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Epitope mapping of a monoclonal antibody that blocks the binding of retinol-binding protein to its receptor

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Summary: To define the receptor binding site of retinol-binding protein (RBP) we have generated monoclonal antibodies (mAbs) to human RBP and examined their ability to interfere with the receptor binding. MAbs to two conserved regions efficiently blocked the binding. No major conformational changes in the protein occurred upon mAb binding, since the mAbs could co-immunoprecipitate the RBP-transthyretin (TTR) complex. One blocking mAb showed reactivity to a synthetic peptide corresponding to one entrance loop of the retinol-binding pocket (amino acid residues 60-70). Thus, our results show that at least one of the entrance loops of the barrel of RBP is located in or close to the receptor binding site. It can also be concluded that the receptor and TTR binding sites involve different regions of RBP.

Vitamin A is transported in plasma as the lipid alcohol, retinol, bound to a specific transport protein, the retinol-binding protein (RBP) [for a review see ref. 1]. RBP is mainly synthesized by the hepatocytes of the liver and is normally secreted as a retinol-RBP complex (holo-RBP) which circulates in plasma bound to a thyroxine transport protein, transthyretin (TTR) [2,3]. In plasma, RBP delivers retinol to specific membrane receptors expressed by cells requiring retinoids for normal function or cells involved in transcytosis of retinol [4-6]. The RBP molecule is, thus, involved in at least three specific molecular interactions, i.e. with retinol, with TTR and with the RBP-receptor. At present, only the binding site of retinol is known in detail [7]. To define the receptor binding site of RBP, we have generated monoclonal antibodies (mAbs) to human RBP and examined their ability to interfere with receptor binding. Here we report the epitope-mapping of a mAb that blocks the binding of RBP to its receptor and that co-immunoprecipitates the RBP-TTR complex.

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MATERIALS AND METHODS

Purification of human RBP and TTR
Human RBP and TTR were purified from outdated plasma by affinity chromatography on immobilized human TTR [8] and human RBP [9], respectively.

Monoclonal antibodies to human RBP
Highly purified human RBP dissolved in PBS was emulsified in an equal volume of complete Freund's adjuvant (Difco, Detroit, USA) and injected into the footpads of Balb/c mice. Each mouse received 15-20 μg of RBP. The immunization procedure and the production of hybridomas were as described [10]. For detection of mAbs reactive towards RBP, the supernatants of the growing hybridomas were screened using an EIA procedure. The conditions used for coating of RBP onto microtiter plates and the subsequent incubations were essentially as previously described [10]. Bound antibodies were visualized using an alkaline phosphatase-labelled goat anti-mouse Ig secondary antibody (Sigma). Positive clones were recloned by limited dilution and expanded. Ascites fluids were generated in Pristane primed Balb/c mice and monoclonal IgGs were isolated by affinity chromatography using protein A-Sepharose (Pharmacia-LKB). The IgG isotypes of the purified mAbs were determined using a mouse typer isotyping kit (Bio-Rad). MAbs with the ability to immunoprecipitate RBP radiolabelled using the Bolton-Hunter reagent [11] (Amersham) were selected for the studies presented here.

Expression of RBP from human, rat and Xenopus laevis and rat TTR in HeLa cells
The cDNAs corresponding to human and X. laevis RBP [12,13] and rat TTR [9] were cloned into the eucaryotic expression vector pCMU [14]. Rat RBP was expressed from a mini-gene cloned into pCMU as described [15]. HeLa cells were transfected with these DNA-constructs using the calcium phosphate procedure, either alone or in different combinations as described [14] and labelled with ^{35}S-methionine (Amersham) for 4 hrs using 0.25 mCi of radioactivity per 10^6 cells. Solubilization of labelled cells, immunoprecipitations and SDS-PAGE were carried out as described [14]. Ten μg of purified Ig of the different mAbs were used in each incubation.

RBP-receptor assay
The RBP-receptor binding assay, employing membrane fractions obtained from bovine retinal pigment epithelial cells, was carried out as previously described [4]. Purified Ig's from the different mAbs (500 ng, 5 μg and 50 μg of Ig per incubation) were preincubated over night at 4°C with 50 ng of human RBP radiolabelled with iodine-125 using the Bolton-Hunter reagent [11]. These mixtures were separately added to RPE-membranes equivalent to 20 μg of total membrane protein, and incubated on ice for 20 min in a total volume of 80 μl. 50 μl of the latter incubation mixtures were used in the ultracentrifugation assay [4]. Non-specific binding of radiolabeled RBP to RPE-membranes was analysed by including a 200-fold molar excess of unlabelled RBP in some assays.

