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G239T mutation in Repeat 1 of human IRBP: Possible implications for more than one binding site in a single repeat

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Purpose: Interphotoreceptor retinoid-binding protein (IRBP) is a four-repeat protein found in the interphotoreceptor space. Each repeat can bind retinoids and fatty acids. The purpose of this study was to examine the effects of the single amino acid substitution, G239T, versus the wild type sequence of human IRBP Repeat 1, on ligand binding at equilibrium, ligand off rates, and protection of retinol from degradation.

Methods: G239T was created by site-specific mutagenesis, expressed in E. coli, and purified. E. coli expressed wild type Repeat 1 (EcR1) and G239T were subjected to thermal denaturation and analyzed by circular dichroism spectroscopy. We compared the ligand binding properties by fluorescence enhancement of retinol and 16-anthryloxy-palmitate (16-AP), tryptophan quenching of the proteins by different ligands, binding competition assays, protection of retinol from degradation, and stopped-flow kinetics to measure transfer of ligands to and from model membranes.

Results: Circular dichroism, fluorescence, and absorbance spectroscopy of G239T and EcR1 showed similar wavelength scans. G239T exhibited about three-fold less fluorescence of bound all-trans-retinol or 13-cis-retinol versus EcR1. Retinol quenching of intrinsic protein fluorescence was reduced by 37% in G239T versus EcR1. Other retinoids used as quenchers produced no difference between intrinsic protein fluorescence of either G239T or EcR1; all exhibited saturable high affinity binding to each protein. Docosahexaenoic acid (DHA) served as a competitive inhibitor of retinol fluorescence enhancement with EcR1. However, DHA did not alter retinol fluorescence with G239T. 16-AP exhibited about 30% higher levels of fluorescence enhancement when bound to G239T versus EcR1. EcR1 prevented oxidative damage of all-trans-retinol whereas G239T provided much less protection. Each protein could accept 9-cis-retinol from small unilamellar vesicles (SUVs) as measured by stopped flow kinetics. Off rates were the same in comparing G239T and EcR1 as acceptors.

Conclusions: Despite the general similarity in shape between G239T and EcR1 and the nearly identical binding behavior with some ligands, distinct differences exist in the ligand binding properties of G239T and EcR1. Fluorescence enhancement/ quenching and retinol protection experiments suggest that retinol binding is reduced by about 50% in G239T versus EcR1. The data suggest that either: (1) EcR1 contains two binding sites for retinol and G239T has lost one site or (2) EcR1 has a single binding site that is altered in G239T to reduce retinol binding. Results of all the experiments were consistent with the first model while some of the data were not consistent with the second model. Thus, it is possible that position 239, found in Domain B2 of IRBP Repeat 1, is located in or near one of two retinol binding sites.

Interphotoreceptor retinoid-binding protein (IRBP) is a retinoid and fatty acid binding protein located in the space between photoreceptor cells and the retinal pigment epithelium. IRBP may be involved with the translocation of visual cycle retinoids [1-4], though possibly as a buffer [5,6] or even an inhibitor [7,8]. IRBP has a four-fold repeat structure [9-11]. Each repeat in IRBP appears to contain two domains, A and B [12]. Domain B may be further resolved into B1 and B2 [12]. IRBP is a member of a sequence related protein family (the C-terminal proteases, CTPs) with a conserved domain for cleaving C-terminal hydrophobic amino acids related to Domain B [13,14]. Domain A of IRBP is not similar to the CTPs but instead may be related to a canonical protease inhibitor, eglin c [12]. Each Repeat of human IRBP can bind retinoid and fatty acid ligands [15-17].

Determining the stoichiometry of ligand binding to IRBP has been problematic with some reports suggesting as few as one [3,18] or two retinol binding sites [19,20] to as many as 6.5 fatty acids bound per single polypeptide [21]. Our recent data suggest that there is at least one site that binds retinoids and fatty acids per Repeat [16,17]. Even within a single Repeat or Module [22], there may be more than one equivalent binding site as data show that Module 4 of Xenopus IRBP has 2.2 binding sites, a central fragment of Module 4 of Xenopus IRBP contains 1.34 binding sites, and the C-terminal third of this Module contains about 0.7 binding sites [22]. Thus, it is possible that there are two or more binding sites in a single human repeat.

In this report, we sought to compare the ligand binding properties of a particular point mutant in IRBP Repeat 1
(G239T) with the corresponding wild type sequence. This mutation was chosen because this glycine is well-conserved, near at active site, and predicted to be at the end of a β-strand [12]. This lesion caused reduced binding of retinol but had little effect on binding of other ligands. While it was not our intent to directly measure binding stoichiometry, our studies suggest a model in which there are two types of ligand-binding sites in a single repeat. Further, the single point mutation, G239T, resulted in the loss of one type of binding site for retinol, but the other site was unaffected by the single substitution.

METHODS

Ligands: The ligands (Table 1) were purchased from Sigma (St. Louis, MO) or Molecular Probes, Inc. (Eugene, OR) and dissolved in 100% ethanol (Midwest Grain Products of Illinois, Pekin, IL). Concentrations were estimated using the noted extinction coefficients (ε, in dimensions of A/M/cm) at the stated wavelength. All ligands were prepared and used under subdued red light employing a Kodak 1A safelight filter (Eastman Kodak, Rochester, NY) with a 25 W incandescent bulb.

