



Effects of dispersed point substitutions in Repeat 1 of human interphotoreceptor retinoid binding protein (IRBP)

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Purpose: The purpose of this study was to measure the effects of mutations on the retinol binding capability of human Repeat 1 of interphotoreceptor retinoid-binding protein (IRBP). First, we predicted important functional amino acids by several computer programs. We also noted the lack of shared functions between Tail-specific protease (Tsp) and IRBP, which bear sequence similarity, and this aided in predicting functional residues. We analyzed the effects of point substitutions on the retinol and fatty acid binding properties of Repeat 1 of human IRBP at 25 and 50 °C.

Methods: To find residues critical to retinol binding that might affect function, a series of thirteen mutations were created by site-specific mutagenesis between positions 140 and 280 in Repeat 1 of human IRBP. These mutants were expressed, purified, and tested for binding properties. The conformations of the proteins were examined by circular dichroism (CD) scans.

Results: Seven of the mutations exhibited reduced binding capacity, and five were not expressed at high enough levels to assess binding activity. Four of the mutants were purified, and their CD scans were very similar to those of Repeat 1. Only one of the mutations did not affect binding, folding, or expression when compared to wild type Repeat 1.

Conclusions: Several IRBP mutants containing point mutations retained native structure but lost retinol binding function. The data suggest that retinol binding is affected by many different amino acid substitutions in or near a binding pocket. That even a single point substitution can profoundly affect binding without affecting overall conformation suggests that much of Domain B (from amino acid positions 80 to 300) is involved with ligand binding. This excludes three previously proposed IRBP-retinol binding mechanisms: (1) retinol binds to a small portion of the protein repeat, (2) retinol can bind to any hydrophobic patch in the protein, and (3) native conformation is not required for retinol binding to the repeat.

Mammalian interphotoreceptor retinoid-binding protein (IRBP) is a large glycoprotein (about 140 kDa) with a four fold repeat structure [1]. IRBP is known to bind various retinoids and fatty acids [2-8] but the role of IRBP in retina physiology remains poorly understood [9,10]. Each of the four Repeats contains about 300 amino acids with the first 80 corresponding to Domain A and the last 220 corresponding to Domain B, and each repeat is known to bind at least one retinoid or fatty acid [11,12]. In this article, we mutated amino acids in Domain B of Repeat 1 to identify some of the amino acids needed to bind ligands such as retinol.

We predicted amino acids involved in ligand binding by *E. coli* expressed human Repeat 1 (EcR1) in a companion paper [13], taking into consideration work by Baer and coworkers [2,14] on the fourth Module of *Xenopus* IRBP. In this paper, we describe the construction of thirteen substitution mutations in EcR1 (Mutants 1-13) and their retinol binding properties.

As demonstrated previously [11,12], individually expressed human IRBP repeats each have the ability to bind retinoids and fatty acids. Experiments demonstrate that EcR1 has the highest retinol fluorescence enhancement of the four Repeats, and has the same K_d value for retinol as whole human IRBP expressed in baculovirus. Consequently, considerable functionality of whole four repeat IRBP protein may come from Repeat 1, thus we felt it best to study this particular repeat. We mutated EcR1 residues that are well conserved and that were predicted to be located directly after putative β -strands (the possible location of a binding site [15]). The amino acids fitting both criteria might be in a ligand binding site of either an α/β or $\alpha + \beta$ class protein [15].

We scattered the mutations throughout the 110-300 region of the human Repeat 1, as Baer et al. [2] showed that no retinol binding activity was associated with Domain A in *Xenopus* Module 4. After making these substitutions (Mutants 1-13) with the intent of perturbing function but without affecting the gross structure of the repeat, we assessed their functional and structural properties by testing the solubility of the mutants, examining their conformation, and measuring the effects of the mutations on retinol's interaction with each protein. We conclude that many amino acids in Domain B are involved with retinoid binding, as various single amino acid changes throughout the 140-300 region affect retinoid bind-

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ing. These data rule out three models in which (1) only a few critical amino acids are needed for the protein to bind retinoids, (2) tertiary structure is not important for ligand binding, and (3) retinol binds to any hydrophobic patch in Repeat 1.

METHODS

Site directed mutagenesis of *EcR1*: Thirteen point mutations (Figure 1) were made in *E. coli* expressed human Repeat 1 (*EcR1*) sequence using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Gel purified oligonucleotide primers (Table 1) for both strands of the target DNA containing the mutation of interest were extended by the high fidelity DNA polymerase *Pfu* via low number temperature cycling. These primers were 27 to 34 bases in length, with the mutations centrally located. The primers were at least 40% G/C, terminate in at least one G or C, and had a melting temperature of ~10 °C above the optimum extension temperature for *Pfu* of 68 °C as determined by the formula: $T_m = 81.5 + 0.41(\%GC) - 675/N - m$, where N is the primer length in bases and m is the percent mismatch. Generally the primers were in excess (125 ng each) and the template concentration was varied from 12.5 to 50 ng. Primers were purified from 15% polyacrylamide, 7 M urea, 1x TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3) gels. Oligonucleotides were identified by UV shadowing, extracted, ethanol precipitated, and resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA.

A typical QuikChange 50 µl PCR reaction consisted of 25 ng pLexR1 plasmid template DNA, 5 µl 10x reaction buffer (100 mM KCl, 60 mM (NH₄)₂SO₄, 200 mM Tris-Cl (pH 8.0), 20 mM MgCl₂, 1% (v/v) Triton X-100, 100 µg/ml nuclease free bovine serum albumin), 125 ng each mutant oligonucleotide, and 1 µl of a 10 µM dNTP mix (2.5 µM each dNTP) overlaid with mineral oil in a thin walled 600 µl microfuge tube (Stratagene). The tubes were heated to 95 °C for 30 s followed by 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min. The intact pLexR1 plasmid is 3843 bp in length. The extension time (8 min) was calculated as about 2

min/kb. *Pfu* polymerase does not displace the oligonucleotide primers and the resultant product was a mutated plasmid with unligated and staggered nicks. Completed PCR reactions were cooled on ice, 10 units *DpnI* were added under the mineral oil, mixed, centrifuged briefly, and incubated for 1 h at 37 °C. There are 20 *DpnI* sites (5'-GmA/TC-3') within the pLexR1 plasmid. *DpnI* restriction endonuclease digestion selectively eliminates the *E. coli* host *dam* methylated parental template and not newly synthesized, nonmethylated mutant DNA strands. Theoretically, upon transformation into competent GI724 *E. coli*, the nicked mutant plasmids were ligated and should be the sole transformants.

