gene are associated with autosomal recessive childhood-onset severe retinal dysrophy, Leber’s congenital amaurosis (LCA), and some forms of retinitis pigmentosa (RP).8-11 The homozygous RPE65 knockout mouse has shown photoreceptor degeneration and diminished rod response in ERG.11 These findings indicate that intact RPE65 is essential for maintaining physiological functions of the RPE and retina.

The physiological function of RPE65 is presently uncertain. Several lines of evidence suggest that it may play a role in retinoid processing.5,11 In the retina of the RPE65 knockout mouse, regeneration of the rod visual pigment rhodopsin is impaired, although apoprotein opsin is available, indicating the absence of 11-cis retinal. In the RPE of the knockout mouse, retinyl ester overaccumulates. Ester saponification shows that all the retinyl ester is in the all-trans form, whereas the 11-cis ester is absent.11 These results suggest that the regeneration of 11-cis retinal is blocked at the isomerization-hydrolisis step, supporting the hypothesis that this protein is essential for isomerohydrolase activity in the visual cycle of retinoid metabolism.11 Recent results from Van Hooser et al.12 have shown that feeding the RPE65 knockout animals with 9-cis retinal restores the ERG, which supports the premise that the absence of RPE65 impairs the retinoid metabolic process.

To study this protein further, we expressed human RPE65 in S99 cells with the baculovirus system and purified native RPE65 from the bovine RPE.

METHODS

Cloning of Human RPE65 and Preparation of the HRPE65/pFastBacHTa Donor Plasmid

The HRPE65 cDNA was reverse transcribed and amplified by PCR from human RPE RNA on a thermal cycler (Robocycler Gradient 40; Stratagene, La Jolla, CA) using a commercial RT-PCR system (Titan One- Tube; Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. The primers were designed to amplify the HRPE65 coding region according to the previously reported HRPE65 sequence (GenBank accession number U8991).13 The sense primer (5'-CGAGATCCATGTCTATCCAG GTTGAG-3') contained an EcoRI site, and the antisense primer (5'-CGAAGCTTGCGTTAGATGCTA-3') contained a HindIII site to facilitate cloning. The RT-PCR product was cloned into a vector (pFastBacHTa; Life Technologies, Gaithersburg, MD) at EcoRI and HindIII sites in frame with a 6× histidine tag. The reading frame and orientation of the insert were confirmed by DNA sequencing.
Production of Recombinant HRPE65 Baculovirus

A baculovirus expression system (Bac-to-Bac; Life Technologies) was used to express HRPE65. Recombinant bacmid DNA was generated in Escherichia coli, isolated, and analyzed according to the manufacturer’s instructions. Sf9 cells were seeded into six-well plates at a density of 9 x 10^3 viable cells per well. The cells were allowed to attach for 1 hour at 28°C before transfection with HRPE65 bacmid DNA in a suspension (Cellfectin; Life Technologies), according to the manufacturer’s instructions. The virus was harvested from the cover medium at 72 hours after transfection. The recombinant virus was clarified by centrifugation at 100g for 10 minutes and subsequently stored at 4°C. The virus was titered with a kit (BacPAK Baculovirus Rapid Titer; Clontech, Palo Alto, CA), according to the manufacturer’s instructions.

Expression and Purification of rHRPE65

Sf9 cells were infected with a multiplicity of infection (MOI) of 5 or 10 and were harvested at 48 or 72 hours after infection by centrifugation at 100g for 10 minutes at 4°C. Cells were resuspended in 1X binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, [pH 7.9]) containing 0.1% Triton X-100, and 0.5% n-dodecyl-β-D-maltoside (NDDM). The supernatant was centrifuged at 30,000 rpm for 10 minutes. The supernatant was reserved for purification of the membrane-associated form, and the pellet was solubilized in 1X binding buffer supplemented with 0.1% Nonidet P-40 and 6 M guanidine HCl for purification of the non-membrane-associated form.

