Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein

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Introduction

Retinoids (vitamin A and its analogs) are required for cellular proliferation, differentiation and apoptosis (Morris-Kay and Sokolova, 1996; Napoli, 1996). With the exception of vision, the all-trans- and 9-cis-isomers of retinoic acid are the active retinoid forms needed to support retinoid-dependent processes. Retinoic acid isomers regulate gene expression by binding to specific nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), that function as ligand-dependent transcription factors (Chen and Evans, 1995; Kurokawa et al., 1995; Leblanc and Stunnenberg, 1995; Mangelsdorf et al., 1995; Pfahl and Chytil, 1996). Expression of >300 genes is influenced by retinoic acid availability (Gudas et al., 1994; Clagett-Dame and Plum, 1997). The retinoid used in the visual cycle is 11-cis-retinal (Wald, 1968), which forms a Schiff’s base with opsin in photoreceptors to generate rhodopsin, the visual pigment. When excited by light, 11-cis-retinal isomerizes to all-trans-retinal. This light-catalyzed isomerization alters the conformation of opsin to expose G-protein-binding sites (Gregory-Evans and Bhattacharya, 1998), which induces a cascade of reactions resulting in a transmembrane hyperpolarization of the rods and/or cones (Baylor, 1996). This signal serves as the stimulus for second order neurons in the retina, which then transmit information to the next layer of neurons. Visual information is integrated in the central nervous system (Kelsey, 1997).

All retinoids present in the body originate from the diet (Blaner and Olson, 1994). Dietary retinoids are packaged by the intestine as retinyl ester, along with other dietary lipids, in chylomicrons. The majority of dietary retinoid is cleared by and stored within the liver (Vogel et al., 1999). To meet tissue retinoid needs, the liver secretes into the circulation retinol bound to a 21 kDa protein, retinol-binding protein (RBP). Crystallographic analysis of retinol–RBP from different species reveals a single retinol molecule buried within a highly conserved eight-stranded β-barrel structure (Soprano and Blaner, 1994; Newcomer, 1995). The retinol-binding site of RBP consists principally of amino acid residues coded by exons III, IV and V. The genomic organization of the RBP gene is also highly conserved across species (Soprano and Blaner, 1994).

Retinol-RBP is found in a 1:1 molar complex with a 55 kDa protein, transthyretin (TTR) (Monaco et al., 1995). This complex formation prevents retinol–RBP excretion by the kidney (Soprano and Blaner, 1994). The mechanism through which tissues acquire retinol from circulating retinol–RBP is subject to considerable debate. Membrane receptors for holo-RBP have been reported in the retinal pigment epithelium (RPE), in the placenta and in several other tissues and cells (Vogel et al., 1999). Such receptors could provide cells with a mechanism to regulate retinol uptake.

In the mouse embryo, RBP is synthesized as early as 7 days post-coitum (d.p.c.) by the visceral yolk sac,
consistent with a role for RBP in retinol delivery during embryogenesis (Johansson et al., 1997; Sapin et al., 1997). Developmental abnormalities were indeed induced in 7.5 d.p.c. embryos treated in vitro with antisense RBP mRNA (Bavik et al., 1996). RBP is also expressed in first trimester human placental membrane and in fetal liver (Marks et al., 1991).

Furthermore, except during severe vitamin A deficiency and in some disease states, retinol–RBP concentrations in the blood are maintained within narrow limits (Goodman, 1984; Helms et al., 1986; Matsuo et al., 1987; Holm et al., 1988; Winckler et al., 1989).

Based on these data, RBP is postulated to be essential for maintaining retinoid-dependent signaling pathways within the body.

To determine the role of RBP in retinoid physiology, we generated a mouse RBP knockout strain by targeted disruption of the genomic locus. The resulting RBP–/– mice are viable and fertile, but have reduced blood retinol levels, a marked but reversible impaired retinal function during the first months of life and impairment of retinol mobilization from hepatic stores.

Results

Targeted disruption of the RBP gene and generation of RBP–/– mice

A mouse RBP genomic fragment was used to construct a targeting vector. We replaced 591 bp of the RBP gene with a neomycin resistance cassette (PMC1neo), beginning 252 bp upstream of the TATA box and extending through exon I, intron I, exon II and 10 bp of intron II. The herpes thymidine kinase gene (PMC1tk) was inserted in the vector for counter-selection against non-homologous recombinants (Figure 1A). The targeting vector was linearized and electroporated into 129/Sv ES cells. After selection with G418 and gancyclovir, 570 resistant clones were isolated, expanded and screened by Southern blot. Genomic DNA was digested with BamHI, HindIII and EcoRI and hybridized with four different probes: fragment A, encompassing exons III and IV; fragment B, containing exon X; the mouse RBP cDNA described below; and a 0.7 kb PstI fragment of the PMC1neo cassette. Figure 1B shows a Southern blot with fragment A as probe indicating targeting of an RBP allele. This was confirmed by hybridizations with fragment B, the Neo probe and the mouse RBP cDNA (data not shown). The clone was injected into C57BL/6J blastocysts to generate male chimeras, scored by coat color. Chimeric mice were then bred with wild-type C57BL/6J females and germline male chimeras, scored by coat color. Chimeric mice were clone was injected into C57BL/6J blastocysts to generate probe and the mouse RBP cDNA (data not shown). The confirmed by hybridizations with fragment B, the Neo cassette. Figure 1B shows a Southern blot with fragment A, encompassing exons III and IV; fragment B, HindIII fragment that co- migrated with the wild-type 129/Sv allele. This fragment represents an RBP genomic rearrangement that occurred after exposure of the embryonic stem (ES) cells to the targeting vector (see below). Heterozygotes (RBP+/–) were then mated to generate RBP knockout homozygotes (RBP–/–) (Figure 1D). The RBP–/– mice were born at the expected Mendelian frequency, indicating that RBP is not essential for normal prenatal development. Furthermore, RBP–/– adults are viable and fertile.
Expression of the RBP gene was analyzed by Northern blot. Figure 2A shows RBP mRNA in total liver RNA from mice of all three genotypes. No wild-type RBP transcript was detected in RBP+/+ mice. However, a transcript of ~1300 nucleotides (~400 nucleotides larger than RBP mRNA) was shown in RBP+/+ and RBP−/− mice. Western blot analysis of blood samples from mice of all three genotypes, using a rabbit polyclonal anti-rat RBP antiserum, revealed no wild-type 21 kDa RBP protein in RBP+/+ and RBP−/− mice, the 28 kDa mutant protein (m), the 14 kDa TTR monomer and the cross-reacting light chain IgG (ig) and the 14 kDa TTR monomer are also indicated.