Transformation of competent cells with mutated plasmid: GI724 cells were made chemically competent [16]. Five to ten µl *DpnI* digested PCR reaction were mixed with 100 µl GI724 competent cells and transformed [16]. GI724 is wild type for some *E. coli* restriction and modification systems and therefore greater amounts of plasmid were added than in strains routinely used for transformation. For other transformations XL-1 competent cells were used.

Sequence Determination: The presence of a mutation was tested by sequencing using ³⁵S-α-dATP and a T7 Sequenase 2.0 DNA Sequencing kit (Amersham, Arlington Heights, IL) following the chain terminator method [17]. Putative Mutants 1 through 5 were examined using the LexR1-1 primer, Mutants 6 through 11 were sequenced with the LexR1-2 primer, and Mutants 12 and 13 were sequenced using the LexR1-3 primer. The LexR1 primers are underlined in the *EcR1* nucleotide sequence in Figure 1. The pLexPROM primer, 5'-CACCTACCAACAATGC-3', was used to sequence the P_L promoter and corresponds to bases 2068 through 2084 of the pLex vector. The pLex Forward primer, 5'-GGTGACGCTCTTAAAAATTAAGCC-3', was used to determine the sequence just after the PL promoter, which includes the multiple cloning site, the *Bam*H1 cloning site and the 5' end of the insert. This primer corresponds to bases 2222 through 2245 of the pLex vector. The *AspA* Reverse primer, 5'-TGTAACGACGCGCCAGTGC-3', corresponds to vector bases 2507 through 2488, and was used to determine the sequence of the transcription terminator following the insert.

Large scale protein preparation: Typical protein preparations consisted of 8 flasks of 1.5 liters induction media, a minimal tryptophan-free media (1x M9 salts, 0.2% (w/v) casamino acids, 0.5% (w/v) glucose, 1 mM MgCl₂, and 100 µg/ml ampicillin), grown at 30 °C. Protein expression was induced by addition of tryptophan and a rise in temperature to 37 °C, once a sufficient cell density had been reached. We used 30 µg/ml tryptophan for *EcR1* and 45 µg/ml for the mutants of *EcR1*. Cultures were induced at A₅₅₀ of 0.6 and were harvested 4 h after induction. Cells were collected by centrifugation at 6500 x g for 7 min. Cell pellets were stored at -80 °C.

Proteins were isolated by lysing cells, separating soluble from insoluble proteins, and successively extracting proteins based on the method of Kripl [18] modified by omitting EDTA

TABLE 1. MUTAGENESIS PRIMERS

Mutant	Primer	Size	Amino acid Changed	Primer sequence (5' to 3')
1	R1TSP1U	28 b	V116N	GC GG GTGGACAGCAACCGGGCCAGGAG
2	R1TSP2U	32 b	L147A	CGCCTTAGTGCTGGATGCCGGCACTGCACAG
3	R1TSP3U	34 b	R148D	CTTAGTGCTGGATCTCGATCACTGCACAGGAGGC
4	R1TSP4U	30 b	G152A	CCGGCACTGCACAGCGGGCCAGGTCTCTGG
5	R1TSP5U	27 b	G153A	CACTGCACAGGACCGCCAGGTCTCTGGC
6	R1TSP6U	28 b	L208A	GGATGTGGTGGTCCGACACAGCAGCCAG
7	R1TSP7U	28 b	E218A	CAGGGGCGTGCCCGCCGACATCGCGCAC
8	R1TSP8U	27 b	T237A	GTGGGCGAGCGGGCGGGGGAGGGGGCC
9	R1TSP9U	29 b	G239T	CGAGCGGACTGGGACCGGGGCGGTGGACC
10	R1TSP10U	28 b	T249A	CCGGAAGCTGAGGCGGGCGGAGTCTGAC
11	R1TSP11U	34 b	E251A	GAACTGAGGATAGCGCGTCTGACTTCTCTTC
12	R1TSP12U	29 b	G278A	GTGGGAGGGCAGCGCGGTGCTGCCCTGTG
13	R1TSP13U	28 b	P281A	GCAGCGGGGTGCTGCGCTGTGTGGGAC

Primers listed are designated U for upper strand. The sequence for the mutant amino acid is in red. Primers for the lower strand are the reverse complements of the corresponding upper strand primers. Both an upper and lower strand primer must be used for each PCR reaction.

and DTT. The final potassium thiocyanate washing step of the Krippel method was omitted because no proteins were detected in the supernatant after this extraction step. The precise methods for each mutant differed based on the subcellular localization of the expressed protein as detailed below. After extraction, the proteins were purified by metal ion affinity chromatography.

Purification of Mutants 6-8 and 10-13: Cell paste (25 to 40 g) was resuspended in 180 ml of ice cold buffer containing 50 mM Tris (pH 8) and 5% (v/v) glycerol (Buffer A). The pH was adjusted to 8.0 with several drops of 10 N NaOH to optimize lysis, and 200 mg lysozyme (Sigma, St. Louis, MO) were added. The cells were incubated on ice for 15 to 30 min with occasional mixing, 25 ng/ml DNase I (Sigma) plus 125 ng/ml RNase (Sigma) were added, and incubation on ice continued

for another 15 to 30 min. If the solution of lysed cells was still viscous, the solution was homogenized. The lysate was centrifuged at 90,000 x g at 10 °C for 20 min. This first supernatant (SN1) contained soluble proteins. The pellet was resuspended by homogenization in 150 ml Buffer A plus 0.05% (w/v) sodium deoxycholate (Sigma), and NaCl added to 1 M. The mixture was stirred 1 to 2 h at room temperature, then centrifuged as above to yield supernatant 2 (SN2). The second pellet was resuspended by homogenization in 50 to 75 ml ice cold Buffer B (50 mM Tris, pH 8) plus 1.5% (w/v) n-octyl- β -D-glucopyranoside (OBDG, Inalco, San Luis Obispo, CA) and centrifuged as above to produce supernatant 3 (SN3). The third pellet was resuspended by homogenization into 25 to 30 ml Buffer B (8 M Urea, 10 mM Tris, 0.1 M NaPO₄, pH 8) used to denature the mutant protein. The solution was centrifuged and the final urea-insoluble pellet discarded. The last solution after centrifugation was called supernatant 4 (SN4).

Purification of EcR1 and Mutant 4: EcR1 and Mutant 4 were found in SN1, thus none of the pellet extraction steps after the first centrifugation were needed to purify these two proteins, and SN1 was directly applied to a metal ion column as described later.

