Unique Biochemical Nature of Carp Retinol-binding Protein

N-LINKED GLYCOSYLATION AND UNCLEAVABLE NH₂-TERMINAL SIGNAL PEPTIDE*

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Retinol transport and metabolism have been well characterized in mammals; however, very little is known in fish. To study the mechanism by which fish retinol-binding protein (RBP) is able to remain in plasma besides its small molecular size, we isolated RBP cDNA from a carp liver cDNA library. Comparison of the deduced amino acid sequence with that of known vertebrate RBPs showed that carp RBP has high homology to the other cloned vertebrate RBPs, but it lacks the COOH-terminal tetrapeptide, RNL(S)L, which is most likely involved in the interaction with transthyretin in mammalian RBPs. In addition, the primary structure of carp RBP contains two consensus N-linked glycosylation sites that represent a unique feature. We have obtained experimental evidence, by in vitro and in vivo expression experiments, that both sites are indeed glycosylated. We have also characterized the protein as a complex type N-linked glycoprotein by lectin binding assay, neuraminidase and endoglycosidase H and F digestion. Inhibition of glycosylation by tunicamycin treatment of transfected cells caused a great reduction of RBP secretion. Since kidney filtration of anionic proteins is less than half of that of neutral protein of the same size, this finding strongly suggests that the amount of carp RBP filtration through kidney glomeruli may be reduced by a glycosylation-dependent increase in the molecular size and negative charge of the protein. A second unique feature of carp RBP as secretory protein is the presence of a nonconserved NH₂-terminal hydrophobic domain, which functions as an insertion signal but is not cleaved cotranslationally and remains in the secreted RBP.

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‡ The abbreviations used are: RBP, retinol-binding protein; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Endo, N-glycosidase; ER, endoplasmic reticulum; PAI-2, plasminogen activator inhibitor-2; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; TTR, transthyretin; PCR, polymerase chain reaction.

Retinol binding protein (RBP, 1 molecular mass of about 20 kDa) is the specific blood carrier of vitamin A (retinol) that transports retinol from its storage site, the liver, to the various vitamin A-dependent tissues, where it is internalized and metabolized to its active form, retinoic acid. Retinoic acid is an important transcription modulator involved in the regulation of proliferation and differentiation of many cell types, as well as in fetal morphogenesis (1).

RBP is synthesized mainly by hepatocytes and secreted when bound to retinol (molar ratio of 1:1) and to a 55-kDa homotetrameric protein, the transthyretin (TTR) (2–4). The association of RBP with TTR stabilizes the complex and at the same time increases the size of the RBP-retinol complex, thus lengthening the time of RBP-retinol circulation in plasma, by preventing easy filtration of the relatively small RBP molecule through the kidney (5).

RBP has been isolated and characterized from different species: human (6), rat (7), rabbit (8), chicken (9), Xenopus (10), and others (11, 12). In some species, the structure has been resolved by x-ray diffraction (13, 14). These studies show that RBP is an extremely conserved protein, sharing strong homology among the species studied, particularly in the regions involved in retinol binding, interaction with TTR and tertiary structure organization.

The structure and function of TTR have also been analyzed in detail (14, 15). These studies revealed that in the circulating complex one RBP molecule binds to a TTR tetramer and that the affinity of the interaction is much higher when RBP is in the “holo” form, charged with retinol. Furthermore, a putative interaction domain with TTR has been identified in human RBP (15).

RBP was purified by column chromatography from rainbow trout plasma, and its primary and three-dimensional structures were analyzed, as well as its binding affinity for human TTR (16, 17). The amino acid sequence and the tertiary structure are highly conserved, but the in vitro affinity of trout RBP for human TTR appears to be much lower than that of mammalian RBP. Moreover, no circulating RBP-TTR complex was found in trout plasma by gel filtration techniques. A thyroid hormone-binding protein displaying amino acid sequence homology with higher vertebrate TTR was recently isolated from salmon serum (18). Cloning of sea bream TTR provided experimental evidence that TTR is expressed in fish (19), in contrast to previous findings obtained by other investigators through radiolabeled thyroxine binding assays (20). These observations indicate that, in fish, RBP transports retinol without forming a complex with TTR, raising the question on how such a small molecular mass RBP-retinol complex can be retained in plasma and escape kidney filtration.