**Table I.** Serum levels of RBP in RBP+/+, RBP+/− and RBP−/− mice determined by RIA

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RBP (mg/dl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP+/+</td>
<td>3.5 ± 0.62</td>
<td>10</td>
</tr>
<tr>
<td>RBP+/−</td>
<td>2.1 ± 0.60</td>
<td>10</td>
</tr>
<tr>
<td>RBP−/−</td>
<td>ND</td>
<td>10</td>
</tr>
</tbody>
</table>

The values given represent mg of rat RBP equivalents and are means ± SD. ND = not detectable (<0.1 mg/dl). n = the number of male mice used.

**Characterization of the function and structure of the 28 kDa protein**

We reasoned that the 28 kDa mutant protein possessed little of the intact structure of RBP since the protein was not detected by an RIA carried out under non-denaturing conditions. Consequently, it was unlikely that the protein acted to transport retinol in RBP+/− mice. We confirmed this possibility through in vivo studies. We specifically asked whether the 28 kDa mutant protein functions to transport retinol in the RBP−/− mice. Since the small amounts of retinol present in the circulation of RBP−/− mice arise from recent dietary retinol intake (see below), six 3-month-old male RBP+/+ and RBP−/− mice were each given by gavage a dose of [3H]retinol in peanut oil. Ten hours after administration of this dose, a time when all chylomicron retinyl ester should be cleared from the circulation (Weng et al., 1999), the mice were sacrificed and pools of RBP+/+ and RBP−/− serum were obtained. Both serum pools (53103H c.p.m. in 0.5 ml of RBP+/+ serum and 35203H c.p.m. in 0.5 ml of RBP−/− serum) were fractioned on the same Sephacryl S-200 gel filtration column. Fractions from the two column runs were taken for analyses of 3H c.p.m. by scintillation counting, [3H]retinol by HPLC and the presence of RBP or the 28 kDa mutant protein by Western blot analysis. As can be seen for RBP−/− serum (Figure 3), the concentration of the 28 kDa mutant protein was greatest in the two fractions (fractions 17 and 18) corresponding to the void volume of the column and progressively declined to an undetectable level by fraction 24. Immunoreactive 28 kDa protein could not be detected in any subsequent column fractions. Since tetrameric TTR would be expected to elute from this gel exclusion column with an elution volume corresponding to approximately those of fractions 30–35, the 28 kDa mutant protein cannot be bound to TTR in the circulation. 3H c.p.m. first appeared in fractions 21 and 22 and reached a maximum level in fractions 25 and 26. This major peak of 3H c.p.m. accounted for >90% of the 3H c.p.m. applied to the column. A second minor peak of 3H c.p.m. (<10% of the 3H c.p.m. applied to the column) could be detected in column fractions 36–41. HPLC analysis of pooled fractions 21–29 indicated that the 3H c.p.m. present in these fractions were present as [3H]retinol. For wild-type serum (Figure 3C), the main peak of 3H c.p.m. and immunoreactive RBP co-eluted in fractions labeled M1 and M2 in Figure 3C, at an approximate volume at which the RBP–TTR complex would be expected to elute. A second minor peak of 3H c.p.m. and immunoreactive RBP was also present in later fractions (Figure 3C, lane L) that eluted with the approximate volume expected for uncomplexed RBP. Neither 3H c.p.m. nor immunoreactive RBP could be detected in fractions constituting the void volume (Figure 3C, lane V). Based on these data, we concluded that the 28 kDa protein must be present in the blood as part of a high molecular weight aggregate that does not function to transport retinol in the circulation. This conclusion was supported further by another in vivo binding experiment. Briefly, RBP+/+ and RBP−/− mice were injected with [3H]retinol, blood was taken and the percentage of circulating [3H]retinol bound to RBP or to the 28 kDa mutant protein was determined. The results indicate that in the blood of RBP+/+ mice, 10% of the total radioactivity was bound to RBP, whereas
in the blood of RBP–/– mice the percentage of radioactivity associated with the 28 kDa mutant protein was at the experimental background level (1%; data not shown).

