

Identification and Modulation of a Growth Hormone-Binding Protein in Rainbow Trout (*Oncorhynchus mykiss*) Plasma during Seawater Adaptation

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A soluble protein that specifically bound ¹²⁵I-human growth hormone (hGH) was identified in rainbow trout plasma, using HPLC-gel filtration. The binding affinity of the protein for hGH was $1.2 \times 10^9 \text{M}^{-1}$. ¹²⁵I-rainbow trout GH (tGH) was also able to bind to the protein albeit with a lower affinity ($6.6 \times 10^7 \text{M}^{-1}$) than hGH. Crosslinking experiments using ¹²⁵I-hGH revealed two specific bands of 150 and 130 kDa. The complex ¹²⁵I-hGH-BP could be precipitated by a monoclonal anti-GH receptor antibody, suggesting a close relationship between the plasma GH-BP and the GH receptor. A fourfold increase in the hGH binding to the GH-BP was shown 48 h after transfer of the fishes from freshwater to seawater. The increase in binding was related to a high binding capacity without significant changes in binding affinity. These results suggest a potential role of this related GH-BP as an index of GH effects during seawater adaptation in salmonids.

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Growth hormone (GH)² and prolactin (PRL), with somatolactin (SL) in fish and placental lactogen in mammals, belong to the same family of hormonal peptides (Rand-Weaver and Kawauchi, 1993). The major functions of GH in all species studied are control of metabolism and growth of skeletal and soft tissues. In salmonids, it is now established that GH enhances survival and improves hypoosmoregulatory ability during seawater adaptation (Clarke *et al.*, 1977). The hypoosmoregulatory effect of GH in seawater is independent of its growth promoting effect (Collie *et al.*, 1989). In teleost fishes, as in other vertebrates, the mechanism of GH action involves GH binding to membrane receptors. Few data are available concerning functional interactions between GH and its receptor in fish. It has been suggested that the homodimerization of GH receptor is a characteristic both in mammals and in fishes (Calduch-Giner *et al.*, 1997). GH receptors are present in many organs and most abundantly in the liver (Gray *et al.*, 1990). To assess the target organs for GH in salmonids, a homologous radioreceptor assay for GH was established for coho salmon and rainbow trout (Sakamoto and Hirano, 1991; Yao *et al.*, 1991). In the trout, GH receptors were

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² Abbreviations used: GH, growth hormone; PRL, prolactin; SL, somatolactin; GH-BP, GH-binding protein.

identified in liver, gill, intestine, and posterior kidney. Scatchard analysis showed the presence of a single class of GH receptors characterized by high affinity and low capacity. The number of receptors in the gill, intestine, and kidney was about 3–6% of that in the liver (Sakamoto and Hirano, 1991). These results indicate that liver and osmoregulatory organs are potential targets for GH actions. Receptor numbers in the liver increased after transfer to seawater, suggesting the likelihood of at least partial mediation by the liver of the osmoregulatory actions of GH (Sakamoto and Hirano, 1991). An interaction of one or more factors, such as hormonal and nutritional status, in association with GH receptor turnover may occur, contributing to the successful adaptation of salmonids to the marine environment.

In mammals, a soluble form of the GH receptor has been identified (Baumann, 1991). The growth hormone-binding protein (GH-BP) which represents the extracellular domain of the GH receptor (Leung *et al.*, 1987). Serum GH-BP has been identified in numerous domestic animals, from poultry to mammals (Davis *et al.*, 1992). Regulation of the expression of GH-BP appears to be also under the control of multiple hormonal, nutritional, or environmental factors, although its biological function remains to be clarified (Postel-Vinay, 1996).

A soluble protein has been identified in rainbow trout (*Oncorhynchus mykiss*) plasma which specifically binds human GH (hGH) with higher affinity and lower capacity than the homologous rainbow trout GH (tGH). Circulating GH-BP was identified in both freshwater- and seawater-adapted trout. During seawater adaptation a fourfold increase in GH-binding capacity occurred (48 h after transfer), declining thereafter. Thus, judged by the binding of labeled hGH to circulating GH-BP, the present data provide new avenues to study GH effects at the receptor level and its potential involvement during seawater adaptation in salmonids.

