Specificity of Binding of all-*trans*-Retinyl Ester to RPE65[†]

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ABSTRACT: Membrane-bound RPE65 (mRPE65) is a binding protein for all-*trans*-retinyl esters, which are the substrates for the isomerization reaction that completes the visual cycle. RPE65 is essential for rhodopsin regeneration and, hence, for vision. As RPE65 appears to be part of the rate-limiting pathway in the visual cycle, specific antagonists of the molecule will be important in evaluating its full physiological role. The protein is known to stereoselectively bind all-*trans*-retinyl esters (tREs), with dissociation constants in the 50 nM range. This study explores the overall binding specificity of RPE65 with respect to both retinoids and other isoprenoids in an effort to define the specificity of binding, and to begin the process of designing specific antagonists for it. The nature of the specificity directed toward the three main structural elements (retinoid, linker, and acyl moieties) in the tRE molecule is reported. In the all-*trans*-retinyl ester series, binding affinity increased as a function of the hydrophobicity of the fatty acyl group. In the linker region, binding affinities were little affected by amide, ketone, and ether replacements for the carboxy ester moiety of the naturally occurring tRE ligand. Finally, modifications in the all-*trans*-retinoid moiety are also tolerated. For example, E,E-farnesyl palmitate binds with approximately the same affinity as does all-*trans*-retinyl palmitate. Other isoprenoid analogues also bind, as do truncated retinoids in the β-ionone series. Therefore, mRPE65 is a moderately specific retinoid binding protein directed at long chain all-*trans*-retinyl esters.

The vertebrate visual cycle is comprised of those biochemical reactions which begin with the photoisomerization and hydrolysis of the 11-*cis*-retinal Schiff base chromophore of rhodopsin in the photoreceptors to all-*trans*-retinal, and ends with the processing of all-*trans*-retinyl esters (tREs)[1] in the retinal pigment epithelium (RPE) to form 11-*cis*-retinol (al) [cROL(AL)] (Scheme 1). The initiation of the phototransduction cascade by the bleaching of rhodopsin has been intensively studied over the years (*1*−*5*). The key event here is the photochemical cis−trans photoisomerization, leading to the functionally active conformer(s) of rhodopsin (Meta II) (*6*−*8*). Meta II initiates the cascade of biochemical events leading to photoreceptor hyperpolarization.

For vision to proceed after bleaching, 11-*cis*-retinal must be regenerated biochemically in the eye. The biochemical reactions that carry out the enzyme-catalyzed trans → cis conversion are confined to the retinal pigment epithelium (RPE) (*9*, *10*). Although not all of the components that comprise the visual cycle have been identified, it is thought that the isomerization process requires a minimal three-component system in the RPE (Scheme 1) comprised of lecithin retinol acyl transferase (*11*) (LRAT), mRPE65 (*12*, *13*), a binding protein for all-*trans*-retinyl esters (tREs) (*14*−*16*), and an isomerohydrolase activity (IMH), which processes tREs into 11-*cis*-retinol (*17*−*19*).

RPE65 is a critical component of the minimal three-component system required for isomerization. RPE65 is a major protein expressed in the retinal pigment epithelium (RPE) (*12*, *13*). The protein plays an essential role in visual cycle function as revealed by knockout studies in mice. These studies show that RPE65 knockouts are unable to synthesize 11-*cis*-retinoids, but instead accumulate hydrophobic tREs (*20*). Studies using retinoid-based affinity biotinylation of RPE65 have revealed its function as a tRE binding protein (*14*). Quantitative fluorescence measurements showed that the membrane-associated form of RPE65 (mRPE65), which is S-palmitoylated at three cysteine residues, stereospecifically and saturably binds tRP with a K_d of 47 nM (*15*, *21*). tROL and 11-*cis*-retinoids are bound weakly (*15*, *21*). The binding of tREs by mRPE65 is functionally significant because it is important for their processing by IMH (*16*). mRPE65 has been reported not to possess intrinsic isomerase activity itself, but tRP bound to the protein is processed to 11-*cis*-retinol by the addition of added RPE membrane-associated IMH (*16*). In aggregate, these studies leave little doubt that mRPE65 is a specific tRE binding protein which has an essential role in mobilizing hydrophobic tREs, thus

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[1] Abbreviations: BIP, β-ionoacetyl palmitate; BIHE, β-ionoacetyl hexadecyl ether; CNS, central nervous system; cROL(AL), 11-*cis*-retinol(al); DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FP, E,E-farnesyl palmitate; GGP, E,E,E-geranylgeranyl palmitate; GP, E-geranyl palmitate; IMH, isomerohydrolase; LRAT, lecithin retinol acyltransferase; mRPE65, membrane-bound RPE65; NPR, *N*-palmitoyl-all-*trans*-retinamide; PBS, phosphate-buffered saline; RHE, all-*trans*-retinyl hexadecyl ether; RPE, retinal pigment epithelium; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; sRPE65, soluble RPE65; tRA, all-*trans*-retinyl acetate; tRE, all-*trans*-retinyl ester; tRH, all-*trans*-retinyl hexanoate; tRP, all-*trans*-retinyl palmitate; tROL, all-*trans*-retinol; tROH, vitamin A.