We also investigated the primary structure of the mutant 28 kDa protein following trypsin digestion and very sensitive matrix-assisted laser desorption/ionization-mass spectroscopic (MALDI-MS) analysis (Henzel et al., 1993). For this purpose, an immunoprecipitate of the 28 kDa protein was made employing the same rabbit anti-rat RBP antiserum used to detect the 28 kDa protein by immunoblot analysis. This immunoprecipitate was subjected to SDS–PAGE. Following staining of a lane of this polyacrylamide gel for protein with Coomassie Blue, the major protein band present on the gel, corresponding to the 28 kDa protein, was excised from the gel. The excised protein band was then subjected to proteolytic digestion with trypsin. The peptide fragments generated upon proteolysis subsequently were fractionated by MALDI-MS. Figure 4 provides the profile obtained from the mass spectrometer for these peptides and Table II provides a listing of the masses (m/z) that would be expected for peptides generated following complete and partial digestion of mature serum RBP (the arrows in Figure 4 provide the positions where these RBP fragments would be expected to be located upon mass spectroscopy). As can be seen from inspection of Figure 4 and Table II, none of the peptides detected upon MALDI-MS analysis showed masses (m/z) that would be expected for peptides generated from complete or partial digestion of native RBP. Considering that MALDI-MS has a low limit of detection of <500 fmol per peptide fragment, we conclude from this primary analysis that the mutant protein does not share extensive stretches of identity with RBP. To investigate further the primary sequence of the 28 kDa protein, we also carried out an analysis of the N-terminal sequence of the 28 kDa mutant protein (Ferrara et al., 1993). This analysis revealed, for the first 15 N-terminal amino acids of the 28 kDa protein, no similarity in the primary sequences for mouse RBP and the 28 kDa protein. No other similarities were detected for the 28 kDa protein and any other known sequences in the Swiss Protein Database (data not shown). Based on this sequence information for the 28 kDa protein, we hypothesize that it probably represents a frameshift fusing a small portion of RBP to an unknown open reading frame. The resulting polypeptide contains only a small stretch of the original RBP sequence. Accordingly, although the mutant DNA and mRNA were detected by Southern and Northern blot analysis, the mutant protein was recognized by the rabbit but not by the sheep anti-RBP antiserum.

**Serum and tissue levels of retinol and retinyl esters in RBP–/– mice**

Serum and tissue retinol and retinyl ester levels were measured by reverse phase HPLC for 13-week-old male and female animals. Serum retinol levels in RBP+/+ mice were 50% those of RBP+/+ mice, whereas the levels in RBP–/– mice were 12.5% of wild-type (Table III). No statistically significant differences in hepatic total retinol levels between RBP–/– and RBP+/+ mice were observed (data not shown). Thus, at this age, the absence of RBP dramatically reduces serum retinol levels, but does not impair accumulation of hepatic retinol stores. No statistically significant differences in total retinol concentrations were observed in kidney, spleen, testis and ovary in 13-week-old RBP+/+ and RBP–/– mice (data not shown).

Serum and hepatic total retinol concentrations were also measured in 3-week- and 5-month-old RBP+/+ and RBP–/– mice. Serum levels of retinol were consistently low.
Fig. 4. MALDI-MS analysis of a trypsin digest of the 28 kDa mutant protein. The numbers on the figure represent the masses (m/z) for each of the tryptic fragments obtained after digestion of the 28 kDa protein and separation by mass spectroscopy. Only tryptic fragments with a mass (m/z) of >1000 are given in the figure. The arrows indicate the position where peptides generated upon complete or partial tryptic digestion of native mouse RBP would be expected to be found. A listing of the masses of RBP tryptic fragments generated upon complete or partial digestion of mouse RBP is provided in Table II. This mass spectroscopic analysis is able to detect peptides with a low limit of detection of <500 pmol. Thus, for peptides with masses (m/z) ranging between 1000 and 5000, as shown in this figure, we would expect to be able to detect peptides if they were present at a level as low as 0.5–2.5 ng.

Table II. MALDI-MS analysis

<table>
<thead>
<tr>
<th>Position</th>
<th>Predicted mass</th>
<th>Experimental mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>118–157</td>
<td>4654.996</td>
<td>4288.94</td>
</tr>
<tr>
<td>108–139</td>
<td>3858.192</td>
<td>3586.89</td>
</tr>
<tr>
<td>140–167</td>
<td>3913.135</td>
<td>3118.48</td>
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<tr>
<td>49–76</td>
<td>3064.393</td>
<td>2586.70</td>
</tr>
<tr>
<td>81–105</td>
<td>2819.173</td>
<td>2466.67</td>
</tr>
<tr>
<td>79–103</td>
<td>2799.142</td>
<td>2356.92</td>
</tr>
<tr>
<td>118–139</td>
<td>2649.807</td>
<td>2201.93</td>
</tr>
<tr>
<td>81–103</td>
<td>2543.822</td>
<td>2145.00</td>
</tr>
<tr>
<td>48–68</td>
<td>2408.667</td>
<td>2099.30</td>
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<tr>
<td>49–68</td>
<td>2280.493</td>
<td>1913.02</td>
</tr>
<tr>
<td>140–157</td>
<td>2024.212</td>
<td>1855.77</td>
</tr>
<tr>
<td>106–117</td>
<td>1486.774</td>
<td>1696.76</td>
</tr>
<tr>
<td>36–47</td>
<td>1383.635</td>
<td>1664.97</td>
</tr>
<tr>
<td>38–48</td>
<td>1284.543</td>
<td>1608.42</td>
</tr>
<tr>
<td>158–168</td>
<td>1242.334</td>
<td>1494.76</td>
</tr>
<tr>
<td>108–117</td>
<td>1227.408</td>
<td>1403.44</td>
</tr>
<tr>
<td>19–28</td>
<td>1196.323</td>
<td>1258.23</td>
</tr>
<tr>
<td>38–47</td>
<td>1156.369</td>
<td>1145.00</td>
</tr>
<tr>
<td>158–167</td>
<td>1086.146</td>
<td>1072.74</td>
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<tr>
<td>172–179</td>
<td>1019.160</td>
<td>1016.163</td>
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<tr>
<td>69–78</td>
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</tbody>
</table>

Comparison between the masses of the peptides obtained after tryptic digestion of the RBP protein (predicted mass) and those of the peptides obtained after tryptic digestion of the 28 kDa protein (experimental mass). The mass value (m/z) is expressed in Da. The first column indicates the position of the cleavage sites in the RBP protein.

