The transfer of transthyretin and receptor-binding properties from the plasma retinol-binding protein to the epididymal retinoic acid-binding protein

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Members of the lipocalin superfamily share a common structural fold, but differ from each other with respect to the molecules with which they interact. They all contain eight β-strands (A–H) that fold to form a well-defined β-barrel, which harbours a binding pocket for hydrophobic ligands. These strands are connected by loops that vary in size and structure and make up the closed and open ends of the pocket. In addition to binding ligands, some members of the family interact with other macromolecules, the specificity of which is thought to be associated with the variable loop regions. Here, we have investigated whether the macromolecular-recognition properties can be transferred from one member of the family to another. For this, we chose the prototypical lipocalin, the plasma retinol-binding protein (RBP) and its close structural homologue the epididymal retinoic acid-binding protein (ERABP). RBP exhibits three molecular-recognition properties: it binds to retinol, to transthyretin (TTR) and to a cell-surface receptor. ERABP binds retinoic acid, but whether it interacts with other macromolecules is not known. Here, we show that ERABP does not bind to TTR and the RBP receptor, but when the loops of RBP near the open end of the pocket (L-1, L-2 and L-3, connecting β-strands A–B, C–D and E–F, respectively) were substituted into the corresponding regions of ERABP, the resulting chimaera acquired the ability to bind TTR and the receptor. L-2 and L-3 were found to be the major determinants of the receptor- and TTR-binding specificities respectively. Thus we demonstrate that lipocalins serve as excellent scaffolds for engineering novel biological functions.

Key words: vitamin A, lipocalin, protein engineering, RBP–ERABP chimaera, RBP receptor.

INTRODUCTION

Vitamin A is transported in the plasma as retinol bound to a specific 21 kDa carrier protein, called retinol-binding protein (RBP). The majority of RBP circulates in the plasma as a macromolecular complex with another protein, transthyretin (TTR) [1]. There is considerable evidence to suggest that under physiological conditions RBP delivers retinol to vitamin-A-requiring cells via specific cell-surface receptors [2–10]. Thus RBP is involved in multiple molecular interactions: it binds retinol and interacts with TTR and the cell-surface receptor.

The structural elements that determine the binding of retinol to RBP have been identified through elucidation of the three-dimensional structure of RBP by X-ray diffraction [11,12]. The polypeptide chain of RBP is folded into an orthogonal β-barrel, made up of eight anti-parallel β-strands (labelled A–H; Figure 1). The interior of the barrel, known as the retinol-binding pocket, is lined with hydrophobic residues that make specific contacts with the retinol and determine the ligand specificity. The barrel is open at one end and closed at the other. The open end of the barrel, through which retinol presumably enters and exits the binding pocket, is made up of three loops, which connect the β-strands A and B, C and D, and E and F. These loops are refered to as L-1, L-2 and L-3, respectively, in this paper (Figure 1).

The molecular basis of RBP–TTR interaction was first investigated by examining the effect of point mutations on TTR binding and deletions introduced into loops of RBP [13]. The authors demonstrated that all the three loops (L-1–L-3) near the open end of the β-barrel interact with TTR, with L-3 making the strongest contact. These findings are in agreement with the structural data subsequently obtained by X-ray diffraction studies of the chicken [14] and human [15] RBP–TTR complexes.

The third binding property of RBP, which is perhaps the most physiologically important but the least well understood, is that with the cell-surface receptor. In some cell types, such as those from the pigment epithelium [3,9], intestine [2], placenta [5,6] and testis [7], this interaction results in the transfer of retinol to the cell, with the resultant apo-form of RBP returning to circulation. In others, such as hepatocytes [4,8], retinol uptake appears to involve endocytosis of the retinol–RBP complex. Structure–function studies [13], using mutants of RBP and the placental receptor, showed that loops L-2 and L-3, which participate in the RBP–TTR interaction, are also involved in binding to the receptor. The affinities of the loops for the receptor, however, were found to be the reverse of those for TTR; that is, L-2 binds to the receptor more strongly than L-3.

Although the mutagenesis studies [13] showed that mutation of RBP loops decreases or abolishes the binding of RBP to its receptor, one could argue that these effects are a consequence of structural changes elsewhere in the protein, rather than the direct effect of the residues mutated. One approach to rule out this possibility would be to implant these loops into a protein that has a similar overall fold to RBP but which lacks the TTR- and

Abbreviations used: ERABP, epididymal retinoic acid-binding protein; RBP, retinol-binding protein; TTR, transthyretin; PEG 8000, poly(ethylene glycol) 8000.