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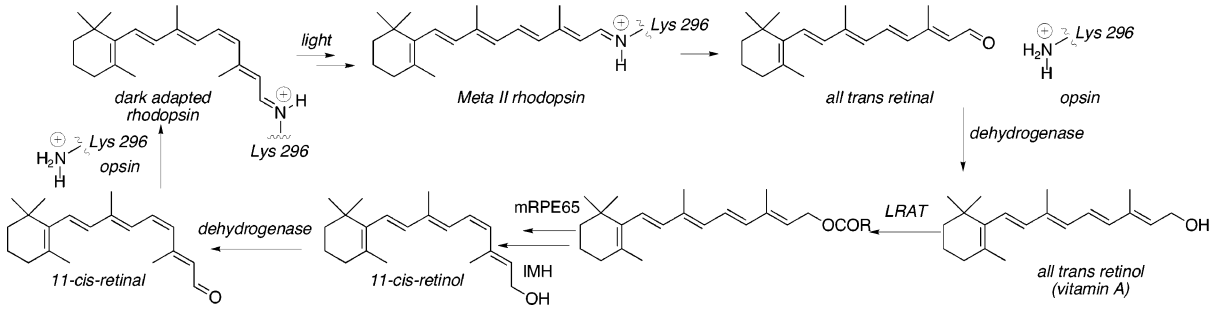
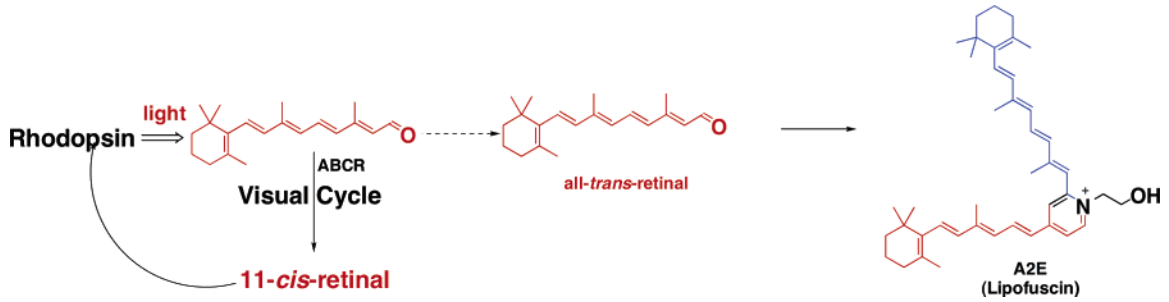
Scheme 1: Mammalian Visual Cycle



Scheme 2: Formation of Lipofuscin A2E



allowing them to enter the visual cycle. In addition, RPE65 is of regulatory significance in the operation of the visual cycle. The protein plays an important regulatory role as a gatekeeper, controlling retinoid flux in the visual cycle by switching between mRPE65 and a soluble form of RPE65 (sRPE65) which preferentially binds tROL rather than tRE (*21*). The flow of all-*trans*-retinoids can be directed to storage or processing into 11-*cis*-retinoids by this switch (*21*). This requires that RPE function be part of the rate-limiting step in the operation of the visual cycle.

Specific antagonists directed against RPE65 are likely to be of substantial interest for defining the precise role of this protein in the temporal regulation of visual cycle function. It has been shown that 13-*cis*-retinoic acid (Accutane), a drug used in the treatment of acne, binds to and inhibits mRPE65 (*22*), in addition to inhibiting 11-*cis*-retinol dehydrogenase (*23*). One of the functional consequences of inhibition of mRPE65 by 13-*cis*-retinoic acid is the inhibition of rhodopsin regeneration (*24*). This observation, along with other data, supports a role for RPE65 in the rate-limiting step in the visual cycle (*24*−*26*). Specific RPE65 antagonists may also be of interest as potential drugs for the treatment of certain forms of macular degeneration.