MATERIALS AND METHODS

Animals and Blood Sampling

Immature yearling rainbow trout (*O. mykiss*) weighing 280–320 g were obtained from the Cornec fish farm hatchery (Brest, France) and transported to the IFREMER station (Brest). They were maintained in

Swedish tanks ($2 \times 2 \times 1$ m) containing 2000 liters of running freshwater at 12–13°C, under natural photoperiod, for 2–3 weeks before study (April 1996). Fish were starved 24 h before seawater transfer. Otherwise, fish were fed a ration of 1.5% body wt/day with commercial trout moist pellet, using an automatic feeder. Rainbow trout were transferred to seawater replacing the freshwater with a seawater supply into the tank, reaching full seawater condition (salinity: 35.5‰) within half an hour. After decapitation of unanesthetized fish, they were weighed and blood samples were collected from the dorsal aorta into heparinized tubes. The samples were centrifuged (4°C) at 3000g for 10 min, and the plasma was aliquoted and stored at –20°C until assayed.

Materials

Recombinant hGH was obtained from Serono Laboratories (Boulogne, France), ovine PRL (oPRL) was from NIDDK-16, National Hormone and Pituitary Program (Bethesda, MD), bovine GH (bGH) was from W. Baumbach, American Cyanamid Corporation (Princeton, NJ), recombinant trout GH (tGH) was from Pharos (Belgium) according to Rentier-Delrue *et al.* (1989a), recombinant rainbow trout PRL (tPRL) was according to Mercier *et al.* (1989), recombinant tilapia GH (tiGH) was according to Rentier-Delrue *et al.* (1989b), rainbow trout somatolactin (tSL) was according to Rand-Weaver *et al.* (1991), and tilapia PRL was according to Swennen *et al.* (1991).

Carrier-free ^{125}I -Na was purchased from Amersham International (Buckinghamshire, UK), disuccimidyl suberate (DSS) and Iodogen were from Pierce (Rockford, IL), and protein PAK 300 sw column was from Waters (Milford, MA). Iodination of hGH was performed using Chloramine-T (Lesniak *et al.*, 1973). ^{125}I -tGH was obtained by the Iodogen method (Salacinski *et al.*, 1981). Labeled hormones were purified on Sephadex G75 column and eluted with 0.1% bovine serum albumin/0.05 M phosphate buffer, pH 7.5. Specific activities of radiolabeled hormones ranged from 80 to 140 $\mu\text{Ci}/\mu\text{g}$ (2.96–5.18 MBq/ μg).

GH-Binding Protein Assays

GH-binding activity in plasma was measured as described by (Tar *et al.*, 1990). Briefly, after filtration

through 0.45-mm Millipore minifilters, different volumes of plasma were incubated for 20 h at 4°C in 100 μ l of 0.1% bovine serum albumin/0.1 M phosphate buffer, pH 7.0, containing 1×10^5 cpm [125 I]hGH (16 h at 4°C) or [125 I]tGH (3 h at 20°C) in the absence or presence of various concentrations of unlabeled GH. These incubation conditions were determined in preliminary experiments as giving the highest binding level for hGH and tGH, respectively, and they did not affect the affinity. Elution was performed isocratically using a degassed buffer (0.1 M, Na₂SO₄/0.1 M phosphate buffer, pH 7.0) pumped at a rate of 0.5 ml/min. Radioactivity was recorded on line by using a Berthold LB 504 gamma detector connected to a computer. The binding of labeled GH is expressed as the radioactivity in peak I divided by the total radioactivity (peaks I + II): I (125 I-GH-BP), II (free 125 I-GH). Scatchard analyses were performed using the program Ligand (Munson and Rodbard, 1980).

Cross-Linking Experiments

Cross-linking of 125 I-hGH to the plasma trout GH-binding protein was achieved as follows: a 50- μ l aliquot of plasma was incubated with 125 I-hGH (10^6 cpm) in 25 mM phosphate buffer, pH 7.4 (final volume, 65.5 μ l). Parallel incubations were performed with an excess (2 μ g) of native hGH. After 12 h at 4°C, disuccinimidyl suberate in dimethyl sulfoxide was added at a final concentration of 0.5 mM. After 15 min at room temperature, 7 μ l of 1 M Tris-HCl, pH 6.8, was added. Sample buffer, containing 2-mercaptoethanol (5% final) and SDS (1% final), was added to the mixture. Half of each sample (50 μ l) was heated for 5 min at 100°C and resolved by 7% SDS/PAGE along with prestained M_r standards under reducing conditions by the procedure of Laemmli (1970). Gels were dried and scanned using a PhosphorImager (Molecular Dynamics).