Purification of Mutant 9: Mutant 9 was found in SN4, and the lysis, deoxycholic acid, and NaCl solubilization steps were combined, followed by an OBDG solubilization step, which was used to remove contaminating proteins prior to urea denaturation. Mutant 9 containing cell pellets were resuspended in Buffer A and incubated on ice with lysozyme for 20 min prior to addition of deoxycholic acid and further incubated for 20 min. RNase and DNase were added and the 0 °C incubation continued until the viscosity was reduced. NaCl (1 M) was added to the solution prior to the first centrifugation. Thus, the first and second supernatants of the standard procedure have been combined. The protein preparation then proceeded as above.

Immobilized metal ion affinity chromatography: For soluble EcR1 and Mutant 4 batch purification under native conditions, SN1 was incubated at 4 °C on a rocking platform with 2 to 3 ml buffer equilibrated Ni-NTA (nickel ion nitrilotriacetic acid) superflow resin (Qiagen, Chatsworth, CA) and 0 to 15 mM imidazole, pH 8 (Sigma) for 1 h. The resin-supernatant slurry was loaded into a column (Bio-Rad, Hercules, CA) and the flow-through fraction collected. The column was washed 3 times with 10 bed volumes of 50 mM NaPO₄, 300 mM NaCl, 20 mM imidazole, pH 8. Finally, the protein of interest was eluted with 2 to 3 resin bed volumes of the above buffer with 250 mM imidazole.

Mutants 6-13 were batch purified under denaturing conditions. Urea soluble proteins from SN4 were bound to 2 to 3 ml of buffer-equilibrated resin for 1 h at 4 °C on a rocking platform. The column was loaded and the flow through fraction collected as above. The resin was then washed with 10 bed volumes of Buffer B, 10 bed volumes of Buffer C (Buffer B adjusted to pH 6.3), 2 to 3 volumes of Buffer D (Buffer B

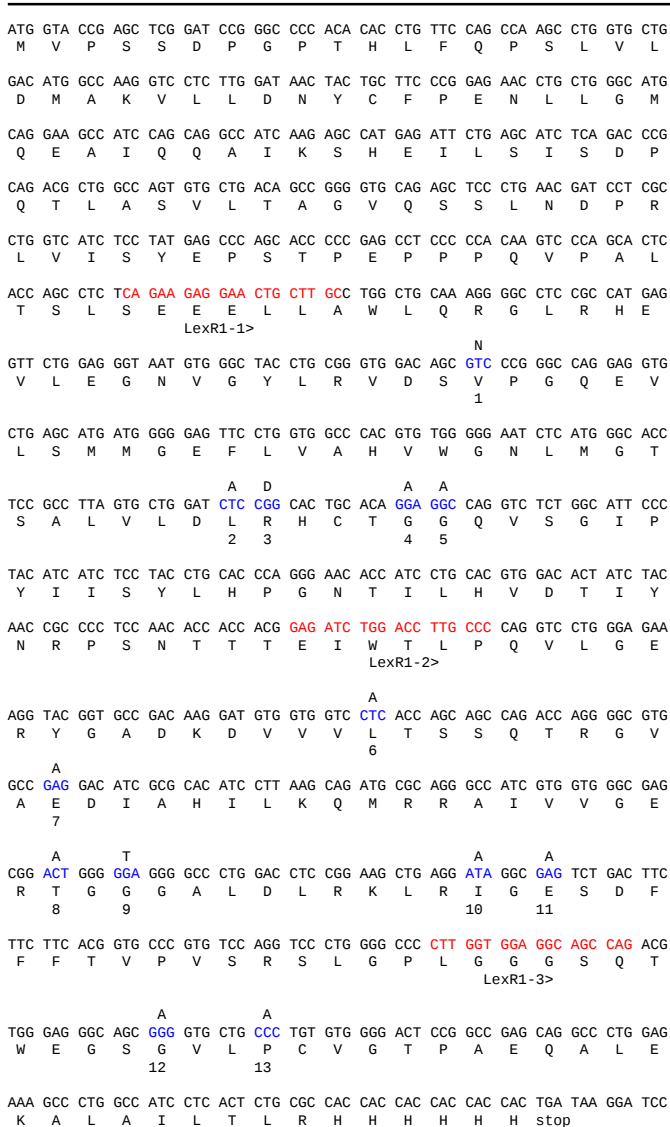


Figure 1. Sequence of IRBP Repeat 1. Mutant amino acids are just above the wild type amino acids being mutated. Primers are in red with names below the sequence. The wild type codons changed by site directed mutagenesis are in blue.

adjusted to pH 5.9), and ultimately eluted with 2 to 3 bed volumes of Buffer E (Buffer B adjusted to pH 4.5). Generally, the IRBP protein of interest eluted beginning at pH 5.9 (Buffer D), but much of the protein remained bound until elution with Buffer E. Urea buffers were prepared and used within 2 weeks and were pH-adjusted just before use. If an eluted protein was contaminated with other *E. coli* host proteins, another nickel or a cobalt resin column was run.

Protein Storage: Purified proteins were stored at 4 °C for denatured samples and either -20 or 4 °C for soluble samples including 10 mM 2-mercaptoethanol. Mutant protein samples were stored in the urea denatured state.

Purification of SlyD: SlyD was fortuitously purified from a contaminated preparation of Mutant 10 protein. The elution fractions pH 5.9 through 4.5 from a nickel ion column were combined and brought up to pH 8 with concentrated NaOH prior to exposure to cobalt resin for 1 h at room temperature. Elution at pH 6 was performed and only SlyD appeared to bind and elute from the cobalt resin under these conditions. These affinity purifications were done under denaturing conditions (7 M urea). Dialysis was performed on the SlyD sample as for the mutant EcR1 proteins as described below.

Renaturing purified proteins: Purified proteins were dialyzed at 4 °C overnight against at least three changes of greater than or equal to 500 volumes of 300 mM NaCl, 50 mM NaPO₄, pH 7 buffer in dialysis tubing (MW cutoff of 12-14 kDa, Gibco-BRL, Gaithersburg, MD). Concentrations within the dialysis bags were 1 to 2.5 µM protein. The first dialysis buffer and the starting protein solution were supplemented with 10 mM 2-mercaptoethanol. Renatured proteins were centrifuged for 10 min at 10,000 x g to remove precipitate. The protein concentration and sample turbidity were estimated by absorbance at 280 and 320 nm. Samples were used in experiments immediately after dialysis.

SDS-PAGE analysis and western blotting: Monitoring of expression over time and determination of the protein preparation fraction with the majority of mutant EcR1 protein was accomplished via standard SDS-PAGE analysis and western blotting. One gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250. The proteins on the matching gel were transferred to nitrocellulose (Protran BA85, Schleicher & Schuell, Keene, NH). Blot-transferred membranes were incubated with primary antibody solution (1: 1000 dilution of the H3B5 monoclonal antibody, which was a gift from L. Donoso, Wills Eye Hospital, Philadelphia, PA) specific for human IRBP amino acids 361-367. While the mAb is specific for the sequence AASEDPK in Repeat 2 of human IRBP [19], it cross reacts with EcR1 [11].