The specific inhibition of visual cycle function and RPE65 may be of substantial medical utility for the inhibition of formation of the lipofuscins in the A2E family and the treatment of macular degeneration, a leading cause of blindness (*27*−*29*). The highly retinotoxic lipofuscins are formed during the operation of the visual cycle (Scheme 2) (*28*). Inhibition of excessive flux through the visual cycle would be an ideal approach to preventing A2E formation. The retinoic acids can inhibit lipofuscin accumulation in animals (*29*), but are unlikely useful as drugs because of their known very substantial toxicities, which includes teratogenicity and CNS and dermatological toxicities (*30*). Therefore, it is of some interest to begin to determine which features of tREs are essential for potent binding to mRPE65. These studies will both explore the chemical basis of specificity for mRPE65 and serve as a starting point for the design of specific and nontoxic antagonists of mRPE65 as possible drug candidates for the treatment of macular degeneration. Using a specific and quantitative fluorescence assay, the essential structural features required for binding of ligand to mRPE65 are determined.

EXPERIMENTAL PROCEDURES

Materials

Frozen bovine eye cups devoid of retinas were purchased from W. L. Lawson Co. (Lincoln, NE). EDTA, phenyl-Sepharose CL-4B, tROL, tRP, tRA, and Trizma base were from Sigma-Aldrich. DTT was from ICN Biomedicals Inc. Anagrade CHAPS was from Anatrace. HPLC grade solvents were from Sigma-Aldrich Chemicals. Anti-RPE65 (NFIT-KVNPETLETIK) antibody was obtained from Genmed Inc. Broad spectrum EDTA-free protease inhibitor cocktail was obtained from Roche Biosciences. The precast gels (4 to 20%) for SDS−PAGE, BenchMark prestained molecular weight markers were from Invitrogen. DEAE-Sepharose was from Amersham Biosciences. All reagents were analytical grade unless specified otherwise.

Methods

Purification of mRPE65. mRPE65 was extracted and purified from the bovine eye cups using a procedure described previously (*31*). The purity of RPE65 was verified by silver staining and Western blotting.

Syntheses of the mRPE65 Ligands. All the work with retinoids was conducted under dim red light. Unless otherwise specified, the column used in HPLC was a 5 μm PVA-Sil (250 mm × 4.6 mm, YMC−Waters Corp) normal phase HPLC (NP-HPLC) column, and the mobile phase was hexane and dioxane (93:7, v/v) with a flow rate of 1.5 mL/min. NMR spectra were recorded on a Varian 200 MHz NMR instrument.

all-trans-Retinyl hexanoate (tRH) was prepared by the published procedure (*32*).

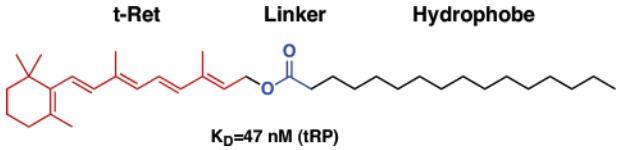
N-Palmityl-all-trans-retinamide (NPR) was synthesized as follows. Isobutyl chloroformate (1.2 equiv) and *N*,*N*-diisopropylethylamine (2 equiv) were added to a solution of all-*trans*-retinoic acid (1 equiv) in dichloromethane (5 mL/mmol of acid) at 0 °C and stirred for 2 h. Hexadecylamine (1.5 equiv) was added and stirring continued for 2 h. The solution was then diluted with dichloromethane (5 mL), washed with a saturated aqueous solution of $NaHCO_3$, NH_4Cl, and NaCl, and dried with sodium sulfate. The solvent was evaporated, and the compound was purified first by silica column chromatography followed by the HPLC method: HPLC retention time 3.53 min; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.13 (dd, J = 15, 15 Hz, 1H), 6.38−6.10 (m, 4H), 5.75 (s, 1H), 3.84 (t, J = 6 Hz, 2H), 2.39 (s, 3H), 2.02 (s, 6H), 1.93−1.86 (m, 2H), 1.71 (s, 3H), 1.65−1.40 (m, 4H), 1.27−1.20 (m, 30H), 0.99−0.96 (s, s, 6H); UV−vis (EtOH) λ_{max} 373 nm; ESI found [M + Na] m/z 546.4635, $C_{36}H_{61}ON$ requires [M + Na] m/z 546.4635.

