Identification of serum GH-binding proteins in the goldfish (*Carassius auratus*) and comparison with mammalian GH-binding proteins

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Abstract

The present study constitutes the characterization of a specific, high-affinity GH-binding protein (GHBP) in the serum of a teleost, the goldfish (*Carassius auratus*). GH-binding assay and ligand blotting techniques were employed to identify GHBPs in goldfish serum and hepatocyte culture medium. The binding characteristics and apparent molecular weights (M_r) of goldfish GHBPs were also compared with those of rabbit and rat. LIGAND analysis identified a single class of high-affinity and low-capacity binding sites for iodinated recombinant carp GH (rcGH) in the goldfish serum, with an association constant (K_a) of $20.1 \times 10^9 \text{ M}^{-1}$ and a maximum binding capacity (B_{max}) of 161 fmol ml^{-1} serum. A single class of binding sites for iodinated recombinant sea bream GH and bovine GH (bGH) was also found in goldfish serum, but with a much lower affinity than that of rcGH. The binding affinity for iodinated bGH in rabbit and rat sera was found

to be similar to that reported previously. Ligand blotting revealed multiple forms of GHBPs in sera of goldfish, rabbit and rat with M_r ranging from 70 kDa to 400 kDa and 27 kDa to 240 kDa under non-reducing and reducing conditions respectively. A prominent band with M_r of 66 kDa and a minor band with M_r of 27 kDa were observed to occur in sera from all three species under reducing conditions. Iodoacetamide promoted the shedding of three GHBPs with M_r of 25, 40 and 45 kDa from the cultured goldfish hepatocytes. The appearance of all bands was completely inhibited by the presence of excess unlabeled rcGH. Our results provide clear evidence that a GHBP exists in the goldfish and indicate that more information on teleost GHBPs is needed if the physiology of growth in teleosts is to be fully understood.

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Introduction

Circulating growth hormone-binding proteins (GHBPs) have been identified and characterized in the serum of a number of mammalian species, including the mouse (Peeters & Friesen 1977, Smith *et al.* 1989), rabbit (Ymer & Herington 1985), rat (Baumbach *et al.* 1989, Amit *et al.* 1990, Massa *et al.* 1990), dog and pig (Lauteric *et al.* 1988), sheep (Amit *et al.* 1992, Davis *et al.* 1992), goat (Jammes *et al.* 1996), guinea pig (Ymer *et al.* 1997), and human (Baumann *et al.* 1986, Herington *et al.* 1986). In mouse and rat, GHBP is derived by translation of an alternatively spliced growth hormone receptor (GHR) mRNA lacking the appropriate transmembrane and intracellular domains of the GHR (Baumbach *et al.* 1989, Smith *et al.* 1989). In humans and rabbits, GHBP is considered to be largely generated from proteolytic cleavage of the membrane-anchored receptor (Barnard & Waters 1997). The mechanistic details of proteolytic shedding of the human and rabbit GHRs remain unclear, although a recent study in the IM-9 lymphocyte culture (Alele *et al.* 1998) indicated the involvement of a metalloprotease.

In other vertebrate groups, studies on GHBPs are very limited. There are reports of serum GHBPs in avian (Vasilatos-Younken *et al.* 1991, Davis *et al.* 1992), a reptilian (Sotelo *et al.* 1997), and a teleost (Sohm *et al.* 1998) species. The goal of the present study was to investigate the GHBPs in the circulation of a teleost, the goldfish (*Carassius auratus*). GH-binding assay and ligand-blotting techniques were employed to identify GHBPs in goldfish serum and from cultured hepatocytes. The binding characteristics and molecular sizes of goldfish GHBPs were also compared with those of rabbit and rat. Our results provide clear evidence that a GHBP exists in the goldfish, as in other vertebrates, and indicate that the physiological role of GHBP in teleosts needs to be investigated in future studies.

Materials and Methods

Experimental animals

Goldfish of the common or comet varieties were maintained as previously described (Zhang & Marchant 1996).