Synthetic peptides and epitope mapping
Synthetic peptides corresponding to amino acids 4-19, 27-35, 46-54, 60-70, 89-101, 120-128, 139-153, and 172-182 were synthesized using an ABI 430 peptide synthesizer (Applied Biosystems Inc.) using solid phase synthesis with t-BOC chemistry [16, 17], and analysed using high pressure liquid chromatography and plasma desorption mass spectrometry. The peptides were dissolved in 70 % formic acid at a concentration of 10 mg/ml. For the EIA, the peptides were diluted with PBS to a final concentration of 10 μg/ml and microtiter plates coated overnight at 4°C (50 μl/well). Following washing in PBS containing 0.1% Tween 20, the plates were incubated with the monoclonal antibodies diluted to 1mg of Ig per ml in PBS containing 1% bovine serum albumin for 1hr at room temperature and then overnight at 4°C. The plates were extensively washed as above and bound antibodies were detected using an alkaline phosphatase-labelled goat anti-rabbit IgG secondary antibody (Sigma Chemicals). p-Nitrophenylphosphate was used as the substrate and product formation was estimated at 405 nm using a microtiter plate reader.

The three dimensional structure of human RBP

The coordinates for the three dimensional structure of human RBP [8] was obtained from the Brookhaven collection of three dimensional protein structures. The structure was displayed as described [18].

RESULTS AND DISCUSSION

Characterisation of monoclonal antibodies to human RBP

Several mAbs displayed strong immunoreactivity to human RBP using an EIA procedure and were able to immunoprecipate human RBP radiolabelled with the Bolton-Hunter reagent. In contrast, the mAbs reacted poorly, if at all, to human RBP labelled with chloramin T. None of the mAbs recognised purpurin [19], a retinol-binding protein with about 50 % amino acid identity to RBP. To examine whether the mAbs cross-reacted with RBP from different species, an eucaryotic expression vector containing the cDNAs for human and X. laevis RBP, and a minigene encoding rat RBP were separately transfected into HeLa cells and the cells were labelled with ^{35}S-methionine. Expressed RBPs were then subjected to immunoprecipitation using the mAbs and analysed by SDS-PAGE (Figure 1). The mAbs could be divided into three groups based on the immunoreactivity to the different RBPs. One mAb from each group was selected for further studies. The immunoreactivity and the isotype determinations of these mAbs are summarized in Table 1. The mAb K14 recognized all three species of RBPs and the autoradiogram (Figure 1) revealed that the migration of human, rat and X. laevis RBPs differ slightly (human RBP M_r 21,000; rat RBP M_r 20,000; X. laevis M_r 18,500). The mAb L48 reacted with the two mammalian RBPs, whereas L40 was specific for human RBP. Thus, at least three different epitopes are represented among these mAbs.

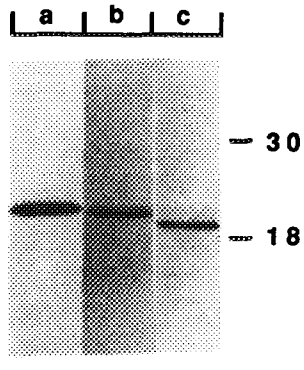

Figure 1. Immunoprecipitation and SDS-PAGE analysis of human, rat and X.laevis RBP. Expression vectors containing the cDNAs for human (lane a), a minigene for rat RBP (lane b) and a cDNA for X. laevis RBP (lane c) were separately transfected into HeLa cells. Transfected cells were labelled with ^{35}S-methionine and expressed RBP was subjected to immunoprecipitation using Ig-fractions from the different mAbs. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. MAb K14, which immunoprecipitated RBP from all three species, was used in the shown experiment. The migration of the molecular weight markers is given in kDa.