The elucidation of the mechanisms by which vitamin A is distributed to target tissues in fish is extremely important to optimize aquaculture conditions. In fact, different fish species grown in aquaculture exhibit a high rate of skeletal malformations during embryonic development, and this phenomenon...
might be related to an incorrect supply of some nutrients in the diet. In particular, vitamin A or, more exactly, its active metabolite retinoic acid plays an essential role in modulating some step of antero-posterior axis formation during embryogenesis by regulating Hox gene expression. Moreover, since it regulates proliferation and differentiation of various cell types, including chondrocytes, it is directly involved in skeleton development (21–24).

As a first step in the study of the possible mechanisms by which RBP-retinol is retained in fish plasma without forming a complex with TTR, a cDNA clone for RBP was isolated from a carp liver cDNA library and characterized using in vivo and in vitro expression systems.

We present in this paper the experimental evidence that the protein has two unique features; one is the presence of two N-linked sugar moieties, and the second is the presence of a nonconserved uncleaved NH2-terminal signal peptide. Such features may play an important physiological role in the proper intracellular transport of the protein and in decreasing its plasma clearance through the kidney.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody against c-Myc epitope tag and all chemicals unless specified otherwise were obtained from Sigma Italia (Milan, Italy). Nick translation kit, nylon membranes, pGEX glutathione S-transferase fusion vector, and protein G-agarose were obtained from Amersham Pharmacia Biotech (Rainham, United Kingdom). Plaque lifts (Nytan filters) and nitrocellulose filters were from Schleicher & Schuell GmbH (Dassel, Germany). The carp liver cDNA library (Uni-ZAPTM XR Vector) and ExcAssist/SOL-R system were from Stratagene (Heidelberg, Germany), and Dynabeads, oligo(dT)20 were from Dynal (Oslo, Norway). SuperSignal Substrate was from Pierce Europe B.V. (Oud Beijerland, The Netherlands). Restriction enzymes and T3 RNA polymerase were from Promega Corp. (Madison, WI). Endoglycosidase H (EC 3.2.1.96), N-glycosidase F kit (EC 3.5.1.52), neuraminidase from Vibrio cholerae (EC 3.2.1.18), and DIG glycancitation kit, RNA inhibitor, dog pancreas microsomes, and Fugu gene6 were obtained from Roche Diagnostics S.p.a. (Monza, Italy). QIA-QUICK DNA purification/extraction kit was from Qiagen GmbH (Hilden, Germany). [35S]Methionine-cysteine ([35S]-Protein Labeling Mix, 1,000 Ci/mmol) was from PerkinElmer Life Sciences (Les Ulis, France). Culture media, fetal calf serum, antibiotics, glutamine, and amino acids were from HyClone (Logan, UT). Plasmid pBlueScript, located downstream and upstream of the cloned cDNA and used as a vector, was obtained from Roche Diagnostic S.p.a. (Monza, Italia). QIA-QUICK DNA purification/extraction kit, RNA inhibitor, dog pancreas microsomes, and Fugu gene6 were obtained from Roche Diagnostics S.p.a. (Monza, Italy). QIA-QUICK DNA purification/extraction kit was from Qiagen GmbH (Hilden, Germany). [35S]-Methionine-cysteine ([35S]-Protein Labeling Mix, 1,000 Ci/mmol) was from PerkinElmer Life Sciences (Les Ulis, France). Culture media, fetal calf serum, antibiotics, glutamine, and amino acids were from HyClone (Logan, UT). Plasmid pCMV/Myc/ER was obtained from Invitrogen (Carlsbad, CA). RI-100 rainbow trout liver cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). 10–15% acrylamide gradient precast gels were purchased from Novex (Carlsbad, CA).

cDNA Library Screening—1 × 106 plaques were screened with a human RBP cDNA, as probe, labeled with [α-32P]CTP by a nick translation kit. Plaque lifts (Nytran filters) were hybridized overnight at 58 °C and washed twice with 2× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS, plus twice with 1× SSC, 0.1% SDS. Filters were subjected to autoradiography. Positive clones were isolated and excised from phage DNA with ExcAssist/SOL-R system; the inserted DNA fragments were sequenced by a conventional deoxynucleotide chain termination method (25) using the universal primers (T3, T7) of pBlueScript, located downstream and upstream of the cloned cDNA and some synthetic oligonucleotides as primers.

