Lack of effect of RPE65 removal on the enzymatic processing of all-*trans*-retinol into 11-*cis*-retinol in vitro

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Abstract RPE65 is a major membrane associated protein found in the vertebrate retinal pigment epithelium (RPE). Various studies have shown this protein to be essential for visual function, possibly at the level of the processing of retinoids. The pigment epithelium is the anatomical site in which the visual chromophore 11-*cis* retinal is generated. The two critical RPE enzymes in the isomerization pathway are lecithin retinol acyl transferase (LRAT) and isomerohydrolase, which processes all-*trans*-retinyl esters into 11-*cis*-retinol. Both enzymes are membrane bound. It is shown here that RPE65 can be largely extracted (90–95%) from RPE membranes by 1 M KCl by itself, or with added detergent CHAPS. The almost quantitative extraction of RPE65 from RPE membranes has little or no effect on in vitro LRAT and isomerohydrolase activities in quantitative enzymatic assays using RPE membranes, suggesting that RPE65 may not have an important role to play in the enzymatic processing of all-*trans*-retinol into 11-*cis*-retinol in vitro.

Key words: Visual cycle; Isomerohydrolase; RPE65; Vitamin A

1. Introduction

Visual pigment regeneration in the vertebrate eye absolutely requires the resynthesis of 11-*cis*-retinal, the visual chromophore, in the retinal pigment epithelium (RPE). The salient enzymological steps which occur in the RPE involve the esterification of all-*trans*-retinol (vitamin A) by lecithin retinol acyl transferase (LRAT) and the processing of the resultant all-*trans*-retinyl esters to 11-*cis*-retinol by an isomerohydrolase [1]. The 11-*cis*-retinol is oxidized by a retinol dehydrogenase to afford 11-*cis*-retinal [2]. Both LRAT and isomerohydrolase activities are membrane bound, and both have resisted purification. However, a substantial amount is known about LRAT; its kinetic mechanism of action has been solved [3], structure-activity studies have been performed on its substrates [4], and powerful, specific reversible and irreversible inhibitors have been designed for it [5]. The specific, active site directed covalent labeling of the approximately 25 kDa enzyme has led to its identification and cloning [6]. Moreover, the 11-*cis*-retinol dehydrogenase has also been cloned and sequenced [2]. A major gap in our understanding of the visual cycle is in the identification of the enzyme(s) involved in the isomerohydrolase reaction.

Recently, much attention has been paid to a major RPE membrane associated protein referred to as RPE65 [7–9]. This protein, which is unique to the RPE, has been suggested to be a possible component of the isomerohydrolase [10]. The fact that RPE65 is uniquely associated with the RPE is of interest because the RPE is the predominant, if not sole, site of 11-*cis*-retinoid biosynthesis [11]. Interestingly, mutations in the RPE65 protein are associated with human blindness [12,13], and knockout mice appear not to be able to synthesize 11-*cis*-retinoids, although they still appear to be able to synthesize retinyl esters (D. Bok, personal communication). This phenotype is quite intriguing and suggests an important general role for RPE65 in retinoid processing in the vertebrate eye. However, there is no direct and quantitative information that bears on the question of whether RPE65 has a specific role to play in the isomerization event per se. Since RPE65 can neither be the 11-*cis*-retinol dehydrogenase nor LRAT (their sequences have already been determined), it is possible that RPE65 is the isomerohydrolase [10] or it may play some hitherto undefined role in retinoid processing.

We have developed a highly quantitative in vitro assay system for retinoid isomerization using bovine RPE membranes [14]. In this system, 11-*cis*-retinol biosynthesis (from added all-*trans*-retinol) is powerfully and stereospecifically feedback inhibited by the product 11-*cis*-retinol [14]. RPE65 is found in the RPE microsomal membranes used in these studies. RPE65 can be removed from RPE membranes by salt and detergent. In the current study, the effect of RPE65 removal on 11-*cis*-retinol biosynthesis is determined using these membranes to quantitatively assess whether or not this protein is essential in the crucial portion of the visual cycle in which vitamin A is converted into 11-*cis*-retinol. The studies reported here indicate that RPE65 does not have an important role in this process.