Table 1. Some characteristics of three monoclonal antibodies to human RBP

mAb	Ig subtype	Immunoprecipitation of RBP from Human	Rat	Xenopus
K14	IgG3	+	+	+
L40	IgG2b	+	-	-
L48	IgG2a	+	+	-

Monoclonal antibodies blocking the binding of RBP to its membrane receptor

To analyse whether the mAbs would interfere with the binding of RBP to its membrane receptor, fixed amounts of radiolabelled RBP were mixed with various amounts of purified Ig fractions (0, 0.5, 5 and 50 μg per incubation) from the different mAbs and incubated overnight. These incubations were separately added to RPE-membrane fractions and the ability of RBP to bind to the membrane receptor was estimated by the ultracentrifugation assay [4]. Both K14 and L48 efficiently interfered with the binding of RBP to the RBP-receptor, whereas L40 had no effect (Fig.2). The two mAbs with ability to block binding are directed against conserved epitopes since they cross-react with rat RBP (and in the case of K14 also with X. leavis RBP) (Table 1). Unless these antibodies induce major conformational changes in RBP that disrupt the receptor binding, these epitopes should be located in or close to the RBP-receptor binding site.

The membrane receptor and TTR binding sites involve separate regions of RBP

To examine whether RBP would retain affinity for TTR after antibody binding, HeLa cells were transfected with expression vectors containing the cDNAs for human RBP and

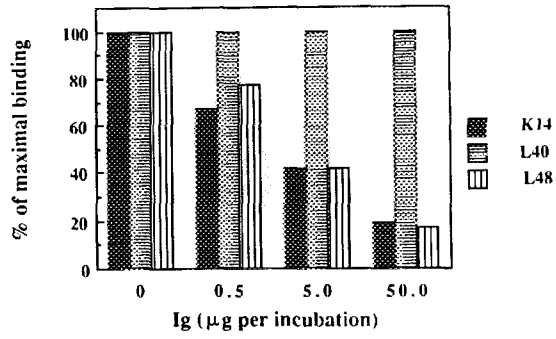

Figure 2. Binding of RBP to the RBP-receptor in the presence of Ig from three mAbs to human RBP.
Ig fractions from three mAbs to human RBP were separately incubated with radiolabeled RBP and the formed RBP-Ig complexes were analysed as to their ability to bind to the RBP-receptor using a membrane binding assay [4]. Non-specific binding was estimated by performing receptor binding assays in the presence of a 200-fold molar excess of unlabelled RBP. The values represent the means of duplicate analyses and the individual values differed by less than 10 %.

rat TTR. The transfected cells were labelled with ^{35}S-methionine and formed RBP-TTR complexes were subjected to immunoprecipitation using Ig from the mAbs and resolved by SDS-PAGE. All three mAbs immunoprecipitated RBP bound to TTR (Fig.3). Since both K14 and L48, which efficiently interfere with the binding of RBP to its membrane receptor, can co-immunoprecipitate RBP and TTR, these data show that no major conformational changes are induced by the antibodies. Given the size of an antibody it seems unlikely that the TTR-binding site is located close to the antibody epitope. In agreement with our previous finding that TTR does not block RBP-receptor binding [4], these data suggests that the membrane receptor and TTR-binding sites involve separate regions of RBP. In contrast, Sivaprasadarao and Findlay [6] have reported that high concentrations of TTR block the binding of RBP to the RBP-receptor expressed in human placenta suggesting the possibility that TTR and the RBP-receptor share the same binding site of RBP. It should be noted that the high concentration of TTR necessary for receptor blockage in that study [6] would exclude a direct competition. Instead, the results might be explained by a slight contamination of RBP in the TTR preparation used [6] or that the RBP-TTR complex binds less efficiently to the membrane receptor.

Based on molecular dynamics simulations of the holo and apo forms of RBP, Åqvist et al. [20] suggested that the entrance loops of the barrel might be the TTR-binding site. In part this suggestion was based on earlier tryptophan modification experiments and on retinoid reconstitution experiments [21,22]. Other putative TTR-binding sites in RBP involve the highly conserved N-terminal region, the -S-S-bridged loop between residues 120-129 and the highly conserved C-terminal region of the α-helix [23]. Our analyses of

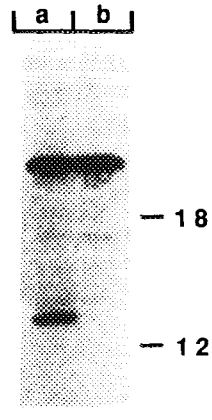

Figure 3. Immunoprecipitation and SDS-PAGE analysis of the RBP-TTR complex. HeLa cells were transfected with expression vectors containing the cDNAs encoding human RBP and rat TTR (lane a) or human RBP only (lane b). Transfected cells were labelled with ^{35}S-methionine and expressed proteins present in the medium were subjected to immunoprecipitation using Ig from the mAbs. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. The autoradiogram for L48 is shown. Identical autoradiograms were seen with K14 and L40 (data not shown). The migration of the molecular weight markers is given in kDa.

a chimeric RBP containing the N-terminal region of purpurin, which does not bind TTR, have shown that TTR-binding is not conferred by the N-terminal region alone [24]. Possibly, the antibody epitope and the TTR-binding site might be located on different sides of the protruding structure formed by the three entrance loops.