Stopped Flow Kinetic Experiments: Kinetic experiments employed an SFA-20M stopped flow mixing apparatus (HiTech Scientific, Salisbury, UK) attached to a Spex FL2T2 (Instruments SA, Inc., Edison, NJ) spectrophorometer. One barrel of the stopped flow apparatus was filled with phosphatidylcholine small unilamellar vesicles (SUVs) prepared by ethanol injection [23]. The concentration of the SUVs was determined after ashing the phosphatidylcholine by inorganic phosphate assay [24]. The other barrel was filled with protein. The ligand was mixed in advance with either the protein or the SUVs depending on the direction of transfer [17]. For retinol transfer, excitation was set to 330 nm and emission to 479 nm, and transfer from protein to SUVs was measured by recording a change in retinol fluorescence. For 9-cis-retinal as ligand, excitation was set to 280 nm and emission to 336 nm and a change in fluorescence quenching of the protein was measured. Data were analyzed by curve fitting to a double exponential as described previously [17].

TABLE I. LIGANDS USED IN THE STUDY

<table>
<thead>
<tr>
<th>Ligand name</th>
<th>Catalog number</th>
<th>Purity</th>
<th>Molar extinction coefficient</th>
<th>Wavelength</th>
</tr>
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<tbody>
<tr>
<td>all-trans-retinol</td>
<td>R-7632</td>
<td>&gt;95%</td>
<td>46,000</td>
<td>325 nm</td>
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<td>9-cis-retinal</td>
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<td>&gt;95%</td>
<td>36,080</td>
<td>373 nm</td>
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<tr>
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<td>R-2625</td>
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<td>45,000</td>
<td>351 nm</td>
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<tr>
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<td>&gt;90%</td>
<td>48,390</td>
<td>328 nm</td>
</tr>
<tr>
<td>all-trans-retinal</td>
<td>R-2500</td>
<td>&gt;95%</td>
<td>44,000</td>
<td>373 nm</td>
</tr>
<tr>
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<td>R-3255</td>
<td>&gt;98%</td>
<td>39,800</td>
<td>354 nm</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>D-2534</td>
<td>99%</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>16-anthroyloxy-palmitic acid</td>
<td>A-39*</td>
<td>90%</td>
<td>8,200</td>
<td>361 nm</td>
</tr>
</tbody>
</table>

*From Molecular Probes catalog; all others from Sigma catalog

Figure 1. SDS PAGE and monoclonal antibody detection of G239T and EcR1. EcR1 and G239T were cloned, expressed, and purified as previously described [12]. The samples were run on two 10% SDS polyacrylamide gels [34]. In lane 1, EcR1 was mixed with molecular weight markers and the sizes of the markers are shown on the left. In lane 2 purified G239T was loaded. In Lane 3, about ten times the amount of G239T was loaded. (A) shows a Coomassie Brilliant Blue stained gel. (B) shows an immunoblot stained with the monoclonal antibody H3B5 [35]. The results showed that G239T and EcR1 migrated with the same mobility and gave an estimated mass of 36,400 Da. This is in close agreement with the calculated molecular mass of 34,500 Da. This is also compatible with the calculated molecular mass of 34,500 Da. Both EcR1 and G239T showed strong reactivity with the monoclonal antibody specific for Repeats 1 and 2 of human IRBP [35]. The G239T preparations were >90% pure as indicated by the lack of other detectable bands on the protein stained gel. However, an apparently degraded product of G239T, with an estimated molecular mass of 27,500 Da was detected by immunostaining in Lane 3 of B. This band is virtually undetectable in the protein-stained gel.

RESULTS

The results are presented in three groups of experiments: (A) protein structure, (B) binding at equilibrium, (C) putative IRBP functions.

(A) Protein structure: Experiments 1-3 were conducted to control properties of EcR1 versus G239T at the level of protein structure. The hypothesis being tested in these three experiments was that G239T and EcR1 protein preparations possess similar three dimensional shapes, given that they differ...
fer by only one amino acid substitution in the polypeptide and given that they are expressed from identical vectors in the same strain of *E. coli*.

**Experiment 1. Identity of G239T mutant protein:** As shown in Figure 1, G239T and EcR1 reacted with monoclonal antibody H3B5 [27], which is specific for human IRBP Repeats 1 and 2 [16]. G239T and EcR1 had the same mobility on SDS PAGE gels (Figure 1A). Microsequencing of the first 4 amino acids (data not shown) of G239T showed that the purified G239T protein had the same N-terminal sequence as EcR1. Also, nucleic acid sequencing of the G239T expression clone showed that the only difference between this clone and the wild type counterpart lay in the substitution at position G239 (data not shown).

**Experiment 2. Circular dichroism of EcR1 and G239T:** The importance of glycine at position 239 to the structural integrity of the protein was assayed by circular dichroism (CD) spectral analysis shown in Figure 2. The spectra of EcR1 and G239T were roughly the same at 25 °C. Upon thermal denaturation at 95 °C, the final spectra were similar with a 50% decrease in the CD signal (compared to 25 °C), especially near 205-230 nm.

