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Presence of Insulin-Like Growth Factor Binding Protein (IGF-BP) in Rainbow Trout (*Oncorhynchus mykiss*) Serum

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ABSTRACT Recombinant human IGF-I (hIGF-I) binding to rainbow trout serum fractions was studied in vitro. Binding protein preparations, from human and trout serum (hBP and tBP), which were obtained using size exclusion chromatography (AcA 54) under acid conditions, were used to characterize IGF binding protein. Mammalian protein binding assay, using hIGF-I as labeled hormone, was optimized (pH, Tris concentration, bound-free ^{125}I-hIGF-I separation conditions by charcoal) for trout serum. The association kinetics (T = 4°C) of specific binding presented a plateau from 20–30 h, and nonspecific binding remained stable for the same time. Percentage of ^{125}I-hIGF-I which was able to bind to tBP was at least 55%. In the same incubation conditions, hIGF-I binding to trout BP had a higher affinity (Ka = 2.8 10^{10} M^{-1}) than those obtained with human BP preparation (Ka = 0.17 10^{10} M^{-1}) which contains four molecular forms of BPs. The binding was saturable but its capacity was fourfold lower in trout (Bmax = 410 ng/ml of serum) than in human (Bmax = 1868 ng/ml) serum. Binding to trout BP is very specific for human IGF-I and for trout IGF serum extract. hIGF-II crossreacts partially but no binding was observed with porcine insulin. When trout serum was chromatographed on an analytic AcA 54 column (1 × 100 cm), the maximum hIGF-I activity was found in a peak which corresponded to 55 kDa. Two other peaks (34 and 23 kDa) also bound hIGF-I, but with a much lower activity. In Western-ligand-blot from SDS-PAGE, binding of ^{125}I-hIGF-I to trout serum presented a major band (MW = 41.5 kDa) similar to the biggest form of IGF-BP-3 from human serum. These results suggest that IGF binding protein(s) exists in trout serum but the majority of IGF binding activity is carried only by one form (41.5 kDa), in contrast to mammals which have four forms of BP with a similar magnitude of serum concentration. © 1993 Wiley-Liss, Inc.

In fish, numerous studies on the hormonal control of body growth have outlined the essential role of growth hormone (GH) (Donaldson et al., '79; Le Bail et al., in press). GH could directly act on peripheral organs because GH receptors are present in most of the tissues investigated (Gray et al., '90; Hirano, '91; Yao et al., '91; Perez-Sanchez et al., '91; Le Gac et al., '92). In mammals, it is well established that GH effects on skeletal growth are also mediated by IGF-I. The little information available at present in fish corroborates the existence of a similar regulation (Bern et al., '91). Evidence for an indirect action of GH via a serum factor was first reported in rainbow trout *(Oncorhynchus mykiss)* by Komourjian and Idler ('78) and more recently in eel *(Anguilla japonica)* by Duan and Inui ('90). In vitro, mammalian IGF-I is able to increase the incorporation of $^{35}SO_4$ in ceratobranchial cartilage from eel (Duan and Hirano, '90, '91), coho salmon *(Oncorhynchus kisutch)*, mudsucker *(Gillichthys mirabilis)*, and striped bass *(Morone saxatilis)* (Bern et al., '91). Whether the effect of IGF is specific or not is unknown because insulin is also active, sometime at lower doses (Duan and Hirano in Bern et al., '91). Mammalian IGF-I is also active in vivo on the growth rate of coho salmon, but only if it is administered using constant infusion in food limited fish (Bern et al., '91).

IGF-I was detected in trout (Daughaday et al., '85; Bautista et al., '90) and in sea bream *(Sparus auratus)* (Funkenstein et al., '89) using mammalian radioimmunoassay, and in tilapia *(Oreochromis mossambicus)* and Atlantic salmon *(Salmo salar)* using rat radioreceptor assay (Drakenberg et al., '89). But the partial validation of these techniques for fish introduced a doubt. The presence of IGF-I was definitively demonstrated in coho salmon by Cao et al. ('89) giving the complete sequence of its mRNA which had 80% homology with human IGF-I.

The action of IGF-I is modulated by insulin-like growth factor binding proteins (IGF-BP) which are

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found in extracellular fluids of mammals (Hossenlopp et al., '87; Clemmons, '90; Sara and Hall, '90). The IGF-BP family consists of six forms, having around 40% homology in their amino acid sequence (Shimasaki and Ling, '91). They differ by their affinity to IGF-I and IGF-II and by their variation of expression in different tissues or with development stages and with hormone influence. In adult human serum, the major form is IGF-BP-3 revealed in Western-ligand-blot by two bands of 38.5–41.5 kDa corresponding to two glycosilated forms of the same protein (Hossenlopp et al., '87). In fish, none of these IGF-BPs has yet been characterized.

The aim of this study was to characterize the presence of IGF-BP in the serum of rainbow trout using recombinant human IGFs. As a first step, mammalian protein binding assay, using hIGF-I as labeled hormone, was optimized (pH, Tris concentration, bound-free ^{125}I-hIGF-I separation conditions by charcoal) for trout serum.