One receptor-blocking mAb shows immunoreactivity to a synthetic peptide correponding to one entrance loop of RBP

Based on the three-dimensional structure of human RBP [7] and the amino acid sequence differences between human, rabbit, and rat RBPs [23], synthetic peptides corresponding to all potentially antigenic sites of human RBP were synthesized. An unrelated peptide as well as purified human RBP and TTR were included as controls. As shown in Table 2, K14 and L40 did not show specific immuno-reactivity to any of the peptides. In contrast, L48 showed distinct immunoreactivity to the peptide corresponding to amino acid residues 60-70 although the reactivity was weaker than that to intact RBP. These residues correspond to one of the entrance loops and it is conserved in the mammalian RBPs, a fact which is consistent with the ability of L48 to recognize both human and rat RBPs. As seen in Fig. 4, the three entrance loops (residues 27-35, 60-70 and 89-101) form a protruding structure in RBP and we conclude that at least one of these is located in or close to the RBP-receptor binding site.

During the preparation of this manuscript it was reported that a mutation in the loop region corresponding to amino acid residues 60-70 of RBP greatly affected the ability of the recombinant protein to recognize human placental RBP-receptors [25] and this mutant was able to bind both retinol and TTR, consistent with the data presented in this study.

Table 2. Epitope mapping of monoclonal antibodies to human RBP using synthetic peptides

	Antibody		
Peptide	K14	L40	L48
Control	0.030±0.003	0.000±0	0.000±0
4-19	0.014±0.007	0.000±0	0.000±0
27-35	0.016±0.004	0.000±0.002	0.000±0.002
46-54	0.038±0.009	0.000±0.002	0.000±0
60-70	0.018±0.005	0.000±0	0.215±0.009
89-101	0.000±0.001	0.005±0.002	0.000±0
120-128	0.029±0.001	0.000±0.001	0.034±0.006
139-154	0.030±0.003	0.000±0.001	0.000±0
172-182	0.025±0.009	0.030±0.002	0.017±0.002
RBP	1.217±0.121	2.550±0.060	3.303±0.018
TTR	0.014±0.007	0.000±0	0.000±0

Microtiter plates were separately coated with 9 different peptides and the immunoreactivity of the monoclonal antibodies to human RBP was determined with each peptide using an EIA procedure as outlined in Materials and Methods. Bound antibodies were visualized using alkaline phosphatase-labelled second antibodies using p-nitrophenylphosphate as the substrate. The formation of p-nitrophenol was measured at 405 nm. Intact RBP served as a positive control and purified human TTR was included as a negative control. The data are means of quadruplicate analyses ± SEM.

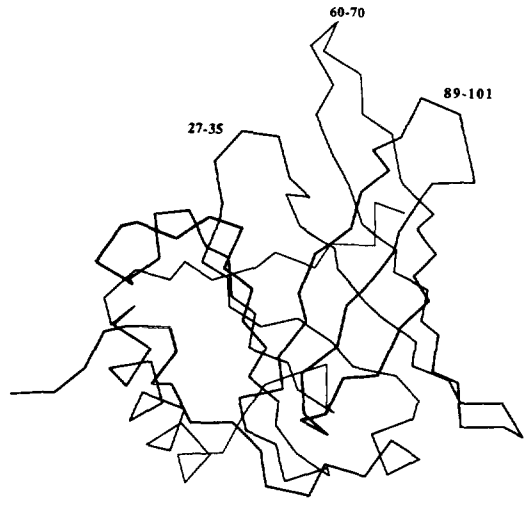

Figure 4. Visualisation of the three loops surrounding the entrance of the retinol binding pocket in human RBP.

The C_α-backbone in human RBP is shown [8]. The three entrance loops (residues 27-35, 60-70 and 89-101) surrounding the opening of the retinol-binding pocket are indicated. The N-terminus is to the very left in this projection while the C-terminal region is located behind the β-barrel.

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