The goldfish were anesthetized by immersion in 0.005% (w/v) tricaine methanesulfonate (MS222). Blood samples were obtained from each fish by inserting a 25 gauge needle attached to a disposable syringe into the caudal vasculature. The blood was centrifuged at 10 000 *g* and serum was collected and stored at -20°C . Serum from female New Zealand White rabbits and male Wistar rats was kindly provided by Drs K Prasad and A Richardson (Departments of Physiology and Anatomy, University of Saskatchewan). All animals were used in accordance with guidelines established by the Canadian Council on Animal Care.

Hormones and reagents

Recombinant carp (*Cyprinus carpio*) GH (rcGH) and sea bream (*Sparus aurata*) GH (rsbGH) were kindly provided by Dr A Gertler (The Hebrew University of Jerusalem, Israel). The rcGH used in the present study was the form containing five cysteine residues (Fine *et al.* 1993). Bovine GH (bGH) and rat prolactin (rPRL) were gifts from the National Hormone and Pituitary Program (NIADDK, Baltimore, MD, USA). Carp prolactin (cPRL) was a gift from Dr R E Peter (University of Alberta, Edmonton, Alberta, Canada). Electrophoresis equipment and reagents were purchased from BioRad Co. (Mississauga, Ontario, Canada). Ultrogel AcA54, iodoacetamide, cell culture reagents, and all other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Iodination and binding studies

Iodination of the GHs was performed as previously described, using the lactoperoxidase method (Zhang & Marchant 1996). The specific activity of the labeled hormones was routinely more than 100 $\mu\text{Ci } \mu\text{g}^{-1}$ when determined using self displacement (Zhang & Marchant 1996) in liver membrane receptor-binding assay.

Serum-binding studies were carried out using modifications of a published method (Ymer & Herington 1985). Binding of goldfish, rabbit and rat sera with iodinated hormones was performed at $21\text{--}23^{\circ}\text{C}$ for 4 h using a 10 mM phosphate assay buffer containing 10 mM MgCl_2 , 0.02% (w/v) sodium azide, and 0.1% (w/v) BSA, at pH 6.5 in a final volume of 250 μl . Goldfish, rabbit and rat sera were used at a final protein concentration of 800 μg . Separation of bound ligand from free ligand was performed by gel filtration on AcA54 mini-columns (0.8×30 cm) at $21\text{--}23^{\circ}\text{C}$ (Ymer & Herington 1985). Data were subjected to LIGAND (Munson & Rodbard 1980) analysis to determine the association constant (K_a) and maximum binding capacity (B_{max}). When appropriate, displacement curves were also analyzed using the ALLFIT (De Lean *et al.* 1978) computer program.

All binding assays were carried out at pH 6.5, slightly lower than is routinely used in mammalian binding assays

(Ymer & Herington 1985). This change was necessary as the optimal binding of ^{125}I -rcGH to all serum-binding proteins, defined as the highest specific binding with a non-specific binding of less than 5%, was found to occur at pH 6.5 (data not shown). This is similar to our previous finding for the binding of ^{125}I -rcGH to goldfish liver membranes (Zhang & Marchant 1996). Maximal binding of ^{125}I -bGH to rabbit and rat serum was found to occur at pH 7.2, but was only slightly reduced ($<1\%$) at pH 6.5, with no alteration in the shape of the ^{125}I -bGH displacement curve (data not shown). Thus, an assay buffer pH of 6.5 was selected in order to allow direct comparisons to be made between rcGH and bGH in all species.

Ligand blotting of serum GHBP

Ligand blotting of GHBPs from goldfish, rabbit and rat sera was performed using a slight modification of published methods (Hocquette *et al.* 1990, Vasilatos-Younken *et al.* 1991). Briefly, 20 μg serum protein was separated by SDS-PAGE on a 7.5% gel under both reducing and non-reducing conditions (Laemmli 1970). Under the reducing conditions, serum proteins were treated with 100 mM dithiothreitol (DTT) or 5% (v/v) β -mercaptoethanol prior to separation. Prestained molecular weight standards (BioRad, Richmond, CA, USA) were separated in another lane of the gel. Separated proteins were transferred to nitrocellulose (0.45 μm pore size) using a BioRad mini transfer unit with Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol, at pH 7.4). The nitrocellulose membrane was washed with 3% Nonidet P-40 in Tris saline (10 mM Tris, 150 mM NaCl, pH 7.4) for 30 min and placed in blocking buffer (Tris saline containing 2% skim milk powder, 1% BSA, and 0.1% Tween-20, pH 7.4) for 2 h. The nitrocellulose membrane was then incubated with approximately 200 000 c.p.m. tracer in 50 ml assay buffer in the presence or absence of excess unlabeled GH (10 $\mu\text{g}/\text{ml}$) for 24 h at room temperature. Finally, the nitrocellulose membrane was washed, dried, and exposed to X-ray film (Kodak X-Omat AR5 or Biomax MS1) for 4 days at -70°C .