Plasmid Construction—Carp RBP cDNA was amplified by PCR using two oligonucleotides that contained NheI upstream of the initiator methionine codon and NotI just before the termination codon. After digestion with NheI and NotI, the cDNA was cloned into pClneo-Myc (modified from pClneo) to construct pClneo-carpRBPmyc. pEGFP-carp putative signal peptide, defined as “chimeric protein” or “carp signal peptide,” was constructed by ligating to pEFGP-N3 vector the carp RBP NH2-terminal region (26 amino acids) amplified by PCR using two oligonucleotides containing NheI upstream and SalI downstream. Plasmid pGEX-carpRBP was constructed by ligating to pGEX vector the carp RBP sequence amplified by PCR using two oligonucleotides containing BamHI upstream and EcoRI downstream. The resulting fusion protein is formed by glutathione S-transferase followed by carp RBP.

RNA Preparation and Northern Blot Analysis—For Northern blot analysis, total RNA was extracted as described by Chirgwin et al. (26) from different carp tissues (liver, intestine, brain, kidney, and female gonads). Polyclonal RBP antibody was then isolated from total RNA on Dynabeads, oligo(dT)20. Polyclonal RBP antibody samples were resolved by electrophoresis in agarose, 1.5% formaldehyde gel and transferred onto nylon membranes, which were then hybridized with 32P-labeled carp RBP cDNA and with rat actin cDNA for normalization under standard conditions (27).

Cell Culture, Transfection, and Metablic Labeling—Carp liver cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4% glutamine, 100 units/ml penicillin-streptomycin, 1% nonessential amino acids, and 10% fetal calf serum. RI-100 rainbow trout liver cells were grown in Medium 199 with 10% fetal calf serum.

Transfection was performed on cells at 80% confluence in 35-mm plates. 3 μl of Fugene6 was mixed with 97 μl of serum-free medium in an Eppendorf tube, 1 μg of DNA was added, and the mixture was incubated for 15 min at room temperature. The mixture was added to each plate in 2 ml of complete medium, and the plates were incubated at 37 °C for 24 h.

Cells were washed with phosphate-buffered saline (PBS), supplied with methionine-cysteine-free DMEM and incubated at 37 °C for 30 min for starvation of the two amino acids. After this period, 100 μg/ml 35S-protein labeling mix was added to fresh methionine-cysteine-free DMEM (300 μl/plate) and the cells were incubated for the times indicated in the figure legends.

Antibody Preparation and Immunoprecipitation—Antibody against purified trout RBP was raised in mice. This antibody has been proven to recognize carp RBP by Western blotting but not by direct immunoprecipitation. Antibody against carp RBP was therefore raised in rabbit by injecting the fusion protein glutathione S-transferase-carpRBP; the fusion protein, expressed in Escherichia coli cells, was extracted from inclusion bodies (27) by boiling in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μg/ml bromphenol blue, 10 μg/ml β-mercaptoethanol) and separated by 13% SDS-PAGE. The corresponding gel band was then cut and eluted overnight in 10 μl Tris-HCl, pH 8.

Labeled cells were harvested in 1 ml of cold radioimmunoprotein assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100), and samples were subjected to immunoprecipitation as previously described (3). Immunoprecipitates were dissolved in the desired amount of sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 μg/ml bromphenol blue, 10 μg/ml β-mercaptoethanol), incubated in boiling water for 5 min, and then analyzed on 13% SDS-PAGE, followed by sodium salicylate fluorography (28).

Western Blotting—Proteins fractionated on SDS-PAGE as described above were transferred on a nitrocellulose filter using 25 mM Tris, 192 mM glycine, pH 8.3, buffer containing 20% methanol. The blot was stained with Ponceau S to check the extent of transfer, destained with PBS, and immunostained as described (3). Finally, the blot was incubated with SuperSignal substrate (Pierce) and exposed to X-ray films for appropriate time.