(12.5%) in both age groups of RBP−/− mice (data not shown). Three-week-old RBP−/− and RBP+/+ mice had equivalent liver total retinol concentrations (data not shown). However, 5-month-old RBP−/− mice had significantly higher hepatic retinol and retinyl ester levels than age- and sex-matched RBP+/+ animals (Table IV). Thus, although serum retinol levels remain low with increasing age, RBP−/− mice accumulate more hepatic total retinol than wild-type controls. These observations suggest that retinol mobilization from hepatic stores may be compromised in RBP−/− mice.

Table III. Serum retinol levels in RBP+/+, RBP+/− and RBP−/− mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Retinol(a) (μg/dl)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP+/+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>25.6</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>16.5 ± 3.8</td>
<td>4</td>
</tr>
<tr>
<td>RBP+/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>15.6</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>9.5 ± 2.9</td>
<td>4</td>
</tr>
<tr>
<td>RBP−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>2.7 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>3.1 ± 1.1</td>
<td>4</td>
</tr>
</tbody>
</table>

aRetinol levels are determined by reverse phase HPLC and are expressed as μg/dl of serum ± SD (when ≥2 mice were analyzed). n = numbers of 13-week-old male (M) or female (F) mice analyzed.

RBP−/− mice have impaired retinal function

Because of the unique role of retinol in vision, we measured retinol levels in the eye cups of RBP+/+ and RBP−/− mice at different ages by reverse phase HPLC. At 3 weeks, eye cup retinol content in RBP−/− mice was only 25% of wild-type. The eye cup retinol content of RBP−/− mice increased with age at the same rate as controls, but remained significantly lower for as long as 13 weeks.
with time

Table IV. Hepatic levels of retinol and retinyl esters in 5-month-old RBP\(^{+/+}\) and RBP\(^{-/-}\) mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Retinol(^a) (μg/g)</th>
<th>Retinyl esters(^b) (μg/g)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP(^{+/+})</td>
<td>19.8 ± 6.5</td>
<td>700 ± 309</td>
<td>5</td>
</tr>
<tr>
<td>RBP(^{-/-})</td>
<td>27.7 ± 19.6</td>
<td>1798 ± 1278</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\)Retinol levels are determined by reverse phase HPLC and are expressed as μg/g of tissue.

\(^b\)Retinyl esters are determined by reverse phase HPLC and are expressed as retinol equivalents/g of tissue. The levels of each individual retinyl ester (retinyl palmitate, retinyl oleate, retinyl stearate and retinyl linoleate) were summed to provide the total retinyl ester levels. Values are given as means ± SD. 

\(n\) = numbers of age- and sex-matched mice analyzed.

The retinal impairment of RBP\(^{-/-}\) mice reverses with time

Dark-adapted ERG analysis of mice of different ages showed that the vision of RBP\(^{+/+}\) animals progressively improved and approached that of wild-type animals. Figure 5D illustrates representative ERG responses of a RBP\(^{-/-}\) mouse at different ages. Whereas the response at 4 weeks of age showed the typical abnormalities observed in young RBP\(^{-/-}\) mice, ERG profiles from the same mouse 4 weeks of age showed the typical abnormalities observed at 24 weeks of age had a normal appearance, with a deep b-wave amplitude of RBP\(^{-/-}\), RBP\(^{+/+}\) was needed to elicit a threshold b-wave. Comparison of the b-wave amplitude of RBP\(^{-/-}\), RBP\(^{+/+}\) and wild-type mice (Figure 5C) reveals a 100-fold decreased (2 log units) sensitivity of the RBP knockout mice to light. The response to a maximal stimulus was about half of normal.

Analysis of retinol-derived metabolites in the eye cups of RBP\(^{-/-}\) mice

Homogenates prepared from eye cups of 3-week-old RBP\(^{+/+}\) and wild-type animals were assayed by normal phase HPLC for 11-cis-retinal, the retinol-derived metabolite required in the visual cycle, and retinyl ester. The level of 11-cis-retinal in age- and sex-matched RBP\(^{+/+}\) mice averaged 57.3 ± 9.9 ng/pair of eye cups (\(n = 5\)), whereas the level in age- and sex-matched RBP\(^{-/-}\) animals was only 10.7 ± 6.4 ng/pair of eye cups (\(n = 5\)). The low concentration of 11-cis-retinal is entirely consistent with the abnormal visual phenotype. This difference was still observed in 8-month-old RBP\(^{+/+}\) animals compared with age- and sex-matched wild-type mice (87 ± 16 ng/pair of eye cups in RBP\(^{+/+}\) mice and 49 ± 19 ng/pair of eye cups in RBP\(^{-/-}\) animals; \(n = 4\)). Furthermore, retinyl ester levels were extremely low in 8-month-old RBP\(^{-/-}\) animals, averaging 13 ± 5 ng/pair of eye cups, compared with 168 ± 32 ng/pair of eye cups in RBP\(^{+/+}\) mice (\(n = 4\)). In contrast, eye cup retinol levels were identical in adult animals (53 ± 7 ng/pair eye cups in RBP\(^{+/+}\) mice; 56 ± 13 ng/pair eye cups in RBP\(^{-/-}\) mice).