all-trans-Retinyl hexadecyl ketone (RHK) [3,7-dimethyl-1-(2,6,6-trimethylcyclohex-1-enyl)pentacosa-1,3,5,7-tetraen-9-one] was synthesized as follows. Hexadecyl iodide (1.1 equiv) was dissolved in tetrahydrofuran (2.2 mL) and cooled to −78 °C followed by the addition of *tert*-butyllithium (2 equiv) and stirred for 2 h. all-*trans*-Retinal (1 eq) dissolved in tetrahydrofuran (1 mL) was added to the reaction mixture and stirring continued for 30 min, after which it was warmed to room temperature. The reaction was quenched with saturated aqueous ammonium chloride and the mixture extracted with hexane. The combined extracts were washed with brine, dried with $MgSO_4$, and evaporated by reduced pressure to give the alcohol. Dess−Martin periodinate (1.1 equiv) was added to a solution of alcohol in dichloromethane (25 mL/mg of alcohol) at room temperature and stirred for 10 min. The reaction mixture was then treated with a sodium thiosulfate/sodium bicarbonate solution (1:1, v/v, 10% sodium thiosulfate and aqueous saturated sodium bicarbonate) and stirring continued for an additional 10 min. Water was added and the reaction mixture extracted with hexane, washed with brine, and dried with $MgSO_4$, and the combined extracts were evaporated under reduced pressure. The crude product was first purified with silica column chromatography followed by HPLC: HPLC retention time 2.57 min; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.35 (dd, J = 13, 15 Hz, 1H), 6.17−5.93 (m, 4H), 5.35−5.25 (m, 1H), 2.41 (t, J = 6 Hz, 2H), 2.01−1.90 (m, 2H), 1.99 (s, 3H), 1.79 (s, 3H), 1.69 (s, 3H), 1.47−1.10 (m, 32H), 1.01 (s, 3H), 0.90 (s, 6H); UV−vis (EtOH) λ_{max} 362 nm; ESI found [M + Na] m/z 531.4542, $C_{36}H_{60}O$ requires [M + Na] m/z 531.4536.

all-trans-Retinyl hexadecyl ether (RHE) [2-(9-hexadecyloxy-3,7-dimethylnona-1,3,5,7-tetraenyl)-1,3,3-trimethylcyclohexene] was prepared by the published route (*33*).

β-Ionoacetyl palmitate (BIP) [hexadecanoic acid 3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienyl ester] and its corresponding ether (BIHE) [2-(5-hexadecyloxy-3-methylpenta-1,3-dienyl)-1,3,3-trimethylcyclohexene] were synthesized from β-ionoacetyl alcohol, obtained from β-ionone by a literature preparation (*34*) using the same synthetic protocol as for the corresponding retinoids. β-Ionoacetyl palmitate (BIP): HPLC retention time 2.35 min; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.10 (d, J = 15 Hz, 1H), 6.02 (d, J = 16 Hz, 1H), 5.52 (t, J = 8 Hz, 1H), 4.70 (d, J = 8 Hz, 2H), 2.50−2.20 (m, 3H), 2.18−1.86 (m, 1H), 1.82 (s, 3H), 1.68 (s, 3H), 1.66−1.50 (m, 4H), 1.38−1.12 (m, 26H), 1.01 (s, 6H), 0.82 (s, 3H); UV−vis (EtOH) λ_{max} 264 nm. β-Ionoacetyl palmityl ether (BIPE): HPLC retention time 9.22 min [in the YMC-PVA SIL NP, 250 mm × 10 mm column and with a mobile phase of hexane and dioxane (93:7) with a flow rate of 5 mL/min]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.20−5.90 (m, 2H), 5.66 (t, J = 9 Hz, 1H), 4.70 (d, J = 9 Hz, 2H), 3.13 (d, J = 6 Hz, 2H), 2.18−1.80 (m, 2H), 1.58 (s, 3H), 1.42 (s, 3H), 1.40−1.36 (m, 32H), 1.16 (s, 6H), 0.86 (s, 3H); UV−vis (EtOH) λ_{max} 235 nm.

Scheme 3: Three Elements in the Structure of all-*trans*-Retinyl Palmitate (tRP)



Geranyl palmitate (GP), farnesyl palmitate (FP), and geranylgeranyl palmitate (GGP) were prepared by published procedures (*35*−*37*).

Fluorescence Binding Assays. mRPE65 in PBS and 1% CHAPS (pH 7.4) was used in the fluorometric titration studies. All titrations were performed at 25 °C. The samples in PBS buffer were excited at 280 nm, and the fluorescence was scanned from 300 to 500 nm. Fluorescence measurements, using 450 μL quartz cuvettes with a path length of 0.5 cm, were taken at 25 °C on a Jobin Yvon Instruments, Fluoromax 2 apparatus employing the right-angle detection method.

The fluorescence of the protein solution was measured after equilibrating it at 25 °C for 10 min. The sample was then titrated with a solution of retinoid dissolved in DMSO in the absence of any overhead light, and the solution was mixed thoroughly before fluorescence measurement. In each titration, to a 300 μL solution of the protein was added an equal amount of retinoid, typically 0.3 μL, and the solution was thoroughly mixed before it was allowed to equilibrate for 10 min prior to the recording of the fluorescence intensity. The addition of DMSO (0.1% per addition) did not have any effect on the fluorescence intensity. The binding constant (K_D) was calculated from the fluorescence intensity as described previously (*15*, *21*).

