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RPE65, the Major Retinal Pigment Epithelium Microsomal Membrane Protein, Associates with Phospholipid Liposomes

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The retinal pigment epithelium (RPE)-specific protein RPE65 is the major protein of the RPE microsomal membrane fraction. Though RPE65 lacks transmembrane domains or signal peptide, detergents are required for its maximally effective solubilization in isotonic buffers. However, in 0.75–1.0 M KCl, RPE65 is as soluble without detergent, indicating a peripheral membrane association. We wished to understand why this non-membrane-inserted protein was so closely associated with RPE microsomal membranes. To explore the possible involvement of interactions with phospholipids, an isotonic salt-soluble extract of RPE was incubated with phosphatidylcholine (PC)/phosphatidylserine (PS)/phosphatidylinositol liposomes and centrifuged to sediment the liposomes. RPE65 cosedimented with the liposome pellet. RPE65 also cosedimented with synthetic dipalmitoyl-, 1-palmitoyl, 2-docosahexaenoyl-PC or dipalmitoyl-PS liposomes. Incubation with 1 mM Ca^{2+} or 1 mM EGTA had no effect, indicating a Ca^{2+}-independent association. A spectrophotometric assay showed that this interaction of RPE65 with phospholipid vesicles resulted in increased light scattering, consistent with phospholipid vesicle aggregation. Resonance energy transfer experiments showed that any putative aggregation occurred without subsequent vesicle fusion. This PC affinity was further confirmed by incubation of RPE extract with dimyristoyl-PC-immobilized artificial membrane (IAM.PC) matrix. The RPE65 selectively bound and was elutable with 2% detergent. This RPE65–phospholipid liposome association may explain the solubilization characteristics of RPE65 and may be related to the function of RPE65 and to its physical association with the RPE smooth endoplasmic reticulum. © 1997 Academic Press

Key Words: **phospholipids; liposomes; retinal pigment epithelium; Ca^{2+}-independent; microsomal membranes.**

The photoreceptor neurons of the vertebrate retina and the retinal pigment epithelium (RPE)[3] constitute a complex that is highly interdependent developmentally and functionally. Both contain highly specialized membrane components central to their common purpose of maintaining the visual cycle: the RPE with its extensive, vesiculated smooth endoplasmic reticulum where the enzymes involved in visual cycle retinoid metabolism and isomerization are localized; and the outer segment membranes containing rhodopsin and the other components of the visual transduction cascade. While many of the photoreceptor outer segment membrane visual cycle-associated proteins have been characterized, few visual cycle-associated proteins of the RPE have been characterized (for review, see Ref. 1). The visual cycle enzymes of the RPE are localized in the microsomal membrane fraction, of which the smooth endoplasmic reticulum is a major component (1). In addition, the RPE phagocytoses and degrades shed outer segment discs (2). However, very little is known of the proteins involved in this or in the subsequent

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[3] Abbreviations used: BSA, bovine serum albumin; Chaps, 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; IAM.PC, phosphatidylcholine-immobilized artificial membrane; NBD, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl); OS, outer segment(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RPE, retinal pigment epithelium.

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recycling of the outer segment components. It is clear from the processes involved that specialized proteins may exist that subserve these RPE-specific membrane-related functions.