MATERIALS AND METHODS

Chemicals

Recombinant human insulin-like growth factor 1 (hIGF-I) and 2 (hIGF-II) were a gift from CIBA-GEIGY (Switzerland). Porcine insulin (I-3503), bovine serum albumin (BSA A-8531), carbonic anhydrase (AnC C-7025), and cytochrome-c (CytC C-7150) were purchased from Sigma.

^{125}I-hIGF-I was prepared using the method previously described for ^{125}I-sGH (Le Bail et al., '91). The specific radioactivity (SA = radioactivity content/protein content) varied between 80 and 160 μCi/μg. Labelled hormone was stored at –20°C in glycerol (v/2v).

IGF binding protein and IGF preparations

Serum was obtained 4 weeks after ovulation from female trouts *(Oncorhynchus mykiss)* weighing 1–4 kg. The fish had been kept at 10°C and fed ad libitum. Human serum was a gift from Blood Transfusion Center (Rennes, France).

Binding protein and IGF preparation, from human and trout sera, were done at 4°C. Dissociation of IGF from its BP was obtained under acid conditions: 20 ml of serum were incubated with 20 ml acidic buffer (0.15 M NaCl, 1 M CH_3COOH, pH 2.5) for 1 h. Then it was chromatographed on a size exclusion AcA 54 (IBF, France) column (5 × 52 cm) using the same acidic buffer. Proteins eluted in the exclusion volume, which correspond to the BP fraction, and proteins coeluted with ^{125}I-hIGF-I (used as marker), which correspond to the IGF fraction, were separately concentrated to 20 ml in an ultrafiltration cell (Amicon Diaflo with a PLGC disc polysulfone Millipore membrane; D = 76 mm, MW = 10 kDa). pH was adjusted to 7.6 with 2 N NaOH, and the medium was desalted using a G25 Sephadex column (Pharmacia Corp.) in 50 mM Tris-HCl buffer as the eluent. These preparations (hBP, tBP, and tIGF) were aliquoted and frozen at –20°C until assayed.

Repartition of the ^{125}I-hIGF-I binding activity was studied in trout serum chromatographed on a longer AcA 54 column (5 × 100 cm) using the same elution conditions described above. The protein concentration of each fraction (15 ml) was estimated reading its absorbtion at 280 nm (Uvikon 930 Spectrophotometer, Kontron Instrument). A 2 ml aliquot of each fraction was lyophilized and then 1 ml of 50 mM Tris-HCl buffer was added. After centrifugation, the supernatant was desalted using a PD 10 column (Pharmacia Corp.) in 50 mM Tris-HCl buffer as the eluent. Binding activity to hIGF-I of each fraction was tested according to the technique described below. To estimate the molecular weights of peaks which bind hIGF-I, the column was calibrated using bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome-c (12.3 kDa).

IGF protein binding assay

IGF binding assay was performed using a protocol similar to that described by Binoux et al. ('84). Fifty microliters of iodinated hIGF-I (20,000 cpm) in Tris-HCl buffer (50 mM Tris, 1% BSA, pH 7.6) were incubated (24 h, 4°C) with 100 μl of BP preparation and with 350 μl of Tris-HCl buffer (200 mM Tris, 1% BSA, pH = 9), with cold hIGF (500 ng/ml) added (nonspecific binding = NSB) or omitted (total binding = TB) or with 350 μl of serial dilutions of trout IGF preparation (tIGF). At the end of incubation, 0.5 ml of suspended charcoal (5 g of charcoal in 100 ml of 50 mM Tris-HCl, pH 9, preincubated 4 h at 4°C with 0.5% BSA) were added to separate bound and free ^{125}I-hIGF-I. After 75 min at 4°C, tubes were centrifuged (4,000*g*, 15 min) and the charcoal pellets (free IGF) were counted in an automatic gamma counter (Packard Multi-Prias 2).

Calculation

The specifically bound (SB) radioactivity was calculated by subtracting the pellet radioactivity of the nonspecific binding tube (NSB) (mean of duplicate determination) from the pellet radioactivity of the total binding tube (TB) (mean of duplicate determination).