Origin of the residual serum retinol in RBP\(^{-/-}\) mice

To ask whether the retinol in the circulation of RBP\(^{-/-}\) mice arose from recent dietary intake, we placed these and control animals on a vitamin A-deficient diet. After 1 week, plasma retinol in RBP\(^{-/-}\) mice fell from 12.5% of wild-type to undetectable levels (Figure 6C), whereas wild-type animals on the same diet maintained constant plasma retinol concentrations (data not shown). This indicates that the residual serum retinol in the knockout mice derives from recent dietary intake. Note that RBP\(^{-/-}\) mice fed the vitamin A-sufficient diet showed a low peak of serum retinyl palmitate on HPLC analysis (Figure 6B). A similar peak, which derives from chylomicron-bound dietary retinyl ester, was observed for RBP\(^{+/+}\) mice fed a control diet but not for RBP\(^{-/-}\) mice maintained on a vitamin A-deficient diet (data not shown). The RBP\(^{-/-}\)
Fig. 6. Effects of vitamin A-deficient diet on plasma retinol levels and on retinal function. Representative reverse phase HPLC profiles for plasma retinol from RBP<sup>+/+</sup> (A) and RBP<sup>/−</sup> (B) mice consuming a control vitamin A-sufficient diet and from a RBP<sup>+/−</sup> mouse (C) consuming a vitamin A-deficient diet for 1 week after maintenance throughout life on a vitamin A-sufficient diet. These age-matched mice were not fasted prior to sacrifice. For each of the HPLC profiles, the white arrow indicates the expected position of retinol, which elutes with a retention time of ~2.2 min. The peak eluting with a retention time of ~1.65 min corresponds to the solvent benzene and the peak eluting at 2.6–2.7 min corresponds to the internal standard retinyl acetate. In (B), the peak eluted with a retention time of ~19.2 min corresponds to retinyl palmitate. The scale of the vertical axis for each panel (A, B and C) is identical (absorbency units at 325 nm). The horizontal axis for the profile from RBP<sup>/−</sup> mice consuming the control diet (C) is scaled differently to demonstrate the retinyl palmitate peak. (D) Relationship between the amplitude of the b-wave of the ERG in μV, in response to white light stimuli at the maximal intensity, as a function of the age of the mice. Analysis of RBP<sup>+/+</sup> ( ), RBP<sup>+/−</sup> ( ▲ ) and RBP<sup>/−</sup> ( △ ) mice fed a vitamin A-sufficient diet and RBP<sup>/−</sup> ( △ ) mice fed a vitamin A-deficient diet. Values are given as the mean ± SE for three to five animals each per group. Error bars indicate the standard error.

Table V. Hepatic levels of total retinol in RBP<sup>+/+</sup> and RBP<sup>/−</sup> mice after 7 days on a vitamin A-deficient diet

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Retinol&lt;sup&gt;a&lt;/sup&gt; (μg/g)</th>
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</thead>
<tbody>
<tr>
<td>RBP&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>control</td>
<td>281 ± 69</td>
<td>5</td>
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<tr>
<td>RBP&lt;sup&gt;/−&lt;/sup&gt;</td>
<td>deficient</td>
<td>50 ± 22</td>
<td>5</td>
</tr>
<tr>
<td>RBP&lt;sup&gt;/−&lt;/sup&gt;</td>
<td>control</td>
<td>177 ± 75</td>
<td>5</td>
</tr>
<tr>
<td>RBP&lt;sup&gt;/−&lt;/sup&gt;</td>
<td>deficient</td>
<td>241 ± 63</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Retinol is determined as total retinol pool (retinol + retinyl esters) by reverse phase HPLC and is expressed as μg/g of tissue (retinyl ester concentration is determined as retinol equivalents/g of tissue). Values are given as means ± SD.

<sup>n</sup> = numbers of age- and sex-matched mice analyzed.

Animals maintained for 7 days on the deficient diet and sex- and age-matched RBP<sup>/−</sup> mice maintained for the same period on the control diet had equivalent hepatic total retinol levels (Table V). In contrast, hepatic total retinol concentrations fell significantly in RBP<sup>+/+</sup> mice maintained for 7 days on the vitamin A-deficient diet compared with sex- and age-matched RBP<sup>+/+</sup> mice maintained on the control diet (Table V). That the vitamin A-deficient diet induces a decline in serum retinol in RBP<sup>/−</sup> mice without a compensating decrease in hepatic total retinol levels indicates that RBP is required to mobilize retinol from hepatic retinoid stores.

Effect of the daily dietary intake on the visual phenotype

To test the influence of dietary vitamin A intake on the visual phenotype, we monitored retinal function in RBP<sup>/−</sup> and RBP<sup>+/+</sup> mice maintained on vitamin A-deficient or vitamin A-sufficient diets, starting at the time of the weaning (Figure 6D). At weaning (21 days of age), RBP<sup>/−</sup> mice had a significantly lower b-wave than either wild-type or heterozygous mice. After 63 days on the control diet, the dark-adapted ERG response of RBP<sup>/−</sup> mice was normal. In contrast, the b-wave amplitude of RBP<sup>/−</sup> mice kept on a vitamin A-deficient diet progressively decreased (Figure 6D), their ERG threshold rose, the amplitude of the a-wave decreased and the falling phase was more prolonged. The ERG response of RBP<sup>+/−</sup> mice kept on a vitamin A-deficient diet remained unchanged throughout the experiment (data not shown). After 130 days on a vitamin A-deficient diet, in RBP<sup>/−</sup> mice the b-wave amplitude fell to undetectable levels (Figure 6D). However, retinal histological analysis of these mice by light and electron microscopy revealed no retinal abnormalities (data not shown).