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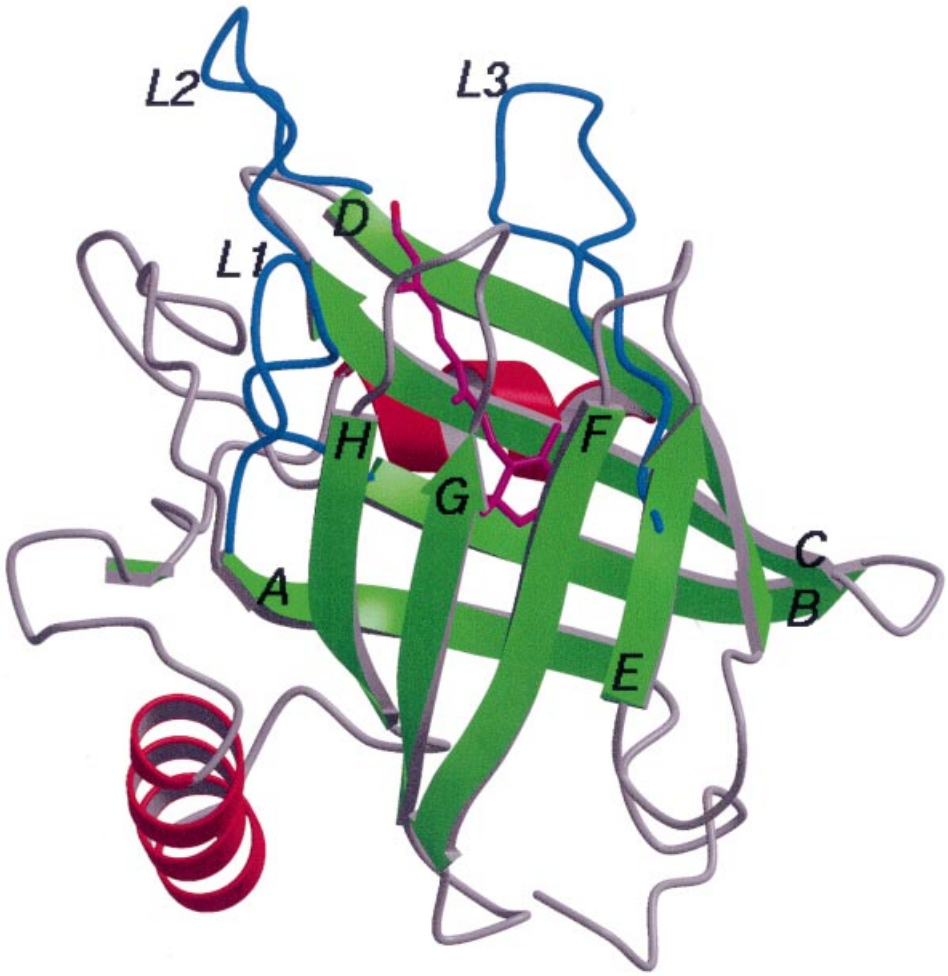

Figure 1 Structure of the ERABP–retinoic acid complex

Strands (labelled A–H) are coloured green, helices are coloured red and the loops are in grey. Retinoic acid is shown as a purple stick model. The blue ribbon represents the position of loops L-1–L-3 in RBP after superposition on the holo-ERABP structure.

receptor-binding properties, and to demonstrate that these properties can be conferred on the recipient protein.

Towards this end, we chose the epididymal retinoic acid-binding protein (ERABP) [16–18]. ERABP shows a remarkable similarity to RBP in the structure of the β-barrel, but displays considerable differences in the structure of the loops that form the entrance to the ligand-binding pocket [18] (see Figure 1). Consistent with these differences, here, we find that ERABP is incapable of binding to TTR and the RBP receptor. However, when the loops of RBP are substituted, ERABP acquires the ability to bind TTR as well as the receptor.

EXPERIMENTAL

Materials

All-*trans*-retinoic acid, poly(ethylene glycol) 8000 (PEG 8000), goat γ-globulins, dibutyl phthalate and TTR were purchased from Sigma. Dinonyl phthalate was from BDH. Restriction and modification enzymes were purchased from Boehringer Mannheim or Promega. *Pfu* DNA polymerase was purchased from Stratagene. All other reagents and chemicals were of appropriate grade and obtained from Sigma, Pharmacia or BDH. *Escherichia coli* XL1-Blue (Stratagene) and BL21 (DE3) (Novagen) were used for general cloning and expression purposes respectively.