Affinity constant (Ka) and binding capacity

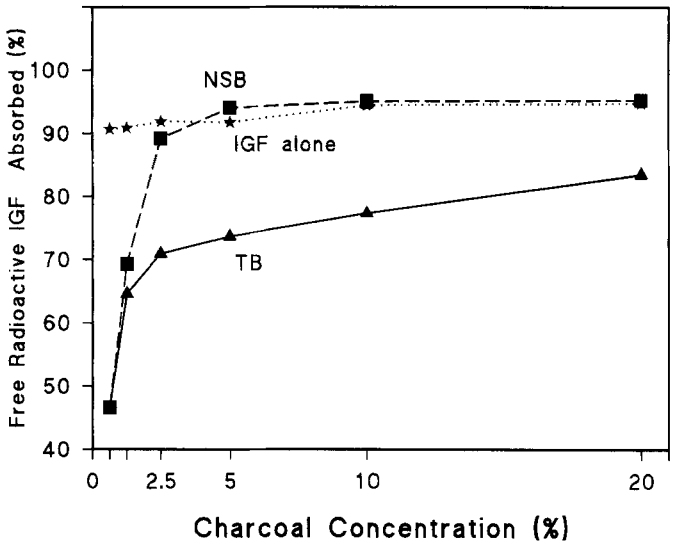


Fig. 1. Effect of charcoal concentration (in % of buffer added) on the adsorbtion of free ^{125}I-hIGF-I. Radiolabeled hIGF-I (20,000 cpm/50 µl) was incubated (24 h, 4°C) without (free IGF) or with 100 µl of trout binding protein preparation (tBP) and 350 µl of buffer, with cold hIGF (500 ng/ml) added (NSB) or omitted (TB). At the end of incubation, 0.5 ml of buffer containing increasing concentration of charcoal (preincubated under an excess of BSA) was added; then the tube was vortexed and incubated (30 min, 4°C) before centrifugation. The supernatant was counted and expressed as a percentage of total ^{125}I-hIGF-I added.

(Bmax) were estimated from the Scatchard transformation (1949) of inhibition curves for specific binding of ^{125}I-hIGF-I to serum binding protein preparation (BP).

Western-ligand-blot

Western-ligand-blot was performed according to Hossenlopp et al. ('86). Briefly, human, fetal calf, or trout serum was fractioned by SDS-PAGE (11% acrylamide slab gel) under nonreducing conditions according to Laemmli ('70). After electrophoresis, the gel was soaked in Tris-glycine buffer (15 mM Tris, 120 mM glycine, pH 8.3, 15% methanol) for 30 min and placed on a nitrocellulose sheet (0.45 µm BA 85; Schleicher and Schüll, FRG) and sandwiched between Whatmann 3MM filter paper equilibrated with the same buffer. Electroblotting was performed under a constant voltage, 150 V, at 15°C for 3 h using a Protean II (Biorad).

After transfer, the nitrocellulose membrane was soaked at 4°C, 30 min in saline buffer (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.05% sodium azide) supplemented with 3% NP_{40}, then 2 h with buffer supplemented with 0.5% gelatine and finally 10 min with buffer containing 0.1% of Tween 20. The nitrocellulose membrane was then incubated overnight at 4°C with 400,000 cpm ^{125}I-hIGF-I in a sealed plastic bag with 3 ml saline, 0.1% BSA (for 100 cm^2 blot), with (NSB) or without (TB) cold hIGF-I (1 µg/ml). Thereafter, the membrane was washed at 4°C, twice for 15 min in Tween 0.1%, and one time with saline. The blot was dried and then exposed for 4 days at –80°C to Fuji X-ray film and two Dupond Cronex Li-Plus intensifying screens.

The molecular weights (MW) of trout IGF-BP are determined by comparison with fetal calf and

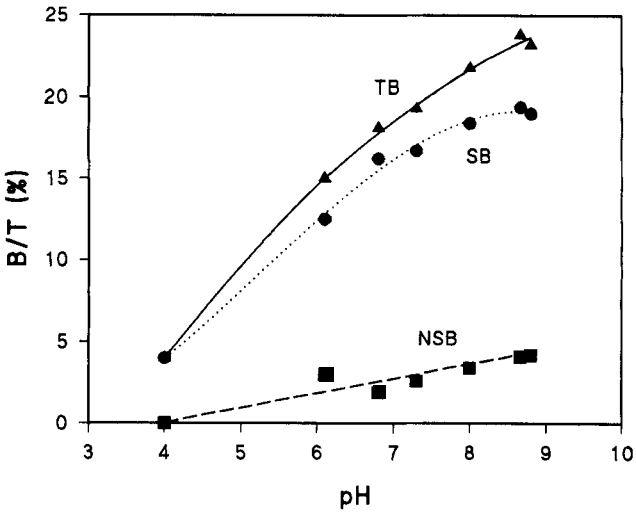


Fig. 2. Effect of pH on total (TB), specific (SB), and nonspecific (NSB) binding of ^{125}I-hIGF-I to trout serum binding protein preparation. pH 7–9 buffers were prepared using 150 mM Tris-Base and Tris-HCl solutions. Lower pH solutions were obtained by using 150 mM CH_3COOH. The other incubation conditions are described under Materials and Methods.