**Experiment 3. Intrinsic protein fluorescence wavelength scans:** After excitation at 280 nm, EcR1 and G239T both had emission maxima at 336 nm and the scans were very similar in shape (data not shown). Denaturation of G239T in 8 M urea yielded a maximum at 358 nm, as does EcR1 (data not shown) and baculovirus-expressed whole IRBP [15].

In summary, these data and those of the previous two experiments suggest that the gross structure of the G239T mutant is similar to wild type EcR1.

**(B) Ligand binding of retinoids and fatty acids:** Experiments 4-9 were conducted to compare the ligand binding properties of G239T and EcR1 and to test the effects of the single amino acid substitution under equilibrium binding conditions.

**Experiment 4. Binding of 16-AP:** We examined the fluorescence enhancement of 16-anthroyloxy-palmitate (16-AP) on mixing with G239T or EcR1 under equilibrium conditions. As shown in Figure 3, when 16-AP was mixed with either G239T or EcR1, fluorescence increased rapidly in the range of 0 to 100 nM and then paralleled the ligand in buffer control at higher concentrations of ligand. Fluorescence enhancement was about 30% greater with G239T. These high affinity and

![Figure 2. Circular dichroism measurements of EcR1 and G239T. Experiments were carried out in 300 mM NaCl, 50 mM NaPO₄, pH 7. CD spectroscopy employed a Jasco J-715 spectropolarimeter (Easton, MD), and an RTE-111 temperature controller (Neslab, Portsmouth, NH). Spectra were collected in 0.5 mm path length cells from 200 to 260 nm. 3 µM samples were used, heated at about 1°C per min, and spectra were obtained at 25°C and 95°C. To assure temperature equilibrium was reached, samples were incubated for 2 min at each scanning temperature before the spectrum was recorded. The profiles of the two spectra showed minima at about 208 nm and a broad shoulder up to about 225 nm on both EcR1 and G239T. While the spectra were not identical in shape, they appeared similar, suggesting a grossly similar three dimensional shape. After high temperature treatment the CD spectra were very similar.](http://www.molvis.org/molvis/v6/a8)

![Figure 3. 16-AP fluorescence enhancement. Aliquots of 16-AP were added into independent preparations of 1.0 µM G239T (average from three independent preparations, represented by the green triangles), 1.0 µM wild type EcR1 (red squares), or buffer without protein (black circles). Fluorescence enhancement of 16-AP was measured by excitation at 363 nm and emission at 432 nm. In this and all other equilibrium binding experiments, the buffer was 300 mM NaCl, 50 mM NaPO₄, pH 7.0, which afforded the best stability for G239T. In this and other binding experiments (Figures 3 and 6-12), the abscissa represents the accumulated total concentration of the ligand added to the cuvette; the ordinate represents fluorescence intensity measured with a photon counting spectrofluorometer in photons counted per second (cps). 0.5 or 1 µl aliquots of the ligand (dissolved in ethanol) were added to 700 µl volume of protein solution in the cuvette, making 10-15 additions of the ligand. The final ethanol concentration did not exceed 2.5% (v/v).](http://www.molvis.org/molvis/v6/a8)
saturable interactions were indicative of specific binding of 16-AP with each protein.

**Experiment 5. Wavelength scans of retinol and proteins at equilibrium:** To study whether ligand-protein interactions affected fluorescence maxima, we conducted excitation and emission wavelength scans. An excitation scan (with emission set to 479 nm) of wild type EcR1 or G239T plus a ten-fold molar excess of all-trans-retinol revealed an excitation maximum for each protein at ~330 nm (Figure 4). The most prominent difference between the two profiles was the 50% lower fluorescence intensity of G239T. Also, there was a second peak at 310 nm in G239T where a shoulder had appeared in EcR1.

Interestingly, an emission scan (exciting at 330 nm) yielded different maxima for EcR1 and G239T (Figure 5). The emission maximum of ~450 nm for G239T was substantially blue shifted compared to the 479 nm maximum of EcR1. A small shoulder on EcR1 was visible at about 440-450 nm. (The source of the shoulder was not Raman scattering of water as that was found at 375 nm). The other important difference was the greatly reduced fluorescence intensity of G239T versus EcR1. The emission intensity of G239T was reduced about 2.7 fold compared to EcR1.

**Experiment 6. Fluorescence enhancement of all-trans-retinol and 13-cis-retinol at equilibrium with EcR1 or G239T:** As shown in Figure 6 and Figure 7, EcR1 and G239T exhibited fluorescence enhancement with all-trans-retinol and 13-cis-retinol at submicromolar concentrations, indicating high affinity. The curves asymptotically approach a line parallel to “ligand alone” at concentrations higher than 1 µM, suggesting saturation. Note that the contribution of free ligand to fluorescence was not subtracted from the protein-plus ligand samples. Either ligand mixed with G239T exhibited much lower fluorescence enhancement than EcR1, by about 3-fold. The magnitude of the fluorescence enhancement was larger with all-trans-retinol (Figure 6) than 13-cis-retinol (Figure 7), about 40,000 cps versus 12,000 cps, with EcR1 as the ligand binding protein, but the relative response of G239T was the same. However, the retinol-G239T fluorescence signal was clearly greater than that of retinol alone (Figure 6B) and control proteins (Figure 6C).