Immunoprecipitation of GH-BP

Trout plasma samples (200 μ l) were incubated with 125 I-hGH (2×10^5 cpm, 300 μ l) in the presence or absence of unlabeled hGH in 0.1% bovine serum albumin/25 mM Tris, pH 7.5/10 mM MgCl₂. After 16 h at 22°C, 1 μ g/1 μ l monoclonal antibody (mAb 263) directed against the extracellular domain of GH-R

(Biogenesis, Barnard *et al.*, 1985) was added to the medium, and incubation was continued for an additional 5 h. Five-hundred microliters of γ -globulin (0.1%) and 25% (v/v) polyethylene glycol (final concentration 12.5%) in 25 mM phosphate buffer, pH 7.4, was added. As a positive control, rabbit hepatic membranes were used. The membranes were diluted in 25 mM Tris buffer, pH 7.4/2.5% Triton X-100 at final protein concentration of 6 mg/ml. After centrifugation for 30 min at 15,000g, 200 μ l of the supernatant (solubilized receptor) was incubated respectively with labeled hGH and mAb263 as described for trout plasma. The tubes were centrifuged, the supernatant was discarded, and the radioactivity of the pellet was measured in an LKB counter.

Statistics

Statistical analysis of the results was based on the Duncan's multiple range test, comparing experimental groups. Results are expressed as means \pm SEM.

RESULTS

Characterization of GH-BP in Trout Serum

The elution profile of labeled 125 I-hGH incubated with 2 μ l plasma of rainbow trout is shown in Fig. 1A. Two peaks (I and II) were identified. Peak I (20% of total radioactivity) represents the complex of 125 I-hGH-binding protein. The elution time of the radioactive peak occurred at 14 min. This peak is abolished by an excess (1 μ g) of native hGH used to calculate the non specific binding (Fig. 1A). Peak II consisted of free 125 I-hGH. Specific binding of 125 I-hGH increased from 10 to 70% of the total radioactivity with plasma volumes from 1 to 25 μ l. Fifty percent inhibition of 125 I-hGH binding to the BP was obtained with 2 ng/incubation of hGH (Fig. 2A). Competition with homologous or heterologous hormones showed that only tGH competed with 125 I-hGH with a low apparent affinity ($IC_{50} = 0.75$ μ g/incubation). Very limited competition was observed with tSL ($IC_{50} = 20$ μ g/incubation). No inhibition was detected with excess (50 μ g) tPRL, tiPRL₁₇₇ and tiPRL₁₈₈, oPRL, tiGH, or bGH (Fig. 2A). No binding of 125 I-oPRL was observed after

incubation with either 2 or 50 μ l of serum (data not shown). The affinity and the capacity of the GH-BP for 125 I-hGH as evaluated by Scatchard plot analysis are $1.2 \times 10^9 \text{ M}^{-1}$ and $3.48 \times 10^{-10} \text{ M}$, respectively (Fig. 2A).

A similar HPLC elution profile was observed when 2 μ l of plasma was incubated with 125 I-tGH (Fig. 1B). This peak is abolished by an excess (1 μ g) of native hGH (data not shown), indicating that tGH and hGH probably bind to the same molecular size BP in rainbow trout serum. Complete inhibition of 125 I-tGH binding was obtained using 50 μ g of unlabeled tGH with $\text{IC}_{50} = 20 \mu\text{g}/\text{incubation}$. Characteristics of tGH binding estimated by Scatchard plot analysis, confirmed the low affinity and the high capacity (Fig. 2B).