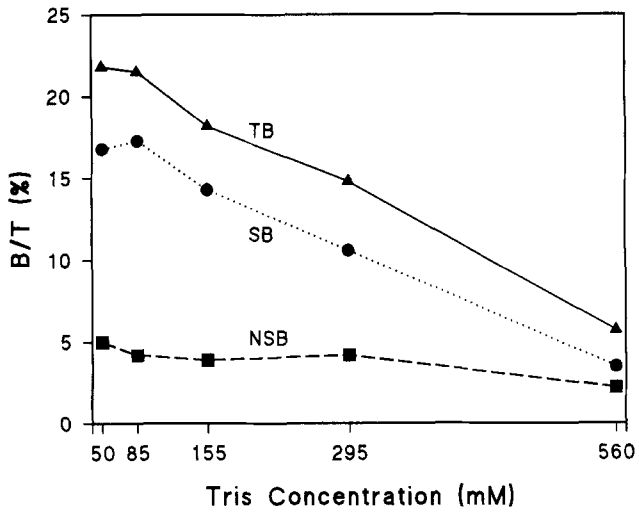


Fig. 3. Effect of Tris concentration on total (TB), specific (SB), and nonspecific (NSB) binding of ^{125}I-hIGF-I to trout serum binding protein preparation. Increasing concentrations of Tris-HCl were used for buffer preparations (pH 9). The other incubation conditions are described under Materials and Methods. Tris concentration was expressed as final concentrations in millimoles.

human IGF-BP for which the molecular weights are well established (Hossenlopp et al., '87).

RESULTS

Separation and incubation conditions

Increasing concentrations of charcoal were tested for the separation of free and bound ^{125}I-hIGF-I after incubation (Fig. 1). At all the concentrations tested, more than 90% of the free labeled hIGF-I was adsorbed by charcoal if the incubation was done without binding protein preparation. When labeled hIGF-I was incubated with tBP and an excess of cold hIGF-I, the maximum fixation of IGF-I was obtained with concentrations of charcoal of more than 2.5%. Five percent of charcoal gave the best separation between free and bound hIGF-I and was thus retained for the following studies. Increasing concentrations of BSA (from 1.25–20 mg/ml) or trypsin inhibitor (from 0.12–2 mg/ml) or ascorbic acid (from 0.12–2 mM) in the incubation medium had no effect on both nonspecific and specific binding (data not shown). No difference was observed on specific and nonspecific binding using increasing time (from 15–120 min) of separation with charcoal (data not shown).

Figure 2 shows the effect of incubation buffer pH on binding of ^{125}I-hIGF-I to trout binding protein preparation. Maximum specific binding occurred between pH 8 and pH 9. The nonspecific binding increased slowly with pH.

Nonspecific binding was not significantly modified by Tris concentration in the medium of incubation (Fig. 3). Concentration of Tris higher than 85 mM decreases the specific binding. Taking in account this data, the most favorable conditions appeared to be 150 mM Tris (for a high buffering capacity) and pH 8.5, and these were used for the following studies.

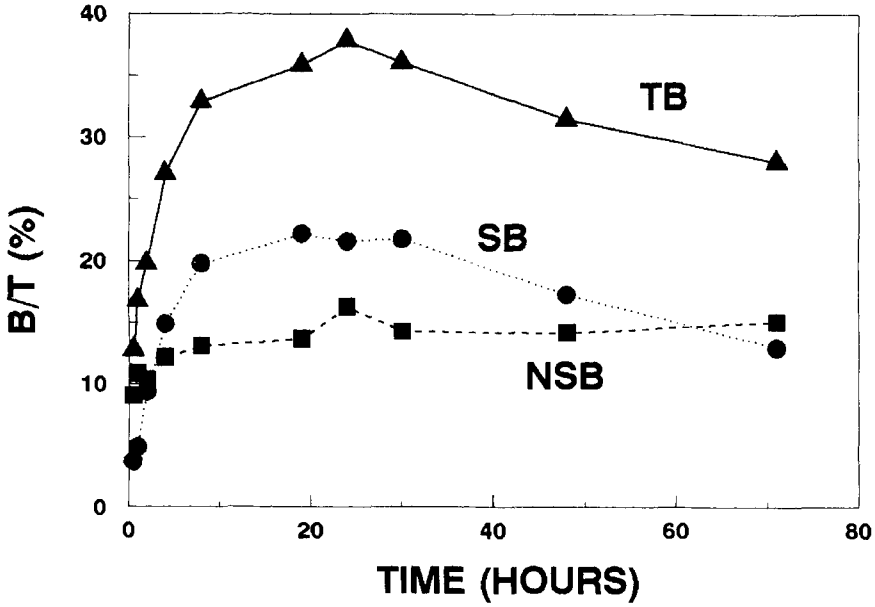


Fig. 4. Association kinetics of ^{125}I-hIGF-I to trout serum binding protein preparation. Binding assay procedure is described under Materials and Methods. NSB, nonspecific binding; SB, specific binding; TB, total binding. Binding was expressed as a percentage of total ^{125}I-hIGF-I added.