2. Materials and methods

2.1. Materials

Frozen bovine eye cups were obtained from J.A. and W.L. Lawson Co in Lincoln, NE. [11,12-$^3H_2$]All-*trans*-retinol (specific activity 30.0 Ci/mmol) was purchased from DuPont-New England Nuclear. 11-*cis*-Retinal was acquired through the National Eye Institute. 11-*cis*-Retinol was synthesized by the reduction of 11-*cis*-retinal with sodium borohydride. 13-*cis*-Retinol and fatty acid-free bovine serum albumin were purchased from Sigma. Ascorbic acid (vitamin C) and DL-α-tocopherol (vitamin E) were obtained from Fluka. HPLC-grade solvents were purchased from J.T. Baker.

2.2. Preparation of pigment epithelium membranes

The procedure for the preparation of bovine retinal pigment epithelium membranes is described in previous literature [14]. The membranes were irradiated with UV (365 nm) for 5 min on ice prior to use to destroy endogenous retinoids.

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Abbreviations: LRAT, lecithin retinol acyl transferase; DTT, dithiothreitol; DMSO, dimethylsulfoxide; RPE, retinal pigment epithelium

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2.3. Preparation of $^3H_2$-all-trans-retinol/vitamin E/DMSO mixture

In each assay, a $^3H_2$-all-*trans*-retinol/vitamin E/DMSO mixture was prepared to prevent decomposition of the $^3H_2$-all-*trans*-retinol. All procedures were performed under dim red light unless mentioned otherwise. Vitamin E was added to 50 μl of $^3H_2$-all-*trans*-retinol (1 mg of vitamin E per 1 ml of $^3H_2$-all-*trans*-retinol). This mixture was dried with nitrogen gas and redissolved in 25 μl of DMSO.

2.4. Lecithin:retinol acyltransferase (LRAT) and isomerohydrolase assays

The conversion from $^3H_2$-all-*trans*-retinol to $^3H_2$-all-*trans*-retinyl ester by LRAT was monitored at different time intervals (0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, and 45 min) according to published procedures [14]. Radioactivity was counted with an on-line Bertold LB 506-C HPLC radioactive monitor interfaced with an IBM 386 computer. Calibration of the system was done by extracting and injecting defined amount of [11, 12-$^3H_2$]-all-*trans*-retinol.

Radioactive retinoids were analyzed on a 5 μm PVA-Sil column (250×4.00 mm, YMC) with 7% dioxane in hexane at a flow rate of 1.5 ml/min. A standard mixture of isomeric retinols were coeluted (monitored at 325 nm) to identify the isomeric retinoids. Each LRAT and isomerohydrolase assay was performed three times to calculate standard deviations.

2.5. Extraction of RPE65 with 1 M KCl

200 μl of suspended bovine RPE membrane in 0.1 M phosphate buffer, pH 7.5 was centrifuged at 174 000×*g* for 15 min at 4°C. The supernatant was discarded, and the pellet was sonicated in 1 ml of TBS (150 mM NaCl, 10 mM Tris base (pH 7.2), 5 mM EDTA and 1 M KCl) on ice with a Branson sonifier for 1 min (3×20 s) intermittently for 5 min. The homogenate was then centrifuged at 174 000×*g* for 15 min at 4°C using a Beckman TL-100 table top ultracentrifuge. The resulting supernatant and the pellet were divided for analysis of LRAT and isomerohydrolase activities as well as for Western analysis. The pellet was resuspended in 200 μl of TBS. For Western blotting 2 μl of pellets and 20 μl of supernatant were loaded onto a 12% SDS-PAGE gel. The loading volumes of the pellet and supernatant fractions were determined by the relative volumes of initial prepared RPE membrane fractions. Control indicates non-treated RPE membranes, and TBS treated pellet and supernatant indicate RPE membrane treated in TBS without 1 M KCl. The overall recoveries of RPE65 in the treated sample compared to untreated control is approximately 100%.