**Experiment 7. Quenching of intrinsic protein fluorescence by the putative binding of retinoids and other ligands:** Retinol and retinoic acids were used to quench protein fluorescence of EcR1 and G239T. In Figure 8, EcR1 was more quenched than G239T using all-trans-retinol as the ligand, 83% versus 52% at saturation, respectively. In contrast, all-trans-retinoic acid (Figure 9) and 13-cis-retinoic acid quenched both proteins equally. In Figure 9 and Figure 10 the amount of quenching was about 66% of the total intrinsic fluorescence of EcR1 or G239T without the ligands. Also, in these three figures, half maximal quenching occurred at submicromolar concentrations, suggesting high affinity interactions of ligand and protein. The curves appeared to plateau or approach an asymptote at low ligand concentrations of 1-3 µM, suggesting saturation.

![Figure 4. Retinol excitation scan of G239T and EcR1. An excitation scan from 250 to 400 nm of 1 µM wild type EcR1, G239T, or buffer plus 10 µM retinol was carried out (emission set to 479 nm). The excitation maxima for all samples was 336 nm. The red line and squares represents EcR1, the green line and triangles represents G239T, and the black line and circles represent buffer lacking protein.](http://www.molvis.org/molvis/v6/a8)

![Figure 5. Retinol emission scan of G239T and EcR1. Emission scans from 400 to 600 nm of 1 µM wild type EcR1, G239T or protein-free buffer plus 10 µM all-trans-retinol were carried out with excitation set at 330 nm. The scan of retinol-EcR1 (red line) revealed a maximum at 479 nm (label “B”) and a small shoulder at 440-450 nm (label “A”). G239T (green line) complexed with retinol exhibited a maximum at about 450 nm, and the peak height was much lower. Retinol in buffer without protein is represented by the black line. The comparatively high concentration of free retinol (~9 µM) partly masks the small fluorescence enhancement contributed by the ~1 µM G239T-retinol complex indicated by the green line. One explanation of the shift in emission maxima is that the mutation causes a reduction in fluorescence intensity and shifts the maximum to 450 nm within a single ligand binding site. Another possible explanation is that a highly fluorescent site with a maximum at 479 nm is lost, making more apparent a second site (which contributes the shoulder at 450 nm) recognizable as a peak with a maximum at 450 nm in G239T.](http://www.molvis.org/molvis/v6/a8)
Table 2 shows curve fitting results of data taken from another intrinsic protein fluorescence quenching by all-trans-retinol experiment. Curve fitting of this quenching data was carried out as described before [17]. Equating quenching with the amount of ligand binding, we obtained about twice as many retinols bound per polypeptide (1.7 versus 0.8) in comparing EcR1 with G239T. The Kd was also about 3-fold higher for G239T versus EcR1 but still in the submicromolar level.

**Experiment 8. Retinol fluorescence enhancement and competition with DHA:** We tested whether Docosahexaenoic acid (DHA) competitively inhibits the binding of retinol with EcR1 or G239T in retinol fluorescence enhancement assays. Figure 11 shows that DHA appeared to exhibit competitive inhibition with EcR1. In contrast, DHA had no effect on retinol fluorescence enhancement in G239T. Moreover, the EcR1 treated-with-DHA sample curve was virtually identical to G239T with or without DHA treatment.

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**Figure 6.** Retinol fluorescence enhancement by G239T and EcR1. A. Typical titrations of EcR1 and G239T. Four separate G239T preparations and a single EcR1 protein preparation (1 µM each) were titrated with all-trans-retinol and the fluorescence enhancement of retinol was measured as described previously [15-17]. Ligand added to buffer alone (black circles) showed a gradual linear increase in fluorescence, while EcR1 (red squares) revealed a rapid increase of 40,000 cps from 0 to 1.5 µM total retinol concentration, indicative of a high affinity interaction of retinol and the protein. EcR1 plateaued beyond 1.5 µM ligand concentration suggesting saturation. The four preparations of G239T (average shown by the green triangles) exhibited a similar high affinity and saturable interaction with retinol, but the intensity of the fluorescence enhancement was about 3-fold less. B. Typical titrations in the submicromolar range. Three separate G239T preparations (1 µM each) were titrated five times and two EcR1 protein preparations were titrated three times with all-trans-retinol between 0 and 1 µM. A regression was carried out and the black lines indicate the fitted curves. 99% confidence intervals are shown on either side of each curve by the dotted black lines. Free retinol fluorescence is shown by the black circles. The fluorescence of the mixture of retinol and G239T is indicated by the green squares. The fluorescence intensity of EcR1 mixed with retinol is shown by the red triangles. G239T clearly exhibited fluorescence in excess of the buffer control at all concentrations of retinol greater than zero. However, the fluorescence of G239T was not as substantial as EcR1 at concentrations greater than 0.1 µM total ligand concentration. C. Demonstration of specific binding by G239T and EcR1 and no binding by control proteins. Two preparations of G239T (green triangles and red squares), EcR1 (light blue circles), catalase (yellow down-pointing triangles), γ-globulin (dark blue diamonds), and thrombin (violet hexagons, 1 µM solutions) were titrated with retinol from 0 to 1 µM and the fluorescence measured. Representative titrations are shown. Specific binding is defined as a sizable increase in the fluorescence response at low concentration that is saturable, reaching a plateau or paralleling the ligand alone (buffer, black circles). Specific binding was shown with EcR1 by its rapid increase (about 10 times the rate of increase) over retinol alone at low concentrations, in the range of 0 to 0.4 µM, and saturation by its paralleling retinol alone at higher concentration. Likewise, G239T manifested specific binding of retinol by this assay, though showing a lesser enhancement of 2-3 fold over retinol alone. No fluorescence enhancement was detected with catalase, γ-globulin, or thrombin, suggesting that these proteins lack a binding site for retinol and cannot specifically bind this ligand.
Experiment 9. DHA as a putative ligand that can bind to EcR1 and G239T: DHA may be an allosteric affector of 11-cis-retinal binding to bovine IRBP [28]. We asked whether DHA quenches intrinsic fluorescence of either G239T or EcR1 in order to test for a direct effect of DHA on each protein. Figure 12 shows that DHA quenched intrinsic fluorescence of each protein equally. Both proteins were saturated with about 0.4 µM DHA. Beyond this concentration there was a gradual decrease in fluorescence, which may reflect nonspecific effects of DHA on fluorescence. The fraction of intrinsic fluorescence that was quenched was roughly one fourth of the total. This fraction is smaller than the quenching of intrinsic protein fluorescence by the other ligands tested.