Evaluation of GH-BP molecular weight of cross-

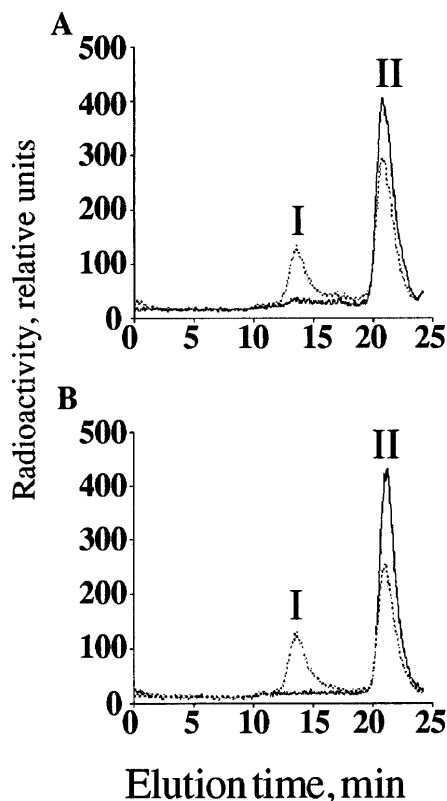


FIG. 1. Elution profile from HPLC-gel filtration column of 125 I-hGH (A) and 125 I-tGH (B) incubated with 2 μ l of rainbow trout plasma. Incubations were performed in the absence (thin line) or presence of 1 μ g hGH or 50 μ g of tGH (thick line). The elution times of complexed 125 I-hGH-BP (A) or 125 I-tGH-BP (B) (peak I) was 14 and 21 min for 125 I-hGH (A) or 125 I-tGH (B) (peak II). For 125 I-hGH, total binding and nonspecific binding were 21 and 4%, respectively (A).

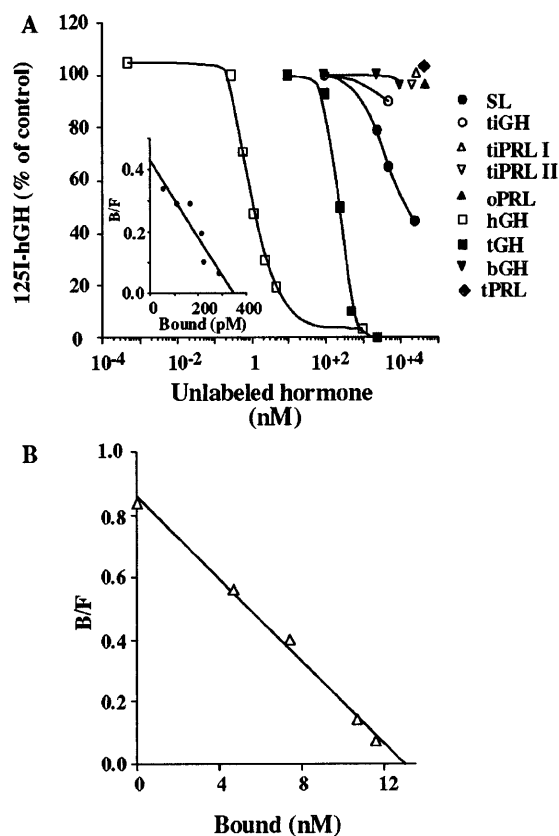


FIG. 2. Effects of increasing concentrations of tGH, tPRL, tSL, oPRL, hGH, bGH, tiGH, and tiPRLs on the binding of 125 I-hGH to rainbow trout plasma (A) and representative Scatchard plots of competition binding experiments with 125 I-hGH (A) and 125 I-tGH (B). (A) 2 μ l of plasma from rainbow trout transferred for 2 days in seawater was incubated for 20 h at 4°C, in the absence or presence of increasing concentrations of unlabeled homologous or heterologous hormones. The binding of 125 I-hGH is expressed as the percentage of the maximal binding. The results are from one representative experiment, repeated two times, and are the mean of duplicate determinations. Maximal binding was 20% of radioactivity. (Inset) One representative Scatchard plot of competition binding experiment with 125 I-hGH from rainbow trout transferred for 2 days in seawater. The affinity and capacity are, respectively, $1.23 \times 10^9 \text{ M}^{-1}$ and $3.48 \times 10^{-10} \text{ M}$. (B) Scatchard plot of competition binding experiment in plasma from rainbow trout transferred for 2 days in seawater. The K_a and B_{max} are $6.6 \times 10^7 \text{ M}^{-1}$ and $1.3 \times 10^{-8} \text{ M}$, respectively.

linked 125 I-hGH-BP was performed by SDS/PAGE under reducing conditions (Fig. 3). Two bands corresponding respectively to complexes of 150 and 130 kDa (lane 1) were revealed. Both are specific, since they were inhibited when an excess of native hGH was added (lane 2). No inhibition was observed with 2 μ g

tPRL, tSL, or tGH (data not shown). To better characterize the tGH-BP, immunoprecipitation studies were carried out using a monoclonal antibody against the GH receptor (mAb263). The antibody immunoprecipitated the complex hGH/GH-BP (20.3% of total radioactivity was immunoprecipitated). These results suggest a close immunological relationship between the membrane GH receptor and the trout plasma GH-BP.