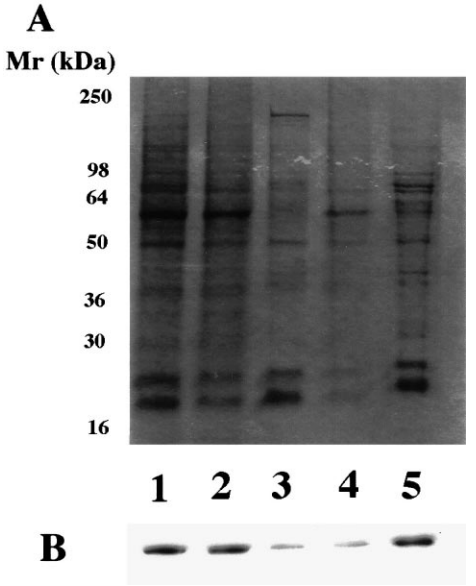

Fig. 1. SDS gel electrophoresis and immunoblot analysis of RPE membranes extracted with 1 M KCl-TBS. The RPE membranes were extracted with 1 M KCl-TBS and separated on a 12% SDS polyacrylamide gel, electrophoretically transferred onto a PVDF membrane, and detected with the antibody against RPE65. A: Coomassie blue. B: Immunoblot. Lane 1, RPE membrane (control). Lane 2, pellet extracted with TBS in the absence of 1 M KCl. Lane 3, supernatant extracted with TBS in the absence of 1 M KCl. Lane 4, pellet extracted with 1 M KCl-TBS. Lane 5, supernatant extracted with 1 M KCl-TBS.

2.6. Extraction of RPE65 with 1 M KCl/0.1% CHAPS

200 μl of suspended bovine RPE membrane in 0.1 M phosphate buffer, pH 7.5 was centrifuged at 174 000×*g* for 15 min at 4°C. The supernatant was discarded, and the pellet was intermittently homogenized in ice water in 1 ml of TBS (150 mM NaCl, Tris base 10 mM (pH 7.2), 5 mM EDTA, 0.1% CHAPS and 1 M KCl) with a bath sonifier (Laboratory Supplier Co.) for 20 min (4×5 min). The homogenate was then centrifuged at 174 000×*g* for 15 min at 4°C using a Beckman TL-100 table top ultracentrifuge. The resulting supernatant and the pellet were split for analysis of LRAT and isomerohydrolase activities, and Western analysis as indicated above. The overall recovery of RPE65 in the treated sample compared to untreated control is approximately 85%.

2.7. SDS-PAGE

12% SDS-PAGE was carried out in a Tris/glycine buffer system [15]. The protein samples for electrophoresis were prepared with 1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol and immediately placed in a boiling water bath for 5 min. A portion of the sample was mixed with 0.2% bromophenol blue and 70% glycerol and was then applied to each gel lane. Gels were stained with 0.1% (v/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol, and 10% (v/v) glacial acetic acid, and destained with 10% (v/v) methanol and 10% (v/v) glacial acetic acid.

2.8. Western analysis

Proteins were separated by 12.0% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) microporous membranes (Millipore)