Goldfish hepatocyte culture and iodoacetamide treatment

Preparation of goldfish hepatocytes was modified from Krumschnabel *et al.* (1994). Goldfish were killed in excess anesthetic (0.05% MS222). Portions of liver were removed under sterile conditions and washed in HEPES buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 24 mM NaHCO_3 , and 15 mM HEPES, pH 7.5) containing 0.1 mg/ml kanamycin. The liver was then minced and incubated with 0.1% collagenase for 2 h at 26°C in an atmosphere of 95% air/5% CO_2 . Digested tissue was filtered through a 75 μm nylon mesh, centrifuged at 50 *g* for 45 s, and the supernatant removed by aspiration. Sedimented hepatocytes were washed three times in

HEPES buffer and resuspended in a serum-free culture medium (Dulbecco's modified Eagles' medium/medium 199, 3:1) containing 0.1 mg/ml kanamycin. Prior to cell seeding, the number of viable cells was counted on a hemocytometer using the dye exclusion method. Only cell preparations with a viability greater than 98% were used for the subsequent experiments. The protein content of hepatocytes was determined using the method of Lowry *et al.* (1951). Approximately 1×10^7 cells were plated in a 2.5 cm culture dish and incubated in a humidified atmosphere of 95% air/5% CO₂. Cell attachment occurred approximately 40 min after seeding. The attachment efficiency of goldfish hepatocytes was routinely 80% or greater. After cell attachment, fresh medium was added and renewed every 2 days thereafter.

In order to release GHBP in the culture medium, goldfish hepatocytes cultured for 3 days were treated with 20 mM iodoacetamide for 1.5 h at 26 °C. Similar iodoacetamide treatment has been reported to release a maximum amount of GHBP into the culture medium from IM-9 lymphocytes (Trivedi & Daughaday 1988, Alele *et al.* 1998). At the end of the incubation, the medium was collected and any remaining cells were removed by centrifugation. Medium protein (20 µg) was separated by SDS-PAGE on a 12% gel (Laemmli 1970) under both reducing and non-reducing conditions. Separated proteins were then subjected to ligand blotting as described above.

Results

GH-binding studies

Binding of ¹²⁵I-rcGH to goldfish serum was dependent on incubation pH, time, and temperature. At the optimal pH of 6.5, equilibrium was reached 3 h after incubation at room temperature (data not shown). The fraction of ¹²⁵I-rcGH, ¹²⁵I-bGH and ¹²⁵I-rsbGH that would bind to an infinite concentration of serum protein was calculated to be approximately 80% of the total radioactivity added. Therefore, total binding was corrected prior to LIGAND analysis as described previously (Zhang & Marchant 1996). Typical elution profiles for the total and non-specific binding of ¹²⁵I-rcGH to goldfish serum are shown in Fig. 1. Three peaks were observed for the total or non-specific binding profile (Fig. 1). Peak I in the total binding profile represented specific GHBP fractions which were inhibited by the presence of excess unlabeled rcGH in the incubation mixture prior to gel filtration. The peak I in the non-specific binding profile represented fractions of non-specific binding of ¹²⁵I-rcGH to goldfish serum. Peak II and peak III represented free ¹²⁵I-rcGH and Na¹²⁵I respectively (Fig. 1). Specific binding of ¹²⁵I-rcGH to serum GHBPs was calculated as the difference between the total and non-specific radioactivity corresponding to the peak I fractions.