To summarize experiments 4-9, equilibrium binding experiments showed that EcR1 and G239T exhibited only minor changes in binding properties with most ligands. However there was a large difference in fluorescence enhancement with all-trans-or 13-cis-retinol, and DHA-retinol competition experiments highlighted these differences. Quenching of intrinsic protein fluorescence confirmed the differences in binding retinol.

(C) Tests of IRBP’s potential roles in vivo: Experiments 10 and 11 compared EcR1 and G239T in assays of putative function. One assay measured protective properties of the proteins in preventing retinol degradation (probably oxidative damage) in aqueous solutions. This was intended to mimic IRBP protecting all-trans-retinol from damage in the interphotoreceptor matrix (IPM). The other assay measured off-rates in transferring retinal from SUVs to protein. This was intended to mimic transfer of 11-cis-retinal from RPE into the IPM.

Experiment 10. Protection of retinol from degradation: Figure 13 shows a test of the ability of G239T and EcR1 proteins to protect retinol from degradation. The integrity of

![Fluorescence quenching by all-trans-retinol](image)

Figure 8. Fluorescence quenching by all-trans-retinol. Three separate G239T preparations (0.7 µM of each protein, average indicated by green triangles) and two individual EcR1 protein preparations (also 0.7 µM, average represented by red squares) were titrated with all-trans-retinol and the quenching of intrinsic protein fluorescence was measured by excitation at 280 nm and emission at 336 nm. Background fluorescence was examined by adding ligand to buffer without protein and is represented by the black circles. Protein fluorescence dropped sharply between 0 and 0.5 µM retinol concentration with all protein preparations, suggesting a high affinity interaction. Defining nonsaturable fluorescence intensity as signal below a line drawn through the last four points of the curves (retinol concentrations of about 2 to 3 µM), the amount of intrinsic protein fluorescence that was quenched was 83% for EcR1 and 58% for G239T. Both proteins demonstrated specific retinol binding compared to the buffer control (as indicated by the low concentrations of ligand needed for the rapid decrease in fluorescence and apparent saturation by 2 µM ligand concentration). Retinol quenched intrinsic protein fluorescence of EcR1 more than G239T. The difference in quenching (58 versus 83%) was attributed to either (1) a lesser amount of retinol that could be bound by G239T, (2) ligand binding to different sites in the proteins, or (3) a different spatial arrangement of tryptophans in the two proteins. The latter explanation seemed unlikely given the sequence identity between EcR1 and G239T (except at position 239) and the similar CD spectra.
retinol was measured as retention of original absorbance at 330 nm. Absorbance was measured for 1 h at room temperature in the dark (except light from the measuring spectrophotometer beam). After 1 h, absorbance at 330 nm was reduced by about 33% in buffer without protein. In the same time span, absorbance was reduced by about 4% when retinol was mixed with EcR1. Two of three G239T protein preparations partially protected retinol (about 10% of absorbance was lost). In a third G239T preparation, retinol absorbance was reduced by about 23%.

**Experiment 11. Transfer of 9-cis-retinal from SUVs to proteins:** To examine the transfer of 9-cis-retinal from SUVs to protein, we measured the quenching of intrinsic protein fluorescence (exciting at 280 nm and emitting at 336 nm). The SUVs consisted of 5 mole percent 9-cis-retinal in phosphatidylcholine vesicles. As shown in Table 3, the off rates (ranging from 2.8 to 4.4 s⁻¹) for 9-cis-retinal leaving the SUVs were not different when using either G239T or EcR1 as the acceptor.

In summary, the last two experiments show that EcR1 protects retinol well. The wild type protein and the mutant both accept 9-cis-retinal efficiently from SUVs. However, the mutant loses some of its capability to protect retinol from oxidative damage (Figure 13).

**DISCUSSION**

The current comparison of the G239T mutant with wild type human Repeat 1 (EcR1) was undertaken to learn whether the notable reduction in retinol fluorescence (Figure 4, Figure 5, and Figure 6) observed in G239T was due to reduced binding of retinol and also whether the effects of the lesion were widespread in binding different ligands. Analysis of our results suggests two models of the retinoid binding sites in G239T and EcR1. These models suggest consequences of the G239T mutation on retinoid protection and extraction from simple membranes.