In Vitro Transcription, Translation, and Treatment of the Products with Endo H and Proteases—Plasmid encoding carp RBP was linearized and then purified using a QIAQuick DNA purification/extraction kit. Transcription and translation were carried out as described in the Promega technical manual. Immediately after the incubation, the translation mixture was chilled on ice, diluted to 100 μl with a buffer containing 0.25 μM sucrose, 50 mM Hepes/KOH, pH 7.5, 140 mM KCl, and 3 mM MgCl2 and then overlaid onto a 200 μl cushion containing 0.5 M sucrose, 50 mM Hepes/KOH, pH 7.5, 500 mM KCl, and 5 mM MgCl2. The step gradient was centrifuged at 65,000 rpm at 4 °C for 60 min in the 100.3 rotor of the Beckman centrifuge TL-100. The pellet was resuspended in 20 μl of 0.25 M sucrose buffer and divided in two samples; one was incubated at 37 °C with endoglycosidase H (Endo H, 2.5 milliunits (specific activity 125 units/mg of enzyme protein)) overnight, and the other was used as untreated control. In the protease protection experiment, the pellet was treated as described previously (29).

In Vitro Deglycosylation of Serum RBP and Neuroaminidase Treatment—Two carp serum samples (2.5 μl each) were mixed with 2.5 μl of Dilution Buffer and boiled for 3 min in the presence of 1% β-mercaptoethanol. After addition of 5 μl of Reaction Buffer to all samples, one of the two samples received 1 μl of α-D-Glucosidase, and the other received 5 μl of Endo F (5 milliunits). All the samples were incubated for 1 h at 37 °C, mixed with 5 μl of SDS gel loading buffer, boiled for 5 min, and then analyzed by SDS-PAGE, followed by immunostaining as described previously (3).

Treatment with neuraminidase from V. cholerae and subsequent analysis were carried out as follows. The immunoprecipitates by anti-
Carp Retinol-binding Protein

**FIG. 1. Amino acid sequence alignment.** Alignment of carp RBP with trout, *Xenopus*, chicken, rabbit, rat, and human RBPs is shown. The putative carp RBP signal sequence at the NH\(_2\) terminus and \(N\)-glycosylation sites are indicated by double line and waved line, respectively. The six cysteines involved in disulfide bonds are indicated by closed rectangles. The amino acids predicted to form the retinol pocket are indicated by open rectangle. Amino acid residues that are identical to those of carp RBP are indicated by bold letters. Cleavage site of the signal peptide of *Xenopus*, chicken, and mammalian RBPs is shown by an arrow.

**RESULTS**

**Isolation of Carp RBP cDNA and Amino Acid Sequence Comparison with Other Species**—The cDNA encoding carp RBP was cloned from a liver cDNA library by hybridization, using a human RBP cDNA as probe under low stringency conditions. Three positive candidate clones were isolated. The largest, of about 800 base pairs, contained an open reading frame corresponding to 213 amino acid residues. To confirm that the cDNA encodes carp RBP, the deduced amino acid sequence was compared with human (6), rat (7), rabbit (8), chicken (9), dog pancreas microsomes, two additional bands were detected at 27 and 30 kDa (lane \(A\)), whereas, unlike in mammals, a significant amount of mRNA is also expressed in intestine. RBP transcription is barely detectable in kidney and brain and absent in female gonads. Therefore, when carp RBP is expressed in specific tissues like mammalian RBP, poly(A)

**Expression of RBP mRNA in Carp Tissues**—To examine whether carp RBP is expressed in specific tissues like mammalian RBP, poly(A)

**Two Consensus N-Linked Glycosylation Sites Are Cotranslationally Glycosylated in Vitro**—As seen in Fig. 1, carp RBP contains two consensus N-linked glycosylation sites in the NH\(_2\)-terminal region. Since none of the vertebrate RBPs sequenced to date have N-linked glycosylation sites, we examined in vitro glycosylation of the carp protein. The cDNA was transcribed in vitro, and the transcript was translated in a rabbit reticulocyte lysate cell-free system. SDS-PAGE analysis of the primary translation product shows migration at a position corresponding to about 24 kDa (data not shown). When the transcript was translated in the presence of dog pancreas microsomes, two additional bands were detected at 29 and 30 kDa (lane \(B\)), corresponding to the expected molecular mass of single and double glycosylated products, respectively. However, when microsomes were added to the translation mixture 90 min following initiation of translation, only one product with a molecular mass of 24 kDa was obtained (data not shown). As the band with the smallest molecular mass
migrated at the same rate as the primary translation product in lane 1, it may be either untranslocated or translocated but unglycosylated RBP. To discern between the two possibilities, we performed a digestion with exogenously added proteases.