Expression and purification of binding proteins

RBP and ERABP were expressed in *E. coli* and purified by affinity chromatography as described previously [19,20]. RBP was expressed as the native protein and purified on TTR-affinity resin [19]. ERABP was expressed as a fusion protein with the streptavidin-binding sequence added to its C-terminus. The resulting recombinant, referred to as ERABP-streptag fusion protein, was purified using streptavidin-resin [20]. The chimaeric proteins (see below) were purified by TTR-affinity chromatography. The concentrations of the purified proteins were determined by measuring the absorbance at 280 nm and using the molar absorbtion coefficients, ϵ, at 46760 $M^{-1} \cdot cm^{-1}$ for RBP and 17400 $M^{-1} \cdot cm^{-1}$ for EBABP and its chimaeras.

Transplantation of RBP loop residues into ERABP

The ERABP chimaeras were generated in the T7 promoter-based expression vector construct, pKS-ERABP [20]. Loops L-1, L-2

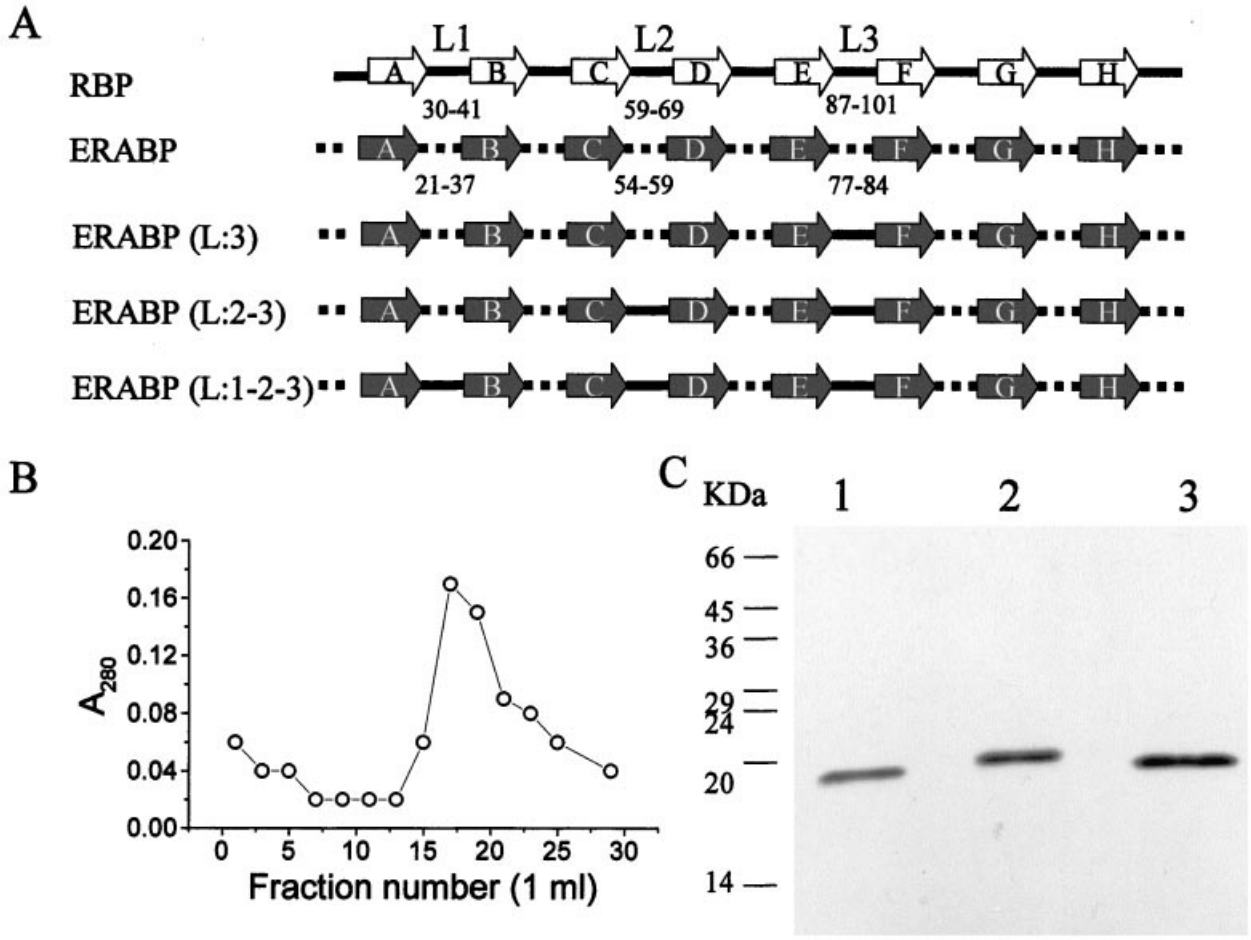

Figure 2 Expression and purification of ERABP–BP chimaeric proteins

(**A**) Schematic representation of the secondary structures of RBP, ERABP and the chimaeric constructs; only the β-sheets (arrows) and the loop regions (solid lines for RBP and dotted lines for ERABP) are shown. The numbers under the loops labelled L-1, L-2 and L-3 refer to positions of amino acid residues in the primary structure. (**B**) Purification of the ERABP(L:1-2-3) chimaera on TTR-affinity resin; the bed volume was 8 ml. Chromatography profiles for other chimaeras were similar (not shown). (**C**) SDS/PAGE of the ERABP chimaeric proteins purified by TTR-affinity chromatography. Lane 1, ERABP(L:3); lane 2, ERABP(L:2-3); lane 3, ERABP(L:1-2-3). Positions of the marker proteins are shown on the left.