We have previously characterized one candidate for such an RPE-specific membrane-related function: RPE65, an RPE-specific 61-kDa nonglycosylated, microsomal membrane-associated protein found in mammal, bird, and amphibian RPE. Although its function is still unclear, it could be involved in a function related to RPE/photoreceptor interaction since it is first expressed late in RPE development, preceding by 1 or 2 days the morphological appearance of rat rod photoreceptor outer segments (3). It is a marker for the terminal differentiation of RPE whose expression is rapidly lost by RPE cells when grown in culture (3). Båvik *et al.* (4–6) ascribe to this protein the function of a retinol-binding protein receptor, though many of its characteristics belie such a function. RPE65 is localized to the cytoplasm and specifically to intracellular membranes (7), rather than having the basolateral distribution expected of a retinol-binding protein receptor. Our previous investigations showed that RPE65, although tightly bound to microsomal membranes, was not an integral membrane protein. Its isolation was facilitated by the use of zwitterionic detergents but it could also be efficiently solubilized by high salt (3). This conclusion is supported by the lack of any prospective transmembrane domains in the primary sequence deduced from the cDNA (3, 6). The only possible sequences consistent with membrane association were putative amino-terminal amphipathic α-helices. A glycosyl phosphatidylinositol anchor can also be discounted since when RPE65 was solubilized in Triton X-114, it partitioned in the detergent phase (3), and it lacks a signal peptide (8). At the same time it is the major protein associated with the microsomal membrane fraction of the RPE and likely has an important structural function along with any other activity it may have. Accordingly, it was of interest to determine the nature of the interaction between RPE65 and the RPE microsome membrane. We decided to explore this through the use of liposomes as model membrane systems. In this paper, we show that RPE65 can interact with phospholipids. RPE65 can be selectively cosedimented with phospholipid liposomes and is the major component protein of the RPE that is so sedimented.

EXPERIMENTAL PROCEDURES

Materials. Natural phospholipids were purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic phospholipids used were purchased from Avanti Polar Lipids (Alabaster, AL). Immobilized artificial membrane phosphatidylcholine (IAM.PC) columns and packing material were obtained from Regis Chemical Co. (Morton Grove, IL). Triton X-114 was purchased from Pierce (Rockford, IL) and Genapol C-100, Chaps, *N*-heptyl-β-D-glucoside, and cholic acid (sodium salt) were from Calbiochem (La Jolla, CA).

Tissue and membrane preparations: Antibodies. Bovine RPE cells were prepared from slaughterhouse eyes as previously described (3). In the various experiments, we used either a total RPE extract or a RPE microsomal membrane preparation. Total RPE extract was obtained by homogenizing fresh bovine RPE cells in buffer A (150 mM NaCl, 10 mM Tris–HCl, pH 7.4), in some cases containing a detergent as indicated. Bovine RPE microsomal membranes were prepared by differential centrifugation. RPE cells were homogenized (10–12 strokes in a Dounce glass homogenizer) in 0.32 M sucrose in 0.1 M phosphate buffer, pH 7.4, and centrifuged at 30,000*g* to sediment unbroken cells, nuclei, mitochondria, lysosomes, and melanin granules. The supernatant was then centrifuged at 105,000*g* for 1 h to sediment the microsomal membrane fraction. The membrane pellet was resuspended in 100 mM phosphate buffer, pH 7.4, and stored at −80°C. To solubilize RPE65 from this membrane preparation, Chaps was added to a final concentration of 0.3% and the mixture incubated at 4°C for 1 h, followed by centrifugation at 105,000*g* for 30 min.

Phospholipid liposome preparation and cosedimentation experiments. The procedure of Genge *et al.* (11) was used to make small unilamellar vesicle liposomes. A mixture of natural phospholipids [2 mg phosphatidylcholine (PC), 1 mg phosphatidylserine (PS), 1 mg phosphatidylinositol (PI), and 2 mg cholesterol] was dried under vacuum for 45 min. Three hundred and fifty microliters of buffer A was added and the mixture was sonicated with a Branson 185 sonifier equipped with a microtip probe for approximately 40 min (15 s sonication followed by 45 s of ice cooling each time) until the solution became translucent. Synthetic phospholipid liposomes were prepared by sonicating together 5 mg of the phospholipid and 2 mg cholesterol, dried from chloroform under a stream of argon, in 1 ml buffer A or 100 mM sodium phosphate, pH 7.5 (4 × 3 min sonication in a cup-horn) . The solution was centrifuged at 12,000*g* for 2 min to precipitate unincorporated lipids and the liposomes were sedimented by centrifugation for 1 h at 100,000*g* at 20°C in a Beckman (Palo Alto, Ca) TL-100 ultracentrifuge, followed by resuspension in 400 μl of buffer. Binding studies following the method of Glenney (12) used either a salt-soluble extract of RPE cells, prepared by homogenizing fresh RPE cells in buffer A, or a 0.3% Chaps extract of RPE cells dialyzed against buffer A (3). In a typical assay 100 μl of RPE extract was added to 100 μl of liposome suspension and the mixture was incubated with rocking at room temperature for 30 min. Dialyzed RPE extract without the addition of liposomes as well as a mixture of 100 μl of 0.02% BSA with liposomes was used as control. These preparations were then centrifuged at 100,000*g* for 30 min at 20°C. The resultant liposome pellet was washed with 100 μl of buffer A and centrifuged at 100,000*g* for 30 min. The supernatant and pellet fractions were analyzed by SDS–PAGE and immunoblotting.