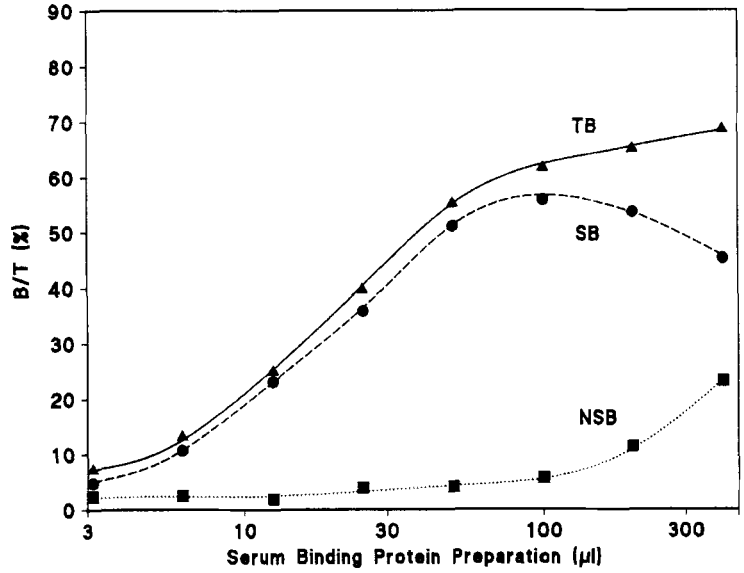


Fig. 5. Effect of increasing amount of trout serum binding protein preparation (5–400 µl) on total (TB), specific (SB), and nonspecific (NSB) binding of ^{125}I-hIGF-I. Binding was expressed as a percentage of total ^{125}I-hIGF-I added.

Association studies

The effect of incubation duration on the hIGF-I–tBP complex formation was studied at 4°C (Fig. 4). Under our incubation conditions, apparent equilibrium of the specific binding was reached after 20 h, remained stable up until 30 h and then decreased. Nonspecific binding appeared relatively constant for 70 h.

Saturation and competition experiments

The percentage of ^{125}I-hIGF-I binding to increasing amounts of trout serum binding protein preparation is shown in Figure 5. Specific binding increases proportionally to the amount of tBP from 3 µl to 50 µl (1 µl of tBP was prepared from 1 µl of serum); it reaches a maximum (55%) at 100 µl and decreases at the higher concentrations. Nonspecific

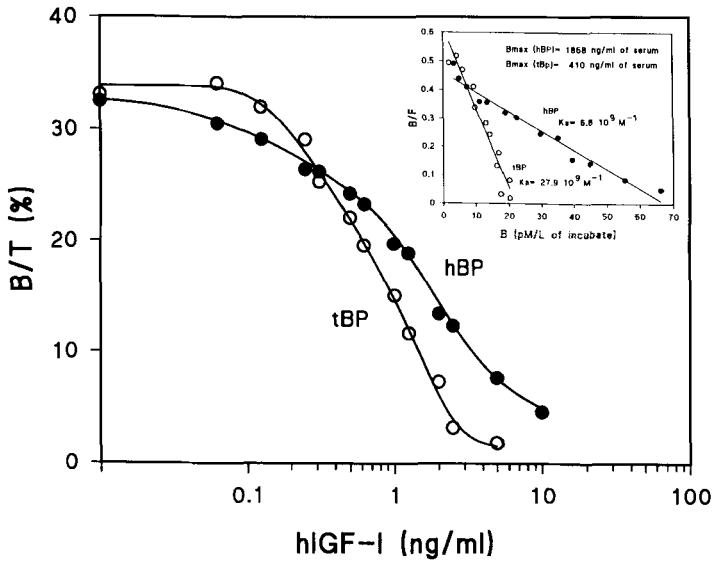


Fig. 6. Competitive inhibition curves for specific binding of ^{125}I-hIGF-I to trout (tBP) and human (hBP) serum binding preparations by increasing hIGF-I expressed in ng/ml. Binding was expressed as a percentage of total ^{125}I-hIGF-I added. The incubation conditions are described under Materials and Methods. Inset: The derived Scatchard plot. Bmax is expressed in ng/ml of serum.

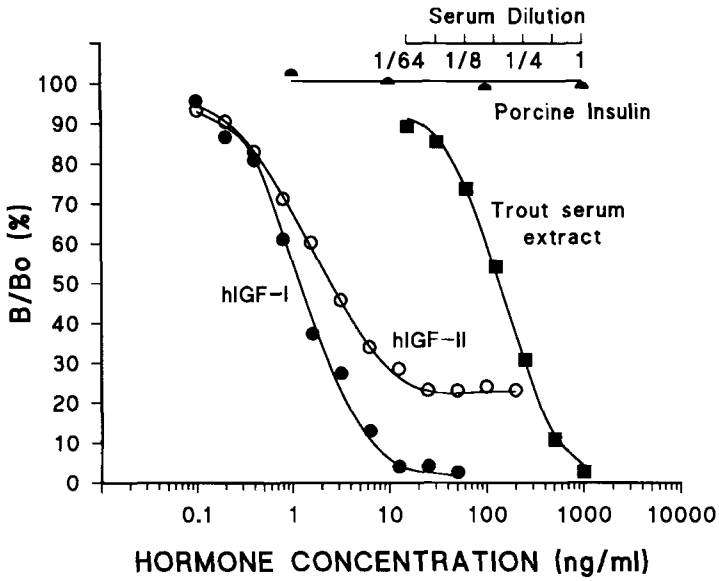


Fig. 7. Competitive inhibition curves for specific binding of ^{125}I-hIGF-I to trout serum binding preparations (tBP) by increasing various hormone or serum preparations expressed in ng/ml. Binding was expressed as a percentage of ^{125}I-hIGF-I specific binding in the absence of competition (Bo). Bo corresponded to B/T = 25%.

binding increases very slowly up to 100 µl of tBP and more quickly with higher concentrations.