Transfer to Seawater

All studies were performed using ¹²⁵I-hGH as a ligand for plasma GH-BP. A fourfold increase in GH binding activity (*P* < 0.01) was observed in the plasma of rainbow trout 48 h after transfer into seawater (Fig. 4A). This increase is related to a greater binding

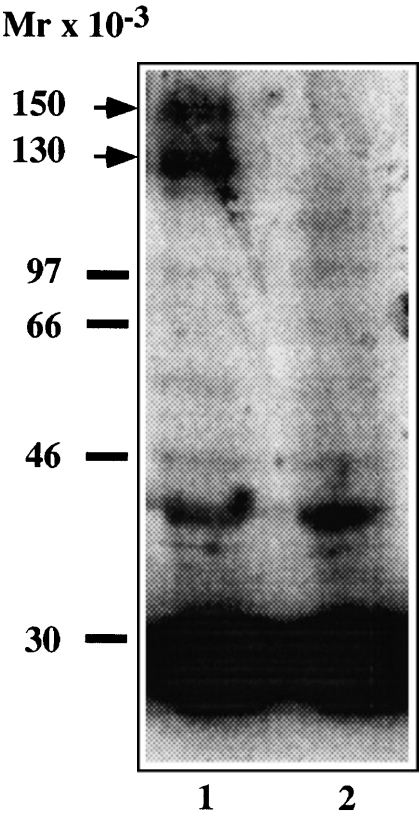


FIG. 3. Autoradiograph of PAGE showing cross-linking of ¹²⁵I-hGH-BP in 25 µl of plasma from freshwater rainbow trout incubated in the absence (lane 1) or presence (lane 2) of 2 µg hGH. Electrophoresis was performed under reducing conditions. Markers of MW standards are indicated by lines (*M_r* × 10⁻³). Arrows indicate the position of specific radiolabeled proteins.