For some experiments liposomes were prepared by a dialysis method (13) using a Mini-Lipoprep device (Dianorm GmbH, Munich, Germany; Sialomed, Columbia, MD) following the procedure of Schwendener *et al.* (14). A mixture of 5 mg of DPPC and 1 mg of cholesterol was dissolved in the presence of 29 mg *N*-heptyl-β-D-glucoside (lipid/detergent molar ratio of 0.13) in 1 ml 100 mM phosphate buffer, pH 7.4, at 50°C. This was dialyzed in the Mini-Lipoprep device against 100 mM phosphate, pH 7.4, in a 45°C water bath overnight. Liposomes were used without further dilution. This procedure yields 79 ± 2-nm diameter liposomes (14). This method was also used to prepare *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE/*N*-rhodamine-PE/DPPC (NBD-PE/N-Rh-PE/DPPC) liposomes for resonance energy transfer studies.

Vesicle aggregation experiments. The aggregation of phospholipid vesicles was monitored as the change in absorbance at 520 nm. Typically, a 100-μl aliquot of liposomes produced by dialysis was mixed with the RPE microsomal extract to be assayed (100 μl) and the mixture immediately placed in a Shimadzu UV160U spectrophotometer. The change in absorbance at 520 nm was followed for 10 min.

Resonance energy transfer. Resonance energy transfer was used to assess vesicle fusion (15). NBD-PE/N-Rh-PE/DPPC vesicles were prepared by dialysis as described above. For each assay 75 μl of NBD-PE/N-Rh-PE/DPPC vesicles was mixed with 75 μl of DPPC vesicles, RPE microsome/DPPC vesicles, or 1% NP-40. These were incubated at 42°C for 15 min and then diluted to 3 ml with 100 mM phosphate, pH 7.4. The emission wavelength spectrum was measured by exciting the samples at 450 nm in an SLM Aminco Model SPF 500 spectrofluorometer.

Binding to IAM.PC. Fifty milligrams of IAM.PC packing material was mixed with 500 μl 0.3% Triton X-114 extract of RPE cells. The tube was briefly centrifuged and the supernatant removed and reserved as filtrate 1. The IAM.PC packing material was washed four times with 500 μl of detergent-free buffer, each time reserving the supernatant (filtrates 2–5). Finally the IAM packing material was washed with 500-μl aliquots of Triton X-114 at concentrations ranging from 0.01 to 3% (filtrates 6–11). Fractions were analyzed by SDS–PAGE analysis and immunoblotting.

SDS–PAGE and immunoblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in 12% gels using the buffer system of Laemmli (16). Gels were stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol/10% acetic acid or blotted onto nitrocellulose (17). The monoclonal antibody RPE9 (10) was used as the primary antibody at a dilution of 1:6700. The secondary antibody used was goat anti-mouse IgG, alkaline phosphatase-conjugate (Gibco-BRL, Gaithersburg, MD), at a dilution of 1:3000.

RESULTS

Phospholipid affinity of RPE65. Incubation of an isotonic salt-solubilized extract of RPE cells with a phospholipid liposome preparation followed by ultracentrifugation resulted in the cosedimentation of the RPE65 protein with the liposome pellet (Fig. 1). The selectivity of this cosedimentation can be seen by the fact that the supernatant of extract incubated with liposomes shows the same pattern as the initial extract minus the 61-kDa band (Fig. 1A). The almost total removal of RPE65 from the RPE extract after incubation with liposomes compared with only slight sedimentation of the protein in the absence of liposomes is suggestive of a high affinity of the protein for the phospholipid liposomes (Fig. 1B). Additionally, BSA was not sedimented following incubation with liposomes (data not shown). It is of interest to note that the 40-kDa band which is immunoreactive with the RPE9 mAb (Fig. 1B) does not cosediment with the phospholipid liposomes.