N-terminal sequence analysis: Proteins for sequence analysis were separated on SDS polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride (PVDF, Millipore, Bedford, MA) membrane. The bands from three identical lanes were visualized by Ponceau S Red staining, cut from the membrane and N-terminal sequence analysis performed on a gas phase sequenator.

Ligands: All-trans-retinol, ($\epsilon = 46,000$ at 325 nm, > 95% pure, Sigma) and 16-anthroyloxy-palmitate (16-AP, $\epsilon = 8000$ at 363 nm, >90% pure, Molecular Probes, Inc., Portland, OR) were dissolved in ethanol and used under subdued red light (Kodak 1A safelight, Eastman Kodak, Rochester, NY).

Fluorescence measurements: Equilibrium fluorescence measurements were performed at room temperature in subdued red light on solutions of EcR1, mutants, and other proteins ranging from 0.7 to 1 µM, according to previously described methods [11,12], except that proteins were assayed in 300 mM NaCl, 50 mM NaPO₄, pH 7.0.

Circular dichroism (CD) measurements: CD spectroscopy employed a Jasco J-715 spectropolarimeter (Easton, MD). Spectra were collected in 0.5 mm path length cells from 200 to 260 nm. UV readings below 200 nm were prevented by NaCl in the buffer (50 mM NaPO₄, 300 mM NaCl, pH 7), which strongly absorbs light at these wavelengths and interfered with recording the CD signals from the proteins. Methods otherwise were as previously described [12].

RESULTS

Mutation of IRBP Repeat 1: We mutated amino acids located in human Repeat 1 predicted to alter retinol binding, and these 13 conserved amino acids included:

- (1) Residues conserved between tail-specific protease (Tsp) and IRBP that were previously found to be important for Tsp function [20]. The amino acids selected for alteration in Mutants 2, 3, 4, 6, 7, 8, 9, 12, and 13 are identical in Tsp and all four repeats of both bovine and human IRBP. Mutant 1 changed a similar but not identical residue conserved in the mammalian IRBP repeats and Tsp.
- (2) Residues more conserved among other members than within IRBP repeats: All were at the very end of or immediately after putative β -strand structures. Residues represented by mutants 5, 10, and 11 met that criteria.
- (3) Residues that were identical in all thirteen members of the IRBP-C-terminal protease (CTP) family. Mutants 2, 3, 4, 9, and 13 met that criterion.
- (4) Residues that are found at the ends of possible β -strands. Mutants 2, 3, 6, and 10 met that criterion.
- (5) Residues that are situated immediately after the predicted β -strands. Mutants 1, 4, 5, 8, 9, and 11 fit this criterion.
- (6) Residues that met none of the above criteria. Mutants 12 and 13 differed because they were at least ten amino acids downstream from a β -strand. Mutant 7 substituted an amino acid nine residues downstream of a hypothesized β -strand structure and within a likely α -helix.

The Baer, et al. alignment [2] of the fourth Repeat of IRBP from various species and several CTPs revealed that the wild type residues matching Mutants 1, 2, 3, 4, 5, 7, and 9 are the same in those chosen sequences, and Mutants 1, 2, 3, 4, and 9 are identical in both Figure 1 and the Baer alignment.

The mutation of conserved residues corresponding to Mutants 2 and 3 have no structural or functional effect on Tsp [20]. Mutants 4, 5, 7, and 8 structurally alter Tsp as deter-

mined by circular dichroism (CD) spectra [20]. The three active site residues discovered in Tsp that retain wild type CD structure but lose protease activity are not conserved in the mammalian IRBP repeats, though Mutant 9 is directly adjacent to one of those active-site residues. If the sequences of Repeat 1 and Repeat 4 (the only repeat sequence broken by introns) are aligned, then Mutants 1 through 3 are in the corresponding Exon 2-like segment, Mutants 4 and 5 are in Exon 3, and the remaining 8 mutations (6 through 13) are in the Exon 4-like portion of Repeat 1.

Several experiments were done to characterize single amino acid substitutions of EcR1. At the DNA level, we se-

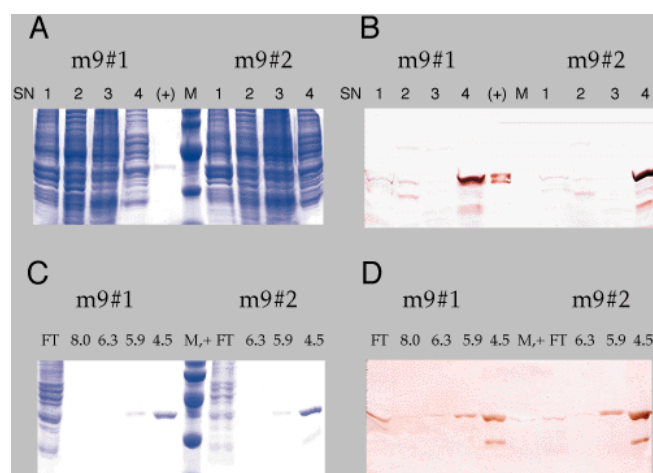


Figure 2. Typical mutant 9 purification. Two separately grown preparations, Mutant 9 number 1 and Mutant 9 number 2, showing supernatants 1 to 4 of the protein preparations and the results of nickel ion affinity purification of SN4 from each preparation. Mutant 9 number 1 and number 2 are individual protein preparations of separate clones with the Mutant 9 G239T substitution. Protein preparation supernatants 1 through 4 (SN 1 to 4) were prepared by differential extraction and centrifugation yielding the several supernatants and a final pellet. An immunoblot with monoclonal antibody H3B5 found Mutant 9 protein mostly in SN4 (urea). Nickel ion affinity purification of SN4 from both Mutant 9 clones, and detection with H3B5 located the mutant protein eluting at pH 5.9 and 4.5, but not with the preceding washes of the column at pH's of 8.0 or 6.3. EcR1 and the mutant EcR1 protein bands ran between the ovalbumin 43 kDa and carbonic anhydrase 29 kDa molecular weight standards. SlyD runs anomalously on SDS PAGE gels [25] and here migrated about the same distance as the 18.4 kDa β -lactoglobulin marker band. According to the manufacturer, the three marker bands mentioned above have apparent molecular weights on gels of 44.7, 28.3 and 19.3 kDa, respectively. Contamination with SlyD was especially evident in the Mutant 9 number 2 pH 4.5 elution fraction on both the SDS PAGE gel and the immunoblot. FT means column flow through, (+) represents EcR1 used as a positive control and M is the marker lane. In Panels A and B, 1 through 4 are the protein preparation supernatants, and in panels C and D, the numbers 8.0, 6.3, 5.9 and 4.5 designate the pH of the denaturing solution used to wash the column or elute the protein from the column. Panels A and C are Coomassie Brilliant Blue stained SDS PAGE gels, and B and D are monoclonal antibody H3B5 stained western blots of samples corresponding to the gels in A and C.

quenced the regions containing the putative mutations and verified that the appropriate changes had been made successfully (data not shown). At the protein level, the mutations were examined to assess (1) their solubility and propensity to aggregate qualitatively, (2) their CD spectra, and (3) their ability to bind retinol as reflected by retinol fluorescence enhancement when the mutant and retinol were allowed to interact by mixing in equilibrium conditions.