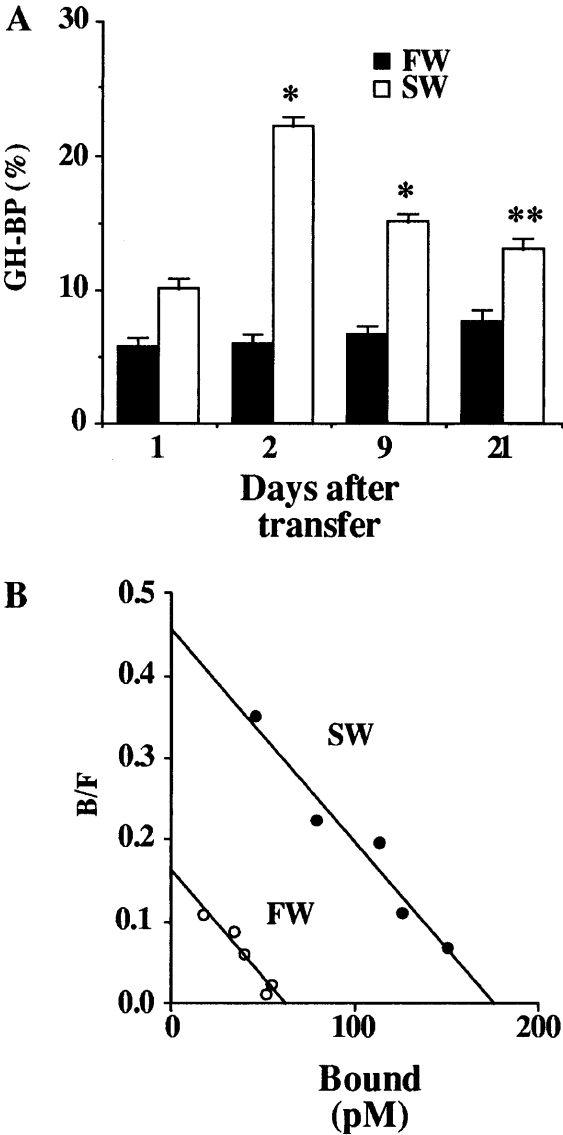


FIG. 4. Time-dependent change of ¹²⁵I-hGH binding to BP (A) and representative Scatchard plots of competition binding experiments with ¹²⁵I-hGH (B) in rainbow trout transferred from freshwater (FW) to seawater (SW). Control fishes (open columns or circles) were compared to transferred fish in seawater (closed columns or circles). (A) GH-BP level is expressed as percentage of specific binding of ¹²⁵I-hGH per 2 µl plasma. Vertical bars represent SEM (*n* = 8). **P* < 0.01 and ***P* < 0.05 compared with both the initial (day 0) and the control fishes transferred in freshwater; (B) representative Scatchard plot experiments using plasma from freshwater (FW) rainbow trout transferred for 2 days and seawater (SW) rainbow trout transferred for 2 days. The *K_a* and *B_{max}* in serum from freshwater control are 1.82 × 10⁹ M⁻¹ and 7.7 × 10⁻¹¹ M. The *K_a* and *B_{max}* using plasma from rainbow trout transferred for 2 days in seawater are, respectively, 2.6 × 10⁹ M⁻¹ and 1.76 × 10⁻¹⁰ M.

capacity as shown in Fig. 4B. The level of GH-BP in plasma of rainbow trout fully adapted to seawater (3 weeks after transfer) was twice ($P < 0.05$) the level found in plasma of freshwater-adapted fish. Although the increase in hGH binding to BP from trout transferred into SW is associated with an increased binding capacity, there is no significant change in binding affinity (FW, $1.69 \pm 0.13 \times 10^9 \text{ M}^{-1}$, $n = 4$; SW, $2.09 \pm 0.18 \times 10^9 \text{ M}^{-1}$, $n = 4$; Fig. 4B).

DISCUSSION

The present study identifies a binding protein with high affinity for hGH in rainbow trout plasma; tGH and tSL appeared to be weak competitors for ^{125}I -hGH binding. No displacement was observed with bGH or tiGH; however, hGH is known to behave as a more potent somatogenic ligand than other GHs for GH-BPs. Also, no displacement was observed with tPRL, tiPRL₁₇₇, tiPRL₁₈₈, or oPRL, suggesting that the BP has no prolactin like activity. Thus, considering the homologous hormones, these binding properties appear to be more related to a putative circulating GH-BP. Similar data with higher binding affinity of heterologous hormones for the GH-BP compared with homologous GH (approximately 100-fold) has been reported in chicken and other domestic animals (Davis *et al.*, 1992). Mutational and structural studies have demonstrated that one hGH molecule binds to two molecules of hGHBP, through two regions called binding sites 1 and 2 (Goffin *et al.*, 1996). Binding site 1 is delimited by a pocket encompassed within helix 1, helix 4, and loop 1 (Fig. 5), whereas binding site 2 is delimited by the opposite site of helix 1 and 3 as well as four residues of the N terminus.

Of the 25 residues identified in hGH as hGHBP binding site 1 determinants (Cunningham and Wells, 1989), 16 are substituted in the tGH; among them 12 are nonconservative substitutions. In helix 4, which contains the most important determinants of binding site 1, five residues of the hGH (Y164, D171, F176, R178, and V185) present such nonconservative substitutions in the tGH (Fig. 5). This would imply that tGH binding site 1 is different from hGH binding site 1. Based on hGH/hGHBP model and since the structure of the tGHR or tGHBP are not known, a working

hypothesis might be that site 1 of tGH has a reduced activity to bind to GHBP than hGH site 1, thus resulting in a decreased affinity of tGH site 1 relative to hGH site 1 for GHBP. Of interest is the limited competition by tSL for hGH binding suggesting that tSL possibly also binds to a membrane receptor belonging to the PRL/GH receptor family. Indeed, no information is available on the type of receptor used by tSL or the physiological role of this hormone in fish (Rand-Weaver and Kawauchi, 1993).