Figure 1 Elution profile of goldfish serum incubated with ¹²⁵I-rcGH following gel filtration on an Ultrogel AcA54 mini-column. An aliquot of 25 µl goldfish serum diluted in 25 µl assay buffer was incubated with approximately 20 000 c.p.m. ¹²⁵I-rcGH in the absence (○, total binding profile) or presence (●, non-specific binding profile) of excess unlabeled rcGH. At the end of incubation, the mixture was eluted with assay buffer at a flow rate of 10 ml/h and 1.5-min fractions were collected. Fraction pools corresponding to peak I were used to calculate bound ¹²⁵I-rcGH. Vo, void volume, Vt, total volume.

LIGAND analysis of ¹²⁵I-rcGH, ¹²⁵I-bGH and ¹²⁵I-rsbGH binding to goldfish serum indicated only a single class of high-affinity and low-capacity binding sites. The K_a for ¹²⁵I-rcGH binding to goldfish serum was approximately tenfold higher than that of ¹²⁵I-bGH and ninefold higher than that of ¹²⁵I-rsbGH (Fig. 2). A single class of high-affinity and low-capacity binding sites for ¹²⁵I-rcGH and ¹²⁵I-bGH was also identified in rabbit and rat serum (Fig. 2). The K_a and B_{max} for iodinated rcGH, bGH and rsbGH in goldfish, rabbit and rat serum are summarized in Table 1.

Displacement of ¹²⁵I-rcGH from goldfish serum by various unlabeled hormones is shown in Fig. 3. The K_s for each hormone was estimated using the LIGAND program in order to determine the relative cross-reactivities of the various hormones with rcGH as the reference; the K_i of unlabeled rcGH was estimated as $12 \pm 4 \times 10^9$ M⁻¹ (mean $\pm$ s.e.m., $n=3$). The relative cross-reactivities of rsbGH, bGH, cPRL, and rPRL were found to be 13.8, 10.9, 1.7, and 0.4% respectively.

Displacement of ¹²⁵I-bGH and ¹²⁵I-rcGH from rabbit serum by various unlabeled hormones is shown in Fig. 4. ALLFIT analysis revealed that all parameters of the bGH and rcGH displacement curves were identical in the ¹²⁵I-bGH and ¹²⁵I-rcGH displacement tests (Fig. 4). Prolactins displayed relatively little displacement of either labeled GH (Fig. 4). When ¹²⁵I-bGH was used, the relative cross-reactivities of bGH, rcGH, rsbGH, rPRL, and cPRL as determined by LIGAND analysis were found to be 100%, 99.7%, 2.0%, 0.2%, and 0.05% respectively. In contrast, in the binding assay of ¹²⁵I-rcGH to rabbit serum, the relative cross-reactivities of rcGH, bGH,

Figure 2 Scatchard plots produced by incubating increasing amounts of ¹²⁵I-bGH, ¹²⁵I-rcGH or ¹²⁵I-rsbGH with goldfish (top panel), rabbit (middle panel) and rat (bottom panel) sera. Data, expressed as mean ± S.E.M. (n=3), were obtained by pooling results from three experiments, each carried out in triplicate and with serum from a different animal.

Table 1 Comparison of bGH-, rcGH- and rsbGH-binding sites in rabbit, rat and goldfish sera. The K_a and B_{max} values were obtained by LIGAND analysis of the results presented in Fig. 2. All data are expressed as means ± S.E.M. (n=3)

Source of sera	Hormone	K _a (10 ⁹ M ⁻¹)	B _{max}	
			(fmol/mg protein)	(fmol/ml serum)
Goldfish	rcGH	20.1 ± 1.8	4.1 ± 0.2	161 ± 7.8
	rsbGH	2.8 ± 0.06	4.1 ± 0.2	158 ± 8.0
	bGH	2.0 ± 0.04	4.0 ± 0.1	162 ± 6.5
Rabbit	bGH	9.1 ± 0.2	49.8 ± 3.2	3540 ± 230
	rcGH	9.5 ± 0.3	46.6 ± 3.7	3310 ± 261
	rsbGH	1.1 ± 0.2	41.8 ± 4.5	2970 ± 316
Rat	bGH	1.2 ± 0.1	83.8 ± 8.9	6030 ± 639
	rcGH	1.1 ± 0.1	80.2 ± 9.8	5780 ± 706
	rsbGH	NA	NA	NA

NA, K_a and B_{max} values are not available due to low (<2%) specific binding of the labeled hormone.