The cell lysate was shaken for 1 hour and then centrifuged at 4°C, 105,000g for 1 hour. The supernatant was mixed with resin (nude-nitrotriacetic acid [Ni-NTA]; Qiagen, Chatsworth, CA) equilibrated by buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, [pH 7.9]). The peptide was conjugated to keyhole limpet hemocyanin (KLH) protein.

Rabbits were subcutaneously injected with an emulsion of 0.3 mg of the peptide KLH and complete Freund’s adjuvant (CFA; Gibco-BRL, Grand Island, NY) and intramuscularly boosted with 0.3 mg of the same emulsion at 3-week intervals. After significant immune responses were developed, the rabbits were killed and the whole serum was collected. Specific antibody to the RPE65 peptide was purified by passing the serum through a column of the epitope peptide coupled to Aminolink beads (Pierce, Rockford, IL), according to a protocol recommended by the manufacturer. The final concentration of the antibody after the column was 50 μg/ml. The anti-whole protein antibody was raised by injection of purified rHRPE65. The protocol for raising this antibody was identical with the one used for the epitope peptide antibody. The final concentration after purification by a protein A column was 5 mg/ml.

Western Blot Analysis

All protein samples were resolved with SDS-PAGE (8-16% tris-glycine gel; Novex, San Diego, CA) and electrotransferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Parsippany, NJ) at 25 V for 2 hours according to the manufacturer’s instructions. The membrane was blocked with 5% (wt/vol) blocking reagent (Blotto; Santa Cruz Biotechnology, Santa Cruz, CA) in TBST (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, and 0.1 mM EDTA), and sonicated for 10 seconds each. The cell lysate was then centrifuged at 39,000g for 10 minutes. The supernatant was reserved for purification of the membrane-associated form, and the pellet was solubilized in 1X binding buffer containing 250 mM imidazole. Fractions (2 ml/each) were collected and analyzed by SDS-PAGE and Western blot analysis as described earlier.

Preparation of Sf-9 Cell Membranes

Sf9 cells (7 x 10^6 cells) were harvested by centrifugation and resuspended in 1 ml of 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 0.1 mM EDTA. Cells were lysed using the freeze-thaw method. Briefly, cells were alternated between an ethanol-dry ice bath and a 37°C water bath for five cycles. Cells were then layered onto a 1.3-ml sucrose cushion (37% wt/vol sucrose in 10 mM Tris buffer [pH 7.4], 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 0.1 mM EDTA) and the sample was spun at 22,000 rpm (53,000g) in a rotor (TL-55; Beckman, Berkeley, CA) for 20 minutes at 4°C. The membrane layer was collected and washed three times as previously described.

Raising Antibodies Specific to RPE65

Rabbits were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two polyclonal antibodies were raised against RPE65: one against a peptide from bovine RPE65 and the other against the whole rHRPE65 protein. The epitope peptide NFTKINPETLETIK of RPE65 was chemically synthesized by the Medical University of South Carolina (MUSC) Protein Sequencing and Peptide Synthesis Facility service using FastMoc chemistry (9-fluorenylmethylcarbonyl [Fmoc] in combination with 2-[(4H-benzotriazol-1-yl)-1,1,3,3-tetramethyllumorion hexafluorophosphate [HBTO] activation) on a peptide synthesizer (model 432; Perkin Elmer Applied Biosystems, Foster City, CA). The peptide was conjugated to keyhole limpet hemocyanin (KLH) protein.