and L-3 of ERABP (Figure 2A) were replaced with the corresponding loops from RBP by PCR using *Pfu* DNA polymerase. First, L-3 of ERABP was substituted with that from RBP. For this, PCR was performed using the following primers: 5′-GGCTACGCCCCAGTACTTCATCTTAAACTTCGCCGGACCATCCCCCTCAGTAGC-3′ and 5′-TCCTTTCTGCAGAAAGGAAATAAAGAAGTTGTTGTTGAAGCCACCGAC-3′. The 3′ ends of these primers contained sequences corresponding to the region of ERABP outside the L-3 and the 5′ ends had one half of the RBP L-3 loop sequence (underlined) to be inserted. The resulting PCR product, which contained the RBP L-3 sequence, but which was depleted of the ERABP L-3 sequence, was ligated and used to transform *E. coli*. Plasmid DNA isolated from the transformants was sequenced to identify the chimaeras.

The resulting chimaeric construct, ERABP(L:3), was next used as a template to introduce the L-2 sequence of RBP using the primers 5′-CAAAAGACGGACTCGGCCGGTGGTCAGGCCAGAAGGTT-3′ and 5′-AATAACTGGGACGTGTGTGTGCTGGAGAAGGTTACA-3′ to generate ERABP(L:2-3). This was then used to introduce the L-1 sequence using primers 5′-GCCCTCGGGGTCCTTAAAGGCAATCTCATACCAGAAGCC-3′ and 5′-CTCTTTCTGCAGGACAACATCGCCATGGTGGTCGAGCTGAAAGAGAAC-3′ to generate the ERABP(L:1-2-3) chimaeric construct. The underlined sequences encode the RBP loop sequences.

Assay for retinoic acid binding and TTR-binding activity

Binding of all-*trans*-retinoic acid to ERABP and its chimaeras was determined as described in [20].

The TTR-binding activity of the binding proteins was assessed using a radioligand competition assay developed for the purpose. TTR (500 nM) was incubated at 37 °C for 15 min with 5 nM ^{125}I-RBP in the absence and presence of increasing amounts of unlabelled competing protein in a total volume of 100 μl of PBS (20 mM sodium phosphate/150 mM NaCl, pH 7.4). At the end of incubation, the tubes were chilled on ice for 2 min and then 50 μl of ice-cold 5 mg/ml goat γ-globulins in 0.1 M sodium phosphate, pH 7.4, were added, followed by 100 μl of 25 % (w/v) PEG 8000. The samples were vortexed immediately, incubated on ice for 25 min and centrifuged at 12000 ***g*** for 8 min at 4 °C. The tubes were overlayered with 200 μl of ice-cold dibutyl phthalate, and centrifuged for an additional 2 min. After freezing

in a dry ice/ethanol bath, the bottoms of the tubes containing the pellets were cut off and counted for radioactivity in a Wallac γ counter. All assays were carried out in triplicate; the S.E. was typically less than 5%. Background precipitation of ^{125}I-RBP was determined in the absence of TTR, and all values were corrected for the background precipitation.

Assay of receptor-binding activity

Binding of ERABP and its chimaeras to placental membranes was measured by testing their ability to compete with ^{125}I-RBP for binding to the receptor. The assays were carried out in triplicate by the oil centrifugation method, as described previously [6]. The data were transformed into the Scatchard form to estimate the equilibrium dissociation constants.