Yield of the mutants versus EcR1: Small scale induced versus uninduced cultures were used to determine if the mutant proteins were being expressed and whether the proteins were recovered from insoluble or soluble fractions derived from *E. coli*. The expressing bacteria were subjected to differential extraction based on the Krippel scheme [18]. A soluble

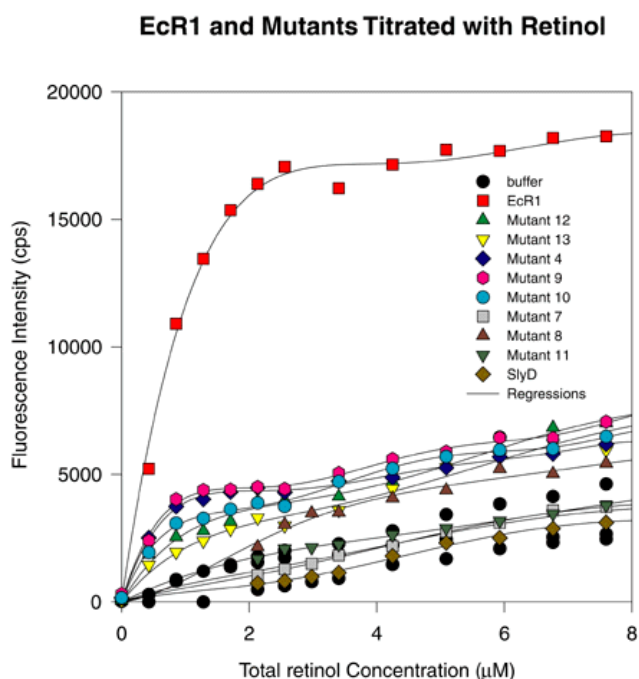


Figure 3. Retinol Fluorescence Enhancement of Mutants 4, 7, 8, 9, 10, 11, 12, 13, SlyD, and EcR1. Aliquots (1 μ M) of EcR1, SlyD, or various mutants were titrated with retinol and the fluorescence enhancement of retinol measured. The abscissa represents total retinol concentration added to the cuvette. Fluorescence was recorded with a photon counting fluorometer and the units on the ordinate represent photons counted per second (cps). Each mutant protein has reduced retinol fluorescence enhancement activity compared to EcR1, suggesting that each mutant has an altered binding site for retinol. SlyD exhibited no fluorescence in excess of buffer alone, suggesting no saturable binding and little if any nonsaturable binding of retinal to SlyD. There may be multiple classes of retinol binding mutants. For example, Mutants 4 and 9 exhibit roughly one-fifth the fluorescence enhancement of the wild type EcR1, while other mutants, for example Mutants 7 and 11, exhibit no detectable fluorescence enhancement. Other mutants seem to exhibit intermediate gradations of fluorescence enhancement. Thus, many different locations in the protein affect retinol binding, and the effects of a point mutation do not fall into a single class.

fraction following lysis was obtained by centrifugation and this first supernatant (SN1) was assayed by examining western blots. Only 1 of the 13 mutants was significantly expressed in the soluble fraction (Mutant 4, G152A). The other 12 mutants were not found in the soluble fraction and many were recovered in the first pellet (data not shown).

Most of the mutants exhibited diminished expression in *E. coli* compared to EcR1. While greater than 20 mg of EcR1 were obtained from 12 L of culture, less than 1 mg was typically obtained from similarly grown mutant EcR1 cultures. Figure 2 shows a typical purification of two mutant preparations. The purification scheme yielded mostly purified proteins. The only visible contaminant, at less than 5%, was SlyD, which did not bind retinol (Figure 3) or 16-AP (Figure 4).

Four of 13 mutant proteins (Mutants 1, 2, 3 and 5) could not be detected on western blots after induction or during typical protein preparation. Sequence analysis of the DNA constructs for these mutants verified the sequence of the insert, promoter, and terminator, suggesting that these four proteins should be expressed unless the expressed polypeptides exhib-

ited an inhibitory effect on protein production or a toxic effect on the bacteria.

Buffer effects on solubility and retinol fluorescence enhancement: Because of the apparent insolubility of some of the mutants in *E. coli*, we tested other buffers to try to stabilize the proteins. Other buffers used for IRBP analysis in the literature [21], such as sodium sulfate (40 mM NaPO₄, pH 7, 1.6 mM EDTA and 160 mM Na₂SO₄) or ammonium bicarbonate (100 mM NH₄HCO₃), did not increase the stability or solubility of a representative mutant. The addition of glycerol or EDTA did not improve stability either; however, none of these changes had any adverse effects on the measurement of retinol fluorescence enhancement of wild type EcR1 or the representative mutant. Qualitatively, the mutant proteins in sodium sulfate or ammonium bicarbonate buffers became turbid faster than the standard buffer (data not shown). Thus, most further experiments were carried out in 50 mM NaPO₄, 300 mM NaCl, pH 7.0, as the standard analysis buffer.

Identity of a contaminant of nickel-column purifications: A contaminating protein was sometimes detected in preparations of some of the mutants. This protein ran at a lower molecular weight than the mutants on SDS-PAGE and was microsequenced by Edman degradation (data not shown). A BLAST search of GenBank yielded a match to SlyD, Swissprot accession number P30856. Given (1) the sequence match at the N-terminal end of the query and subject proteins, (2) the same expected mobility as SlyD on SDS PAGE, and (3) a histidine rich metal binding domain known to exist in SlyD [22-24] with the propensity of the contaminating protein to bind to nickel affinity columns, these data strongly suggested that the contaminant was SlyD.

Importantly, purified SlyD did not enhance fluorescence of all-*trans*-retinol or the fatty acid analog 16-AP in fluorescence titration assays, Figure 3 and Figure 4, respectively, suggesting that SlyD does not bind those ligands. Even so, it is worth warning the scientific community that SlyD is likely to contaminate proteins bearing His-tags that are over-expressed in *E. coli*, and that SlyD theoretically could interfere with subsequent analyses.