In mammals, there is an amino acid sequence identity between the extracellular domain of the membrane GH receptor and the serum GH-BP (Leung *et al.*, 1987). GH-BP is a short soluble form of the GH receptor. Serum GH-BP binds to circulating GH forming a high-affinity complex of 75–80 kDa ($K_a = 3\text{--}9 \times 10^8 \text{ M}^{-1}$) with limited capacity (20–150 ng/ml) (Barnard *et al.*, 1985; Baumann *et al.*, 1986; Baumann, 1991; Tar *et al.*, 1990). The present study only detected hGH-BP complexes of high molecular weight 130–150 kDa. High molecular weight GH-BP has also been detected in human serum. These BPs bind hGH with very low affinity ($10^6\text{--}10^5 \text{ M}^{-1}$) and high capacity (between 2 and 15 $\mu\text{g}/\text{ml}$) and are thought not to be related to the GH receptor (Baumann and Shaw, 1990; Tar *et al.*, 1990). However, there are high molecular weight complexes in murine serum (Smith and Talamantes, 1987) and also in the culture medium of cells transfected with rabbit GH-BP cDNA (Edery *et al.*, 1993). These data suggest that circulating multimers of GH-BP may arise after proteolysis of the membrane bound GH receptor. Therefore, it is possible that the 130- and 150-kDa bands observed after cross-linking or HPLC elution in the present study may correspond to dimers of complexed BP with one or two molecules of hGH (22 kDa). The demonstration that mAb263 monoclonal antibody to the GH receptor is able to immunoprecipitate the hGH-BP complex supports the idea that tGH-BP may represent the extracellular domain of the GH receptor in trout serum.

Two mechanisms for generation of the GH-BPs have been shown. GH-BP may arise from proteolytic cleavage of the membrane receptor (Baumann, 1991; Sotiropoulos *et al.*, 1993) encoded by a single transcript of 4.5 kb (Leung *et al.*, 1987) or by alternative splicing of a primary transcript which gives rise to two different mRNAs (Baumbach *et al.*, 1989). One mRNA of 4.5 kb