RESULTS

Expression and purification of ERABP–RBP chimaeric proteins

Before proceeding to make the chimaeric constructs, we expressed the ERABP-streptag fusion protein in *E. coli* from vectors that allowed its secretion into the periplasm [19]. The fusion protein was purified from the periplasmic extracts of the cells by streptavidin-affinity chromatography and tested for its ability to bind to TTR and the placental RBP receptor. The protein failed to bind TTR and the receptor (see below, Figures 4 and 5). We next sequentially substituted the RBP loops into the ERABP-streptag fusion construct and expressed the resultant chimaeric constructs (Figure 2A) in *E. coli*. Attempts were then made to purify the secreted proteins using both the streptavidin- and TTR-affinity resins. All three chimaeric constructs could be purified using both resins. Figure 2(B) shows a typical chromatographic profile for purification of one of the chimaeric constructs, ERABP(L:1-2-3). Figure 2(C) shows SDS/PAGE analysis of the chimaeric proteins purified to homogeneity on the TTR-affinity resin. In contrast to the chimaeric proteins, the ERABP-streptag fusion protein could not be purified by the TTR-affinity resin (results not shown); this is consistent with the binding data shown in Figure 4 (see below). The finding that the chimaera containing L-3 alone (ERABP-L:3) could be purified by the TTR-affinity resin suggests that L-3 is the major determinant of RBP–TTR interaction. The substitution of RBP loops into ERABP did not alter the ligand-binding ability of the protein: like ERABP, all chimaeric proteins bound retinoic acid (results not shown).

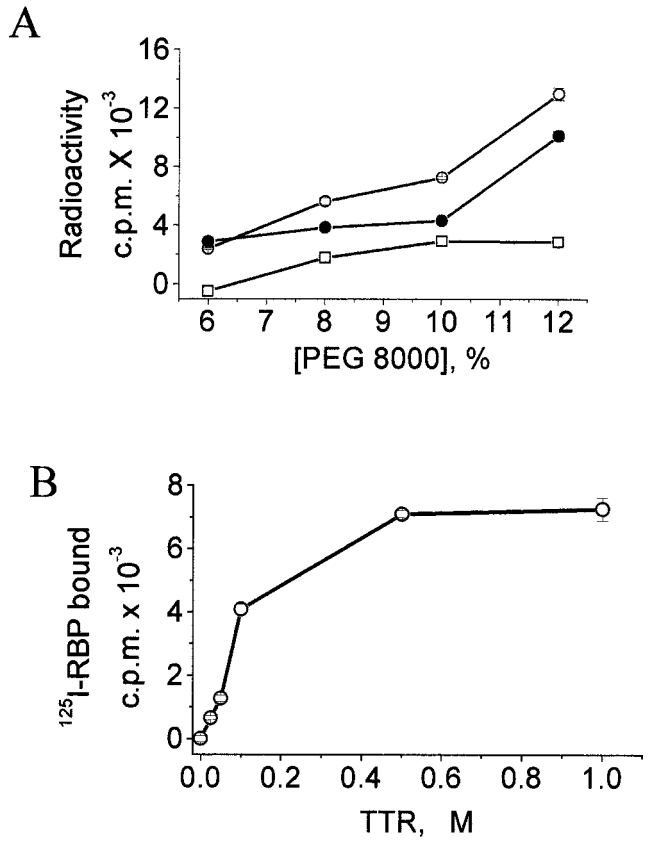

Figure 3 Radioactive assay of RBP–TTR interaction

(**A**) Effect of PEG 8000 concentration on the precipitation of ^{125}I-RBP. ^{125}I-RBP (5 nM) was incubated in the presence (○) and absence (●) of 0.5 μM TTR and then precipitated with various concentrations of PEG 8000 as described in the Experimental section. The difference, representing the precipitated ^{125}I-RBP–TTR complex (□), is also shown. The values represent means ± S.E.M. ($n = 3$). (**B**) Effect of TTR concentration on the precipitation of ^{125}I-RBP. ^{125}I-RBP (5 nM) was incubated with various concentrations of TTR and precipitated with 10% PEG 8000. The data points (means ± S.E.M) represent radioactive counts after correction for background precipitation.

Radioactive assay of RBP–TTR interaction

In order to estimate the relative abilities of the fusion proteins to bind to TTR, we developed a sensitive radioactive assay. This was found to be necessary because the published methods, employing fluorimetric assays [21], require milligram quantities of the interacting proteins, and the expression of ERABP and the chimaeric constructs in such quantities proved to be difficult.

The assay was similar to that used for the assay of detergent-solubilized RBP receptor described previously [22]. It is based on the observation that at lower concentrations, PEG 8000 precipitates the RBP–TTR complex completely, but only a small fraction of RBP. This is seen in Figure 3(A), which shows that the amount of ^{125}I-labelled RBP precipitated at and above a PEG concentration of 8% was greater when TTR was present in the assay mixture than when it was omitted. The difference in the amount of radioactivity precipitated in the presence and absence of TTR was taken as a measure of the amount of ^{125}I-RBP–TTR complex. The difference remained unchanged between PEG concentrations of 10% and 12%, suggesting that 10% PEG is enough to precipitate the complex completely; 10% PEG was, therefore, used in the subsequent assays.