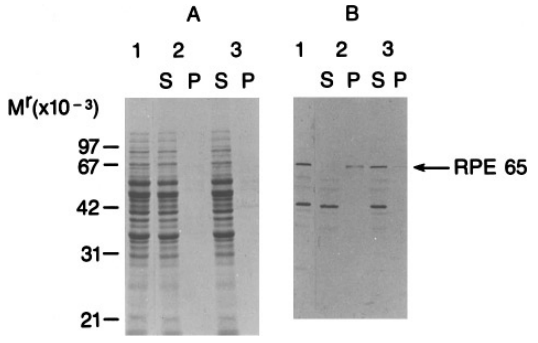

FIG. 1. Cosedimentation of RPE65 with mixed phospholipid liposomes. An isotonic salt-soluble extract of RPE cells was incubated with or without mixed phospholipid liposomes and centrifuged to pellet the liposomes. (A) Twelve percent SDS–PAGE gel stained with Coomassie blue; (B) immunoblot reacted with RPE9 mAb. Lane 1 is an isotonic salt-soluble extract of RPE cells; lane 2 is an RPE extract incubated with liposomes; lane 3 is an RPE extract incubated without liposomes. Equivalent amounts of pellet and supernatant were loaded on a 12% SDS–PAGE gel. The supernatant after centrifugation is designated S, while the liposome pellet is P.

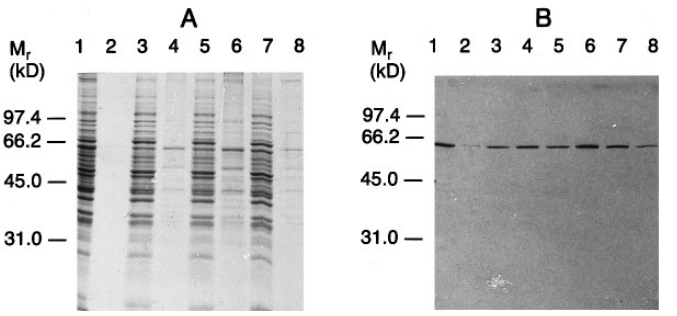

FIG. 2. Cosedimentation of RPE65 with synthetic phospholipid liposomes. A 0.3% Chaps extract of RPE cells was incubated with synthetic phospholipid liposomes for 1 h at room temperature and centrifuged. Equivalent amounts of supernatant and pellet were loaded on a 12% SDS–PAGE gel. (A) Coomassie blue-stained gel; (B) immunoblot reacted with RPE9. Lanes are 1, no liposomes, supernatant; 2, no liposomes, pellet; 3, dipalmitoyl-PC, supernatant; 4, dipalmitoyl-PC, liposome pellet; 5, 1-palmitoyl, 2-docosahexaenoyl-PC, supernatant; 6, 1-palmitoyl, 2-docosahexaenoyl-PC, liposome pellet; 7, dipalmitoyl-PS, supernatant; and 8, dipalmitoyl-PS pellet.

In addition, dialyzed, detergent-extracted RPE65 was incubated with synthetic phospholipids with defined acyl moieties found in the RPE. In these experiments, 0.3% Chaps-extracted protein, dialyzed against buffer A, was incubated with dipalmitoyl-PC, 1-palmitoyl, 2-docosohexaenoyl-PC, and dipalmitoyl-PS. It was found (Fig. 2) that RPE65 cosedimented with all three synthetic phospholipid liposomes. The variation in cosedimentation of RPE65 with these synthetic phospholipid liposomes does not appear great enough to indicate a strong head-group preference, at least between PC and PS. Furthermore, even if the detergent-extractable and salt-extractable RPE65 represent two separate populations of this protein, both will cosediment with phospholipids. The difference in degree of cosedimentation between the experiments in Figs. 1 and 2 is probably due to the relative amounts of RPE65 in each extract; since less RPE65 is extractable using the low salt extraction method, a relative total cosedimentation may be achieved.

