Rpe65 Is a Retinyl Ester Binding Protein That Presents Insoluble Substrate to the Isomerase in Retinal Pigment Epithelial Cells*

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Photon capture by a rhodopsin pigment molecule induces 11-cis to all-trans isomerization of its retinaldehyde chromophore. To restore light sensitivity, the all-trans-retinaldehyde must be chemically re-isomerized by an enzyme pathway called the visual cycle. Rpe65, an abundant protein in retinal pigment epithelial (RPE) cells and a homolog of β-carotene dioxygenase, appears to play a role in this pathway. Rpe65−/− knockout mice massively accumulate all-trans-retinyl esters but lack 11-cis-retinoids and rhodopsin visual pigment in their retinas. Mutations in the human RPE65 gene cause a severe recessive blinding disease called Leber’s congenital amaurosis. The function of Rpe65, however, is unknown. Here we show that Rpe65 specifically binds all-trans-retinyl palmitate but not 11-cis-retinyl palmitate by a spectral-shift assay, by co-elution during gel filtration, and by co-immunoprecipitation. Using a novel fluorescence resonance energy transfer (FRET) binding assay in liposomes, we demonstrate that Rpe65 extracts all-trans-retinyl esters from phospholipid membranes. Assays of isomerase activity reveal that Rpe65 strongly stimulates the enzymatic conversion of all-trans-retinyl palmitate to 11-cis-retinol in microsomes from bovine RPE cells. Moreover, we show that addition of Rpe65 to membranes from rpe65−/− mice, which possess no detectable isomerase activity, restores isomerase activity to wild-type levels. Rpe65 by itself, however, has no intrinsic isomerase activity. These observations suggest that Rpe65 presents retinyl esters as substrate to the isomerase for synthesis of visual chromophore. This proposed function explains the phenotype in mice and humans lacking Rpe65.

Light perception in vertebrates is mediated by a group of G protein-coupled receptors called the opsins. Most opsin pigments contain 11-cis-retinaldehyde (11cRAL) as the light-absorbing chromophore. Absorption of a photon induces 11-cis to all-trans isomerization of the chromophore, resulting in the activated species, metarhodopsin II. After a brief period, metarhodopsin II decays to yield apo-rhodopsin and free all-trans-retinaldehyde (aRAL). Before light sensitivity of the pigment can be restored, the aRAL must be chemically re-isomerized to 11cRAL by a metabolic pathway called the visual cycle. Most steps in this pathway take place within cells of the retinal pigment epithelium (RPE) adjacent to the photoreceptors. The key step in this pathway is all-trans to 11-cis isomerization of the retinoid, which is catalyzed by an enzyme activity called isomerohydrolase (IMH). IMH has been shown to use fatty acyl esters of retinol as a substrate (1, 2), harnessing the energy of ester hydrolysis [ΔG = −5 kcal/mol (3)] for the endothermic conversion of all-trans-retinal (aRAL) to 11-cis-retinol (11cROL) (+4.1 kcal/mol, Ref. 4). IMH has never been purified or cloned.

Leber’s congenital amaurosis (LCA) is a severe and relatively common autosomal recessive disease that results in blindness at birth. LCA is frequently caused by mutations in the RPE65 gene (5, 6). Rpe65, the product of this gene, is an abundant protein of unknown function in cells of the RPE (7). Rpe65 has high affinity for phospholipid membranes but contains no membrane-spanning domains (8, 9). Mice with a knockout mutation in rpe65 massively accumulate all-trans-retinyl esters (aRE) in their RPE and have no detectable 11-cis-retinoids (10). Photoreceptors in rpe65−/− mice are morphologically normal but contain only apo-rhodopsin instead of rhodopsin pigment. These observations led to speculation that Rpe65 may be IMH.

In the current study, we sought to define the function of Rpe65 and its relationship to IMH. We show by several approaches that Rpe65 specifically binds all-trans-retinyl palmitate (aRP), the most abundant retinyl ester in RPE cells (11). Further, we present data suggesting that aRP bound to Rpe65 is the substrate for IMH.