Immunolocalization of rHRPE65 in Sf9 Cells

Sf9 cells were infected with HRPE65-AcNPV with an MOI of 2 while in midlog phase (2 x 10^6 cells/ml). Cells were harvested 48 hours after infection by centrifugation at 100g for 5 minutes at 4°C. The culture media were aspirated, and the cells were washed twice with 1X PBS. Cells were fixed and permeabilized in 2 ml 2% p-fluorophenylalanine PFA/0.1% Triton X-100 for 30 minutes on ice. Fixed cells were washed twice with 1X PBS and blocked with 1% horse serum for 1 hour at 4°C. Cells were then harvested at 100g and resuspended in mouse anti-histidine tag antibody (1:1000; Calbiochem, La Jolla, CA) or in anti-RPE65 peptide antibody (1:250). The primary antibodies were allowed to bind for 1 hour at 4°C. After two washes with 1X PBS, the cells were resuspended in FITC-conjugated donkey anti-mouse or Cy3 conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:100. After a 1-hour incubation at 4°C, the cells were washed twice with 1X PBS, placed onto poly-L-lysine coated slides, and viewed under a fluorescence microscope.

Specific ELISA for RPE65

ELISA was performed using the sandwich avidin-biotin method. The 96-well plates (model 3590; Costar, Cambridge, MA) were coated with the purified anti-RPE65 peptide antibody (2 μg/ml in PBS, 100 μl/well) at 4°C overnight and blocked with 200 μl/well of PBS containing 1% BSA at 37°C for 1 hour. After three washes in the washing solution (PBS containing 0.1% Tween-20), 100 μl of the standard (3.5-200 ng/ml) and samples were added to each well in duplicates and incubated at 37°C for 1.5 hours. After the plate was washed with the washing solution three times, 100 μl of 1 μg/ml biotinylated anti-RPE65 antibody was added to each well and incubated at 37°C for 1 hour. The plate was washed again with the same solution three times, and then 100 μl of 1 μg/ml peroxidase-avidin (Jackson ImmunoResearch) was
added to each well and incubated at 37°C for 30 minutes. The plate was washed five times with the washing solution and once with PBS. The freshly made substrate (ABTS containing 1 tablet per 10 ml and 10 μl of 30% hydrogen peroxide in citrate-phosphate buffer [pH 4.0]) was added to each well and incubated for 30 minutes. The plate was read in an ELISA reader (Titertek Multiskan Plus; LabSystems, Helsinki, Finland) with a 405-nm optical filter. The concentration of RPE65 was obtained by using purified rHRPE65 as the standard. Immunodiffusivity was confirmed by parallel dilution curves. The yield of the expression was calculated from four independent baculovirus infection and expression experiments.

**Purification of Bovine RPE65 Using Affinity Chromatography**

The purified anti-RPE65 peptide antibody was coupled to a protein A column using a kit (ImmunoPure Protein A IgG Orientation; Pierce), according to the manufacturer’s instructions. Microsomal and soluble proteins were prepared from fresh bovine RPE by differential centrifugation, as described previously. To purify the microsomal RPE65, the microsomal proteins were applied to the RPE65 antibody column followed by a wash with 10 bed volumes of 50 mM sodium borate (pH 8.2) containing 0.3% CHAPS. RPE65 was eluted with 0.1 M glycine-HCl (pH 2.8) containing 0.3% CHAPS.

To purify the cytosolic RPE65, the supernatant from the first 100,000g centrifugation in the microsomal preparation was applied to the antibody column, followed by a wash with 10 bed volumes of 50 mM sodium borate (pH 8.2). The bound protein was eluted with 0.1 M glycine-HCl (pH 2.8).

**Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry**

Fifty micrograms rHRPE65 or bovine RPE65 was dried and dissolved in 7 μl 98% formic acid and 2 μl hexafluoro-isopropanol. After vortexing, a 2-μl aliquot of this mixture was mixed with 2 μl of 10 mg/ml sinapinic acid in 4:6 methanol-water. One microliter of each mixture was spotted on the MALDI plate and allowed to dry. The molecular weight of each sample was measured on a MALDI mass spectrometer (Voyager DE linear mode MALDI-Time-of-Flight [TOF]; PerSeptive Biosystems, Framingham, MA). The instrument was calibrated (Calibration Mixture 3, containing bovine insulin [5734 Da] and horse apomyoglobin [16,852 Da]; PerSeptive), and the calibration was confirmed with BSA. The mean molecular mass was calculated from multiple independent measurements.

