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Identification and Characterization of GH Receptor and Serum GH-binding Protein in Chinese Sturgeon (*Acipenser sinensis*)

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Abstract Chinese sturgeon, a kind of cartilage ganoid, has a history of over one billion years and it is called the living fossil of aquatic biology since it keeps some evolutionary trace. Here, we characterized the growth hormone receptor (GHR) and serum growth hormone binding protein (GHBP) of Chinese sturgeon. It was shown that GHR was expressed in various tissues, mainly in hepatic, kidney and intestine tissues. GHR on the hepatic membrane has high and specific affinity for bream GH (brGH) and Scatchard analysis of the binding data showed a single class of high affinity binding site with an association constant K_a of 3.1×10^9 M^{-1}. A specific band around 94 kD was detected by SDS-PAGE in cross-linking studies of membrane receptors. After incubation of Chinese sturgeon serum with ^{125}I-brGH, a ^{125}I-brGH-GHBP complex was identified by Sephadex G-75, indicating that in the serum exists GHBP specially binding to brGH.

Key words Chinese sturgeon; growth hormone receptor; growth hormone binding protein; ligand binding

Growth hormone (GH) can stimulate bone and cartilage cell proliferation and influence carbohydrate and lipid metabolism. The binding of GH to its specific receptor (GHR) on the surface of target cells will induce dimerization of GHR, which allows the cytoplasmic region of GHR to interact and trigger downstream signaling and gene expression [1,2].

GHR belongs to the cytokine receptor superfamily, and is expressed in many tissues such as the liver, muscle, adipose tissue, cartilage, and brain. The highest level of GHR was generally found in liver [3,4]. GHRs on hepatic cell membranes have been extensively investigated in higher mammalian species butless in non-mammalian vertebrates. Genetic analysis shows that GHR-encoding gene evolves relatively fast. The function of GHR may differ among species as certain functionally conserved amino acid residues were often substituted during evolution [5]. In our previous report, GHR of hepatic cell membrane from several fresh water fish was detected, and a high level of GHR was found on the hepatic cell membranes of the snakehead fish [6]. In addition, it was known that two isoforms of GHR, a membrane-bound short GHR and a soluble growth hormone-binding protein (GHBP), were identified in many species [7,8]. GHBP might be important for GH function, especially maintenance of GH concentration in blood [9].

Chinese sturgeon, a kind of cartilage ganoid with a history of over one billion years, is called the living fossil of aquatic biology for maintaining some evolutionary trace, and therefore listed as the first-class protected animal due to its academic value and its exiguity. It is very important to study the structure and function Chinese sturgeon GHR and GHBP.

In present study, we characterized the growth hormone receptor (GHR) and serum growth hormone binding protein (GHBP) of Chinese sturgeon, the ultimate goal of which is to clone GHR cDNA and to further elucidate the difference between Chinese sturgeon GHR/GHBP in structure and function and those from other fish or

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mammalian.

Materials and Methods

Materials

Recombinant bream growth hormone (brGH), chicken GH (cGH) and ovine prolactin were gifts from Prof. Chueng in Department of Biochemistry, University of Hong Kong, China. Recombinant human growth hormone (hGH) was from Prof. Guo in the Institute of Biochemistry and Cell Biology (SIBS, CAS). Sephadex G-75 and G-200 were purchased from Pharmacia (Uppsala, Sweden). Iodogen and disuccinimidyl suberate (DSS) were from Pierce (Rockford, IL). [^{125}I]NaI was from Amersham (Buckinghamshire, UK). Chinese sturgeon was purchased from market in Shanghai, China and authenticated by Scientific Research Institutes, Shanghai Fisheries University.

Iodination of bream growth hormone

GH was iodinated according to the method described previously [10]. In brief, a 1.5-ml polypropylene tube coated with 10 μg iodogen in $CHCl_3$ was allowed to air dry. Five micrograms brGH were mixed with 1 mCi [^{125}I]NaI in 30 μl 0.2 M HEPES buffer, pH 7.2, and the mixture was allowed to react for 7–8 min with stirring at 25 °C. The reaction was terminated by the addition of 0.3 ml of 50 mM HEPES buffer, pH 7.2, and the mixture was fractionated on a 1 cm×50 cm Sephadex G-75 column by eluting with 50 mM HEPES buffer, pH 7.2, containing 0.2% bovine serum albumin (BSA). The peak of the radiolabeled GH was pooled, aliquoted, and stored at –20 °C. The specific activity of this fraction ranged from 80 to 120 μCi/μg as determined by the trichloroacetic acid (TCA) precipitation method.