EXPERIMENTAL PROCEDURES

Expression of Bovine Rpe65 in Baculovirus-infected Sf9 Cells—We followed the complete coding sequence of bovine Rpe65 into pFastBac bacmid (Invitrogen) and used it to transfect Spodoptera frugiperda (Sf9) cells following the manufacturer’s procedure. Following infection, adherent cells were lysed in phosphate-buffered saline, pH 7.0, 1% CHAPS, and protease inhibitor mixture (Roche Diagnostics) and centrifuged at 100,000 × g for 30 min. The detergent-soluble fractions were analyzed by gel filtration (Zorbas GF-250) on an Agilent 1100 series liquid chromatograph (mobile phase = 20 mM NaHPO4, 130 mM NaCl, flow = 1 ml/min, 280 nm detection). Proteins eluting at M r 158–43 kDa were collected and concentrated using Centricon 30,000 molecular

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weight cut-off (MWCO) ultrafiltration devices (Millipore). The retained proteins were separated on an AKTA FPLC (Amersham Biosciences) using a Mono Q anion exchange column equilibrated with 25 mM Tris-OAc, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, 10 mM NaOAc, flow = 1 ml/min. A gradient to 0.5 mM NaOAc over 60 min was initiated 10 min after sample injection. Collected gradient fractions were analyzed by SDS-PAGE and Western blotting to confirm the presence of Rpe65. For reconstitution studies, partially purified Rpe65 or control proteins from non-expressing S/9 cells were exchanged into reconstitution buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA) using 30,000 MWCO ultrafiltration devices. In some experiments the detergent-soluble fractions were used without further purification.

**Bovine Rpe65 Antibody**—The synthetic peptide from bovine Rpe65, NFITKINPETLETIK, was coupled to keyhole limpet hemocyanin and used without further purification.

**Gel Filtration and Immunoprecipitation**—Protein samples were resolved by SDS-PAGE on a 4–12% gradient gel and transferred to polyvinylidene difluoride membranes (Millipore) in a semidyry transfer apparatus (BioRad) according to the manufacturer’s instructions. Bands were detected using enhanced chemiluminescence according to the manufacturer’s instructions (Kirkegaard & Perry laboratories).

**Retinoid Preparation and Synthesis**—Retinoids were obtained from Sigma (arRP, aRLO, arRAL, and β-carotene) or were synthesized from 11cRLO (11cRP and 11cROL) according to published procedures (12). All retinoids were purified by HPLC to >98% purity (ratio of sample peak area to total chromatogram peak area) and quantified by UV-vis spectroscopy using published molar extinction coefficients (13). Purified retinoid stocks were stored in ethanol at −80 °C prior to use.

**Gel Filtration and Immunoprecipitation**—Divided quartz cuvettes (containing two compartments separated by a quartz window) were used for all gel filtration and reconstitution binding studies. An absorbance baseline (400–250 nm) was established in which each cell contained a protein sample (0.5–1.0 mg/ml of detergent-solubilized Rpe65 in 10 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% CHAPS) in one compartment and the test retinoid (arRP, aRLO, β-carotene, 11cRP, 11cRLO, arRAL, or 11cRAL) at 40 μM in the same buffer in the adjacent compartment. The sample cell was mixed and absorbance measurements were taken at the indicated times using the un-mixed cell as a reference (temp = 30 °C). Control studies were performed in an identical fashion using either heat-denatured protein or lipid-free bovine serum albumin (1 mg/ml).

**Gel Filtration and Immunoprecipitation**—[1-14C]palmitate-arRP (80 μl, 2 μCi) was added (in DMP at 0.25% v/v) to detergent-solubilized proteins from either non-transfected S/9 cells or S/9 cells expressing Rpe65. Samples were rocked overnight at 4 °C and centrifuged at 45,000 × g, 30 min to remove particulate debris. Sample aliquots from the binding mixtures were analyzed by gel filtration chromatography (described above) with on-line photo-diode array and radiometric (14C) detection (H2TR FSA, Packard Instruments) and in immunoprecipitation studies. Briefly, 50 μl of Rpe65 IgG-Aff-Gel 10 beads were incubated with 30 μl of sample (~50 μg of protein) in phosphate-buffered saline/0.3% CHAPS at 4 °C overnight. Similar incubations were performed on control samples containing retinoid alone (C1) and protein alone (C2). Following incubation, the mixtures were centrifuged at 15,000 × g, 30 s and the supernatant (unbound) fractions were collected. Retinoids were washed three times with 500 μl of CHAPS buffer, buffered saline/0.3% CHAPS and resuspended in 50 μl of of 2× Laemmli buffer (4% SDS). Aliquots of each fraction were taken for scintillation counting and for Western blot analysis. Reactivity in C1 was used to correct for nonspecific retinoid binding, while C2 was used to confirm immunolabeling of Rpe65.