RESULTS

Specificity of Binding of Ligand to mRPE65. Studies of binding of tRP, the major retinyl ester found in the RPE, to membrane-bound RPE65 (mRPE65) revealed a dissociation constant of 47 nM (*15*, *21*). In the studies presented here, we focused on the importance of three elements of tRP structure in binding to mRPE65 (Scheme 3). The three elements consist of the hydrophobic acyl moiety, the linker element, and the *trans*-retinoid moiety. The three salient elements shown in Scheme 3 are considered, and their contributions to the binding affinity for mRPE65 are determined.

Initial experiments focused on the importance of the hydrophobic fatty acyl moiety in binding to mRPE65. This is an obvious moiety to probe, since it is already known that mRPE65 binds tROH relatively weakly compared to tRP (*15*,

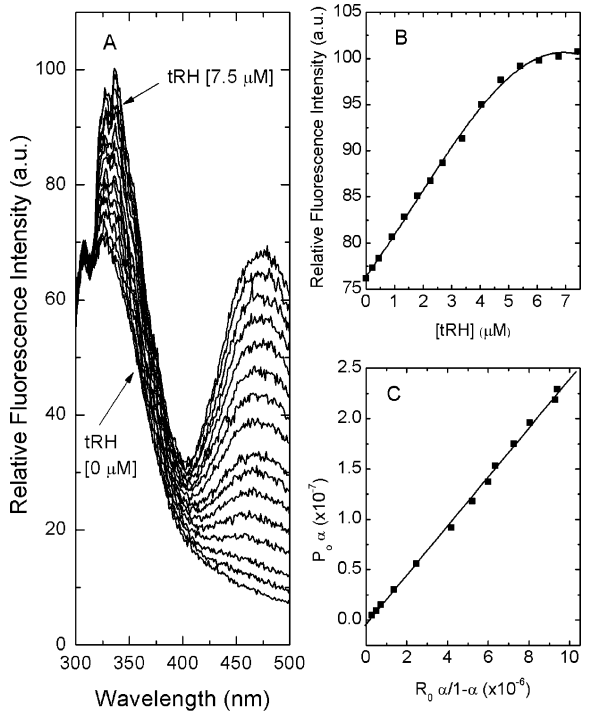


FIGURE 1: Fluorescence titration of mRPE65 with all-*trans*-retinyl hexanoate (tRH). The excitation wavelength was at 280 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 0.24 μM mRPE65 in 100 mM phosphate-buffered saline (150 mM NaCl) (pH 7.4) and 1% CHAPS. Panel A shows the emission spectra of mRPE65 with increasing concentrations of tRH. Panel B shows the change in the fluorescence intensity at 328 nm with increasing concentrations of tRH. Panel C shows the linear square fit plots of the relation $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$ for the titration of mRPE65 vs tRH.

Table 1: Binding of Analogues to mRPE65[a]

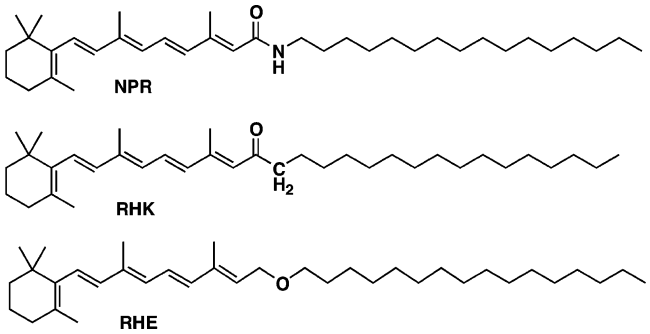
analogue	$K_D \pm$ SD (nM)
all-*trans*-retinyl palmitate (tRP)	47 ± 3[b]
all-*trans*-retinyl hexanoate (tRH)	162 ± 7
all-*trans*-retinyl acetate (tRA)	1300 ± 42
N-palmityl all-*trans*-retinamide (NPR)	50 ± 4
all-*trans*-retinyl hexadecyl ketone (RHK)	34 ± 3
all-*trans*-retinyl hexadecyl ether (RHE)	25 ± 4
β-ionoacetyl palmitate (BIP)	153 ± 23
β-ionoacetyl hexadecyl ether (BHE)	156 ± 23
geranyl palmitate (GP)	301 ± 30
farnesyl palmitate (FP)	63 ± 1
geranylgeranyl palmitate (GGP)	213 ± 7

[a] Binding studies with the analogues were carried out as reported in Materials and Methods. [b] This value was previously reported (*15*, *21*).