Preparation of cell membranes and serum protein

Preparations were carried out according to the method previously described [11] at 0–4 °C unless otherwise stated. Fresh tissues were rinsed and minced in ice-cold 0.25 M sucrose and homogenized in 8–10 volumes of cold 0.25 M sucrose, centrifuged at 600 *g* for 10 min. The supernatant was centrifuged at 10,000 *g* for 30 min, and the pellet was discarded. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgSO_4$, and centrifuged at 35,000 g for 45 min. The pellet was suspended in 50 mM HEPES buffer, pH 7.2, containing 2 mM phenylmethylsulfonyl fluoride (PMSF), homogenized and centrifuged; this process was repeated twice. Finally the pellet was resuspended in 50 mM HEPES buffer, pH 7.2, containing 2 mM PMSF, 1 μg/ml pepstatin A, aprotinin and leupeptin each, and stored at –80 °C.

Blood sample was obtained from Chinese sturgeon by inserting a 25-gauge needle attached to a disposable syringe into the caudal vein. The blood was centrifuged at 10,000 *g* for 10 min and collected serum was stored at –20 °C. The protein concentration of the membrane preparation and serum was determined by the Lowry method modified by Hartree [12] using BSA as standard.

Ligand binding

Ligand binding assay followed the method described previously [13]. Assay was performed in 4-ml polystyrene tube. 200 μg membrane protein in 200 μl assay buffer (25 mM HEPES, pH 7.2, 10 mM $CaCl_2$, 0.2% BSA, 1 mM PMSF) was incubated with 100 μl ^{125}I-labeled brGH (approximately 100,000 cpm) with or without unlabeled hormone in a final volume of 0.5 ml for 24 h at 25 °C. The reaction was terminated by adding 2 ml of ice-cold 25 mM HEPES buffer, pH 7.2, containing 0.2% BSA. The reaction mixture was then centrifuged at 3000 *g*, 4 °C for 20 min, and the supernatant was decanted. The pellet was washed twice with the same buffer, and the radioactivity was measured using a gamma counter. Non-specific binding was determined in the presence of an excess of unlabeled brGH (5 μg/tube). Specific binding was calculated by subtracting the nonspecific binding from the total binding. All assays were performed in triplicate.

Binding of ^{125}I-brGH to serum GHBP

Serum-binding study was carried out according to a published method [14] with modifications. Binding of Chinese sturgeon serum with iodinated hormone was performed at room temperature for 2 h in 10 mM phosphate assay buffer containing 10 mM $MgCl_2$, 0.02% sodium azide (*W*/*V*), and 0.1% BSA, pH 7.0, at a final volume of 250 μl. The serum was used at a final protein concentration of 800 μg. Separation of bound ligand from free ligand was performed by gel filtration on Sephadex G-100 (0.8 cm×30 cm) at 24 °C. Plot analysis was made after gamma counting.

Crosslinking studies

^{125}I-labeled brGH (1,000,000 cpm) was incubated overnight at 25 °C with 1 mg Chinese sturgeon hepatic cell membrane in 800 μl assay buffer containing 25 mM HEPES, 10 mM $CaCL_2$ 1 mM PMSF [15]. A parallel incubation was performed with an excess of unlabeled hormone (25 μg/tube). DSS dissolved in dimethyl sulfoxide was added to a final concentration of 0.5 M. After react-

ing for 20 min at 25 °C, 0.1 ml of 1 M Tris-HCl, pH 6.8, was added and the tube was spun at 10,000 *g* for 5 min. The pellet was solubilized in Laemmli sample buffer and analyzed by SDS-PAGE. The gels were dried and exposed for 8 days using Kodak X-OMAT AR5 film with intensifying screens.

Gel filtration

Membrane preparation was solubilized by adding Triton X-100 to a final concentration of 1% (*W*/*V*) with stirring. After stirring for 60 min, the solubilization was centrifuged at 235,000 *g*, 4 °C for 90 min. The clear extract between the pellet and the floating lipid layer was carefully removed. PMSF and $CaCl_2$ were added to final concentration of 1 mM and 10 mM, respectively. 4,000,000 cpm ^{125}I-brGH was incubated with 0.5 ml of above extract overnight at 25 °C, then loaded onto a Sephadex G-200 column (1 cm×100 cm) previously equilibrated with elution buffer (50 mM Tris-HCl, pH 7.2) containing 0.1 M NaCl, 10 mM $CaCl_2$ and 0.1% Triton X-100.

Results

Tissue distribution of Chinese sturgeon GHR

The distribution of GHR in Chinese sturgeon different tissues was shown in Fig. 1. The highest level of GHR was in liver, which was consistent with that ofsome other vertebrates [16]. However, the relatively high level of GHR in kidney and in intestine, and very low or even undetectable level of GHR in adipose, muscle and brain were found, which were very different from those of others.

Competition for binding of ^{125}I-brGH to the Chinese sturgeon hepatic membrane by unlabeled hormone

As shown in Fig. 2, specific binding of ^{125}I-brGH to Chinese sturgeon hepatic membrane was inhibited by unlabeled brGH in a dose-dependant manner. When the ratio of bound brGH versus free brGH was plotted as a function of bound brGH using Scatchard analysis, a straight plot was obtained, which revealed a single class of high affinity brGH binding site with association constant K_a of 3.1×10^9 M^{-1} for Chinese sturgeon hepatic membrane. Results presented in Fig. 2 showed that hGH exhibited a low inhibition of ^{125}I-brGH binding and bovine GH, chicken GH and ovine prolactin nearly did not inhibit ^{125}I-brGH binding.