**Fluorescence Resonance Energy Transfer (FRET) Studies and Fluorescence Microscopy**—The binding/internalization of arRP in Rpe65 and control proteoliposomes was determined by FRET analysis where the emission of arRP (~400–500 nm) was used to excite the BODIPY-PC or pyramine fluorophores. arRP was added (in DMF, 0.1% v/v) to the washed proteoliposomes at 2 mol% relative to total phospholipids, and the samples were rocked at 33 °C. Aliquots of the binding mixtures were removed at the indicated times and analyzed on an Agilent liquid chromatograph equipped with an on-line fluorescence detector (flow rate = 0.5 ml/min, temp = 25 °C). The instrument executed two injections with a 1-min interval between injections. During the first injection, excitation was set to 325–340 nm and emission was detected using a 400–430 nm filter. A second injection was set to 410–500 nm for BODIPY-PC proteoliposomes or 510 ± 10 nm for pyramine proteoliposomes. For the second injection, excitation was set to 460 ± 10 nm, emission acquisition parameters were unchanged. Spectral data for all runs were acquired in the region of 400–600 nm to confirm FRET spectra. The association of arRP with liposomes containing either S/9-Rpe65 or S/9 β-protein was examined by phase contrast fluorescence microscopy on a Zeiss Axiovert 135 inverted microscope using a Plan-NeoFluor 40 ×/0.75 objective and appropriate filters (DAPI/ Hoechst/AMCA, 360 ± 40 nm exciter, 460 ± 25 nm emitter) (Chroma Technology).

**Preparation of Proteoliposomes**—Microsomal proteins were prepared from homogenates of bovine RPE as previously described (17). Microsomal membranes (5–10 mg/ml) were treated with 365-nm UV light (5 min on ice) to destroy endogenous retinoids, and were homogenized in solubilization buffer (25 mM Tris-OAc, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 1 μM leupeptin, 0.5% Genapol X-100). The sample was rocked for 1 h at 4 °C followed by centrifugation at 175,000 × g, 35 min. Solubilization buffer in the supernatant fraction was exchanged for reconstitution buffer as described above (final protein concentration = 2–4 mg/ml, final detergent concentration = 0.25%). The protein sample was added to an equal volume of PL suspension containing dioleoyl phosphatidylcholine/dioleoyl phosphatidylethanolamine/phosphatidyl inositol/phosphatidic acid (25:50:5:10, mol/mol, respectively) reconstituted in dioleoyl phosphatidyl ethanolamine. The sample was incubated for 1 h at 4 °C, bound Rpe65 or S/9-Rpe65 proteoliposomes was determined by FRET analysis where the emission of arRP (~400–500 nm) was used to excite the BODIPY-PC or pyramine fluorescent dyes. arRP was added (in DMF, 0.1% v/v) to the washed proteoliposomes at 2 mol% relative to total phospholipids, and the samples were rocked at 33 °C. Aliquots of the binding mixtures were removed at the indicated times and analyzed on an Agilent liquid chromatograph equipped with an on-line fluorescence detector (flow rate = 0.5 ml/min, temp = 25 °C). The instrument executed two injections with a 1-min interval between injections. During the first injection, excitation was set to 325–340 nm and emission was detected using a 400–430 nm filter. A second injection was set to 410–500 nm, emission acquisition parameters were unchanged. Spectral data for all runs were acquired in the region of 400–600 nm to confirm FRET spectra. The association of arRP with liposomes containing either S/9-Rpe65 or S/9 β-protein was examined by phase contrast fluorescence microscopy on a Zeiss Axiovert 135 inverted microscope using a Plan-NeoFluor 40 ×/0.75 objective and appropriate filters (DAPI/ Hoechst/AMCA, 360 ± 40 nm exciter, 460 ± 25 nm emitter) (Chroma Technology).

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were preincubated for 5 min at 37 °C. Rewarmed polyethylene glycol was added to 10% w/v, and incubation was resumed for 1.5 h at 37 °C. Samples were quenched with 200 μl of chilled methanol and 100 μl of water. Retinoids were extracted into hexane (2 × 500 μl) and analyzed by HPLC as previously described (17).