G239T, with a glycine to threonine substitution at position 239, was chosen for study because amino acid 239 is conserved in all sequence-related family members [12,22], is adjacent to a known active site residue in Tail Specific Protease (TSP), a distant relative of IRBP [14], and is located after a putative β-strand where an active site residue might be expected [12].

**Protein structure:** If G239T retains the structure of wild type EcR1, then any change in ligand binding of G239T should not be due to a gross three dimensional change, but rather to a more subtle effect. While the CD spectra of G239T and EcR1 (Figure 2) are not identical, and G239T denatures at a lower...
temperature, the data nonetheless suggest that at least some or perhaps most of the native structure is retained in G239T. This is supported by the similar tryptophan fluorescence spectra of G239T and EcR1. Lakowicz [29] suggests that tryptophan emission scans are sensitive to the three dimensional spatial arrangement of tryptophans in a protein. The intrinsic protein fluorescence spectra of G239T and EcR1 were very similar and exhibited the same maximum at 336 nm, consistent with the relative positions of the buried tryptophans in the two proteins being the same. These two lines of evidence suggest that the G239T substitution does not cause a gross change in shape.

Even so, a case could be made for a smaller difference in the conformations of G239T and EcR1. The CD spectra are similar but not identical and G239T denatures at a lower temperature. This could argue that G239T produces a change in protein conformation that affects retinoid binding without G239 being in a retinol-binding site.

**Binding at equilibrium with ligands other than retinol:** If we equate the ligand-protein interactions observed at equilibrium in Figures 3 and 8-11 with the binding of ligand to a specific binding site in the protein, then several retinoids and fatty acids can bind specifically to EcR1 and G239T. The interactions were all saturable and the interactions occurred at low concentrations, indicative of high affinity binding events. G239T bound a subset of ligands well (including 16-AP, and all-trans- and 9-cis-retinoic acids; Figure 3, Figure 9, and Figure 10) suggesting that G239T behaves like EcR1.

The difference between the effect of the mutation on retinol binding versus fatty acid binding where both EcR1 and G239T exhibit saturable binding of 16-AP was striking and

<table>
<thead>
<tr>
<th>Protein</th>
<th>n</th>
<th>Kd</th>
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<tr>
<td>G239T</td>
<td>0.814 ± 0.146</td>
<td>0.644 ± 0.090</td>
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<tr>
<td>EcR1</td>
<td>1.713 ± 0.068</td>
<td>0.221 ± 0.033</td>
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</table>

The curve fitting analysis was carried out on the average of two wild type EcR1 samples and three individual preparations of G239T. For both EcR1 and G239T, n equals the number of binding sites for retinol, and Kd is the dissociation coefficient in µM. The data is listed as the mean ± the standard deviation.

Figure 11. Effect of DHA on fluorescence enhancement of retinol. DHA (11 µM) was premixed with three individual preparations of 1.1 µM G239T (average indicated by the violet hexagons with DHA, green triangles without DHA) and 1.1 µM EcR1 (blue diamonds with DHA, red squares without DHA) and fluorescence enhancement was measured. DHA had little effect on G239T fluorescence enhancement but had a significant effect on EcR1, reducing fluorescence intensity by about 70% at 1 µM total retinol concentration. However, at higher concentrations (otherwise saturating conditions with retinol in the 7-8 µM range), the fluorescence intensity was about the same as the binding curve without DHA. Thus, the shape of the binding curve with DHA suggested that DHA was a competitive inhibitor of the retinol-EcR1 interaction. On the other hand, with or without DHA, G239T binding curves were virtually identical, suggesting little obvious effect of DHA on the interaction of retinol with G239T.

Figure 12. Binding of DHA to EcR1 and G239T. DHA was titrated into three individual preparations of 1.0 µM G239T (average represented by the green triangles) and 1.0 µM wild type EcR1 protein (red squares) and fluorescence quenching was measured by exciting at 280 nm and measuring emission at 336 nm. Background fluorescence of DHA in buffer without protein is represented by the black circles. DHA appeared to interact with G239T and EcR1 equally well. Saturable binding was defined as fluorescence above a line through the last four points of a given curve. Fluorescence below that line was taken to be nonsaturable phenomena. Thus, a rapidly decreasing component (between 0 and 0.4 µM total DHA concentration) of the difference between the binding curve and this line showed a high affinity interaction between protein and DHA. The fraction of the initial intrinsic protein fluorescence that was quenched by DHA was about 20%. This fraction was much smaller than with any other ligand in this study.
suggests that the binding sites for fatty acid and for retinol in Repeat 1 are at different locations in the molecule.

**Retinol fluorescence enhancement:** An important distinguishing characteristic between G239T and EcR1 was the reduced retinol fluorescence of the mutant. This reduction occurred with both all-trans-retinol and 13-cis-retinol. Fluorescence is lower with 13-cis- versus all-trans-retinol, suggesting that the polyene chain plays an important role in fluorescence intensity. However, the relative drop in intensity comparing G239T with EcR1 is the same for both 13-cis and all-trans, suggesting that the head group and the β-ionone ring play important roles in ligand binding.