To compare the affinity constant (Ka) to hIGF-I between human and trout serum binding protein, competitive inhibition curves were used with a constant B/T ratio (Fig. 6). The Scatchard derived plot revealed, in the two species, an apparent single class of binding sites with a binding affinity 4 times greater in trout (Ka = 2.8 10^{10} M^{-1}) than in human (Ka = 0.7 10^{10} M^{-1}). Inversely, maximum binding capacity per ml of serum was 4 times lower in trout (410 ng/ml) than in human (1,868 ng/ml). When incubation was carried out with a fixed concentration of labeled hIGF-I, specific binding to trout BP could be competitively inhibited by increasing the concentration of unlabeled hIGF-I (Fig. 7). The concentration of cold hIGF-I required to obtain 50% displacement of the tracer was about 1 ng/ml. The inhibition curve from hIGF-II was partial and unparallel to the hIGF-I curve, but 50% displacement was obtained with a concentration two-fold higher. Porcine insulin had no effect on binding. Increasing amounts of trout serum extract induce a parallel and full inhibition curve which suggests the presence of a factor related to human IGF-I.

IGF-I binding activity in trout serum chromatographic fractions

When trout serum (20 ml), after acidification, was chromatographed using an AcA 54 column (100 × 5 cm), three major protein peaks (OD at 280 nm) were observed (Fig. 8). The first one (fraction 32), which corresponded to the void volume and contained the major quantity of protein, had no significant binding activity to ^{125}I-hIGF-I. The second peak (55 kDa) eluted had the maximum IGF-I binding activity of the serum, and the third peak (fraction 110) coeluted with the ^{125}I-hIGF-I. Between fractions 70 and 100, two other peaks (34 and 23 kDa) bound hIGF-I, but with a much lower activity than peak 2 (55 kDa).

Western-ligand-blot

Results obtained using Western-ligand-blot from SDS-PAGE of fetal calf, normal adult human, and trout sera incubated with ^{125}I-hIGF-I are presented in Fig. 9. Fetal calf serum presented 5 bands which bound a similar amount of labeled hIGF-I. The two heavier bands (41.5 and 38.5 kDa) corresponded to IGF-BP-3. IGF-BP-2, IGF-BP-1, and IGF-BP-4 corresponded to the 34 kDa, 30 kDa, and 24 kDa bands, respectively. Human serum had the same forms but with a stronger signal for IGF-BP-3. Trout serum presented only two bands. The major one had

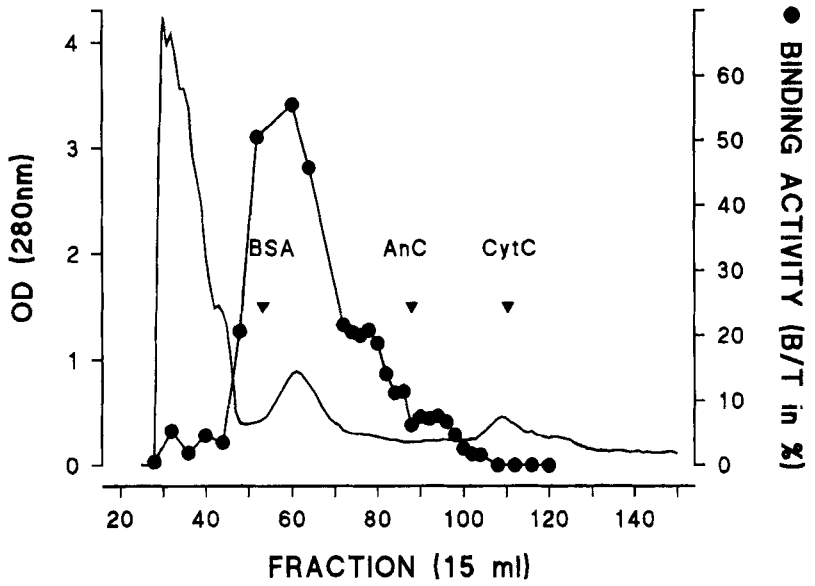


Fig. 8. hIGF-I binding activity in trout serum. After acidification by 1 M acetic acid 0.15 M NaCl (1v/1v), 20 ml of trout serum was chromatographed on an AcA 54 (100 × 5 cm) column equilibrated in 1 M CH_3COOH, 0.15 M NaCl, 0.1% BSA. Protein concentration of eluent was estimated by its absorbance (OD) at 280 nm. hIGF-I binding activity of fractions (100 μl) was tested according to the protocol described under Materials and Methods and expressed as a percentage of total ^{125}I-hIGF-I added (B/T). Arrows indicate substances used for column characterization: bovine serum albumin (BSA; 66 kDa), carbonic anhydrase (AnC; 29 kDa); and cytochrome-c (CytC; 12.3 kDa).

a molecular weight similar to 41.5 kDa IGF-BP-3, and the minor one was a little heavier (45.7 kDa). A total absence of signal was observed in the lanes corresponding to nitrocellulose incubated with an excess of cold hIGF-I.