The visual phenotype is suppressed by a human RBP transgene

To prove that the visual impairment of the RBP<sup>/−</sup> mice was due to the absence of RBP, we generated a mouse...
characterization of the MCK-hRBP mouse strain. (A) The MCK-hRBP construct used for microinjection is depicted. The construct was assembled from a human RBP cDNA (1 kb Clal fragment), the promoter of the muscle creatine kinase gene (3.3 kb PstI–ClaI fragment) and a bovine polyadenylation site (0.35 kb XhoI fragment) by conventional techniques. The positions of the BssHII restriction sites defining the polylinker of the vector are also indicated. (B–D) Demonstration of binding between hRBP and TTR in the peripheral blood of the transgenic mice. Mouse serum (20 μl diluted to 1 ml in PBS) containing as size markers blue dextran (5 mg/ml) and cytochrome c (5 mg/ml) was size-fractionated on a 1×100 cm column of Bio-Gel P-100 flowing in PBS. Included fractions (~0.5 ml) were collected starting with the first fraction containing the blue dextran (fraction 1) and ending with the last fraction containing cytochrome c (fraction 35). The fractions were assayed by RIA for both hRBP and mRBP using specific antibodies (Blaner, 1990). The first RBP-containing-fraction (fraction 2) was the second to contain blue dextran and represents RBP bound to TTR. RBP not bound to TTR would elute in fractions 25–35. Note that the concentrations of hRBP in the peak fractions (maximum 5000 ng) were in the non-linear range of the RIA. (B) Elution profile observed for mRBP in wild-type serum: essentially all RBP is bound to TTR. (C) Elution profile for mRBP in MCK-hRBP transgenic serum: mRBP elutes in association with TTR. (D) Elution profile for hRBP in MCK-hRBP transgenic serum: hRBP elutes in association with TTR.

Strain that overexpressed human RBP cDNA under the control of the muscle-specific creatine kinase (MCK) promoter (Levak-Frank et al., 1995). Some of the characteristics of this transgenic strain are illustrated in Figure 7 and in Table VI; a fuller description will appear elsewhere (L.Quadro, W.S.Blaner, V.Colantuoni and M.E.Gottesman, in preparation). Human RBP is expressed in muscle but not in the liver or the eye cups for this transgenic mouse strain. The human RBP functions like the endogenous mouse RBP: it binds retinol, forms a complex with mouse TTR and transports vitamin A to target tissues (Figure 7). The transgenic animals were crossed with the RBP–/– mice to obtain a strain that overexpressed human RBP in the mouse RBP null background. Electroretinograms of such animals at 4 weeks of age were indistinguishable from those of wild-type mice (data not shown). Thus, human RBP entirely suppresses the visual impairment of the RBP–/– mice. This result confirms and extends the results with the RBP+/– mice which lack a visual phenotype. We conclude that the visual defect in the RBP–/– is indeed the result of RBP insufficiency (and not, for example, due to some activity of the mutant protein).

**Discussion**

To understand the physiological role of RBP, we generated RBP knockout mice and followed these animals for 8 months. The mutant mice totally lack functional RBP but are viable and fertile. However, they show reduced levels of plasma retinol (12.5% of wild-type) and two other major defects: abnormal vision through the first several months of life and an inability to mobilize retinol from hepatic stores.

The viability and fertility of the RBP–/– animals was unexpected, since RBP is the sole specific carrier for retinol in the circulation of adult animals and in the developing embryo (Soprano and Blaner, 1994; Bavik et al., 1996; Johansson et al., 1997; Sapin et al., 1997; Barron et al., 1998). Evidently, there is plasticity in retinoid transport pathways. In fact, circulating retinoic acid levels (data not shown) and the uptake of chylomicron retinyl ester are normal in the knockout mice.

Normal vision and photoreceptor function require retinol and its specific eye metabolite, 11-cis-retinal. Vision is a critical physiological function in higher vertebrates, essential for survival and fitness in the wild. The impor-
tance of RBP in maintaining visual responsiveness is consistent with the high degree of homology of the RBP gene and the conservation of RBP protein and gene structure among different species. RBP knockout animals are born with impaired retinal function, but when fed a standard chow diet, which provides a sufficient and steady source of retinol, the animals acquire normal vision by 5 months of age. The RBP−/− mice are, therefore, able to use an alternative pathway(s) to acquire eye retinol. The alternative pathway(s) functions poorly in utero and early in life, since 21-day-old mice display low eye retinol levels (Figure 5A). However, the retinol content in the eye cups of the RBP−/− mice rises at the same rate as in RBP+/+ animals after weaning (21 days; Figure 5A). Thus, the alternative pathway(s), which we believe derives retinol exclusively from recently ingested vitamin A, is adequate to maintain normal visual function in adult animals under laboratory conditions. However, eye retinol stores (retinyl ester) remain low in adult RBP−/− mice kept on a vitamin A-sufficient diet. This further supports the notion that the vitamin A status of these animals is extremely tenuous, particularly with respect to visual function.