The drop in fluorescence intensity of retinol bound to G239T versus EcR1 could be caused by (1) a loss in the number of retinols that bind to G239T, (2) a looser fit of retinol in G239T’s binding site allowing for rotational or vibrational loss of energy that otherwise would be emitted as fluorescence, (3) a change in the hydrophobic environment, which may change quantum yield, or (4) a smaller fraction of G239T in a properly folded state. Point (2) does not seem likely given that too loose a fit might eliminate a high affinity interaction of protein and retinol. We observed high affinity (submicromolar dissociation constants) with both proteins (Table 2), though G239T had a 3-fold higher Kd. We argue against (3) since an increase in hydrophobicity is usually accompanied by an increase in quantum yield [29]. The large blue-shift of the emission maximum from 479 to 440-450 nm from EcR1 to G239T suggested an increase in hydrophobicity, but the fluorescence intensity dropped instead of the expected rise. We argue against (4), and suggest that G239T is mostly folded normally, because the CD spectra (Figure 2) appeared normal, G239T solutions are not turbid at A320 when first prepared, G239T solutions are clear initially by visual inspection, and G239T has normal binding capacity for other ligands (Figure 3, Figure 9, and Figure 10). This leaves alternative (1), the loss of the number of retinols bound as the more likely explanation for the available data.

**Effect of DHA on retinol binding:** DHA is abundant in the rod outer segment and is essential for vision [30]. DHA is an effective inhibitor of retinol binding to bovine IRBP [20]. Noy and coworkers find that DHA causes a rapid release of 11-cis-retinal from IRBP [28].

A second prominent distinguishing characteristic between G239T and EcR1 was the effect of DHA on retinol fluorescence enhancement. We found that DHA was a competitive inhibitor of retinol binding to EcR1, but that DHA had no effect on retinol fluorescence when bound to G239T (Figure 12). The effect of DHA on EcR1 was to reduce retinol fluorescence to that of G239T with or without DHA. These data suggest that retinol and DHA bind to the same site(s) in EcR1, but that a site is lost or substantially altered in G239T.

**Models of the repeat structure and the number of binding sites:** Two models for the number of binding sites in both G239T and EcR1 are proposed. In Model 1, EcR1 possesses two retinol binding sites and the mutant G239T has lost one of the two sites. In Model 2, EcR1 has only one retinol binding site and the mutant has lost half the binding capacity of this single binding site. All our data are consistent with Model 1. While Model 2 cannot be ruled out, not all of our data are consistent with this option.

Results from some previous studies are consistent with either possibility. Other groups have suggested two retinol

### Table 3. Transfer of 9-cis-retinal from SUVs to EcR1 or G239T

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of experiments</th>
<th>Retinal concentration (µM)</th>
<th>SUV concentration (µM)</th>
<th>Off rate (s⁻¹)</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µM G239T</td>
<td>10</td>
<td>6.25</td>
<td>125</td>
<td>3.627 ± 1.418</td>
<td></td>
</tr>
<tr>
<td>1 µM EcR1</td>
<td>5</td>
<td>6.25</td>
<td>125</td>
<td>2.481 ± 0.293</td>
<td></td>
</tr>
<tr>
<td>4 µM EcR1</td>
<td>10</td>
<td>6.25</td>
<td>125</td>
<td>4.40 ± 1.262</td>
<td></td>
</tr>
</tbody>
</table>

SD = Standard Deviation

Figure 13. All-trans-retinol protection. Absorbance of 2 µM retinol in aqueous solution with or without protein was monitored at 330 nm at 25 °C for 1 h. Samples were incubated in total darkness (except the light beam of the spectrophotometer). The samples were not degassed, thus, they were at equilibrium with air and presumed to contain dissolved oxygen. No antioxidants or preservatives were added to the buffer. Retinol without protein (black line) apparently deteriorated rapidly as the A₃₂₀ decreased by 33% in 1 h. A sample containing 1 µM EcR1 (red line, long dashes) decreased in absorbance by about 4%, while retinol mixed with 1 µM G239T (green line, shorter dashes, representing an average of three different preparations) exhibited a decrease in absorbance of about 15%. The results suggested that binding of retinol to EcR1 and G239T protected this ligand from oxidative damage [5,6]. As considered in the Discussion, G239T, because it protected retinol from damage less, appears to bind less retinol.
binding sites in full length intact bovine IRBP [19,20,28,31-33], with one site strongly enhancing retinol fluorescence and the second site not supporting any additional retinol fluorescence. None of these studies mapped the position of these sites within IRBP. The two sites might be in a single repeat, or there could be one site in one repeat and a second site in another.

Other studies seem to favor Model 1. A study with endogenous fatty acids suggests up to 6.5 moles of ligand bound per mole of polypeptide [21]. The excess of 2.5 more moles of fatty acids over the number of repeats in an IRBP polypeptide implies that at least some repeats must possess more than one binding site. Baer and coworkers [22] found that Module 4 of *Xenopus* IRBP contains 2.2 binding sites, a central fragment of Module 4 has 1.34 binding sites and a nonoverlapping end fragment of the same Repeat has 0.73 binding sites. Their data support Model 1.