21). The binding of all-*trans*-retinyl esters of intermediate hydrophobicity to mRPE65 was probed. Here the hexanoate and acetate esters were studied (tRH and tRA, respectively). Figure 1 shows a typical saturable fluorescence binding isotherm for binding of all-*trans*-retinyl hexanoate to mRPE65. The all-*trans*-retinyl esters quench protein fluorescence and exhibit their own fluorescence through energy transfer (*15*, *21*). As can be seen here, and in Table 1, the binding affinities for the less hydrophobic retinyl esters are weaker than for tRP. Affinities seem to be determined, in a graded way, by the hydrophobicity of the fatty acyl chain. This pattern is also observed in non-retinyl ester binding ligands (P. Maiti and R. R. Rando, unpublished experiments).

The importance of the linker element was probed next. Here we studied the binding of the cognate amides, ketones, and ether substitutions in the palmitate series (Scheme 4). The saturable binding curves for the retinyl amide (NPR) and ketone (RPK) are shown in Figures 2 and 3, respectively. In these instances, only the quenching of protein fluorescence is observed. The binding data for the various palmitate equivalents of the amides, ketones, and ethers (RHE) are recorded in Table 1. These data show that an ester linker moiety is not essential for binding of ligand to mRPE65. Moreover, if the palmitoyl equivalents are all compared (Scheme 4 and Table 1), there is only an approximately 2-fold spread in binding affinities among esters, amides, ketones, and ethers. Thus, it is concluded that the linker moiety does not make highly specific contacts with mRPE65.

Scheme 4: Amide, Ketone, and Ether Retinoid Analogues



Previous studies showed that mRPE65 stereoselectively binds tRP when compared to its 11-*cis* congener (*15*, *21*). The importance of the all-*trans*-retinyl moiety for binding

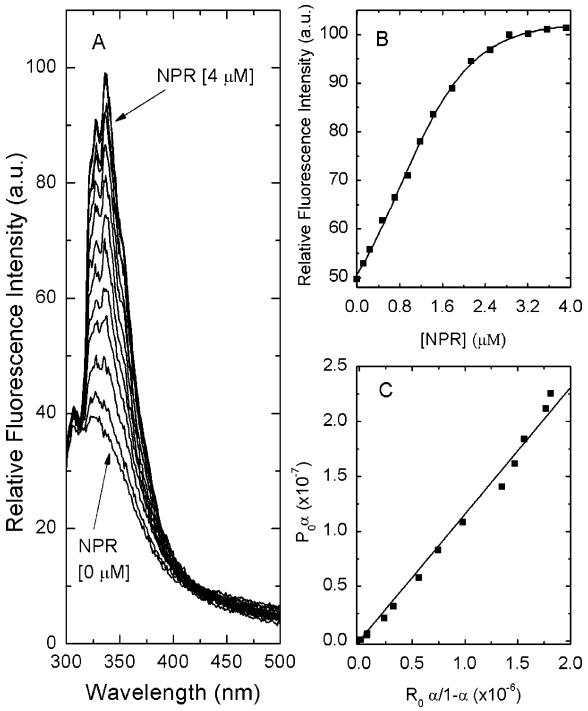


FIGURE 2: Fluorescence titration of mRPE65 with *N*-palmityl all-*trans*-retinimide (NPR). The excitation wavelength was at 280 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 0.24 μM mRPE65 in 100 mM phosphate-buffered saline (150 mM NaCl) (pH 7.4) and 1% chaps. Panel A shows the emission spectra of mRPE65 with increasing concentrations of NPR. Panel B shows the change in the fluorescence intensity at 328 nm with increasing concentrations of NPR. Panel C shows the linear square fit plots of the relation $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$ for the titration of mRPE65 vs NPR.

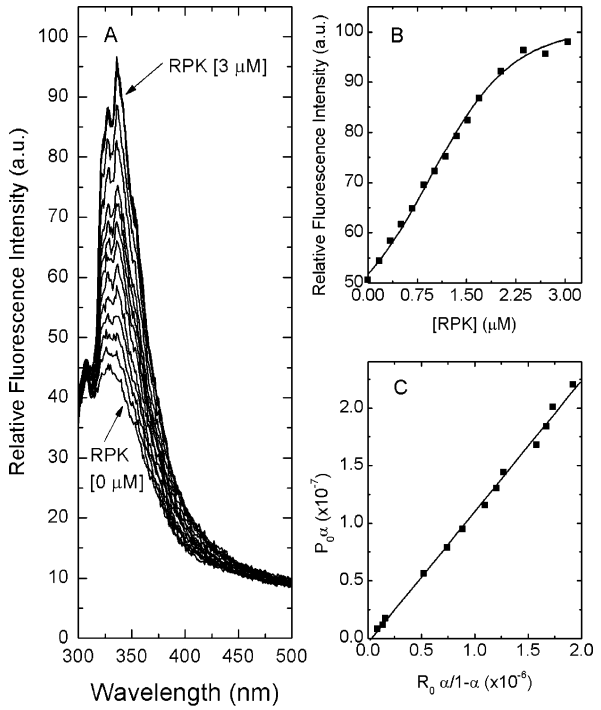
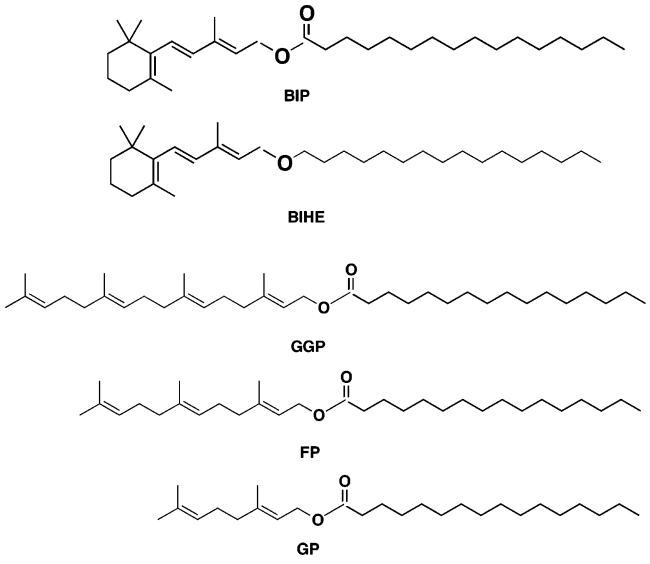