When the products translated in the presence of microsomes were digested with proteases in the absence of detergent, all three bands were protected (lane 4), but none of the band was protected when digested in the presence of detergent (lane 5). This result indicates that all three forms of the protein are cotranslationally translocated into the endoplasmic reticulum (ER) lumen, where they are protected from digestion by exogenously added proteases. This result suggests the interesting possibility that the NH₂-terminal hydrophobic domain (predicted signal peptide) of carp RBP (see Fig. 1) may not be cleaved during cotranslational translocation because the fastest migrating band in lane 4 (indicated by an asterisk) has the same mobility as the primary translation product indicated by an asterisk in lane 1.

To confirm that the two high molecular weight bands represent N-linked glycosylated forms of carp RBP, the products translated in the presence of microsomes were digested with Endo H. This enzyme specifically removes, by cutting between the two N-acetylgalactosamine residues, the “core” carbohydrates added to N-linked glycoprotein in the ER, but does not remove the carbohydrates of complex and hybrid type that are further modified in the Golgi apparatus. As shown in Fig. 3B (lanes 1 and 2), following digestion the two bands with higher molecular mass shifted to the position of the smallest molecular mass (24 kDa, deglycosylated RBP, indicated by an asterisk). These results clearly demonstrate that carp RBP is cotranslationally glycosylated. It is apparent from comparison between panels A (lanes 3 and 4) and B (lanes 1 and 2) of Fig. 3 that the protease-resistant, fastest migrating band (indicated by an asterisk in Fig. 3A, lane 4) migrated at the same rate as the deglycosylated RBP (indicated by an asterisk in Fig. 3B, lane 2). Since the fastest migrating band in Fig. 3A (lane 4) comigrated with the primary translation product (indicated by asterisks in Fig. 3A, lane 1), the deglycosylated form shown in Fig. 3B also appears to have the same molecular mass as the primary translation product. In other words, newly in vitro synthesized carp RBP was cotranslationally translocated in the ER lumen where it was glycosylated, but the NH₂-terminal hydrophobic domain was not cotranslationally cleaved.

NH₂-terminal Hydrophobic Domain Is Not Cleaved during Its Synthesis in Carp Liver—To examine whether the NH₂-terminal hydrophobic domain of carp RBP is cleaved during translocation in vitro, total proteins from carp plasma were digested with the deglycosylating enzyme Endo F (Fig. 3B), which removes the core carbohydrate of all types of N-linked glycoprotein by cutting between asparagine residue and N-acetylgalactosamine. The size of the Endo F-digested RBP was then compared with that of the deglycosylated, microsomal RBP synthesized in vitro (Fig. 3B, lane 2). Western blot analysis of the circulating form of carp RBP was performed using an anti-trout RBP antibody. The results show that undigested carp plasma RBP migrates as a single band of about 32 kDa. However, upon Endo F digestion, the protein appears as a single band that migrates at the same rate as the deglycosylated, microsomal RBP. These data confirm that the NH₂-terminal hydrophobic domain of carp RBP is not cleaved cotranslationally, but remains in the secreted RBP.

It should be pointed out here that the molecular mass of the in vivo synthesized carp RBP is slightly larger than the microsomal form translated in vitro. This can be attributed to post-translational oligosaccharide processing that is carried out in the Golgi apparatus. To examine this possibility, additional analysis was carried out to characterize the terminal carbohydrate of serum carp RBP using the “DIG glycan differentiation kit,” as described in the legend to Fig. 4. This kit contains five different lectins: *Galanthus nivalis* agglutinin (specific to terminal mannose), *Sambucus nigra* agglutinin (specific to Neu5AcO-2–6Gal and Neu5AcO-2–6GalNAc), *Maackia amurensis* agglutinin (specific to sialic acid O-2–3Gal), peanut agglutinin (specific to Galβ-1–3GalNAc), and *Datura stramonium* agglutinin (specific to Galβ-1–4GlcNAc). The results (Fig. 4A) show that, among five lectins, only MAA and DSA bound to carp RBP.
the manufacturer's manual. These include: strips were subjected to five different lectin binding assays according to N is a complex type of N is a complex type of N is a complex type of plasmid encoding a chimeric protein the NH2-terminal 26 amino acids, including the first N-linked glycosylation site of carp RBP, indicating that the terminal sugar moiety is composed of sialic acid (α2–3) and galactose (β1–4), but not mannose. The presence of sialic acid in the secreted RBP was also examined by comparing the migration rate of neuraminidase treated and undigested RBP in native PAGE, because the migration rate of the sialylated protein becomes slower upon digestion with neuraminidase, due to the decrease in negative charge. For this experiment Cos-1 cells were transiently transfected with a Myc-tagged expression construct of carp RBP, and secreted RBP was purified from the culture medium by immunoprecipitation with anti-Myc antibody and digested with neuraminidase from V. cholerae. As positive control, transferrin was digested with neuraminidase and analyzed with the same procedure. Fig. 4B clearly shows that neuraminidase digested RBP (lane 2) migrated slower than undigested RBP (lane 1) as in the case of digested (lane 4) and undigested (lane 3) transferrin. These results led us to conclude that carp RBP is a complex type of N-linked glycoprotein.