For the extraction of the protein from the MALDI plate, high laser power was applied (3000 vs. 1700 U for the standards). The instrument operated in positive ion delayed extraction mode with a low-mass gate of 5000 Da. The accelerating voltage was 25,000 V, the grid voltage was set to 87%, and the guide wire voltage to 0.3%. Each spectrum was the average of approximately 200 scans.

**RESULTS**

**Expression of rHRPE65 in Sf9 Cells**

Sf9 cells were infected with the rHRPE65 recombinant virus at MOIs of 5 and 10 and harvested at 48 and 72 hours after infection. As shown by SDS-PAGE and Western blot analysis using total cellular protein, rHRPE65 was expressed as a major protein in Sf9 cells infected with the rHRPE65 virus but not detected in uninfected cells. Under the same conditions, Sf9 cells infected at an MOI of 10 expressed higher levels of rHRPE65 than did those infected at an MOI of 5. At 72 hours after infection rHRPE65 made up a greater percentage of the total cellular protein than at 48 hours (Fig. 1). The average yield of total rHRPE65 was approximately 10 mg/l of culture.

**Membrane Association of the Recombinant RPE65**

The membranes of infected Sf9 cells were isolated by sucrose gradient centrifugation. As shown by SDS-PAGE and Western blot analysis, rHRPE65 was present in the isolated membrane fraction after a vigorous washing procedure (Fig. 2). Subcellular localization of rHRPE65 in Sf9 cells was also examined by immunocytochemistry using the anti-His tag antibody and anti-RPE65 antibody. Consistent with the isolated membrane preparation, both antibodies detected rHRPE65 on the intracellular side of the cell membrane (Fig. 3). The immunofluorescence was not detected in uninfected cells (data not shown), demonstrating the specificity of the antibody.

**Purification of Two Forms of rHRPE65**

The infected Sf9 cells were treated with 0.3% CHAPS to solubilize the membrane-associated rHRPE65, and the recombinant protein was purified through a Ni column. The purified protein appeared as a single band with an apparent molecular weight of 65 kDa and was recognized by the anti-RPE65 peptide antibody (Fig. 4). An average of approximately 1 mg CHAPS-soluble rHRPE65 was purified from 1 l Sf9 cells. It was noted that the level of CHAPS-soluble rHRPE65 was significantly lower than that in the total cell lysate. A portion of rHRPE65 was detected in the CHAPS-insoluble pellet, possibly in the inclusion bodies (data not shown). The CHAPS-insoluble pellet was solubilized by 6 M guanidine and rHRPE65 purified to apparent homogeneity through the Ni column (Fig. 4). The identity of the purified proteins from both CHAPS and guanidine-soluble fractions was further confirmed by partial peptide sequencing using mass spectrometry.

**Analysis of Molecular Mass of rHRPE65**

MALDI mass spectrometry was used to determine the molecular weight of rHRPE65. The non–membrane-associated form of rHRPE65 (from the CHAPS-insoluble pellet) showed a molecular mass of 64,867 ± 80, which is close to the calculated molecular weight (64,665) according to the amino acid sequence (Table 1). This calculated molecular weight of the recombinant protein differs from that of the native HRPE65 (60,947), because of the presence of six histidine residues and other residues encoded by the vector. In contrast, the membrane-associated form (CHAPS-soluble form) of rHRPE65...
showed a molecular mass of 65,380 ± 150, which is 717 higher than the calculated molecular weight and 513 higher than that of the non–membrane-associated form (Fig. 5, Table 1). The complete amino acid sequence of the non–membrane-associated form was determined by mass spectrometry of the trypsin-digested fragments. The results revealed that the non–membrane-associated form contains the intact peptide sequence of RPE65.