FIGURE 3: Fluorescence titration of mRPE65 with all-*trans*-retinyl palmitoyl ketone (RPK). The excitation wavelength was at 280 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 0.24 μM mRPE65 in 100 mM phosphate-buffered saline (150 mM NaCl) (pH 7.4) and 1% CHAPS. Panel A shows the emission spectra of mRPE65 with increasing concentrations of RPK. Panel B shows the change in the fluorescence intensity at 328 nm with increasing concentrations of RPK. Panel C shows the linear square fit plots of the relation $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$ for the titration of mRPE65 vs RPK.

Scheme 5: Non-Retinoid Analogues of mRPE65 Ligands



affinity was further probed. The binding of two sets of substitutions for the all-*trans*-reinoid moiety was studied. In the initial set, β-ionoacetyl palmitate (BIP) (Scheme 5) along with its ether equivalent (BIHE) was studied. The data for the binding of these analogues to mRPE65 are listed in Table 1. Clearly, the truncated retinoids bind with significant affinities, although the measured affinities are approximately 3-fold lower than for the cognate molecules in the all-*trans*-retinoid series. The fact that the ether analogue does bind shows again that there is flexibility in the nature of the linker element.

To further probe the nature of the specificity directed toward the *trans*-retinoid moiety, three standard isoprenoid substitutions were introduced. The isoprenoid substitutions [E-geranyl (GP), E,E-farnesyl (FP), and E,E,E-geranylgeranyl (GGP)] were studied (Scheme 5 and Table 1). It is clear that these molecules can effectively bind as well. The highest-affinity binding occurs with the E,E-farnesyl palmitate ester.

DISCUSSION

RPE65 is an essential component in visual cycle function and stereoselectively binds long chain tREs with high affinity (*14*−*16*). Physiological studies show that RPE65 knockout mice inappropriately accumulate tREs in oil droplets, a result consistent with RPE65 having an essential role in the mobilization of tREs (*14*−*16*). tREs are exceedingly hydrophobic, with limited solubility in aqueous solutions. This can easily be seen in measurements of intermembraneous transfer of retinoids (*38*, *39*). While retinol(al) undergoes interliposomal transfer at exceedingly rapid rates, tREs are inert to transfer (*38*, *39*). Thus, tREs require a binding protein to mobilize them if they are to enter the visual cycle productively. Since binding proteins are required stoichiometrically, they are required in substantial quantities. The substantial level of RPE65 in RPE membranes (*12*, *13*), coupled with its ability to bind tREs, suggests that mRPE65 has a role in binding to and mobilizing tREs for processing in the visual cycle by IMH. Functional experiments show that the role of mRPE65 in the visual cycle is to specifically bind to and mobilize tREs so that they can be processed by IMH (*16*). It has been suggested by some that RPE65 may actually be the IMH because of the homologous relationship between RPE65 and a carotene oxidase (*40*). In this particular study, the X-ray structure of a carotene oxidase is presented. Model building suggests to the authors that a 13,14:13′,14′-di-*cis*-carotene is on the reaction pathway for oxidation to fully all-*trans*-cleavage products (*40*). The latter fact is a key point, because the oxidase only generates all-*trans* products, and therefore is not an isomerase. It cannot be concluded that RPE65 is an isomerase because it shares homology with a protein which may or may not bind *cis*-containing structures, but which does not generate *cis* products, as determined experimentally (*40*). Furthermore, purified RPE65 does not exhibit catalytic activity toward all-*trans*-retinyl esters (*16*). It is known that RPE65 is necessary for 11-*cis*-retinoid generation in vivo (*20*), but it has not been demonstrated that it is necessary and sufficient. The binding studies described here, especially with respect to the lack of specificity directed toward the linker moiety, are inconsistent with the notion that RPE65 is an enzyme.