From chromatographic studies (Fig. 8), the fraction corresponding to peak 55 kDa analyzed in Western-ligand-blot gave a similar pattern to those obtained with crude trout serum; however, a pool of peaks 34 and 23 kDa gave no signal (data not shown).

DISCUSSION

Human IGF-I, which has 80% homology with salmonid IGF-I (Cao et al., '89) and which has biological activity in fish (Bern et al., '91), appeared to be adapted to show the presence of IGF-BP in rainbow trout serum. However, using a heterologous hormone, such as recombinant human IGF-I, to characterize IGF binding protein in rainbow trout, we have to be cautious about the values (Ka, Bmax) obtained in this study which cannot be considered as absolute.

Previous studies on GH receptor have shown that binding was much more dependent on incubation conditions in trout than in mammals (Yao et al., '91). Taking into account this situation, different pH and Tris concentrations were tested on the binding of ^{125}I-hIGF-I to the trout binding protein. We found that in trout, maximum binding occurred between pH 8 and pH 9 even though in rat (Moses et al., '79) and in man (Binoux et al., '84) it is observed from pH 7 to pH 8 and from pH 5 to 8.5, respectively. In our study, we retained pH 9, which is higher than those (pH 7.5) generally used in mammals (Hintz and Liu, '77; Moses et al., '79; Binoux et al., '84; Armstrong et al., '89). The low binding observed at pH lower than 4 confirms that the buffer (pH 2.5) used in AcA 54 chromatography of trout serum to prepare IGF-BP was adapted for the dissociation of IGF and its BP. For ^{125}I-hIGF-I binding to trout BP, 50–100 mM Tris appeared to be the optimal concentration, as in mammals (Hintz and Liu, '77; Armstrong et al., '89). However, we chose to use a stronger Tris concentration (155 mM) to maintain incubation pH 9, after addition of an acidic medium, such as trout IGF serum extract.

The optimal amount of charcoal (0.5 ml of 5% which corresponds to 25 mg per tube) used to separate free and bound ^{125}I-hIGF-I in trout is similar to that reported in rat (Moses et al., '79) while it is two-fold higher than the quantity described in human (Hintz and Liu, '77; Binoux et al., '84; Martin and Baxter, '86). With this amount of charcoal, no significant effect was observed on specific and nonspecific binding when protein concentration increased in the incubation medium or when duration of separation is modified (from 15–120

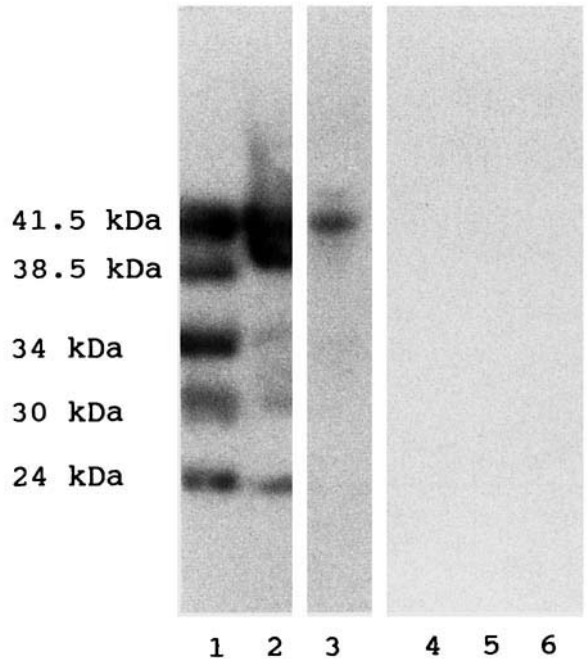


Fig. 9. Serum IGF binding proteins separated by SDS-PAGE and blotted onto nitrocellulose. Fetal calf (3 μl) **(lanes 1, 4)**, normal human (3 μl) **(lanes 2, 5)**, and trout (10 μl) **(lanes 3, 6)** sera were subjected to SDS-PAGE (12.5% acrylamide), followed by blotting onto nitrocellulose (BA 85) as described under Materials and Methods. The nitrocellulose was incubated ovenight at 4°C with 400,000 cpm of ^{125}I-hIGF-I with (lanes 4–6) or without (lanes 1–3) an excess of cold hIGF-I, washed, and autoradiographed. The molecular weights (kDa) of the binding proteins are indicated in the side.

min). The small diminution in specific binding observed with the highest charcoal concentrations could be due to an acceleration of the dissociation and not to an increase of the nonspecific binding, because addition of large amounts of BSA had no effect. Taking into account these data, the modifications we employed from mammal incubation and separation conditions gave stable and reproducible results in our studies.