*Does human Repeat 1 contain two retinol binding sites?:* G239T and EcR1 bound all-trans-retinoic acid, 13-cis-retinoic acid, and 16-AP equally well (Figure 3, Figure 9, and Figure 10). These data suggested that G239T and EcR1 possess one or more ligand binding site(s) that are not affected by the mutation. However, retinol fluorescence decreased by 50% or more. This is consistent with Model 1, above. That G239T bound several ligands as well as EcR1 did, yet only bound half as much retinol, is not as consistent with Model 2, above. Why would there not be similarly large effects on the binding of these other ligands within a single site, given the large effect on retinol binding?

Curve fitting results (Table 2) yielded 1.7 retinol binding sites in EcR1 versus 0.81 in G239T. These data strongly suggest two sites in EcR1 and one residual site in G239T as in Model 1, and are not consistent with Model 2. Model 2 predicts one binding site in EcR1 and half a binding site in G239T. While the 2 to 1 ratio is as expected, each protein had twice the capacity as expected from Model 2.

Fluorescence emission wavelength scans revealed one major peak at 480 nm and a small shoulder at 450 nm of the retinol-EcR1 complex (Figure 5). Scans of the G239T-retinol complex revealed only one peak, but at about the position of the shoulder (440-450 nm) in EcR1, and it appeared that the large 480 nm peak had been lost selectively. The broad and complex spectrum of EcR1 might suggest that it represents the sum of two or more simpler spectra. For example, some of the EcR1’s spectral characteristics might arise from one retinol binding site that has a maximum at 440-450 nm and a second site with a maximum at 480 nm that is several times more intense in fluorescence than the first site. This is consistent with Model 1 where one site (the highly fluorescent site) is inactive in G239T. While fine structure in emission spectra is not unusual for retinol bound to some binding proteins, it seems unlikely that a single altered binding site would selectively lose one peak without a corresponding change in the other peak or shoulder. Thus, this evidence does not support Model 2.

The cause of the decreased binding of retinol by G239T did not interfere with the mutant’s ability to accept retinal from PC SUVs (Table 3). This is consistent with the loss of the one binding site with no effects on the second site that binds retinal. It is less consistent with the mutated single binding site that has lost half its binding capacity for retinol. If there were just one site, some effects on acceptance by the mutated binding site would be expected, yet none were observed.

DHA exhibited a strong competitive effect on retinol fluorescence in EcR1 (Figure 11). DHA exhibited no effect on retinol fluorescence of the G239T mutant. These data are consistent with Model 1 where G239T has lost one site and DHA exhibits its effect solely on the other binding site. It is more complicated to explain how DHA could profoundly and competitively affect retinol fluorescence in a single site in EcR1 (Model 2), yet have no effect on the residual binding capacity of G239T.

*All-trans* and 13-cis-retinol both exhibit fluorescence enhancement in EcR1 and G239T. Although the absolute amount of enhancement is less in 13-cis-retinol, the ratios of the decrease in fluorescence enhancement of EcR1 to G239T are almost exactly the same with the two ligands. This is consistent with Model 1 where G239T has one inactive site. It is more convoluted to explain the identical ratios in the one-site model, given the expectation that the polynene chain (bent versus straight in 13-cis versus all-trans) might behave differently within a single altered but half active binding pocket.

While our experiments do not exclude single-site binding in EcR1 (Model 2), the experiments presented here on G239T and previous studies provide data that are much more consistent with a two-site model (Model 1).

*Kinetic experiments:* Kinetic experiments were conducted to determine whether the mutation at position 239 had an effect on the transfer of retinal from SUVs to protein. The remarkable discovery that IRBP is the only retinoid binding protein that can cause the release of 11-cis-retinol from the apical face of RPE cell cultures [1,2] suggests a unique function (and perhaps a structural domain) of IRBP that is not found in other Vitamin A binding proteins. To mimic in vitro the physiological transfer of 11-cis-retinal from apical RPE plasma membranes to the IPM, our experiments employed PC SUVs containing 5 mole percent 9-cis-retinal and a dilute solution of purified EcR1 or G239T, and the SUVs and protein were mixed in a stopped flow apparatus.

Previously we showed that each Repeat of human IRBP can accept retinal from PC SUVs at a rate faster than from SUVs to BSA [17]. Here we compared retinal transfer rates from PC SUVs to EcR1 or G239T. We found that rates were equally fast, about 2.5-4.4 s⁻¹, in G239T and EcR1, and faster than BSA (1.86 s⁻¹) and the other Repeats (EcR2, 1.17 s⁻¹; EcR3, 2.25 s⁻¹; and EcR4, 1.56 s⁻¹) [17]. This suggests that the loss of retinol binding has had no deleterious effect on retinal transfer. Thus, we propose that components unaffected by the G239T mutation are needed for the faster than expected rates of retinal transfer.
**Summary:** In conclusion, EcR1 appeared to have twice the binding capacity of G239T for retinol. The amino acid substitution at position 239 may be in a retinol binding site, and the change profoundly affects only retinol binding. Probably there are two binding sites for retinol in EcR1 and one residual site in G239T. That a point substitution can have such a large effect in binding without affecting the gross three dimensional shape of the protein suggests that the binding site is a groove or pocket, not a shallow or surface hydrophobic patch of limited selectivity or specificity.

**ACKNOWLEDGEMENTS**

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