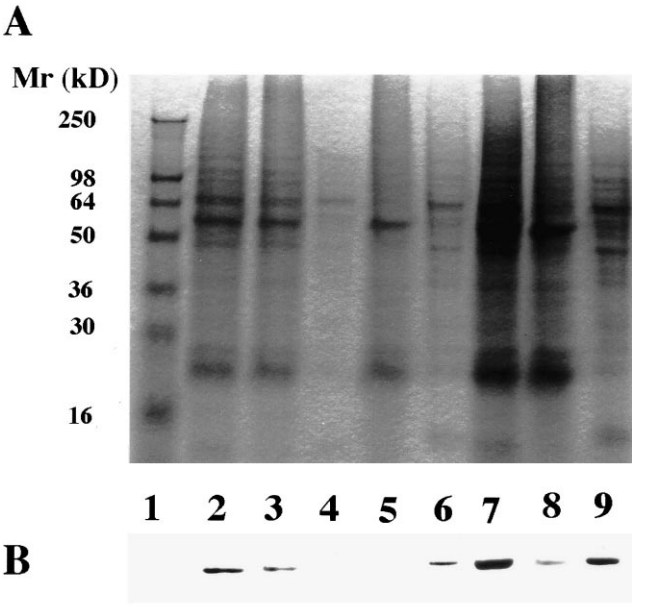

Fig. 2. SDS gel electrophoresis and immunoblot analysis of RPE membranes extracted with 1 M KCl/0.1% CHAPS-TBS. The RPE membranes were extracted with 1 M KCl/0.1% CHAPS-TBS and separated on a 12% SDS polyacrylamide gel, electrophoretically transferred onto a PVDF membrane, and detected with the antibody against RPE65. A: Coomassie blue. B: Immunoblot. Lane 1, marker proteins. Lane 2, 8.0 μg of RPE membrane (control). Lane 3, 6.8 μg of pellet extracted with TBS in the absence of 1 M KCl/0.1% CHAPS. Lane 4, 1.2 μg supernatant of lane 3 extracted with TBS in the absence of 1 M KCl. Lane 5, 4.3 μg of pellet extracted with 1 M KCl/0.1% CHAPS-TBS. Lane 6, 3.1 μg supernatant of lane 4 extracted with 1 M KCl/0.1% CHAPS-TBS. Lane 7, 20.0 μg of RPE membrane (control). Lane 8, 15.0 μg of pellet extracted with 1 M KCl/0.1% CHAPS-TBS. Lane 9, 7.2 μg supernatant of lane 8 extracted with 1 M KCl/0.1% CHAPS-TBS.