TTR-deficient mice also have low circulating serum RBP and retinol levels (5 and 6% of wild-type, respectively) (Episkopou et al., 1993; Wei et al., 1995). However, dark-adapted ERGs from TTR-deficient mice, ranging in age between 3 weeks and 8 months post-partum, were normal (data not shown). Therefore, the absence of the retinol–RBP complex rather than low serum retinol levels per se must be responsible for the impaired retinal function observed in young RBP−/− animals. This raises the question of why young RBP knockout mice have insufficient eye retinol but normal retinol levels in other tissues. Several reports suggest that the RPE expresses a receptor for RBP (Bavik et al., 1993; Nicoletti et al., 1995; Tsilou et al., 1997) which presumably plays a role in the uptake of retinol. Our data clearly demonstrate an RBP-independent mechanism(s) for accumulating eye retinol. However, they do not resolve the question of whether RBP acts through a specific receptor to deliver retinol to the eye.

A knockout of the mouse RPE65 gene has been reported recently (Redmond et al., 1998). The mutant mice have a visual defect. Although once suggested to be the RBP receptor, the phenotype of the RPE65-deficient mouse argues against this notion. The biochemical phenotype of the RPE65-deficient mice is distinctly different from that which we have observed for RBP-deficient mice. The visual defect in the RPE65 mutants is, therefore, related to some function of RPE65 in vitamin A metabolism unrelated to a proposed action in facilitating RBP binding to or uptake by cells.

In addition to an insertion within rbp, our model mice carry an RBP rearrangement that yields a transcript that hybridizes with an RBP probe as well as a 28 kDa mutant protein. Although this protein reacts by Western immunoblot with one of two anti-RBP antibodies, it binds neither retinol nor TTR in the circulation of RBP−/− mice (see Figure 3). Moreover, within the circulation of RBP−/− mice, the 28 kDa protein is present as a high molecular weight aggregate and not as a monomer or bound to TTR. The existence of the 28 kDa mutant protein as a protein aggregate could account for the failure of the sheep anti-RBP antiserum used for RIA analysis to detect the protein under mild non-denaturing conditions whereas the rabbit anti-RBP antiserum used for Western blot analysis was able to detect the 28 kDa protein following denaturation and SDS–PAGE. Furthermore, N-terminal sequencing and very sensitive (with a low detection limit of <500 fmol per peptide fragment) MALDI-MS analysis of peptide fragments generated upon trypsin digestion of the 28 kDa protein revealed no substantial sequence resemblance between the 28 kDa protein and RBP. ERG analyses of RBP+/+ and RBP−/− mice were indistinguishable, indicating that the rearrangement does not induce a gain-of-function mutation. Finally, the impaired retinal function, as assessed by ERG analysis of 4-week-old RBP−/− animals, was suppressed by a human RBP transgene expressed uniquely in skeletal muscle. Consequently, the phenotype of RBP knockout mice is due to the absence of functional RBP.

A similar phenotype recently has been observed in two sisters who lack serum RBP and display low serum retinol levels. The sisters have impaired vision as manifested by night blindness and altered rod function (Seeliger et al., 1999). Genetic analysis of the siblings indicates two compound heterozygous mutations in exons III and IV of the RBP gene, transmitted as a recessive trait (Biesalski et al., 1999). The visual phenotype reported and the impaired release of retinol from the liver stores, revealed by biochemical characterization of these two patients, are similar to what we describe here for the RBP−/− mice. Thus, our mouse strain could be a model for studying this genetic disorder and, eventually, for gene therapy.

The impaired retinal function is not associated with a developmental or structural abnormality in the retina. Indeed, the histology of retina from RBP−/− and RBP+/+ mice at 4 weeks of age, as judged by light microscopy, was indistinguishable (data not shown). The absence of developmental defects in RBP−/− animals indicates that the fetus has sufficient levels of tissue retinoic acid. In fact, retinoic acid and retinol levels in whole newborn (1-day-old) RBP knockout and wild-type mice were identical (data not shown). That the retinoic acid levels are normal is not surprising, since, except for the eye, both fetal and adult RBP−/− animals maintain normal tissue retinol levels in the face of low plasma retinol concentrations.

We find of great interest the fact that by 5 months of age RBP−/− mice accumulate a significantly higher concentration of hepatic retinol than wild-type mice. Furthermore, that the hepatic levels of total retinol of these animals do not fall after a short-term exposure to a vitamin A-deficient diet indicates that RBP−/− mice cannot mobilize hepatic retinoid stores. This suggests that in times of inadequate vitamin A intake, RBP ensures that retinol is available for maintaining normal cellular function. This, we propose, is the major physiological role of RBP.

Materials and methods

Construction of targeting vector

A 0.5 kb RBP mouse cDNA EcoRI fragment lacking exon VI (obtained through the IMAGE Consortium, Mobile, AL) was used to isolate a 14 kb genomic DNA from a mouse λ FixII 129/Sv library. The targeting
vector was constructed by cloning a 6.5 kb EcoRI genomic fragment, extending from 1.5 kb upstream of the start site through the fourth intron, in a Bluescript ISK(-) plasmid in which the Smal site of the polylinker region was eliminated by BamHI-PstI digestion. This recombinant plasmid was then digested with Smal, to eliminate 591 bp of the RBP gene, and a 1.1 kb blunt-ended XhoI-HindIII fragment from the PMC1neo poly(A) gene (Episkopou et al., 1993) was inserted in the opposite orientation. In the S' position, a 1.85 kb AhoI-Hind III blunt-ended fragment of the PMC10k gene (Episkopou et al., 1993) was inserted, in the opposite orientation, into a unique NotI restriction site, also made blunt-ended. DNA was linearized with SacI before use for electroporation in 129/Sv ES cells.