**NH2-terminal Hydrophobic Domain Translocates the Cytosolic Protein GFP to the ER**—The presence of an uncleavable signal peptide is quite unusual in secretory proteins. Comparison of the hydrophobicity profile (30) of carp RBP with that of the two examples found in the literature, i.e. ovalbumin (31, 32) and plasminogen activator inhibitor (PAI-2) (33), shows that the two proteins contain an internal hydrophobic domain that functions as uncleaved signal peptide, while in carp RBP the strongest hydrophobic domain is present in the NH2-terminal region, which consists of 17 amino acid residues (Fig. 1), and displays the typical features of an insertion signal with respect to amino acid composition and its arrangement, which is very similar to that of mammalian RBP.

To test whether the NH2-terminal hydrophobic domain of carp RBP functions as an insertion signal, we constructed a plasmid encoding a chimeric protein the NH2-terminal 26 amino acids, including the first N-linked glycosylation site of carp RBP, in frame with the cytosolic protein GFP, and this construct was transiently expressed in fish tissue culture cells (RI-100), as well as in mammalian Cos-1 cells. A control protein containing a signal sequence and a KDEL sequence in-frame with GFP (pCMV/Myc/ER: termed “ER-GFP”) was used as ER-specific marker. The fluorescence micrographs in Fig. 5 show that the chimeric protein localized in the perinuclear Golgi region and in the ER network spreading to the plasma membrane, both in fish (Fig. 5a) and in mammalian cells (Fig. 5d), while GFP alone remained in the cytosol (Fig. 5c). The chimeric protein was also detected in small cytosolic granules, some of which located very close to the plasma membrane, probably representing secretory granules. When cells expressing the chimeric protein were treated for 2 h with brefeldin A, which reversibly disassembles the Golgi apparatus, a redistribution of the chimeric protein in the ER was detected (Fig. 5e), with a fluorescence pattern similar to that of the ER marker protein (Fig. 5, b and f). These results show that the NH2-terminal hydrophobic domain of carp RBP is able to translocate a cytosolic protein into the ER, functioning therefore as an insertion signal.

To confirm that the chimeric protein is secreted without cleavage of the NH2-terminal hydrophobic domain, cell lysates and culture media of Cos-1 cells expressing RBP-GFP (unglycosylated form, 30 kDa; glycosylated form, 33 kDa), GFP (26 kDa), and ER-GFP (29 kDa) were analyzed by Western blotting using an anti-GFP antibody, and the size of the products was compared by migration in SDS-PAGE. The results (Fig. 6) demonstrate that only the glycosylated chimeric protein is secreted (33 kDa, indicated by a closed arrowhead in lane 5), thus confirming that the NH2-terminal hydrophobic domain defectively functions as an insertion signal. Furthermore, the results clearly demonstrate that the NH2-terminal hydrophobic domain remained uncleaved in the secreted chimeric protein, because the molecular masses of the slower migrating band in lane 2 (indicated by an open arrowhead) and that of the secreted protein (indicated by a closed arrowhead in lane 5) correspond to the unglycosylated (30 kDa) and glycosylated (33 kDa) chimeras, respectively. The high amount of cytosolic GFP found in cells expressing the chimeric protein (thick, faster migrating band in lane 2) can be explained by a much more efficient translation from the internal GFP initiation codon. Interestingly, unglycosylated chimeric protein seemed to be