We next tested the validity of the method by determining the saturation isotherms. Binding of TTR to 5 nM ^{125}I-RBP reached saturation at 0.5 mM TTR (Figure 3B), with 50% maximal binding occurring at 0.09 ± 0.01 μM TTR. Binding of TTR to ^{125}I-RBP was inhibited by increasing concentrations of RBP (Figure 4; see below). Scatchard analysis of the competition data yielded a K_D value of 0.14 ± 0.02 μM, which is in close agreement with the value (0.19 ± 0.1 μM) recently obtained for binding of the first RBP molecule by MS analysis [23].

Binding of ERABP–RBP chimaeras to TTR

Using the radioactive method described above, we tested the ability of the apo-froms of the three chimaeric constructs to compete with ^{125}I-RBP for binding to TTR (Figure 4A). The competition data were transformed to the Scatchard form (Figure 4B) to determine the affinities of interactions of individual chimaeric proteins. Of the three chimaeric proteins, ERABP(L:1-2-3) showed the highest affinity for TTR ($K_D =$

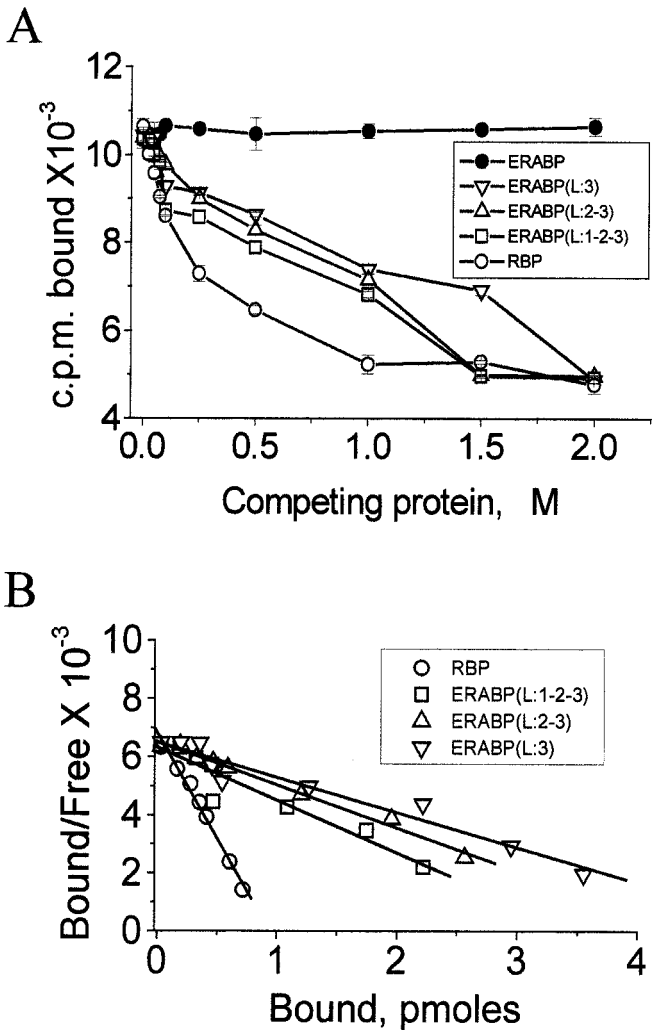

Figure 4 Binding of ERABP–RBP chimaeric proteins to TTR

(**A**) Competitive inhibition of ^{125}I-RBP–TTR complex formation by RBP, ERABP and the chimaeric proteins. ^{125}I-RBP (5 nM) was incubated 0.5 μM TTR in the presence of increasing concentrations of binding proteins, as indicated. TTR-bound radioactivity was assayed as described in the Experimental section. (**B**) Scatchard analysis of RBP–TTR interaction; the competition data (**A**) were transformed and plotted according to the Scatchard equation.