Circular dichroism spectral analyses: Several mutant proteins were scanned by CD for structural alterations as compared to EcR1. Three mutants, Mutants 4, 9, and 10, that did not bind retinol (See Table 2, and retinol binding results considered below) appeared to have CD spectra similar to EcR1 (CD scans not shown). CD measurements (Figure 5) revealed that Mutant 9 had roughly the same CD spectrum as EcR1. As the temperature of either protein was raised, the magnitudes of the scans changed abruptly with transition temperatures of 45 and 60 °C, for Mutant 9 and EcR1 respectively. At temperatures of 80-90 °C the spectra again resembled each other, though with greatly reduced CD intensities.

Binding activity at elevated temperature: To test functionality, a retinol fluorescence enhancement assay was performed with or without a 5 min 50 °C incubation (Figure 6). This temperature was selected as just above the denaturation

Fluorescence Enhancement of 16-AP

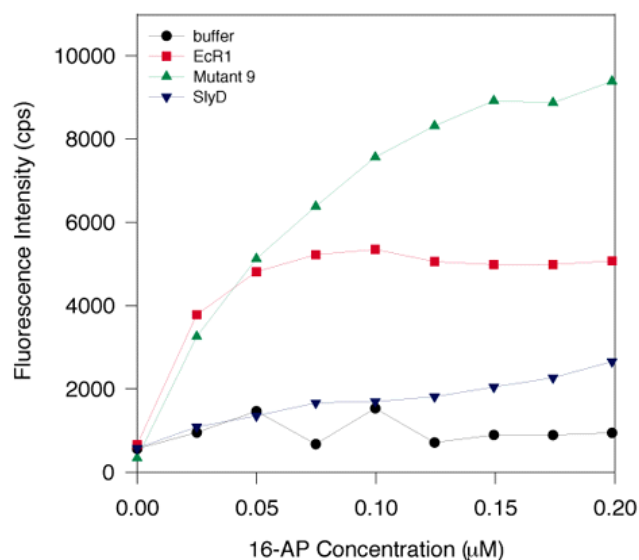


Figure 4. 16-AP Fluorescence Enhancement of Mutant 9, SlyD, and EcR1. Aliquots (1 μM) of Mutant 9, SlyD, or EcR1 was titrated with 16-AP and the fluorescence enhancement of 16-AP measured. The abscissa represents total 16-AP concentration added to the cuvette. Fluorescence was recorded with a photon counting fluorometer and the units on the ordinate represent photons counted per second (cps). Mutant 9 exhibited a significant fluorescence enhancement, while SlyD exhibited either no or only a slight fluorescence enhancement compared to buffer alone. Wild type EcR1 exhibited fluorescence enhancement as previously described with a binding capacity of roughly 0.5 ligands bound per molecule of protein [11,12], and the Mutant 9 binding curve may suggest one 16-AP molecule is bound per polypeptide.

temperature for Mutant 9 and just below the denaturation temperature for EcR1. There was reduced retinol fluorescence enhancement in two Mutant 9 samples after the 50 °C incubation as compared to the same Mutant 9 samples incubated at room temperature. Exposure of EcR1 to a 5 min 50 °C incubation appeared to slightly increase fluorescence enhancement as compared to the room temperature incubated control.

Summary of the thirteen mutations in EcR1: A summary of the characterization of all thirteen mutants is given in Table 2. Expressible mutants were purified to near homogeneity (contaminants less than 5%) and were tested for their ability to bind retinol with the fluorescence enhancement assay. Most of the mutants had significantly reduced retinol fluorescence enhancement (Figure 3), suggesting that each amino acid substitution affected retinol binding. CD spectra were similar to EcR1 for Mutants 4, 9, and 10. The mutants seemed to fall into multiple retinol binding classes. Mutants 4 and 9 exhibited fluorescence enhancement about one-fifth of the amount of EcR1, while Mutants 7 and 12 exhibited no detectable fluo-

rescence enhancement. The other mutants exhibited fluorescence enhancement that was intermediate between one-fifth and zero (Figure 3). One mutant was verified to exhibit excellent binding of another ligand known to bind EcR1: Mutant 9 showed saturable binding of 16-AP and the shape of the binding curve suggests that the K_d for this ligand and Mutant 9 is about the same as for the EcR1-16-AP complex (Figure 4), though with a higher binding capacity than EcR1. While the amount of binding may be double that of EcR1, given that the ligand saturated at about twice the fluorescence intensity, this corresponds to about one molecule of 16-AP bound per molecule of Mutant 9.

DISCUSSION

In a companion paper [13], we analyzed the sequences of the four repeats of human IRBP to predict essential structures and functional domains within the Repeat 1 protein. We found sequence similarity between IRBP Domain A and eglin c, a protease inhibitor that functions by binding tightly to the active

TABLE 2. SUMMARY OF THIRTEEN EcR1 VARIANTS AND WILD TYPE EcR1

Mutant	Substitution	Expressed in E. coli	Soluble in E. coli	Relative expression	Antibody detection	Solubility	WT CD	Fluorescence enhancement
1	V116N	N						
2	L147A	N						
3	R148D	N						
4	G152A	Y	Y	++	Y	++	Y	2+
5	G153A	N						
6	L208A	Y	N	+	Y	-		
7	E218A	Y	N	++	Y/N	++		-
8	T237A	Y	N	++	Y	+		0.5+
9	G239T	Y	N	+++	Y	++	Y	2+
10	I249A	Y	N	++	Y	+	Y	1.5+
11	E251A	Y	N	+	Y	++		-
12	G278A	Y	N	+	Y	++		1.5+
13	P281A	Y	N	++++	Y	+/-		1+
EcR1	none	Y	Y	20+	Y	20+	Y	10+

Codes used in the table: Y = Yes, N = No, blank = not tested, unknown. The number of pluses indicate the relative levels of expression, with 20+ indicating about 20 mg from 12 L of culture and one + indicating roughly 1 mg from 12 L of culture. For solubility 20+ indicates no visually observable turbidity at about 20 µM concentration, one + indicates no visually observed turbidity at about 1 µM protein concentration, intermediate numbers of pluses indicate no turbidity to visual inspection at 2 µM, the +/- indicates some evidence of turbidity at 1 µM. For fluorescence enhancement, 10+ indicates about a 10-fold enhancement of retinol fluorescence with 1 µM protein compared to the fluorescence of 1.5 to 3 µM free retinol, while "-" indicates no additional fluorescence over that of free retinol. Intermediate values of 0.5+ to 2+ indicate subjective relative fluorescence enhancement versus free retinol based on visual inspection of binding curves. Graphs for representative binding curves are presented in Figure 3. Fluorescence Enhancement means retinol fluorescence enhancement, WT CD indicates whether a protein was tested by CD scans and whether the conformation was grossly similar to EcR1. The antibody used (Ab) for detection on western blots was H3B5.

site of a protease. We extended the previous studies of sequence similarities between Domain B of IRBP and Tsp and related proteases across most of the biological kingdoms, and we examined the possibility of shared functions between Tsp and IRBP. The results suggested no shared functions despite sequence similarities, implying that the conservation of amino acid sequence maintains the same structure, not function. Also, we found a conserved pattern of alternating α -helices and β -strands. As a consequence of those studies [13], here we mutated amino acids that might be involved in the function of binding ligands. We made single point substitutions as we thought these would be less invasive in changing tertiary structure in contrast to truncations of several or many amino acids that might greatly affect conformation or tertiary structure.