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** A, lectin staining assay of carp RBP. 2 μl of carp serum (lane 1) or 5 μl each of carp serum subjected to immunoprecipitation with anti-carp RBP antibody (lanes 2–7) were separated by 13% SDS-PAGE and transferred on nitrocellulose by Western blotting as described under “Experimental Procedures.” The nitrocellulose filter was then cut into seven strips. As molecular weight marker, the carp serum sample and one immunoprecipitated carp serum sample were immunostained with anti-carp RBP (lanes 1 and 2, respectively). The remaining five strips were subjected to five different lectin binding assays according to the manufacturer’s manual. These include: G. nigra agglutinin (lane 3), S. nigra agglutinin (lane 4), M. amurensis agglutinin (lane 5), peanut agglutinin (lane 6), and D. stramonium agglutinin (lane 7). B, neuraminidase treatment of serum carp RBP. The anti-Myc antibody immunoprecipitates from the culture medium of Cos-1 cells transiently transfected with pCMV-carpRBPmyc (lanes 1 and 2), and 2 μg of purified human transferrin as the positive control (lanes 3 and 4) were digested or not with neuraminidase and analyzed by native polyacrylamide gel electrophoresis as described under “Experimental Procedures.”

![Figure 5](https://example.com/fig5.png)

**Fig. 5.** NH2-terminal hydrophobic domain of carp RBP targets cytosolic protein GFP to ER in vivo. RI-100 fish cells and Cos-1 mammalian cells were transiently transfected with chimeric protein (NH2-terminal hydrophobic domain of carp RBP-GFP) (a, d, and e), ER-GFP as ER marker (b and f), and GFP as cytosol marker (c). Cells were washed with PBS, fixed with 2.5% paraformaldehyde for 20 min at room temperature, washed again with PBS, and then observed by a Zeiss Axiokop 2 fluorescence microscope.
FIG. 6. Chimeric protein is secreted in the culture medium without cleavage of the NH2-terminal hydrophobic domain (putative signal peptide). Cos-1 cells were transiently transfected with GFP (lanes 1 and 4), chimeric protein (lanes 2 and 5) and ER-GFP (lanes 3 and 6). Cell lysates (lanes 1–3) and medias (lanes 4–6) were fractionated on 13% SDS-PAGE, transferred on nitrocellulose filter, and immunostained with anti-GFP antibody as described under “Experimental Procedures.” The unglycosylated and glycosylated forms of the chimeric protein are indicated by open and closed arrowheads, respectively.

retained in cells (lane 2, slowly migrating band indicated by an open arrowhead), suggesting that N-linked glycosylation may be important for intracellular transport of carp RBP.

Secretion of Carp RBP Is Dependent on N-Linked Glycosylation in Transiently Transfected Cos-1 Cells—To examine this hypothesis, Myc-tagged construct of carp RBP was transiently expressed in Cos-1 cells and metabolically labeled for 4 h in the presence and absence of the core glycosylation inhibitor tunicamycin. The amount of RBP secreted in the two experimental conditions was then compared. As negative control, untransfected Cos-1 cells were treated in the same way (lanes 1 and 2); as positive control, human RBP was transiently expressed in Cos-1 cells. The results in Fig. 7 show that, in the absence of tunicamycin, carp RBP was secreted with almost the same efficiency as human RBP expressed in Cos-1 cells (compare thick bands in lanes 3 and 4 with those in lanes 9 and 10). However, in the presence of tunicamycin, carp RBP was secreted with much less efficiency (compare the thick band indicated by an asterisk in lane 7 and the corresponding faint band in lane 8), suggesting that N-linked glycosylation is important for intracellular transport at the proper speed. As predicted, in brefeldin A-treated cells, the glycosylated protein was immunoprecipitated only from cell lysates (lanes 5 and 6), because brefeldin A blocks transport of newly synthesized proteins from the ER to the Golgi apparatus. Two thick bands can be recognized in lane 3. The faster migrating band migrated at the same rate as the thick band in lane 5 (both are indicated by open circles), and the slower migrating band, indicated by a closed circle, migrated faster than secreted RBP in lane 4. Such migration difference of newly synthesized RBPs may reflect the different extents of glycosylation.