Quantification of RPE65 in Bovine Eyes

The RPE was carefully dissected from bovine eyes, and the microsomal and cytosolic proteins were separated as previously described. RPE65 was detected in both the microsomal and cytosolic fractions of the bovine RPE by Western blot analysis (Fig. 6) and an ELISA specific for RPE65. The ELISA dilution curves of both the microsomal and cytosolic proteins are parallel with the standard curve of purified rHRPE65, suggesting immunologic identity (data not shown). The microsomal fraction contained 7.2 ± 0.4 μg RPE65/eye (mean ± SEM, n = 4) and the cytosolic fraction contained 3.8 ± 1.3 μg RPE65/eye, which is approximately 53% of that from microsomes.

Purification and Measurements of Two Forms of Native RPE65 from the Bovine RPE

RPE65 was purified to apparent homogeneity from both the microsomal and cytosolic fractions of the bovine RPE through immunoaffinity chromatography (Fig. 6). Similar to the recombinant protein, RPE65 from the cytosolic and microsomal fractions showed different molecular mass as measured by MALDI. RPE65 purified from the cytosolic fraction has a molecular mass of 61,161 ± 60, which is close to that of the calculated molecular mass of native bovine RPE65, 60,944, whereas the protein purified from the microsomal fraction of the RPE has a molecular mass of 61,961 ± 170, which is 1017 more than the calculated value and 800 more than the soluble form (Table 1). This result is consistent with the finding for the recombinant protein from Sf9 cells and suggests that the microsomal form of RPE65 contains posttranslational modifications.

**DISCUSSION**

RPE65 plays a critical role in the visual cycle of retinoid metabolism. However, information about the structure of RPE65 has been limited. To obtain RPE65 in a large quantity for structural analysis, we have established an efficient expression and purification system for human RPE65 in insect cells and measured the molecular weights of the purified recombinant and native RPE65 by MALDI.

It has been difficult to purify RPE65 in a large quantity from the RPE, because it requires a large number of fresh bovine eyes, and RPE65 is an unstable protein and is easily degraded during purification. An effective expression system is desirable for analysis of the protein’s structure and determination of its activity. We have previously expressed rHRPE65 in E. coli. However, the protein was insoluble, suggesting that it is not folded or modified properly in E. coli (data not shown). It has
been shown that insect cells offer the same posttranslational processing and modifications as do mammalian cells, including proteolytic processing, secretion, folding, phosphorylation, glycosylation, acylation, and amidation. The expression levels in insect cells are substantially higher than that of mammalian cells. Therefore, we chose the baculovirus expression system to express rHRPE65. This expression system produces a high yield of rHRPE65 and provides a one-step affinity purification through the His tag. Therefore, this expression system is a useful tool for producing a large quantity of purified RPE65 for structural and functional analysis.

RPE65 was identified independently by two groups with different apparent molecular weights of 63 and 65 kDa. The accurate molecular weight of this protein, however, has not been measured previously. This study represents the first measurement of accurate molecular mass of both the recombinant and native RPE65. Although the accuracy of the measurements (−0.3%) was lower than the highest accuracy obtainable with this type of MALDI instrument (0.05%), there were several sources of error that explain it. The standards used to calibrate the instrument (the MALDI standards suggested by the manufacturer) were of lower molecular weight, and the highest possible accuracy may be obtained only within this mass range. The accuracy is lower when a molecular weight is out of this range. Measurements on bacteriorhodopsin (27,068 Da) and bovine rhodopsin (42,002 Da) with the same calibration showed an accuracy of 0.07% and 0.08%, respectively. Because the molecular weight of RPE65 is higher, the obtainable mass accuracy is even lower. There is currently no higher molecular weight standard available to obtain better accuracy for RPE65 measurement. The lower mass accuracy is also a consequence of the higher amount of laser power necessary for the extraction of the RPE65 protein from the sample plate.

The presence of certain posttranslational modifications may cause a significant accuracy decrease as well. The decreased accuracy means that the presence of posttranslational modifications on the nonmembrane form of the protein cannot be verified with these MALDI measurements. However, the membrane-associated forms showed molecular masses significantly higher than that of the molecular weights calculated from their amino acid sequences. This difference was also much higher than the inaccuracy of the measurements, suggesting that both the recombinant and native RPE65 contain significant posttranslational modifications, at least in their membrane-associated forms.