**Effects of Rpe65 on IMH Activities in Mouse RPE—Wild-type (C57BL/6) and strain-matched rpe65—/— knockout mice (10) were reared in 12-h light/dark cycles at 20 °C. Prewarmed polyethylene glycol was added to 10% dioxane/hexane. Retinoids were extracted into hexane (2 × 1 ml) and analyzed by HPLC as described.

**RESULTS**

**Interaction between Rpe65 and aTRP Induces a Change in the UV Absorption Spectrum**—A specific interaction between an apoapoprotein and its light-absorbing ligand invariably induces a change in the absorption spectrum. An indication of this change is the presence of one or more nodal isosbestic points in overlaid spectra from before and after interaction of the ligand with the protein (23). To test for possible binding of aTR to Rpe65, we added samples of aTRP and Rpe65 separately to the two chambers of a divided cuvette. In this and all subsequent experiments, we used baculovirus-infected S9 cells as a source of Rpe65. These cells contained no detectable retinoids, hence the starting Rpe65 protein was in the apo or unbound state. UV absorption spectra were obtained at different times after sample mixing in the cuvette. Incubation of aTRP with Rpe65 caused a reduction in UV absorption at 355 nm and an increase at 280 nm, resulting in an isosbestic point at 295 nm (Fig. 1A). The absorption maximum (λmax) of aTRP dispersed in binding buffer was 355 nm. Thus, incubation with Rpe65 reduced the UV absorption of free aTRP. We repeated this mixing experiment, substituting the geometric isomer, 11-cis-retinyl palmitate (11cRLP) for aTRP. Here, no change in UV absorption was observed during incubation with Rpe65 (Fig. 1B), suggesting that the interaction with aTRP is specific for the all-trans isomer. We used the same spectral assay to test for possible interactions between Rpe65 and 11cRLP or 11cRAL. Similar to 11cRAL, we observed no changes in the UV-absorption spectra of the retinaldehydes upon incubation with Rpe65 (not shown).

**Co-elution of Rpe65 and aTRP by Gel Filtration and Immuno precipitation**—If Rpe65 binds aTR, aTRP should co-elute
with Rpe65 during gel filtration chromatography. We incubated a CHAPS detergent extract of Rpe65-expressing S99 cells with an excess of \[^{14}\text{C}]\text{arRP}. As a control, we incubated a CHAPS extract of non-expressing S99 cells with \[^{14}\text{C}]\text{arRP}. We separated both samples by gel filtration chromatography and monitored arRP elution by measuring \[^{14}\text{C}]-\text{disintegrations per min (dpm)} with an online flow-scintillation detector. We also collected 1-min fractions for analysis by SDS-PAGE. The \[^{14}\text{C}]\text{arRP} eluted in two peaks (Fig. 2A). The first peak was in the column void volume, similar to the profile observed when \[^{14}\text{C}]\text{arRP} in CHAPS was chromatographed alone (not shown) or with a CHAPS extract of non-expressing S99 cells (Fig. 2A).

The second peak of \[^{14}\text{C}]\text{-dpm} was in fractions 17–19 of the Rpe65-expressing sample and contained the preponderance of eluted Rpe65 by protein gel electrophoresis (Fig. 2B). No second peak of \[^{14}\text{C}]\text{-dpm} was observed in the sample derived from non-expressing S99 cells. We obtained UV absorption spectra at the two peaks of \[^{14}\text{C}]\text{-dpm} in the Rpe65 experiment. The spectrum from the first peak showed a \(\lambda_{\text{max}}\) of 325 nm (Fig. 2A, inset), characteristic of arRP in CHAPS. The spectrum from the second peak showed a \(\lambda_{\text{max}}\) of 280 nm, characteristic of proteins, with less intense absorption at 325 nm (Fig. 2A, inset). These data suggest that arRP binds to Rpe65.