DISCUSSION

Mammalian RBPs circulate in plasma bound to the transthyretin tetramer. Since the molecular mass of the complex is about 80 kDa, formation of this complex is able to decrease the extent of glomerular filtration in the kidney (5), whose size limitation is about 50 kDa. However, no RBP-TTR complex was found in trout plasma (16, 17), thus leaving an open question as to the mechanism of plasma retention. To examine whether this is a common feature in fish RBP, we have isolated a candidate cDNA clone for RBP from a carp liver cDNA library and characterized the corresponding protein. The amino acid sequence of carp RBP shows strong homology with that of the other known vertebrate RBPs. All structural and functional domains are highly conserved, with exception of the putative signal peptide sequence and of the COOH-terminal tetrapeptide RNLS/L, that is predicted to stabilize the RBP-TTR complex (15).

In addition, we have found two unique sequence features within the amino-terminal region of carp RBP: the presence of two consensus N-linked glycosylation sites and of an uncleaved signal peptide.

The results of in vivo and in vitro expression experiments have demonstrated that, unlike all other vertebrate RBPs, carp RBP is indeed glycosylated at both sites (Figs. 3, 4, and 7). Lectin binding assays (Fig. 4A), various glycosidase digestions (Figs. 3 and 4B) and synthesis of RBP in the presence of tunicamycin (Fig. 7) reveal that carp RBP is a complex type N-linked glycoprotein. The physiological significance of N-linked glycosylation of carp RBP might be related to a different mechanism for plasma retention in this species. Studies on the effect of electrical charge on the fractional clearance of dextran molecules of various sizes in rats have shown that the negative charges in the glomerular membrane retard the passage of negatively charged molecules and facilitate the passage of positively charged molecules. As one example, filtration of anionic substances 4 nm in diameter is less than half that of neutral substances of the same size (34, 35). Since two N-linked glycosylations increase molecular mass as well as negative charge due to sialylation (as shown in Fig. 4), glomerular filtration of carp RBP is likely to be greatly reduced even without forming a complex with TTR. This possibility will be further examined by measuring the clearance of unglycosylated and glycosylated RBP after intravenous injection.

Recently we have found that sea bream RBP is also a N-linked glycoprotein. It is known that both carp and sea bream have evolved in a different direction than trout, whose RBP is not a glycoprotein, and that the structure of carp kidney is quite different from that of trout. The fact that two out of three N-linked glycosylations increase molecular mass as well as negative charge due to sialylation strongly suggests that this might be a common feature in fish RBP, and, more importantly, that N-linked glycosylation may play an important role in fish RBP function.

The results of the present study (Fig. 7) show that N-linked glycosylation may directly be involved in intracellular transport of carp RBP. We have shown that secretion of carp RBP is greatly reduced by glycosylation inhibitors (compare the amount of secreted RBP in lane 4 with that in lane 6), and four possible explanations can be provided for these results. First,
unglycosylated RBP could be captured by chaperone(s) as a malformed molecule and retained in the ER lumen. Second, the core sugar of RBP may function as a signal for the exit from the ER to the Golgi compartment as is the case for secretory glycoproteins (36), and the unglycosylated form would be retained in the ER lumen. Third, the core sugar of RBP might play an important role in retinol binding and therefore in the conversion of newly synthesized apo-RBP to the holo form. If this were the case, newly synthesized RBP would be held onto the ER membrane through the interaction between the Glc₃Man₃GlcNAc₂ of RBP and the lectin site of calnexin-like lectin (37, 38) until apo-RBP binds retinol to become holo-RBP. This conversion would be the critical step for the exit of RBP from the ER (39–41) and will be further examined by testing whether unglycosylated RBP is present as apo or holo form.

Another unique feature of carp RBP is that the NH₂-terminal hydrophobic domain (residues 1–17) functions as an insertion signal, but is not cleaved cotranslationally and remains in the ER lumen. Third, the core sugar of RBP might play an important role in transport of the nascent chain, induced by glycosylation, may generate a physical force that pulls out the signal peptide from the ER lipid bilayer into the lumen. This interpretation is supported by the observations that both glycosylation sites are located very close to the predicted COOH terminus of the signal peptide and that N-linked glycosylation is generally carried out cotranslationally.

In conclusion, the results reported in the present paper uncovered the existence of novel structure-function relationships in carp RBP. On the basis of these results, it will be very interesting to further investigate which families of fish express N-linked glycosylated RBP and if a more general role can be established for N-linked glycosylation in RBP function.

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