Figure 3 Displacement of 20 000 c.p.m. ¹²⁵I-rcGH from goldfish serum by increasing amounts of various unlabeled hormones. Each point represents the mean ± S.E.M. of triplicate determinations with pooled sera from several animals.

rsbGH, cPRL, and rPRL were found to be 100%, 102.6%, 2.9%, 0.22%, and 0.17% respectively.

Ligand-blotting studies

The serum GH-binding site consisted of multiple bands with high apparent molecular masses (*M_r*) in the goldfish (70, 80, 120, 180, 240, 360, and 400 kDa), rabbit (80, 120, 180, and 240 kDa), and rat (180 and 240 kDa) serum (Fig. 5). Sera from all three species contained the 180 kDa and 240 kDa bands. Two bands with *M_r* of 80 kDa and 120 kDa were also found in both goldfish and rabbit sera. The *M_r* of the labeled bands in all three species was altered under the reducing conditions. The *M_r* of the bands under reducing conditions ranged from 27 to 160 kDa in the goldfish and rat sera and 27 to 240 kDa in the rabbit serum (Fig. 5). A prominent band with *M_r* of 66 kDa and a minor band with *M_r* of 27 kDa were observed to occur in sera from all three species under reducing conditions. The

Figure 4 Displacement of 20 000 c.p.m. $^{125}\text{I-bGH}$ (top panel) or $^{125}\text{I-rcGH}$ (bottom panel) from rabbit serum by increasing amounts of various unlabeled hormones. Each point represents the mean $\pm$ S.E.M. of triplicate determinations with pooled sera from several animals.

appearance of all bands was completely inhibited by the presence of 10 $\mu\text{g/ml}$ of the unlabeled rcGH (data not shown).

Iodoacetamide promoted the shedding of multiple forms of GHBP from the goldfish hepatocyte culture, whereas no GHBP was detected in the conditioned medium in the absence of iodoacetamide (Fig. 6). The GHBP from goldfish hepatocyte culture consisted of three bands with M_r of 25, 40 and 45 kDa (Fig. 6). The appearance of these bands was completely inhibited by the presence of 10 $\mu\text{g/ml}$ unlabeled rcGH (Fig. 6). The M_r of these bands was not altered under reducing conditions.

Discussion

The existence of specific GHBP in the serum and hepatocyte culture of goldfish is confirmed by rcGH binding and ligand-blotting studies. LIGAND analysis of $^{125}\text{I-rcGH}$ binding to goldfish serum indicated a single class of high-affinity and low-capacity binding sites with a K_a of $20.1 \times 10^9 \text{ M}^{-1}$. In mammals, GHBP can be

Figure 5 Autoradiogram of nitrocellulose membrane following ligand blotting of $^{125}\text{I-rcGH}$ to goldfish (lanes A and D), rabbit (lanes B and E) and rat (lanes C and F) sera. Samples were separated by SDS-PAGE under non-reducing (lanes A, B and C) or reducing conditions (lanes D, E and F). Positions of molecular weight standards (kDa) are shown on the left. All bands disappeared when the membrane was incubated with labeled GH in presence of 10 $\mu\text{g/ml}$ unlabeled GH (data not shown).

divided into different types based on GH-binding affinity. Type I and type II GHBP display low-affinity GH binding. Type I GHBP include the mouse and rat, with a binding affinity of $1.2\text{--}3.9 \times 10^9 \text{ M}^{-1}$ (Amit *et al.* 1992). Type II GHBP have even lower GH-binding affinity than type I GHBP, and are found in the sheep (Amit *et al.* 1992, Davis *et al.* 1992), goat (Jammes *et al.* 1996), and cow (Gertler *et al.* 1984, Devolder *et al.* 1993). All type III GHBP have high-affinity binding with GHs ($4.7\text{--}9.2 \times 10^9 \text{ M}^{-1}$), and are present in the rabbit (Ymer & Herington 1985), dog (Lauteric *et al.* 1988), and horse (Amit *et al.* 1992).