To test the effect of the presence or absence of Ca^{2+} on phospholipid vesicle cosedimentation of RPE65, we incubated dialyzed RPE extract and dipalmitoyl-PC in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. No qualitative difference was observed between the Ca^{2+}- and

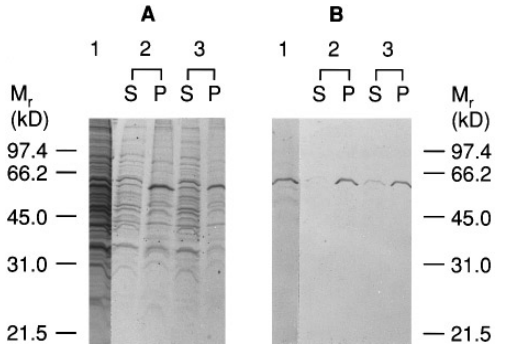

FIG. 3. RPE65 binding to PC liposomes is Ca^{2+}-independent. Chaps extract (0.3%) of RPE cells was incubated with dipalmitoyl-PC liposomes in the presence of either 1 mM Ca^{2+} or 1 mM EGTA. Aliquots were loaded on a 12% SDS–PAGE gel. The pellet samples were four times the equivalent amount of the supernatant samples. (A) Coomassie blue-stained gel; (B) immunoblot. Lane 1, crude RPE extract; lane 2, RPE extract + PC liposomes + 1 mM Ca^{2+}; lane 3, RPE extract + PC liposomes + 1 mM EGTA. The supernatant after centrifugation is designated S, while the liposome pellet is P.

EGTA-incubated liposomes (Fig. 3), indicating that the association of RPE65 to PC is Ca^{2+}-independent.

RPE65 causes possible aggregation but not fusion of phospholipid vesicles. To investigate further the association of RPE65 with liposomes we employed a light-scattering assay to test for aggregation. Addition of a 100-μl aliquot of RPE65 containing 0.075% Chaps extract of RPE microsomal membrane fraction to 100 μl of DPPC liposomes (prepared by dialysis 5 mg DPPC/ml) caused a marked increase in light scattering (Fig. 4, trace 1) compared to controls including buffer and excess irrelevant protein (purified bovine serum albumin; data not shown).

To determine if this possible aggregation is accompanied by vesicle fusion, we employed a resonance energy transfer assay (15). NBD-PE/Rh-PE/DPPC liposomes, DPPC liposomes, and RPE microsomal membrane Chaps extract were incubated at 42°C for 15 min and an emission spectrum was obtained (Fig. 5). No decrease in resonance energy transfer efficiency was detected in the presence or absence of the microsomal membrane fraction compared to the positive control where addition of detergent sharply reduced the efficiency of transfer.

RPE65 binds to IAM.PC. In view of the apparent affinity of RPE65 for phospholipid liposomes, we next investigated whether it would bind to IAM.PC. Since we had previously found that RPE65 partitioned in the detergent-rich phase of phase-separated Triton X-114 (3), initial solubilization studies of RPE cells were performed with this detergent. Using a concentration range of 0.03 to 1.0% it was found that 0.3% Triton X-114 was the lowest effective concentration for solubilization of RPE65 protein (data not shown). IAM.PC matrix (50 mg) incubated with an aliquot of RPE microsomal membrane proteins solubilized in 0.3% Triton X-114 was washed sequentially with a step gradient from 0 to 3% Triton X-114, with brief centrifugation after each wash. It was found (Fig. 6), following initial washes that removed unbound RPE65 (presumably in excess of the binding capacity), that the protein was eluted only at rather high concentrations (1–3%) of Triton X-114. While it was not the only protein to be eluted from the matrix by the detergent, it appeared to