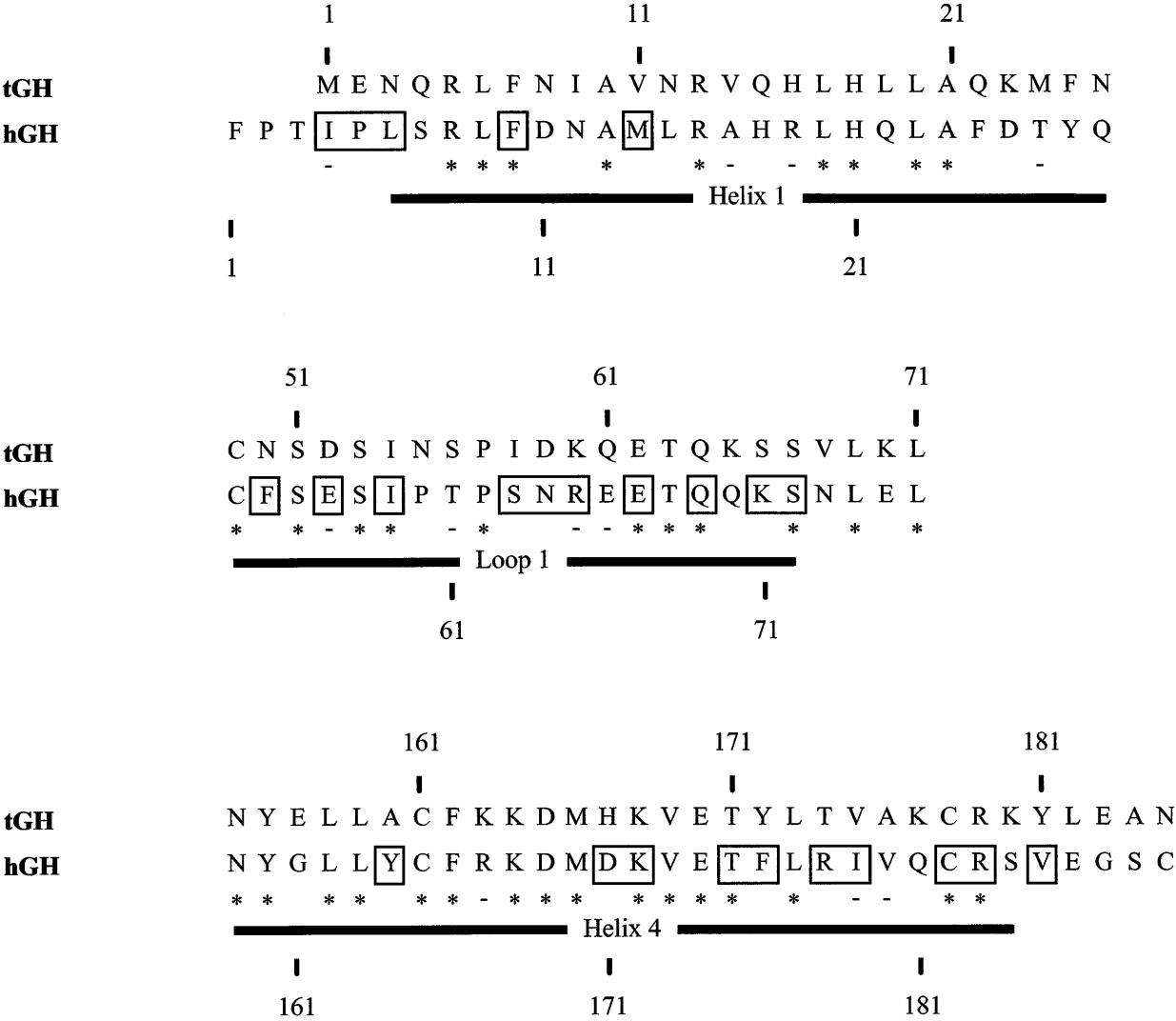


FIG. 5. Comparison of the binding site 1 determinants of hGH and tGH. The three regions constituting the binding site 1 of hGH (N terminus, second half of loop 1 and C terminus) are aligned. Residue numbering *above and below* the sequences refer to tGH and hGH, respectively. Residues involved in hGH/hGHBP interactions are from Cunningham and Wells (1989). Residues reducing the binding by more than twofold are boxed. (*) identical amino acids; (-) conserved amino acids.

encodes the membrane receptor and another of 1.2 kb encodes the GH-BP, i.e., the extracellular domain of the GH receptor lacking the transmembrane and cytoplasmic domains. Molecular cloning of the GHR cDNA should help clarify the mechanism(s) of GH-BP generation in trout.

The role of GH-BP in serum is uncertain in mammals. GH in this complexed form has an increased half-life compared to free hormone (Baumann, 1991). Thus, the hormone bound to the BP is protected from

degradation and represents a hormone “reservoir” in serum. However, GH-BP production by individual tissues could alter independently their own sensitivity to circulating GH or could be regulated under certain physiological conditions, thus modulating GH action.

No kinetic studies have been performed on circulating hGH in rainbow trout. However, in relation to the higher affinity of the BP to hGH relative to tGH found in the present study, it is of interest to note that hGH is more potent than salmon GH in stimulating thyroid

hormone 5'-deiodination in trout liver (Mac Latchy and Eales, 1990; Mac Latchy *et al.*, 1992). Indeed, the interplay between thyroid hormones deiodination and GH is known to be critical for seawater survival of rainbow trout during the early phase of acclimatization (Lebel and Leloup, 1992).

Changes in GH-BP levels in rainbow trout serum during the time course of seawater acclimatization could reflect the up-regulation of hepatic GH receptors. An increased total binding sites for GH occurs 4 days after seawater transfer (Sakamoto and Hirano, 1991a). Circulating GH is significantly increased 24–48 h following seawater transfer and then decreases, reaching levels found in freshwater thereafter (Sakamoto *et al.*, 1990, 1991). Furthermore, 4 days after seawater transfer, both GH metabolic clearance and secretion rate have been shown to be five times higher than those found in freshwater adapted trout (Sakamoto *et al.*, 1990). No information is available on GH receptor turnover in fish. Thus, the low binding affinity of this circulating BP-GH for tGH does not necessarily reflect a physiological function in term of plasma GH clearance. Conversely, it may be that the increased clearance of plasma GH is related to its role played on GH receptor turnover at the cellular level; thus after binding there is an increased GH receptor breakdown in seawater transferred rainbow trout.

Taken together, these results support a biological link between increased circulating GH and a related GH-BP in blood during seawater adaptation in rainbow trout. However, whereas GH binding activity seems to be linked to GH and GH receptor evolution, further studies are needed to assess the regulation and function of this GH-BP in salmonid.

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