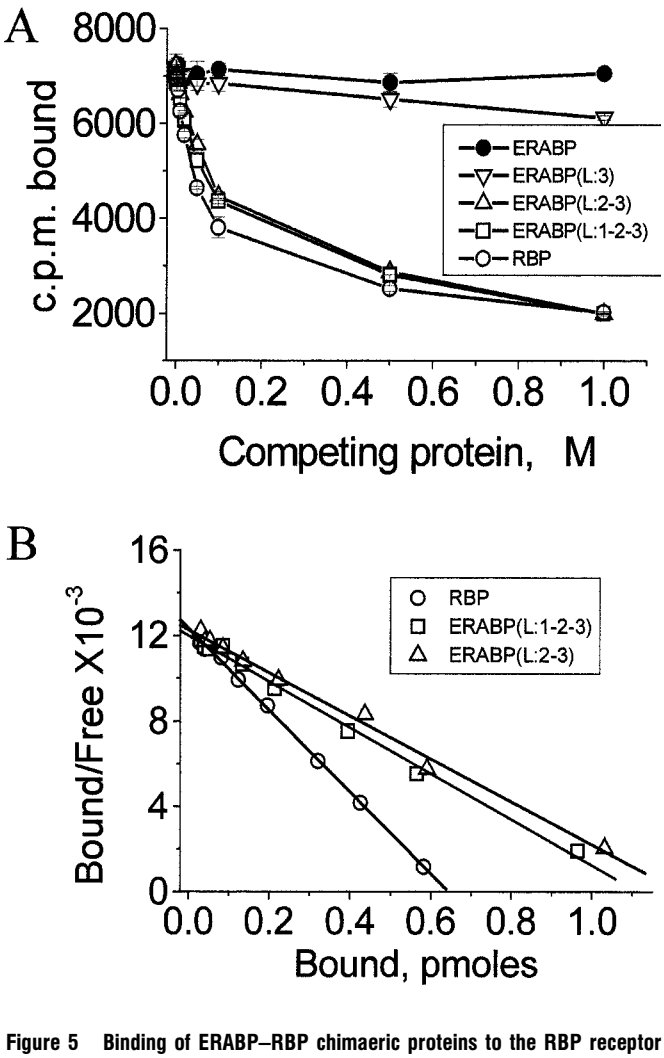

Figure 5 Binding of ERABP–RBP chimaeric proteins to the RBP receptor

(**A**) Competitive inhibition of ^{125}I-RBP binding to placental membranes by RBP, ERABP and the chimaeric proteins. Placental membranes (0.5 mg/ml) were incubated with 2.5 nM ^{125}I-RBP and increasing concentrations of unlabelled proteins, as indicated. The membrane-bound radioactivity was determined as described in the Experimental section. Values represent means $\pm$ S.E.M. (**B**) The competition data from (**A**) were plotted in the Scatchard form.

Table 1 Binding parameters for interaction of RBP and the ERABP chimaeras

Results are means $\pm$ S.E.M., $n = 3$.

	K_D (μM)	
Protein	TTR	Receptor
ERABP	–	–
ERABP(L:3)	0.88 $\pm$ 0.043	Too high*
ERABP(L:2-3)	0.69 $\pm$ 0.026	0.098 $\pm$ 0.01
ERABP(L:1-2-3)	0.53 $\pm$ 0.018	0.092 $\pm$ 0.02
RBP	0.14 $\pm$ 0.02	0.053 $\pm$ 0.008

* The competitive displacement was too low to obtain an accurate estimate of the value.

0.53 $\pm$ 0.018 μM), followed by ERABP(L:2-3) ($K_D = 0.69 \pm 0.026$ μM) and ERABP(L:3) ($K_D = 0.88 \pm 0.043$ μM; Table 1). The difference in the affinity between ERABP(L:3) and that for ERABP with all three loops of RBP [ERABP(L:1-2-3)], however, was rather small, indicating that L-3 of RBP accounts for most of the binding affinity of RBP to TTR. Interestingly, the holoforms of none of the chimaeric proteins showed any binding to TTR (results not shown). This may be due to the carboxyl group of the retinoic acid in the binding pocket altering the conformation of the loops, thereby preventing their binding to TTR. It would be interesting to perform these experiments in ERABP with a binding pocket engineered to bind retinol [20].

Binding of ERABP–RBP chimaeras to the RBP receptor

The receptor-binding properties of the chimaeras were measured by a competition assay using ^{125}I-labelled RBP and human placental membranes. Figure 5(A) shows that the inhibition of ^{125}I-RBP binding by the chimaeric proteins increases as the concentration of the competing proteins increases. Neither the apo- nor the holo-form of ERABP could inhibit the binding of ^{125}I -RBP, even at 1 μM. Chimaera ERABP(L:3) showed slight

inhibition ($\approx 20\%$) of binding at 1 μM. Higher concentrations could not be tested due to the scarcity of purified protein, and hence its affinity for the receptor could not be determined. Chimaeras ERABP(L:2-3) and ERABP(L:1-2-3) both caused effective displacement of ^{125}I-RBP binding with estimated K_D values of 0.098 ± 0.01 μM and 0.092 ± 0.02 μM, respectively (Table 1). Under these conditions, RBP bound to the receptor with a K_D of 0.053 ± 0.008 μM, which is in agreement with the values reported previously [19].

DISCUSSION

Among the members of the lipocalin superfamily so far discovered [24], ERABP shares the highest structural homology with RBP [18]. These two proteins display only minor differences with respect to their ligand-binding specificities: whereas RBP binds all the three physiologically relevant retinoids, including retinol, retinaldehyde and retinoic acid, the specificity of ERABP is restricted to retinoic acid. Based on the structural data, it was proposed that charged residues present near the entrance to the binding pocket restrict the specificity of ERABP to retinoic acid [18]. Experimental evidence in support of this proposition has been presented recently in a study which demonstrated that mutation of the two charged residues to neutral residues is sufficient to extend the specificity of ERABP to retinol and retinal, thereby conferring the ligand-binding specificity of RBP on to ERABP [20].