Summary of the thirteen mutations in EcR1: A summary of the characterization of all 13 mutants is found in Table 2. Most of the mutants had significantly reduced retinol fluorescence enhancement (Figure 3), suggesting that each amino acid substitution affected the retinol binding site. Mutations that affected the retinol binding site support the thought that the binding site is near a cluster formed in three dimensions from the C-terminal ends of parallel β -strands, as in α/β barrel proteins [15]. The locations of these mutant amino acids are not close to each other in the primary sequence, but instead are spread across a large part of Domain B.

We developed several criteria to judge whether the mutant proteins had normal conformation. First, adequate concentration, optical transparency, and lack of a pellet following centrifugation would suggest that the mutant was soluble. Second, if the mutant showed saturable binding in binding titration curves, this would suggest that it was native; proteins beginning to aggregate may bind excess ligand and generate a nonsaturable increase in fluorescence. Similarly, denatured proteins may have exposed hydrophobic regions that may nonspecifically bind ligand, with a gradual increasing amount of fluorescence in excess of the retinol ligand alone. This did not occur with our non-binding mutants. Third, we were able to purify several of the proteins to >95% purity, and these were examined by circular dichroism. They exhibited CD spectra similar to EcR1 (as discussed immediately below), consistent with a soluble and native protein. Thus, we concluded that these proteins were approximately native in conformation and suitable for analysis of their binding functions.

Circular Dichroism studies: Circular dichroism (CD) spectra can be considered rough fingerprints of a protein's native state and can be used to determine if a particular mutation has perturbed wild type structure. While gross changes to secondary and tertiary structure will be detectable, more subtle changes may not be observed by this method; we intended to assess possible gross changes caused by a sequence variation

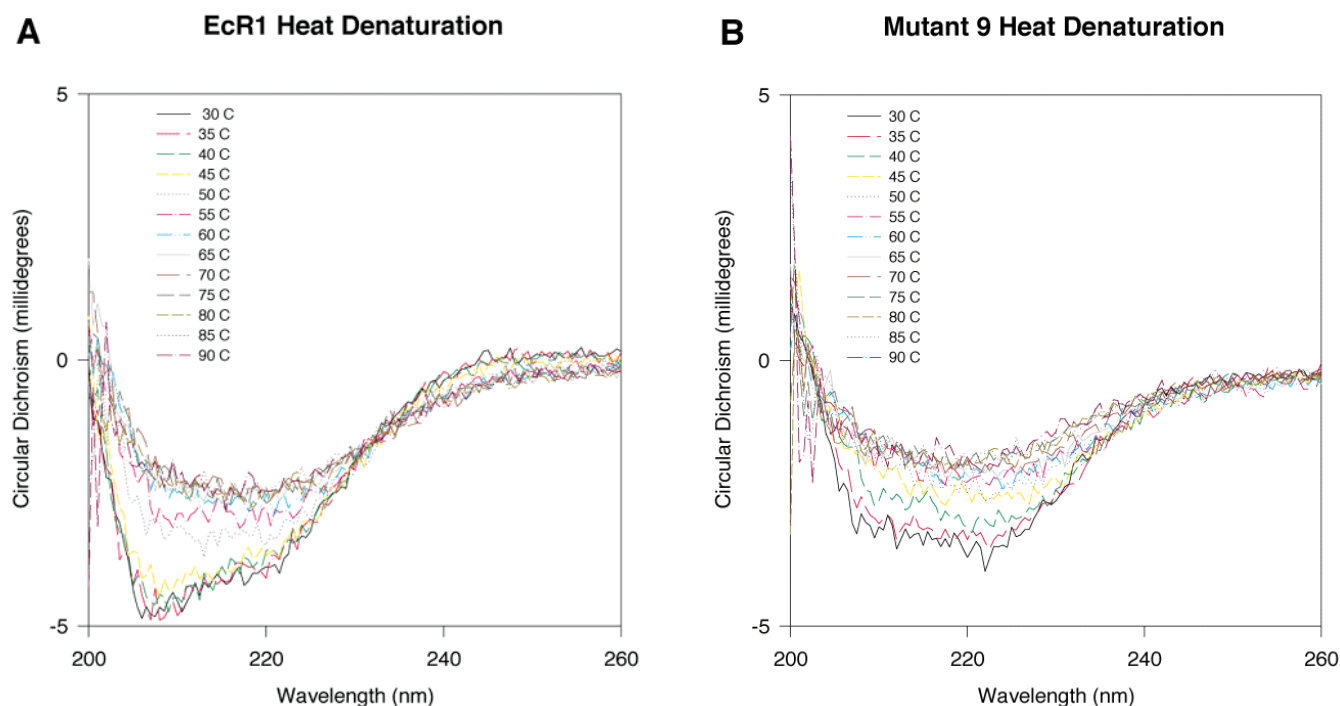


Figure 5. CD measurements of EcR1 and Mutant 9 with increasing temperature. The protein concentration of each sample was 3 μ M, and the buffer was 300 mM NaCl, 50 mM NaPO₄, pH 7. With increasing temperature, each protein began to denature as indicated by the decreasing magnitudes of the CD signals. Sharp changes in the magnitudes of the CD signals at 45 °C for Mutant 9 and at 60 °C for EcR1 suggested that the proteins undergo a transition from a principally native state to a mostly denatured state near these temperatures. There was a residual CD signal in both proteins even at high temperatures (85-90 °C). This signal may represent some remaining secondary structure, perhaps β -strand, which has been reported in other proteins at high temperature.

as compared to the CD spectrum of EcR1. We examined the CD spectra of Mutants 4, 9, and 10, the only mutants for which we could obtain sufficient amounts and purity for CD scans, and compared them to EcR1. While not identical, the three mutant spectra all exhibited a large positive peak at about 195 nm, a large valley at 208 nm, and a negative shoulder at 222 nm, similar to EcR1 and suggesting that they retained the same gross conformation and secondary structure as EcR1. The effects of the mutations suggest that several of the sequence changes result in reduced retinol fluorescence enhancement (Figure 3, Table 2) but that not all of the changes resulted in different CD spectra.