To confirm that arRP binds specifically to Rpe65, we prepared an immunoaffinity matrix containing immobilized IgG from antisera against Rpe65. We incubated CHAPS detergent extracts of Rpe65-expressing and non-expressing S99 cells with an excess of \[^{14}\text{C}]\text{-labeled arRP}. Both samples were incubated with the immunoaffinity matrix. We analyzed the unbound, wash, and eluted fractions for \[^{14}\text{C}]\text{-dpm}. We also did immunoblot analysis of the unbound and eluted fractions with the Rpe65 antibody. Most \[^{14}\text{C}]\text{-dpm} were in the unbound fraction due to the excess of added \[^{14}\text{C}]\text{arRP} (Fig. 2C). This fraction also showed considerable Rpe65 immunoreactivity due to incomplete binding to the immunoaffinity matrix. Importantly, significant \[^{14}\text{C}]\text{-dpm} (~15\%) were present in the Rpe65-containing eluted fraction. \[^{14}\text{C}]\text{-dpm} were scarcely detectable in the equivalent fraction of the non-expressing control sample (Fig. 2C). We confirmed chemical integrity of the eluted \[^{14}\text{C}]\text{arRP} by HPLC analysis following gel filtration and immunoprecipitation. Together these results identify Rpe65 as the protein binding partner for arRP.

Since arROL showed spectral evidence for an interaction with Rpe65 (Fig. 1C), we repeated the gel filtration experiment, substituting \[^{3}\text{H}]\text{arROL} for \[^{14}\text{C}]\text{arRP}. Here, we observed no co-elution of \[^{3}\text{H}]\text{-dpm} with Rpe65 (not shown). This suggests that the interaction of arROL with Rpe65 may be too weak to survive chromatographic separation.

**Rpe65 Solubilizes arRP and Facilitates Its Removal from Membranes.**—Retinyl esters are virtually insoluble in aqueous solutions and thus do not exchange between membrane compartments (25, 26). A potential function for Rpe65 may be to solubilize arRE and to facilitate their removal from membranes. To test this possibility, we employed a novel fluorescent resonance energy transfer (FRET) binding assay in liposomes, using the fluorescent probes, \(\beta\)-BODIPY-500/510 C12-HPC (BODIPY-PC) (27) and pyranine (28). Table I shows the maximal excitation and emission wavelengths for these fluorophores and for arRP in liposomes. The excitation spectra of BODIPY-PC and pyranine overlap with the emission spectrum of arRP. On the other hand, the excitation spectrum of arRP overlaps little with the excitation spectrum of BODIPY-PC or pyranine. To establish conditions of FRET we prepared liposomes containing: (i) BODIPY-PC alone; (ii) BODIPY-PC+ arRP; (iii) pyranine alone; or (iv) pyranine+ arRP. We excited each type of liposome at 325 nm, the excitation max-
fluorescent emissions were 4-fold higher from liposomes containing arRP+ BODIPY-PC or arRP+ pyranine (Fig. 3, A and B). The increased fluorescent emissions in the presence of arRP were due to FRET.

Next, we used the FRET assay to measure Rpe65-dependent uptake of arRP into the lipid bilayer and aqueous interior compartments of liposomes. We prepared four types of liposomes containing partially purified Rpe65 from expressing, or equivalent chromatographic fractions from non-expressing Sf9 cells, and BODIPY-PC or pyranine. Similar quantities of total protein were used in the Rpe65-expressing and non-expressing liposomes. We added free arRP in dimethylformamide (DMF) to media containing each type of liposome, incubated for different times, and measured fluorescent emission at 520 or 510 nm with 325-nm excitation. After a 30-min incubation, the arRP-dependent FRET signal at 520 nm was ~6-fold higher in BODIPY-PC liposomes containing Rpe65 compared with Sf9-controls (Fig. 3C). A similar experiment was performed on pyranine-containing liposomes. Here, the 510-nm FRET signal was also about 6-fold higher in liposomes containing Rpe65 compared with Sf9-controls (Fig. 3D). These results show significantly higher uptake of arRP into the Rpe65-containing compared with Sf9-control liposomes.

The BODIPY fluorophore is coupled to a fatty-acyl chain in phosphatidylcholine, and is thus confined to the internal lipid bilayer. Pyranine is a hydrophilic trisodium salt, confined to the aqueous interior of liposomes. Accordingly, the BODIPY-PC signal with 325-nm excitation is due to FRET from arRP in the bilayer of liposomes while the pyranine signal is from arRP in the aqueous lumina. The onset of FRET was faster in the bilayer than in the aqueous interior of Rpe65-containing liposomes (Fig. 3, C and D). The Rpe65-dependent membrane FRET showed early saturation while the luminal FRET showed sigmoidal kinetics with an initially slow rate of increase. These kinetic data make sense considering that free arRP in the medium must pass through the liposome membrane to reach the aqueous interior.