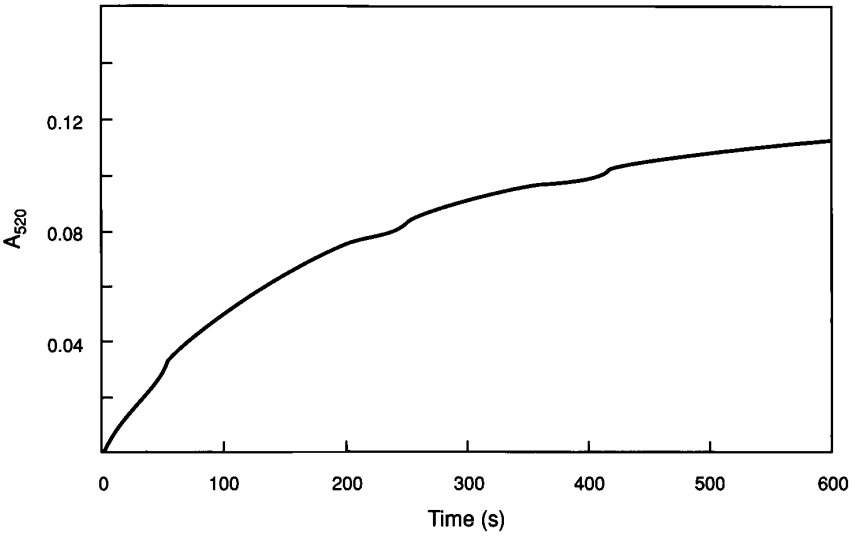

FIG. 4. RPE65 induces a possible DPPC liposomes aggregation. Preincubated 100-μl aliquots of 1:3 dilution 0.3% Chaps (final Chaps concentration 0.075%) extract of RPE microsomal membrane fraction were mixed with 100 μl of DPPC liposomes and immediately placed in a Shimadzu UV160U spectrophotometer (at room temperature) and the change in absorbance at 520 nm followed for 10 min.

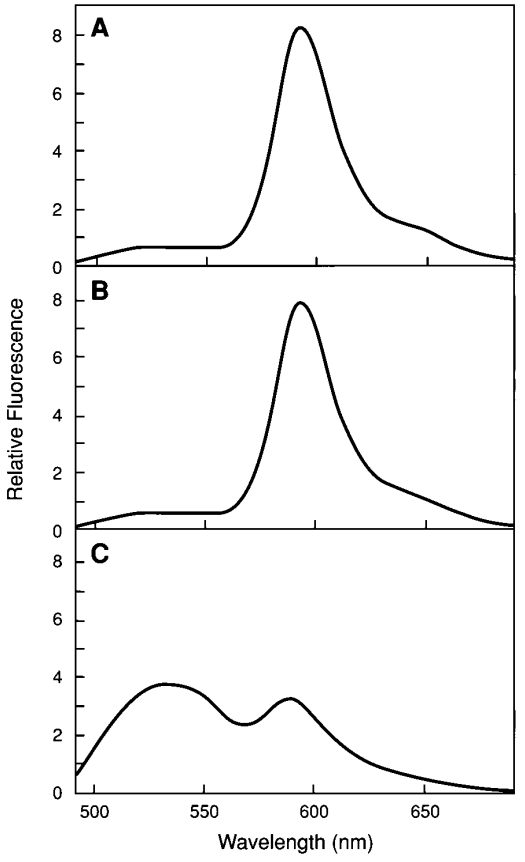

FIG. 5. RPE65-induced vesicle aggregation is not followed by vesicle fusion. A method based on resonance energy transfer was used to assess vesicle fusion. Seventy-five-microliter aliquots of NBD-PE/N-Rh-PE/DPPC vesicles were mixed with 75 μl of (A) DPPC vesicles, (B) RPE microsome/DPPC vesicles, or (C) 1% NP-40. These were incubated at 42°C for 15 min and diluted to 3 ml with 0.1 M phosphate buffer, pH 7.4. The emission wavelength spectrum was measured by exciting the samples at 450 nm.

be the most selectively removed. These high detergent concentrations are very much in excess of the previously determined optimal concentration required for solubilization, indicating a very strong association.