We cannot compare association kinetics of hIGF-I to IGF-BP between mammals and fish because physiological temperatures are different. In our study done at 4°C, apparent equilibrium is reached after 20 h. This kinetic corresponds to those obtained in salmonids with polypeptidic hormone and their receptors (Yao et al., '91; Kanamori and Nagahama, '88; Le Gac et al., '88; Breton et al., '86). A decrease of specific binding is observed after 30 h. Similar phenomena were also noted in fish receptor studies (Hirano, '91; Yao et al., '91; Kanamori and Nagahama, '88). A decrease of the specific binding was also observed when very large amounts of trout BP preparation were incubated with ^{125}I-hIGF-I (Fig. 5). This decrease in specific binding could be explained by the nonspecific binding increase which limited the availability of free hormone. This phenomenon could be the result of protein degradation, but it was not due to oxidation or to enzymatic attack at serum level because ascorbic acid and trypsin inhibitor addition had no effect (data not shown).

Specific binding of ^{125}I-hIGF-I to trout BP was not inhibited by insulin. Inhibition by hIGF-I or by hIGF-II was dose dependent. This confirms that binding to trout serum BP is IGF specific. However, inhibition curves obtained by hIGF-II show a lower affinity to trout serum BP than hIGF-I. IGF fraction from trout serum, which caused an inhibition curve parallel with the hIGF-I curve, confirms the predominance, in trout serum, of a factor or factors more similar to IGF-I than to IGF-II. This result is in agreement with previous detection of IGF-I–like in the serum of rainbow trout (Daughaday et al., '85), Atlantic salmon, *Salmo salar* (Lindahl et al., '85), tilapia, *Oreochromis mossanbicus* (Drakenberg et al., '89; Ng et al., '91) and sea bream, *Sparus aurata* (Funkenstein et al., '89) and the apparent lack of IGF-II–like factor in rainbow trout (Daughaday et al., '85) and tilapia (Drakenberg et al., '89) serum.

The Scatchard plot derived from the inhibition curves suggested only one class of binding site either with human and trout BP. Estimated in the same experiment, the trout affinity constant (Ka = 2.8 10^{10} M^{-1}) appears four-fold higher than human affinity constant (Ka = 0.7 10^{10} M^{-1}). These Ka are in the same order of magnitude as Ka previously observed for IGF-BP-3 in human (Wood et al., '88; Martin and Baxter, '86; Hardouin et al., '87; Hossenlopp et al., '87) and in rat (Barenton et al., '88). Inversely, the number of binding sites was lower in trout (410 ng/ml) than in human (1,889 ng/ml) serum. This low concentration of BP could explain why, in previous work, BP was not detected in fish serum (Binoux et al., '84; Drakenberg et al., '89; Daughaday et al., '85). The insulin-like toxicity of mammalian IGF-I when injected to salmonid (Skyrud et al., '89; Yao and Le Bail, unpublished data) would be the result of a too high level of free IGF-I due to the low binding power of the serum.

Using Western-ligand-blot, analysis of reference serum (i.e., serum BP from normal human and fetal calf) presented five bands which bound ^{125}I-hIGF-I as described in previous studies (Hossenlopp et al., '86; Hardouin et al., '87). Human BP-3 is composed of two forms with molecular weights of 41.5 and 38.5 kDa. With trout serum a major band is observed

which co-migrated with the biggest form of human BP-3. The magnitude of the signal obtained with 10 μl of trout serum is lower than the signal obtained with 3 μl of human serum. These data are in agreement with the low binding capacity previously calculated using the Scatchard plot derived from inhibition curve.

The Western-ligand-blot technique revealed also in trout a minor band having a molecular weight of 45.7 kDa. The Western-ligand-blot reveals also (data not shown) that these two bands (41.5 and 45.7 kDa) correspond to the major binding activity link to a peak eluted at 55 kDa in gel filtration chromatography. These two bands could represent two forms of the same binding protein as is the case of human BP-3. Other minor forms of BPs blurred, with a MW between 30 and 34 kDa, were also detected on the negative with the naked eye, but only faintly. They could be two natural forms of BPs, which correspond to BP-2 and BP-6 in the human, or could correspond to fragments from BP-3 degradation. Proteolysis of BP-3 has been well established in pregnant woman (Hossenlopp et al., '90). Chromatography in acidic condition (Fig. 8) showed also two little peaks (34 and 23 kDa) which presented hIGF-I binding activity. The inhibition curve obtained using hIGF-II suggests the presence of two binding sites in the trout serum BP preparation. Taking into account all these data, it is likely that minor forms of IGF-BP could exist in trout serum. However, their very low concentrations in the serum make a prepurification necessary to confirm their existence and identification.

In conclusion, the results presented here show the presence of at least one specific IGF binding protein in rainbow trout. This is the first characterization of an IGF-BP in fish serum which suggests that it is present in all vertebrates, because previous works have shown its presence in birds (Armstrong et al., '89) and in amphibians (Pancak-Roessler and Lee, '90). Further investigations are necessary to find which mammalian form trout IGF-BP is related to.

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