To rule out that the increased fluorescent signals in liposomes containing Rpe65 versus control proteins were due to different levels of BODIPY-PC or pyranine, we excited both liposome populations at 460 nm, the maximum emission wavelength of arRP, and measured fluorescent emission at 520 or 510 nm. For both probes the emission signals were virtually identical in Rpe65 and Sf9-control liposomes (not shown). Thus, the increased FRET signals in the Rpe65-containing liposomes (Fig. 3, C and D) were not caused by different levels of the probes. It is also unlikely that orientation of the fluorophores contribute to the observed differences between Rpe65 and Sf9-control liposomes. Pyranine, arRP bound to soluble Rpe65, and arRP within the bilayer should experience unrestricted isotropic motion. The motion of BODIPY coupled to phosphatidylcholine is partially constrained. However, this constraint is similar in the Rpe65 and control liposomes.

To confirm Rpe65-dependent uptake of arRP, we analyzed the Rpe65-containing and Sf9-control liposomes by fluorescence microscopy after addition of arRP. We excited with light at 360 nm and imaged using a 435–495-nm bandpass filter. Under these conditions, only direct arRP fluorescence is detected. The control liposomes showed a ring-like pattern of fluorescence (Fig. 3E), suggesting that the arRP was restricted to the lipid membranes. We also consistently observed small fluorescent droplets in the medium surrounding the control liposomes (Fig. 3E). The pattern of fluorescence was dramatically different in Rpe65-containing liposomes. Here, we observed prominent arRP fluorescence in both the membrane and aqueous interior (Fig. 3F). Interestingly, the small fluorescent droplets were never seen in medium surrounding these Rpe65 liposomes. Together, the results presented in Fig. 3 show that Rpe65 not only binds arRE but can also extract them from membranes into the aqueous interior of liposomes. These characteristics suggest a possible function for Rpe65.

**Table I**

| Fluorophore   | Excitation max λ (nm) | Emission max λ (nm) |
|---------------|-----------------------|---------------------|
| arRP          | 325                   | 460                 |
| BODIPY-PC     | 510                   | 520                 |
| Pyranine      | 485                   | 510                 |

**Fig. 3.** Fluorescent resonance energy transfer showing Rpe65-dependent uptake of arRP into liposomes. A, arRP-BODIPY-PC FRET. Emission spectra were obtained with 325-nm excitation from liposomes containing BODIPY-PC alone or BODIPY-PC plus arRP. B, arRP-pyranine FRET. Emission spectra were obtained from liposomes containing pyranine alone or pyranine plus arRP, as in panel A. C, Rpe65-dependent uptake of arRP into lipidosome membranes revealed by arRP-BODIPY-PC FRET. Liposomes containing protein from Rpe65-expressing or non-expressing Sf9 cells plus BODIPY-PC were incubated in media containing free arRP for the indicated times. 520-nm emission was acquired with 325-nm excitation. D, Rpe65-dependent uptake of arRP into the aqueous interior of liposome membranes revealed by arRP-pyranine FRET. Liposomes containing protein from Rpe65-expressing or non-expressing Sf9 cells plus pyranine were incubated in media containing free arRP for the indicated times. 520-nm emission was acquired with 325-nm excitation. E, fluorescence light microscopy of liposomes containing protein from non-expressing Sf9 cells incubated in medium containing free arRP for the indicated times. 520-nm emission was acquired with 325-nm excitation. F, fluorescence microscopy of liposomes containing protein from Rpe65-expressing cells incubated in medium containing free arRP, as in panel E. Note the more uniform fluorescence labeling indicating arRP in both the membrane and aqueous interior of this liposome. The apparent fluorescent intensities of the liposome images in panels E and F do not reflect the absolute levels of arRP.

**Rpe65 Is Required for Isomerization of arRP but Contains No Intrinsic Isomerase Activity**—A potential role for Rpe65 as an arRE-binding-protein may be to present this otherwise insoluble substrate to IMH for isomerization. To test this possibility, we prepared RPE membranes from wild-type (C57BL/6) and...
rpe65−/− knockout mice (10). After extracting to remove endogenous retinoids, the membranes were solubilized in detergent and assayed for production of 11cROL from exogenous aRP substrate plus or minus expressed Rpe65. Representative chromatograms of retinoids formed during these reactions are shown in Fig. 4, A–D. As in previous experiments, we confirmed identification of the retinoids by spectral analysis (insets in Fig. 4, A–C) and co-migration with standards (Fig. 4E). Fig. 4F shows the quantitation of 11cROL synthesized under the four assay conditions. Thirteen pmol of 11cROL were produced by solubilized mouse RPE membranes under the indicated assay conditions. G, histogram showing pmol of 11cROL produced by bovine RPE proteoliposomes from aRP plus non-expressing Sf9 proteins or expressed Rpe65.