DISCUSSION

Our previous work on RPE65 demonstrates that it is a microsomal membrane-associated protein that may be most effectively solubilized by use of detergents or by high salt (3). To better understand the properties of this protein, we wished to study in more detail the nature of the interaction of RPE65 with membranes. Since analysis of the cDNA-deduced primary sequence does not predict any signal peptide or transmembrane domains (8) and since a glycosyl-phosphatidylinositol anchor is excluded (3) by Triton X-114 phase separation (18), the possibility exists that RPE65 associates with membranes by interaction with phospholipids. The region of RPE65 which might function in such an interaction with phospholipids is not known. However, since two potential amphipathic alpha helices are predicted at residues 5–22 and 108–125 by analysis of the protein sequence (8), it is tempting to speculate that these structures might be implicated in the phospholipid interaction that we describe here.

Phospholipids form the largest class of lipids in membranes including those of the RPE (19). Since palmitic acid is the major saturated fatty acid of the RPE and docosahexaenoic acid is one of the two major polyunsaturated fatty acids (19) and one which is actively sequestered by the RPE/photoreceptor OS complex, we elected to study synthetic phospholipids containing palmitic acid at both *sn*-1 and *sn*-2 positions and also PC with palmitic acid at *sn*-1 and docosahexaenoyl at *sn*-2. From our experiments, it appears that RPE65 is the major protein of the detergent-soluble fraction of the RPE that cosediments with phospholipid vesicles. The association of RPE65 to both PC and PS liposomes indicates a lack of specificity for the phospholipid head group. Despite this, there appears to be a greater cosedimentation of RPE65 with the PC species. In addition, this association does not appear to be affected by the presence or absence of Ca^{2+}. Both synthetic PC used have palmitic acid at the *sn*-1 position, but the *sn*-2 acyl group is either palmitoyl or docosahexaenoyl, implying that the *sn*-2 acyl group, at least, is not required for specificity.

The phospholipid association of RPE65 can be differentiated, on the one hand, from the growing number of Ca^{2+}-dependent PS-binding proteins including the annexins (20) and, on the other, from the cytosolic phospholipid-transfer proteins (21), neither group sharing any sequence homology with RPE65. In addition, RPE65 does not share any sequence homology with the bovine seminal plasma protein PDC-109 (22), otherwise known as BSP proteins, which have been shown

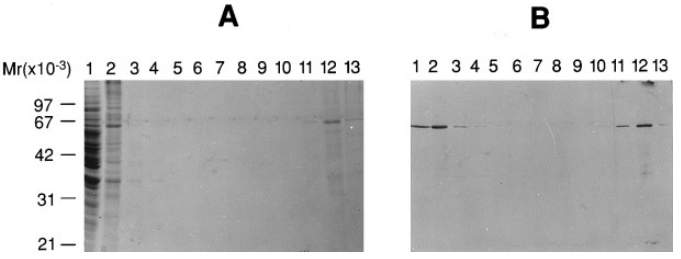

FIG. 6. RPE65 binds to immobilized artificial membrane matrix. A 0.3% Triton X-114 extract of RPE cells was incubated with 50 mg of IAM.PC matrix. The matrix was then washed with buffer containing 0% detergent , followed by washes with buffer containing from 0.01 to 3% Triton X-114. (A) Coomassie blue-stained gel; (B) immunoblot. Lane 1, detergent-free extract of RPE cells; lane 2, unbound material; lanes 3, 4, 5, and 6, 0% detergent wash; lane 7, 0.01% Triton X-114 wash; lane 8, 0.05%; lane 9, 0.1%; lane 10, 0.5%; lane 11, 1.0%; lane 12, 2%; and lane 13, 3%.

to bind to PC and other phospholipids in a Ca^{2+}-independent fashion (23). Despite this, the Ca^{2+}-independent, nonspecific nature of phospholipid binding by RPE65 most closely resembles that of the BSP protein family. In general, however, RPE65 does not share homology with any other phospholipid-binding proteins.