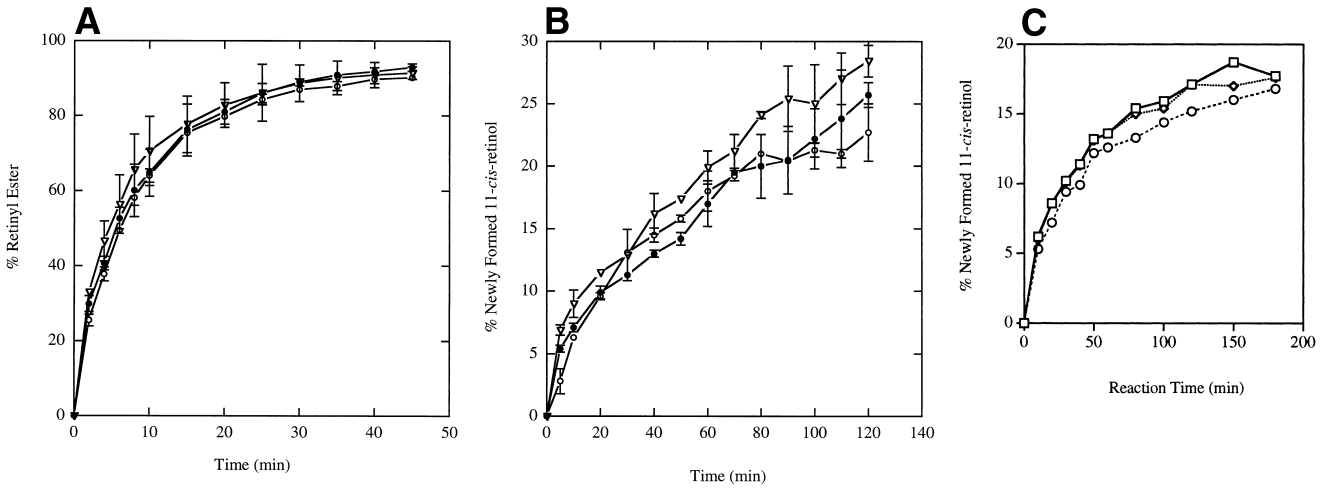

Fig. 3. A: Comparison of LRAT activity between (+) RPE65 and (−) RPE65. Samples were prepared and enzyme activities were assayed according to procedures described in Section 2. Vitamin A concentrations were set at 1.0 μM. The retinyl ester formation with RPE65 (▽), without RPE65 treated with 1 M KCl (●), and without RPE65 treated with 1 M KCl/0.1% CHAPS (○) were carried out at 25°C for up to 45 min. The results represent data obtained from triplicate studies independently performed. B: Comparison of isomerohydrolase activity between (+) RPE65 and (−) RPE65. Samples were prepared and enzyme activities were assayed according to procedures described in Section 2. The 11-*cis*-retinol formation with RPE65 (▽), without RPE65 treated with 1 M KCl (●), and without RPE65 treated with 1 M KCl/0.1% CHAPS (○) were carried out at 37°C for up to 2 h. The results represent data obtained from triplicate studies independently performed. The addition of 0.1% CHAPS to RPE membranes led to a diminution of isomerohydrolase activity of 35–40% in the absence of centrifugation but had no effect on LRAT activities. The isomerohydrolase activities shown in B and C are normalized for this intrinsic inhibition which is unrelated to the removal of RPE65. C: The same experiment as in B except that the vitamin A concentration was 3.0 μM. The formation of 11-*cis*-retinol was determined with RPE65 (□), without RPE65 treated with 1 M KCl (◇), and without RPE65 treated with 1 M KCl/0.1% CHAPS (○) were carried out at 37°C for up to 2 h. The vitamin A concentration in this experiment was 3.0 μM.

in the 25 mM Tris base (pH 8.5), 20 mM glycine, and 20% ethanol using a semi-dry transfer cell (Bio-Rad). The membrane was blocked with 5% non-fat dried milk (Pierce, Rockford, IL) in TBS buffer (25 mM Tris, 150 mM NaCl, pH 7.4, 0.01% Antifoam A and 0.01% thimerosal) for 2 h, and then washed with TBS washing buffer (10 mM Tris, 150 mM NaCl, pH 7.4, and 0.1% Tween 20) for 30 min. For the detection of RPE65, the membrane was incubated with 1:8000 diluted polyclonal antibody against RPE65 obtained from Drs. K. Palczewski and J. Saari for 1 h at room temperature and washed with TBS buffer for 30 min. The second antibody, anti-mouse Ig, horseradish peroxide linked whole antibody from sheep (Amersham Life Science), was diluted 1:4000 and incubated with the membrane for 30 min. After washing with TBS washing buffer for 30 min, the membrane was developed by the enhanced chemiluminescence method (ECL, Amersham Life Science). The percentage of RPE65 from the total amount of pellet and supernatant was taken as 100%. The calculated percentage subtracted each value of pellet and supernatant from the total amount. Quantitation of Western analysis was performed by scanning densitometry using the Optimas 5.2 software package (Optimas Corp., Bothell, WA). Quantitative data are presented as the mean ± S.D. from three independent experiments.