We considered the possibility that the presence of detergent caused some error in our MALDI measurements. Therefore we measured BSA, solubilized both in 0.3% CHAPS and in 6 M guanidine. There was no difference found in the molecular mass from the two measurements (data not shown). Therefore,

<table>
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<th>Protein</th>
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<th>Measurements**</th>
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* Measured mass ± SD.
** The number of measurements averaged.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933588/)
the differences observed in the molecular weight of these two forms of RPE65 cannot be attributed to the effect of detergents. Hamel et al.2,3 demonstrated that RPE65 is not glycosylated, although the sequence contains three consensus glycosylation sites. The protein does not contain a typical myristoylation sequence (Met-Gly) at its N terminus.7 Using biosynthetic radiolabeling of infected Sf9 cells with [3H]-myristate, we have demonstrated that rHRPE65 is not myristoylated (unpublished results). Based on the fact that hydroxylamine failed to remove the protein from the membrane, Bavik et al.8 have suggested that RPE65 is not bound to the membrane by fatty acid acylation. It remains a challenge for the future to reveal the type and site of modifications by the complete mapping of the membrane-associated form of RPE65.

RPE65 is reported to be associated with the microsomal membranes in the bovine RPE.2,19 The present studies demonstrate that rHRPE65 copurifies with the membrane fraction isolated from infected Sf9 cells by sucrose gradient centrifugation, suggesting that it is also associated with the membrane in insect cells. This conclusion was further supported by immunocytochemistry of infected Sf9 cells using anti-His and anti-RPE65 antibodies which both detected the recombinant protein on the cytoplasmic side of the membrane. The mechanism by which RPE65 associates with the membrane is currently unknown. The RPE65 sequence is without the typical hydrophobic transmembrane domains and a signal peptide.5,7 Previous studies have demonstrated that RPE65 is a peripheral membrane associated protein rather than an integral membrane protein.3,7,19 However, experiments with phospholipid vesicles suggest that at least one form of RPE65 may interact directly with phospholipids.19

The posttranslational modification of proteins with hydrophobic lipid-derived substituents is a major route for targeting proteins to membranes.20 The common hydrophobic modifications include fatty acids (myristate and palmitate), isoprenoids (farnesy1 and geranylgeranyl) and glycosyl-phosphatidyl inositol anchors. The modifications in the membrane-associated form of RPE65 may be responsible for its membrane association. The cytosolic forms of native RPE65 have molecular weights similar to the calculated values, suggesting that they are either not modified or are modified to a lesser extent. In the bovine RPE, a substantial amount of RPE65 is present in the cytosolic fraction, approximately half the amount in the microsomes, which has been known as a primary localization of RPE65.2,6 Unlike the native RPE65 in the RPE, however, there were very low levels of recombinant RPE65 in the soluble fraction; instead, a substantial amount was found in the CHAPS-insoluble pellet of Sf9 cells. A possible explanation for this is that in Sf9 cells, unmodified RPE65 may aggregate, forming inclusion bodies, which are not solubilized by CHAPS.

MALDI has revealed that the two forms of RPE65, membrane-associated and non–membrane-associated forms in both the recombinant and native RPE65, have significantly different molecular weights. The difference between the two forms of RPE65 is unknown at this time. However, full-length sequencing of the non–membrane-associated form revealed that the lower molecular weight of this form does not result from the loss of amino acid residues due to partial degradation. It means that the cytosolic form may be a loss of some posttranslational modifications present in the membrane form of the protein or it may be a protein precursor that has already been translated but has not yet been modified. Therefore, the difference in the molecular weight of the two forms is likely to be ascribed to different posttranslational modifications in the two forms. It remains to be determined whether the non–membrane-associated form of RPE65 represents a premature protein or an active form with functional significance.

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