Thermal denaturation affecting ligand binding: Consistent with the apparent denaturation of proteins, we tested whether retinol binding would be lost coincident with the heat denaturation of Mutant 9 (Figure 6). This protein lost its capacity to enhance retinol fluorescence after an incubation at a temperature above its denaturation point (Figure 6). On the other hand, EcR1 incubated at 50 °C did not denature according to the CD scans (Figure 5), and this protein retained full retinol fluorescence enhancement (Figure 6). This experiment

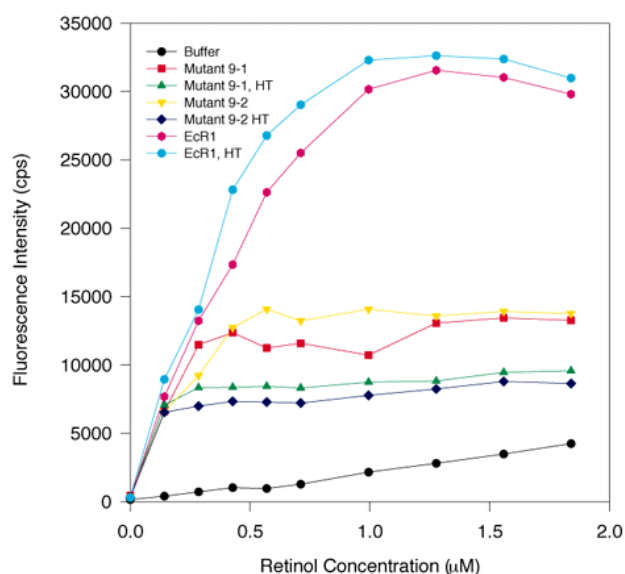


Figure 6. Effect of 50 °C incubation on fluorescence enhancement. 1 μ M of two Mutant 9 preparations (Mutant 9-1 and Mutant 9-2) and EcR1 were tested in a retinol fluorescence enhancement assay with or without a five minute 50 °C preassay incubation (labeled HT for heat treated). The 50 °C incubation had little effect on EcR1 retinol binding. The heat treatment reduced the ability of Mutant 9 to bind retinol. These results are consistent with the hypothesis that retinol fluorescence enhancement is dependent on a native state of the binding protein. For Mutant 9, incubation at 50 °C was above its transition temperature of 45 °C, the incubation substantially denatured this protein, and according to the hypothesis, rendering it less capable of binding retinol. The 50 °C incubation of EcR1 was below its transition temperature of 60 °C, which did not denature the protein, and according to the hypothesis should have no adverse effect on retinol binding.

is consistent with the hypothesis that a native conformation of the protein is needed for retinol to bind to IRBP.

Analysis of the mutants: Several mutations (scattered throughout Domain B) profoundly reduced retinol binding, suggesting that much of this domain is needed for proper retinol binding. These results are somewhat different from the block deletions of Baer et al. [2], who found that a central sequence of amino acids 81-208 from Module 4 of *Xenopus* apparently has two retinol binding sites, though they also suggested that the C-terminal fragment of *Xenopus* may have binding capacity for retinol. Our results showed that a single point mutation near the C-terminal end of the repeat could greatly reduce binding in the entire repeat, whereas Baer et al. [2] showed that even when eliminating the first 80 and last 100 amino acids of the *Xenopus* Module 4, the remainder (81-208) still retained the capability of binding two retinols and has nearly the same binding capacity of the intact Repeat. Possibly our results differ because of the species difference (human vs *Xenopus*) or because of the difference in Repeat (Repeat 1 vs Repeat 4). Baer and co-workers [2] truncated the protein, which might result in a different shape or possibly even a loss in the overall conformation. However, it must be noted that their protein fragments were still soluble suggesting a native conformation. Another possibility is that our point substitutions exert subtle gain-of-function properties that change the interaction of Domain A with Domain B, perhaps preventing ligands from binding to Domain B.

It may be significant that Mutants 1, 2, 3, and 5, which were poorly expressed in *E. coli*, are clustered in the first third of Domain B. Native mutants with reduced binding are clustered in the C-terminal two-thirds of Domain B. There is evidence to suggest that Domain B may consist of two sub-domains, as indicated by the finding of an insertion about half way through Domain B of one of the CTPs. Thus, our findings of a clustering of poor expressers in the first part of Domain B and native but inactive ligand binding mutants in the latter part of Domain B may support the division of Domain B into two distinct structural motifs.

Summary and Conclusions: The lack of shared functions between Tsp and IRBP [13] suggests that the conservation of amino acid sequences between these two proteins is important to the maintenance of an evolutionarily successful tertiary structure that can be adapted for very different biological functions. The apparent fusion of an eglin c-like domain to Domain B of an IRBP/Tsp ancestor may have led to the transformation in function from a protease to a retinoid-binding protein, and this fusion might be an example of exon-shuffling [13]. The Domain A-eglin c similarity, coupled with the already known tight binding of eglin c to proteases, led us to propose a close interaction between Domain A and Domain B in a single IRBP repeat [13]. Those results placed constraints on the choices of amino acids to mutate that would affect retinol-binding functions without interfering with the overall structure of Repeat 1: Mutation of amino acids shared between IRBP and Tsp might cause changes in structure. Also, amino

acids conserved among IRBP repeats might be structurally conserved contact points between Domains A and B. However, conservation of β -strand positions can be used to predict the locations of active site residues and consequently to select candidate amino acids of the retinol binding site.

We conclude from these studies that Domain B is needed to bind retinol and 16-AP. Our studies allow us to rule out several hypotheses about the nature of the binding site in this domain. First, the results rule out the possibility that the binding site for retinol is small, since many amino acids can be mutated individually that affect binding, implying that the binding site is quite large in the context of the domain. Second, that point mutations affect binding also suggests that the binding site is not just a hydrophobic patch on the surface of the protein, as many of the point mutations were not changes decreasing hydrophobicity, and most actually increased hydrophobicity. Third, the results rule out the possibility of a hydrophobic patch hypothesis for another reason: The I249A substitution (Mutant 10) exhibits some binding while the E251A mutation (Mutant 11), just two amino acids away, exhibits no binding activity, suggesting that the precise position of the substitution is the critical variable, not necessarily the hydrophobicity. Fourth, the conformation of the protein is critical, as heat denatured protein loses the ability to bind the ligand: If conformation were not a critical issue, we would expect partially denatured protein to bind substantial quantities of the ligand, and this did not occur.

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