We also measured the effect of Rpe65 on IMH activity in proteoliposomes prepared from detergent-solubilized bovine RPE. First, we induced fusion of these proteoliposomes with a second preparation of liposomes that contained only aRP. Here, 1.3 pmol of 11cROL were produced (Fig. 4G). A similar amount of 11cROL was produced (1.4 pmol) when the aRP liposomes were replaced with liposomes that contained aRP plus non-expressing Sf9 proteins. However, dramatically higher levels of 11cROL were produced (11.3 pmol) when the bovine RPE proteoliposomes were fused with liposomes that contained aRP plus Rpe65 (Fig. 4G). The small amounts of 11cROL produced in the fusion assay with liposomes containing aRP alone or aRP plus non-expressing Sf9 proteins are probably due to endogenous Rpe65 carried-through during preparation of the bovine RPE proteoliposomes. A small amount of 13-cis-retinol (13cROL) was also detected in each assay where 11cROL was produced (Fig. 4, A, B, and D). In a control experiment, we added 11cROL to heat-denatured RPE proteoliposomes. Following incubation, a similar relative amount of 13cROL (plus aRF) was produced, suggesting that the 13cROL in the Fig. 4 experiments is a thermal degradation product of 11cROL.

The results in Fig. 4 establish that IMH activity is dependent upon the presence of Rpe65. To rule out the possibility that Rpe65 is IMH, we incubated liposomes containing expressed Rpe65 plus aRP or aRF and analyzed for production of 11cROL by HPLC. No 11cROL was produced in either assay. We also incubated heat-denatured membranes from wild-type mouse RPE with aRP and Rpe65. Here again, no measurable 11cROL was produced. These data indicate that Rpe65 has no intrinsic isomerase activity.

**DISCUSSION**

In this study we demonstrated that Rpe65 binds atRE. We showed spectrally that apo-Rpe65 and aRF combine to yield a
new molecular species. We showed that aRP co-elutes with Rpe65 during gel filtration chromatography and immunoprecipitation. Also, we showed that liposomes containing Rpe65 take up free aRP from the medium much more rapidly than do control liposomes, and that a significant fraction of the aRP in Rpe65-containing liposomes is found within the aqueous interior. Significantly, we demonstrated that the conversion of aRP to 11cRAL by IMH is dependent on Rpe65, but that Rpe65 alone does not catalyze this isomerization reaction.

Rpe65 is 37% identical to β-carotene-15,15′-dioxygenase (β-CDO) which catalyzes the conversion of β-carotene to aRAL (29, 30). Given this level of identity, the ligand-binding pocket of Rpe65 and the catalytic site of β-CDO may be conserved structural features. Fig. 5 shows the molecular structures for Rpe65 and the catalytic site of β-CDO may be conserved structural features. Fig. 5 shows the molecular structures for Rpe65 during gel filtration chromatography and immunoprecipitation. Also, we showed that liposomes containing Rpe65 take up free aRP from the medium much more rapidly than do control liposomes, and that a significant fraction of the aRP in Rpe65-containing liposomes is found within the aqueous interior. Significantly, we demonstrated that the conversion of aRP to 11cRAL by IMH is dependent on Rpe65, but that Rpe65 alone does not catalyze this isomerization reaction.

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droplets in the media of Rpe65-containing liposomes (Fig. 3F). Thus, Rpe65 on the outer surfaces of liposomes may interact with these lipid droplets to extract arRP. Rpe65 may also dissociate from the liposome membranes to scavenge arRP from these lipid droplets. It is likely that these arRP-containing droplets acquire a skin of phospholipids from the medium, and hence resemble to Rpe65 the surface of a phospholipid membrane at its aqueous interface. Mobilization of arRP in these lipid droplets explains the higher levels of arRP in membranes from Rpe65-containing liposomes (Fig. 3C). Gene therapy of rpe65<sup>−/−</sup> dogs with recombinant virus expressing normal Rpe65 caused disappearance of the arRP inclusions from the treated eye (32). Thus, Rpe65 can mobilize arRP from these lipid inclusions in vivo. The presence of arRP in the aqueous interior of liposomes in the current study (Fig. 3, D and F) is further evidence that Rpe65 extracts arRP from membranes.