Among all the species studied to date, goldfish serum GHBP has the highest K_a value. A recent study (Sohm *et al.* 1998) indicated that binding of rainbow trout GH to homologous serum was of low affinity ($3.8 \times 10^8 \text{ M}^{-1}$), although binding of human GH to rainbow trout serum was of higher affinity ($1.2 \times 10^9 \text{ M}^{-1}$). It is not clear why there are such large variations in the binding affinity of teleost GHBP. One possibility is that there are different categories of serum GHBP in teleosts, as in mammals. However, such variations may also be attributed to differences in hormone purity or bioactivity, assay conditions, or the methods used to calculate hormone-binding parameters.

In goldfish, the affinity of the serum GHBP ($20.1 \times 10^9 \text{ M}^{-1}$) and liver membrane GHR ($19 \times 10^9 \text{ M}^{-1}$; Zhang & Marchant 1996) for rcGH are very similar. This implies a close relationship between goldfish serum GHBP and the liver membrane GHR. A similar K_a for serum GHBP and liver membrane GHR was also found in the rabbit (Leung

Figure 6 Autoradiogram of nitrocellulose membrane following ligand blotting of ^{125}I -rcGH to the culture medium from goldfish hepatocytes treated with (lanes A and C) or without (lane B) iodoacetamide. Samples were separated by SDS-PAGE under non-reducing conditions; the M_r of the bands was not altered under reducing conditions (data not shown). Positions of molecular weight standards (kDa) are shown on the left. All bands disappeared when the nitrocellulose membrane was incubated with labeled GH in the presence of 10 $\mu\text{g}/\text{ml}$ unlabeled GH (lane C).

et al. 1987, Spencer *et al.* 1988), where the GHBP appears to be largely generated from proteolytic cleavage of the membrane GHR (Barnard & Waters 1997). In contrast, the K_a of the rat serum GHBP (Massa *et al.* 1990) was found to be 20-fold lower than that of rat liver GHR (Baxter *et al.* 1980). Rat serum GHBP originates from translation of an alternatively spliced GHR mRNA, rather than directly from proteolytic cleavage of the membrane GHR (Baumbach *et al.* 1989, Smith *et al.* 1989), which may provide one explanation for the difference between the K_a of the serum GHBP and liver membrane GHR in the rat.

In the goldfish GHBP assay, the K_a value for ^{125}I -rcGH was significantly higher than that for ^{125}I -bGH. Analysis of displacement of ^{125}I -rcGH from goldfish serum by various unlabeled hormones also indicates that ^{125}I -rcGH binding to goldfish serum was highly specific for teleost GHs. rsbGH was found to be less potent than rcGH, but was slightly more potent than the mammalian GH. Prolactins were considerably less potent than GHs, suggesting that the goldfish serum GHBPs were specific for somatogenic hormones. This pattern of hormone specificity is similar to the goldfish GHR (Zhang & Marchant 1996), and further indicates that the goldfish GHR/GHBP is very species-specific in terms of hormone binding.

Our results also indicate that rcGH, but not rsbGH, cross-reacts highly with serum GHBPs from the rabbit

and rat. These results are in agreement with our previous findings for GHRs in rabbit and rat liver membranes (Y Zhang & T A Marchant, unpublished observations). High cross-reaction of rcGH with the single class of high-affinity serum GH-binding sites was also evident in the displacement of labeled bGH and rcGH from rabbit serum by various unlabeled hormones. In these experiments, displacement curves from unlabeled rcGH were similar to those of unlabeled bGH. In both the ^{125}I -bGH and ^{125}I -rcGH displacement tests in the rabbit, prolactins were considerably less potent than GHs suggesting that the rabbit binding sites were also specific for GHs.

It is not clear why rcGH but not rsbGH shows high cross-reactivity with mammalian GHRs (Y Zhang & T A Marchant, unpublished observations) and GHBPs (present study). The rcGH used in the present study contains an extra cysteine residue at position 123, as found in the common carp (Fine *et al.* 1993, Law *et al.* 1996). This cysteine does not appear to be directly involved in binding of rcGH to the receptor (Fine *et al.* 1993). Goldfish also possess cDNAs for two GHs; one has cysteine at position 123, whereas this position in the other GH contains a serine (Mahmoud *et al.* 1996). Thus, goldfish and other cyprinid GHs may require a polar amino acid at position 123, which is not necessarily a cysteine (Mahmoud *et al.* 1996). Further studies will be required to fully understand the structure-activity relationship between GH and its receptor or binding protein in teleosts.