Generation of RBP+/− mice
ES cells were manipulated essentially as described previously (Belluscio et al., 1998). 129/Sv ES cells were electroporated with the construct and then placed under positive/negative selection with G418 and gancyclovir (Thomas and Capecchi, 1987). ES cell colonies surviving after 9 days were picked and their genomic DNA was digested with BamHI, HindIII and EcoRI and hybridized with four different probes (see Results and Figure 1). Southern blot analysis was performed using 15 µg of digested genomic DNA, electrophoresed on a 0.8% agarose gel, treated with 0.25 M HCl for 15 min and blotted to Hybond N (Amersham) by capillary transfer in NaOH 0.4 M for 3 h. Hybridizations were performed at 65°C for 3 h in Rapid-hyb Buffer (Amersham) and blots were washed three times at 65°C in 0.1 x SSC, 0.1% SDS before autoradiography. ES cells from the resistant clone were expanded and used to generate chimeras by microinjection into C57BL/6 blastocysts (Papaioannou and Capecchi, 1987). ES cell colonies surviving after 9 days were picked and their genomic DNA was digested with BamHI to eliminate 591 bp of the polylinker region was eliminated by BamHI-PstI digestion. This recombinant plasmid was then digested with Smal, to eliminate 591 bp of the RBP gene, and a 1.1 kb blunt-ended XhoI-HindIII fragment from the PMC1neo poly(A) gene (Episkopou et al., 1993) was inserted in the opposite orientation. In the S' position, a 1.85 kb AhoI-Hind III blunt-ended fragment of the PMC10k gene (Episkopou et al., 1993) was inserted, in the opposite orientation, into a unique NotI restriction site, also made blunt-ended. DNA was linearized with SacI before use for electroporation in 129/Sv ES cells.

RNA and protein analysis
RNA preparation and Northern blot analysis were performed as described (Sambrook et al., 1989); the mouse RBP cDNA described above was used as probe. Rabbit polyclonal anti-rat RBP antiserum (Muto et al., 1972) and rabbit polyclonal anti-rat TTR antiserum (Navab et al., 1989) were used for RIA performed as described (Blaner, 1990).

[^H]Retinol binding assay in vivo
Six 3-month-old male RBP−/− and RBP+/− mice were used to assess the ability of the RBP−/− mutant protein to bind and transport retinol in the circulation. Each was given by gavage a dose consisting of 1 µCi of [^3H]retinol (55 Ci/mmol; New England Nuclear Life Sciences Products, Boston, MA) in 0.1 ml of peanut oil. Ten hours after administration of this dose, a time that is sufficient for all chylomicron retinyl ester to be cleared from the circulation of the mice (Weng et al., 1999), the mice were fasted for 12 h and killed by cervical dislocation. Blood, plasma and red cells were collected into tubes containing 0.25 M HCl for 15 min and blotted to Hybond N (Amersham) by capillary transfer in NaOH 0.4 M for 3 h. Hybridizations were performed at 65°C for 3 h in Rapid-hyb Buffer (Amersham) and blots were washed three times at 65°C in 0.1 x SSC, 0.1% SDS before autoradiography. ES cells from the resistant clone were expanded and used to generate chimeras by microinjection into C57BL/6 blastocysts (Papaioannou and Capecchi, 1987). ES cell colonies surviving after 9 days were picked and their genomic DNA was digested with BamHI to eliminate 591 bp of the polylinker region was eliminated by BamHI-PstI digestion. This recombinant plasmid was then digested with Smal, to eliminate 591 bp of the RBP gene, and a 1.1 kb blunt-ended XhoI-HindIII fragment from the PMC1neo poly(A) gene (Episkopou et al., 1993) was inserted in the opposite orientation. In the S' position, a 1.85 kb AhoI-Hind III blunt-ended fragment of the PMC10k gene (Episkopou et al., 1993) was inserted, in the opposite orientation, into a unique NotI restriction site, also made blunt-ended. DNA was linearized with SacI before use for electroporation in 129/Sv ES cells.

[^H]Retinol and retinyl ester concentrations in plasma and tissues were measured by reverse phase HPLC as described (Blaner et al., 1987; Wei et al., 1995). 11-cis-retinol was measured in eye cups homogenates by normal phase HPLC (Mertz et al., 1997).

Electroretinograms
Dark-adapted ERGs were performed as previously described (Tsang et al., 1996). Dark-adapted ERG responses were obtained from anesthetized mice after their pupils were dilated with 1% phenylephrine HCl. A 30 gauge needle was placed subcutaneously on the forehead to serve as a reference electrode and a ground electrode was placed subcutaneously on the trunk. A saline-moistened cotton wick recording electrode was positioned to contact the cornea. Stimulating light flashes were obtained from a stroboscope (Grass Instruments Inc.). Neutral density filters were placed in front of the aperture to vary the intensity of the flashes. Responses were detected using an oscilloscope and an evoked response-detecting computer in parallel (Nicolet Instruments CA-100). All mice were dark-adapted overnight before ERGs were performed. Stimulation was begun with 4.8 log units of neutral density filtering and the responses were averaged to one flash every second. At high flash intensity, each flash was repeated every 20 s, an interval which, in preliminary experiments, was found to be sufficiently long to exclude interference of one flash on a later one. The duration of one flash was nominally 10 ms.
Diet
Purified retinol-deficient diet (W.F. Fisher & Son, Inc., Diet # 5822C-51) contained ~0.22 IU of retinol/g of diet; purified retinol-sufficient diet (W.F. Fisher & Son, Inc. Diet # 5755C-101) contained 22 IU of retinol/g of diet. All other nutrients were present in the two purified diets at the same concentrations.

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References


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