3. Results and discussion

The studies reported here are straightforward, and depend on determining the effects of RPE65 removal on retinoid processing using bovine RPE membranes. As far as is known, there are two enzymatic activities relevant to the isomerization event in 11-*cis*-retinoid biosynthesis in the RPE, namely LRAT and the isomerohydrolase [1]. RPE65 is known to be a membrane associated protein, but it is not an integral membrane protein and can be solubilized by washing membranes at high pH [16]. However, high pH washing of RPE membranes irreversibly inactivates LRAT and/or isomerohydrolase activities (D.W. Choo and R.R. Rando, unpublished experiments) and thus less harsh methods were sought to solubilize the RPE65. To this end extractions were performed with 1 M KCl, with and without added detergent (CHAPS). Fig. 1A shows data of bovine RPE microsomal membranes extracted with either TBS or TBS containing 1 M KCl. Fig. 1B shows a Western blot of the same experiment detecting RPE65 with a polyclonal antibody directed against this protein (the antibody by itself has no effect on isomerization activities in RPE membranes). Clearly extraction with 1 M KCl markedly solubilizes RPE65. Densitometric determinations show that only 11.7 ± 2.0% of the protein remains membrane bound, the remainder (88.3 ± 2.0%) being found in the supernatant fraction. Adding 0.1% CHAPS to the 1 M KCl further increases the amount of RPE65 extracted, as shown in Fig. 2. In this instance, only 4.9 ± 1.9% of RPE65 remains membrane bound, with the remainder (95.1 ± 1.9%) being solubilized. Increasing the added concentrations of CHAPS proved not to be helpful because of its inactivating effects on the detergent sensitive isomerohydrolase. For example, treatment with 0.3% CHAPS only preserves approximately 10% of the total isomerohydrolase activity. Even treatment with 0.1% CHAPS already reduces total isomerohydrolase activity by approximately 35–40%. Nevertheless, the extraction conditions used are sufficiently mild to allow for an assessment of the putative role of RPE65 in retinoid isomerization in the RPE.

It was straightforward to determine the effects of extraction with 1 M KCl in the presence and absence of 0.1% CHAPS on LRAT and isomerohydrolase activities. Fig. 3A shows that neither extraction had significant effects on membrane associated LRAT activity, and Fig. 3B shows essentially the same behavior with respect to isomerohydrolase activity. In Fig. 3A,B the vitamin A concentrations were set at 1.0 μM. The K_M of LRAT for vitamin A is 0.24 μM [16]. Since retinyl esters generated by LRAT are the actual substrates for the

isomerohydrolase, vitamin A concentrations significantly above 0.24 μM need to be utilized in these experiments. To be more certain that substrate was not limiting, the experiment in Fig. 3B was repeated at 3.0 μM concentration of vitamin A (Fig. 3C). There proved to be virtually no difference in the rates of 11-*cis*-retinol formation in the treated versus untreated samples. Under the conditions of these experiments $<10\%$ of the retinyl esters are processed into 11-*cis*-retinol. It should also be noted that it is neither practical to substantially exceed 3.0 μM concentrations of vitamin A under the conditions employed, nor is it possible to directly add retinyl esters as substrates for the isomerohydrolase due to their limited solubilities.

As discussed above, KCl wash by itself extracts approximately 90% of RPE65 and adding 0.1% CHAPS extracts 95% of the protein. The enzymatic activities were shown to reside only in the membrane fractions under the conditions of the experiments. It should also be mentioned that extraction with 0.3% CHAPS, while allowing the retention of only approximately 10% isomerohydrolase activity in the membrane fraction, quantitatively extracted RPE65 from the membranes by densitometric determinations. Importantly, isomerization activity is never found in the solubilized fraction where RPE65 is found. Therefore, it appears that RPE65 does not have an important general role to play in the enzymatic processing of all-*trans*-retinol into 11-*cis*-retinol in vitro because removing virtually all the RPE65 from the membranes has little effect on 11-*cis*-retinoid biosynthesis. As a corollary to this it is also unlikely that RPE65 represents the isomerohydrolase. The only way that RPE65 could be involved as the isomerohydrolase is if the molecule is in vast excess, so that removal of most of it has little effect on isomerization rates. However, it should also be noted that no isomerization activity is found in the RPE65 containing soluble fractions, again demonstrating that there is no correlation between the presence of RPE65 and retinol processing. It has also been suggested that RPE65 may be directly or indirectly involved in retinoid binding [7]. This would be consistent with the apparent lack of an enzymatic role for this protein in 11-*cis*-retinoid biosynthesis.

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