Multiple proteins have been characterized that bind isomers of retinol and retinaldehyde including CRALBP (24), cellular retinol-binding protein (33), retinol-binding protein (34), and interphotoreceptor retinoid-binding protein (35). However, no binding proteins for retinyl esters have previously been described. The listed binding proteins are thought to stabilize their retinoid ligands and to prevent them from reacting with cellular components. Retinyl esters, however, are relatively inert. What then is the need for an arRP-binding protein such as Rpe65? Retinyl esters represent the principal storage pool of vitamin A (11, 36), and comprise the sole substrate for chemical synthesis of visual chromophore in the RPE (1, 2). The insolubility of arRP in water infers the requirement for a binding protein to extract them from the lipid bilayer and deliver them in a solubilized form to IMH.

Solubilized RPE membranes from rpe65<sup>−/−</sup> mice contained no measurable IMH activity (Fig. 4, C and F). However, we observed virtually complete rescue of this biochemical phenotype with addition of Rpe65 to the assay mixture (Fig. 4, D and F). Similarly, we observed ≈9-fold stimulation of IMH activity in bovine RPE proteoliposomes with addition of Rpe65 (Fig. 4G). The lack of stimulation in the non-expressing S<sup>9</sup>-control experiment shows that the stimulation effect seen with Rpe65 is not caused by extraneous S<sup>9</sup> proteins. A possible interpretation of the data in Fig. 4 is that Rpe65 and IMH are the same protein. However, no 11cROL was produced when Rpe65 was incubated with liposomes containing arRP or aRROL alone, or with aRROL plus heat-denatured RPE proteins from wild-type mice. These observations rule out the explanation that Rpe65 and IMH are the same protein. It remains a formal possibility that Rpe65 is a subunit of a larger IMH complex. The abundance of Rpe65 in RPE cells argues against this possibility. A more likely explanation for the critical dependence of isomerase activity on the presence of Rpe65 is that this protein is required for substrate access by IMH.

Our model for the function of Rpe65 is shown in Fig. 6A. We suggest that the role of Rpe65 is to extract insoluble arRE from ER membrane and present them to IMH. This hypothesis explains the phenotype in rpe65<sup>−/−</sup> mice (Fig. 6B). Synthesis of arRE by LRAT is normal in these mutants (37). However, without Rpe65 to solubilize arRE, IMH is starved for substrate and hence no measurable 11cROL is produced. Instead, arRE accumulate at up to 1,000-fold the level in wild-type RPE due to decreased utilization (38). The arRE saturate the ER membrane and bleed off as lipid droplets, which float above the cytisol following centrifugation (38) and are visible in rpe65<sup>−/−</sup> RPE by electron microscopy (10).

Rpe65 is also present in cone photoreceptors, albeit at much lower levels than in RPE cells (39, 40). Cone-dominant chickens and ground squirrels have been shown to contain 11cRE and arRE in retina in addition to RPE (17, 41, 42). The cellular localization of these retinyl esters within the retina is not known. Recently, a new retinoid pathway that mediates cone-pigment regeneration under daylight conditions was described in chicken and ground squirrel retinas (17). Indirect evidence presented in that study suggests that these esters predominately accumulate in Müller glial cells. However, it is possible that these cells also contain arRE, which may be solubilized by endogenous Rpe65. The presence of Rpe65 in cones may explain the loss of cone function in rpe65<sup>−/−</sup> mice (43, 44).

Recently, Rpe65 was shown to react covalently with a biotinylated derivative of all-trans-retinyl chloroacetate (45). This reaction was competed by addition of aRROL and all-trans-retinyl acetate. Although competition with fatty acyl esters of aRROL such as arRP was not investigated, these data agree with the results presented here.

In summary, we have shown that Rpe65 is a binding protein for arRE. This binding function serves to solubilize arRE, which are otherwise confined to membranes and lipid inclusions. We have also shown that Rpe65 is required for IMH activity but has no intrinsic isomerase activity, suggesting that Rpe65 donates arRE substrate to IMH.

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