In this study, we asked if the other properties of RBP, i.e. its TTR- and receptor-binding properties, can be transferred to ERABP. For this, we first established that ERABP is incapable of binding to TTR (Figure 4) and to the receptor (Figure 5). We then exploited the information available in the literature to engineer the desired functions into ERABP. X-ray diffraction [14,15] as well as mutagenic [13] studies showed that the TTR-binding regions of RBP are associated with the loops (Figures 1 and 2) that form the entrance to the ligand-binding site. The receptor-binding domains of RBP also appear to reside in these loops [13]. Thus we replaced the loops of ERABP with the corresponding loops of RBP, and tested whether these loops confer properties of RBP on to ERABP and, if so, which loops are involved in which function, and to what extent.

The results (Figure 4) demonstrated that substitution of L-3 alone was sufficient to confer the TTR-binding properties on to ERABP. Substitution of the other two loops caused no significant change in the affinity of the chimaera for TTR. The TTR-binding affinities of the chimaeras bearing L-3 were only 4-fold less than that of RBP (Figure 4 and Table 1). These data are consistent with the crystal structure of the RBP–TTR complex, which shows that six out of 14 residues of L-3 are in close proximity to TTR, in comparison with one each from L-1 and L-2 [14,15]. The data are also consistent with our previous report [13] that L-3 in RBP makes the strongest contact with TTR, but are inconsistent with the suggestion that L-2 makes a significant contribution to the binding of TTR. However, it should be noted that in our previous study we mutated the hydrophobic residues, Leu-63 and Leu-64, of L-2 into hydrophilic and charged residues (Ser and Arg, respectively). Since the protein–protein interaction interface in the RBP–TTR complex is hydrophobic in nature, the effect of these mutations was perhaps so drastic that the interactions at the L-3 binding site were also affected.

With regard to the role of loops in binding to the RBP receptor, the results (Figure 5 and Table 1) show that ERABP substituted with L-3 of RBP displayed very low affinity to the receptor (the affinity could not be determined). Addition of L-2 to this chimaera was sufficient to confer binding affinity ($K_D = 0.098 \pm 0.01$ μM) that was comparable with that of the RBP–receptor interaction ($K_D = 0.053 \pm 0.008$ μM; Figure 5 and Table 1). These data are consistent with our previous study [13], which showed that mutation of L-2 residues (Leu-63 and Leu-64) to hydrophilic and charged residues completely abolished the binding of RBP to the receptor, whereas deletion of L-3 residues partially reduced the binding. L-1 does not appear to contribute to the binding.

Whereas the binding of RBP to both TTR and the receptor involves the loops near the entrance to the ligand-binding pocket, the consequences of these interactions seem to be different. Interaction with TTR has been shown to stabilize the binding of retinol to RBP [1]. In contrast, binding to the cell-surface receptor induces the release of retinol from the binding pocket [2,3,6,7,9,10]. It seems therefore that L-2 facilitates the release of retinol, whereas L-3 stabilizes retinol's binding to RBP. X-ray diffraction analyses of the holo- and apo-forms of RBP [12] suggested that L-1 of RBP, in particular Leu-35, controls the movement of retinol into and out of the binding pocket, by switching between the closed and open conformational states. Taken together with the current data, we propose that the interaction of L-3 with TTR stabilizes the closed conformation, whereas binding of L-2 to the receptor stabilizes the open conformation, via a conformational switch of L-1.

In summary, the demonstration that TTR- and RBP-receptor-binding properties can be engineered into EBABP has two important implications. First, it strengthens our previous argument [13] that RBP binds to the receptor via its loops at the entrance/exit site of the retinol-binding pocket, an orientation that would be ideal for smooth transfer of retinol to cellular RBP [10]. Secondly, it illustrates the suitability of lipocalin scaffolds for engineering of novel functions. Lipocalins contain two distinct regions, a structurally conserved central β-barrel, which forms the ligand-binding pocket, and the loops that form the entrance/exit site for the ligand. These loops display considerable variation and are thought to contribute to the specific macromolecular-recognition properties exhibited by some members of the lipocalin superfamily. The ligand-binding pockets of several lipocalins, including ERABP [20] and bilin-binding proteins [25], have been engineered to alter their ligand-binding specificity. In this study, we showed that the variable loops of lipocalins can also be engineered to confer the desired functional properties. Taken together with the previous studies, the present work demonstrates that lipocalin backbones can serve as excellent scaffolds for engineering multiple functional properties with potential biomedical applications.

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