This affinity for liposomes also provided a possible means for its purification using immobilized artificial membrane chromatography (IAM). IAM chromatography (24) is a solid-phase membrane mimetic system that has been successfully used to purify a number of membrane proteins including cytochrome P450s (24) and cholesterol-transfer protein (25). In IAM supports, monolayers of amphiphilic membrane lipid molecules (e.g., dimyristyl-PC) are covalently bonded to silica via the lipid-alkyl chain (26). The protein interacts first with the PC head group and perhaps subsequently with the acyl groups. RPE65 bound quite strongly to the support, being eluted only at the limiting detergent concentration, allowing for the removal of most contaminating proteins, even from a crude extract of RPE cells. These experiments also provided further evidence of the phospholipid affinity of RPE65.

Membrane association, as evidenced by the association of RPE65 with RPE microsomes and by the experiments described here, is likely to be of importance to the function of RPE65. In view of the apparent RPE specificity of RPE65 (3, 8), attention may be focused on possibly RPE-specific functions involving phospholipids, such as possible recycling of OS phagosome-derived phospholipids (27). The mechanism for this enrichment is not known, but is likely to involve specific binding proteins. Other important functions are the transfer of fatty acyl groups from PC to retinol as catalyzed by the lecithin:retinol acyl transferase (LRAT) of the RPE (28, 29) and the isomerohydrolase reaction converting all-*trans* retinyl ester to the 11-*cis* retinol isomer (30).

Phospholipid vesicle aggregation is a valuable model system for a wide variety of important cellular functions. For example, the possible role of the annexin/Ca^{2+}/PS system in exocytosis (31) and the role of myelin basic protein in generating the axonal myelin sheath (32) may be related to the ability of these proteins to aggregate vesicles. The subcellular structure of the outer retina, i.e., the photoreceptor neurons and the RPE cells, is organized around highly specialized membrane structures. Furthermore, membrane vesicles are shed from the photoreceptors, phagocytosed by the RPE, and the components recycled (2). Perhaps there are roles here for proteins competent in recognizing vesicles and capable of inducing aggregation events. The results presented here indicate that a major physical effect of RPE65's association with phospholipid liposomes may be vesicle aggregation. There are two possible explanations for the results presented here (Fig. 4). On the one hand, interaction with lipid may result in the simple self-aggregation of the RPE65 protein resulting in the increase in light scattering observed. On the other hand, RPE65 may interact with the phospholipid vesicles in such as way as to bridge vesicles giving a nucleus which bridges with even more vesicles in a continuing fashion, resulting in a liposome–RPE65 aggregate that is large enough to cause an increase in turbidity. With the present data it is not possible to distinguish between these two possibilities. In the case of the RPE65 results reported here, this putative vesicle aggregation is not accompanied by vesicle fusion such as is seen with the annexin family of proteins (33). Vesicle fusion was assayed for by resonance energy transfer (15). The premise of this experiment is that if vesicle fusion between NBD-PE/Rh-PE/DPPC liposomes and RPE65-loaded DPPC liposomes occurs, then the dilution effect will reduce the efficiency of emission photon energy transfer from the NBD for excitation of the Rh fluorophore. This will result in a decrease in Rh emission and an increase in the untransferred NBD emission. The results of the resonance energy transfer experiments did not show a decrease in Rh emission and so do not support the occurrence of a vesicle fusion event subsequent to an aggregation event. This is not without precedent since vesicle aggregation with lack of membrane fusion is also seen in the cytochrome $P450_{scc}$/dioleoyl-PC system where the binding of the $P450_{scc}$ protein to the membrane provides it with access to its substrate cholesterol and may correlate with the highly vesiculated structure of adrenal and ovarian mitochondria to where cytochrome $P450_{scc}$ is localized and highly abundant (34). If the aggregation hypothesis is correct, the association of RPE65 with phospholipids may represent an analogous mechanism, allowing access of RPE65 to a membrane-associated substrate in the RPE cell. Parenthetically, RPE cells are packed with a very vesiculated smooth endoplasmic reticulum. The subcellular localization of RPE65 is consistent with association with the smooth endoplasmic reticulum, a major component of the RPE microsomal membrane fraction of which RPE65 is the major protein (3). It is reasonable to speculate that the phospholipid association/vesicle-aggregating property of RPE65 may play some role in the function of RPE65 and of the RPE smooth endoplasmic reticulum. Further experiments to identify a possible role are underway.

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