Little is known about the ligand binding specificity of RPE65 other than its known stereoselectively with respect to the all-*trans* configuration (*15*, *21*). In this study, an exploration into the retinoid binding specificity of mRPE65 is initiated to fill this gap in our knowledge about this important retinoid binding protein. Binding studies, which reveal the specificity of protein−ligand interactions, are also important in deciding whether a protein might have an additional activity. For example, if RPE65 were to have an enzymatic activity requiring a carboxy ester moiety, then this

element might be expected to figure importantly in binding affinity. In addition, quantitative binding studies also serve as the basis of specific antagonist design.

In this study, the contributions of the three central elements of tREs to binding to mRPE65 are addressed here. When the role of the acyl moiety was probed it was found that hydrophobicity is important for high-affinity binding. The binding affinities were in the following order: tRP > tRH > tRA. This is in accord with the understanding that vitamin A (tROH) binds only relatively weakly to the protein compared to its palmitate ester (*15*, *21*). It is possible that a palmitate moiety of the mRPE65 directly interacts with the palmitate moiety of the retinyl ester, explaining the increased affinity for the latter.

The role of the ester linker moiety in binding was also probed for its importance. The palmitoylated amide (NPR), ketone (RHK), and ether (RHE) replacements were studied. Surprisingly, it was found that there was very little in the way of specificity directed toward these moieties: they are quantitatively replaceable within a factor of approximately 2, as defined by their affinities for mRPE65. It should be noted that with these analogues, the same effect of hydrophobicity is noted. For example, in the retinyl ether series, while RHE binds with a K_D of 25 nM (Table 1), its hexyl and methyl equivalents bind with K_D values of 151 and 424 nM, respectively (data not shown).

Finally, the importance of the all-*trans*-retinoid moiety was probed by studying two analogues in the β-ionoacetyl series and three isoprenoid substitutions. It is already known that the all-*trans*-retinoid configuration is strongly favored in the tRE series (*15*, *16*). However, latitude in binding specificity is found in the binding of "*trans*" isoprenoids. In this case, the E,E-farnesyl ester (FP) bound with approximately the same affinity as tRP itself. In the isoprenoid palmitate series, binding was maximal with the farnesyl (C15) substitution. Both the E-geranyl and E,E,E-geranylgeranyl analogues were bound with lower affinities. Interestingly, in the isoprenoid series, the more hydrophobic C20 geranylgeranyl derivative bound with a lower affinity than did its farnesyl (C15) counterpart. The two β-ionoacetyl analogues were studied as truncated retinoid analogues. Both β-ionoacetyl replacements bound with appreciable affinities, although the level of binding was reduced when compared to those of their retinoid counterparts.

Altogether, the structure−activity studies show substantial, although not exceptionally high, binding specificity. There is virtually no specificity directed toward the linker moiety, suggesting no highly specific interactions directed toward it and making it unlikely that mRPE65 shows any latent enzymatic activity involving the carboxy ester. However, binding specificity is directed toward the all-*trans* configuration in the retinoid (isoprenoid) and the hydrophobicity of the acyl ester moiety. Given the physiological milieu in which mRPE65 operates, these specificities are, of course, the most relevant.

Because of the essential role of mRPE65 in the visual cycle, antagonists of it could be both mechanistically and clinically important. The studies described here indicate how such molecules might be designed. The retinoic acids bind to mRPE65 (*22*) and also 11-*cis*-retinol dehydrogenase (*23*), and consequently limit the visual cycle (*24*). Because these molecules inhibit two different activities in the visual cycle, they are not useful probes for dissecting the quantitative, physiological importance of either activity in the operation of the visual cycle. A pure mRPE65 antagonist is of much greater interest along these lines. Furthermore, non-retinoid mRPE65 antagonists could be of great interest clinically in preventing lipofuscin formation. It is already known that the retinoids retard the formation of the retinotoxic lipofuscins as a consequence of their abilities to interfere with visual cycle function (*24*, *29*). Lipofuscins in the A2E series (*28*, *41*) are thought to be causative of certain forms of macular degeneration, a leading cause of blindness in industrial countries (*28*, *41*−*43*). While the substantial toxicities of the retinoic acids probably eliminate them from consideration as potential long-term therapies for persons suffering from macular degeneration, nontoxic retinoid surrogates might be ideal candidates. The studies reported here suggest that the design of these kinds of molecules is possible. Studies along these lines are presently ongoing.

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