In the present study, the B_{max} value for serum GHBPs was found to be considerably higher in the rabbit (~ 3300 fmol/ml serum) and rat (~ 6000 fmol/ml serum) than that in the goldfish (~ 160 fmol/ml serum). Amit *et al.* (1992) measured the affinity constants for serum GHBPs in a number of mammalian species, including rabbit, rat, mouse, sheep, cow, horse, cat, monkey, and human, and reported that the B_{max} varied between 140 fmol/ml serum and 19 200 fmol/ml serum (Amit *et al.* 1992). Among other species studied to date, turtle GHBP was found to have a B_{max} of 1080 fmol/ml serum (Sotelo *et al.* 1997), whereas the B_{max} values for serum GHBPs from rainbow trout (Sohm *et al.* 1998) and chicken (Davis *et al.* 1992) were reported to be remarkably higher, ranging from 1.3×10^5 to 1.5×10^6 fmol/ml serum or greater. The physiological significance for such large variations in B_{max} value between different species remains unknown.

Ligand blotting also identified GHBP in goldfish serum and culture medium of goldfish hepatocytes. Goldfish, rabbit and rat sera contained GHBPs of large M_r , migrating from 120 kDa to 360 kDa. These large GHBP complexes in rabbit and rat sera have previously been identified by gel filtration (Ymer & Herington 1985, Baumbach *et al.* 1989, Amit *et al.* 1990, Massa *et al.* 1990). In the present study, DTT or β -mercaptoethanol treatment resulted in the reduction of the M_r of the serum GHBP complexes in all species, suggesting that the large bands observed by ligand

blotting contain disulfide bonds. Similar results have been reported for human serum GHBP (Hocquette *et al.* 1990).

Large M_r GHBPs were also evident in rainbow trout serum (Sohm *et al.* 1998), where 130 and 150 kDa complexes were identified by cross-linking of ^{125}I -hGH to rainbow trout serum. However, the small M_r GHBPs and some of the larger M_r GHBPs in goldfish serum could not be detected by cross-linking of ^{125}I -rcGH to goldfish serum (Y Zhang & T A Marchant, unpublished data). Previous studies utilizing cross-linking techniques were also unable to detect small M_r GHBPs in rabbit serum (Leung *et al.* 1987, Baumann & Shaw 1990). Thus, ligand blotting rather than cross-linking seems to be more appropriate for examining the complete range of serum GHBPs.

In the goldfish, the smallest M_r of serum GHBP under reducing conditions was found to be 27 kDa. A 27 kDa GHBP was also identified in serum of chickens and turkeys (Vasilatos-Younken *et al.* 1991). Previous studies on the primary structure of GHR in rat (Baumbach *et al.* 1989) and mouse (Smith *et al.* 1989) have indicated that the M_r of the extracellular domain of the GHRs is approximately 30 kDa. Thus, the 27 kDa GHBP observed in the present study may represent the extracellular portion of the goldfish GHR. The release of small sized GHBPs from cultured goldfish hepatocytes by iodoacetamide suggests that a mechanism of proteolytic cleavage of membrane GHRs may also be involved in the generation of goldfish GHBP. However, additional studies will be required to determine the nature of the large M_r GHBPs in the goldfish.

The goldfish GHBPs resemble GHBPs from other species by displaying a specific high-affinity and low-capacity binding for GH, presence of multiple forms of GHBPs in the circulation, and a close relationship with hepatic GHRs. Although circulating GHBPs have been found in all species tested to date, the precise physiological role of GHBPs remains unclear (Barnard & Waters 1997). We have previously established and validated a goldfish GH receptor-binding assay (Zhang & Marchant 1996). Together, these results indicate that the goldfish will be a very useful model for studies on the physiological interaction between GH, GHR, and GHBP in teleosts.

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