Gel analysis of ^{125}I-brGH-GHR complexes

Solubilized hepatic membrane by Triton X-100 was incubated with excess ^{125}I-brGH and then separated by Sephadex G-200 (1 cm×100 cm). As shown in Fig. 3(A), there are two peaks in elution spectrum. Peak 1 is GHR-^{125}I-brGH complex and peak 2 is free ^{125}I-brGH. Hepatic membrane was incubated with ^{125}I-brGH in the presence or absence of unlabeled brGH, and then cross-linked by DSS. The membrane was dissolved in binding buffer and then separated on 10% SDS-PAGE. A radioactive band was found and the band of sample without unlabeled brGH was much stronger than that of control with excess unlabeled brGH, indicating that this band was ^{125}I-brGH-

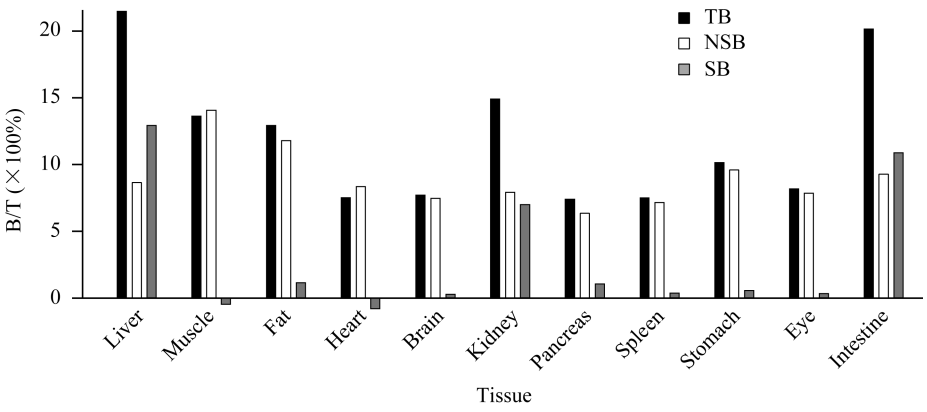

Fig. 1 Total binding (TB), nonspecific binding (NSB), and specific binding (SB) of ^{125}I-brGH to membranes from various tissues of Chinese sturgeon

Cell membranes (200 μg) were incubated with approximately 150,000 cpm ^{125}I-brGH as described in "Materials and Methods". Binding data were expressed as percentages of the total counts added (B/T). Each value represented the mean of three determinations.

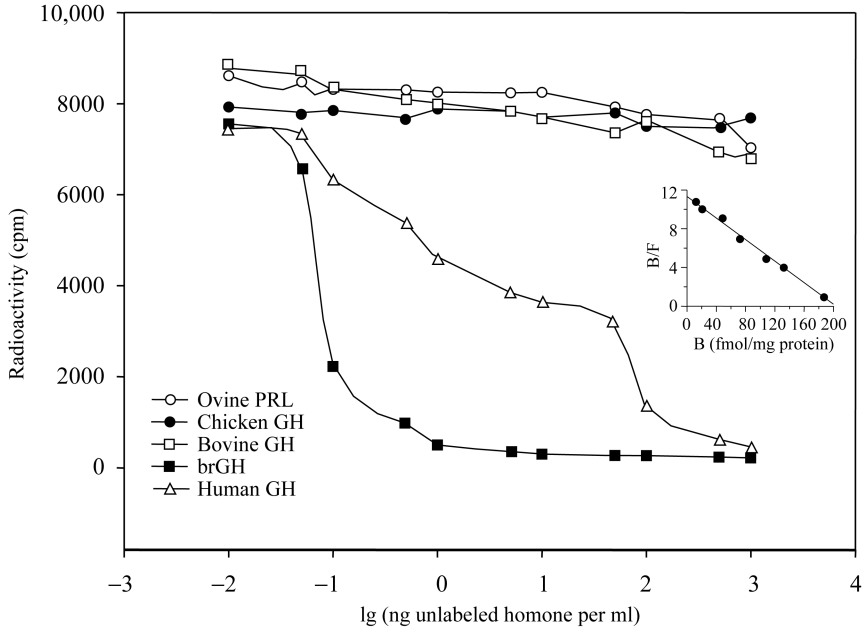

Fig. 2 Displacement of ^{125}I-brGH binding to Chinese sturgeon hepatic membrane by unlabeled hormones

Inner figure is Scatchard plot analysis of the ^{125}I-brGH binding data. B, bound ^{125}I-brGH; F, free ^{125}I-brGH.

GHR complex.

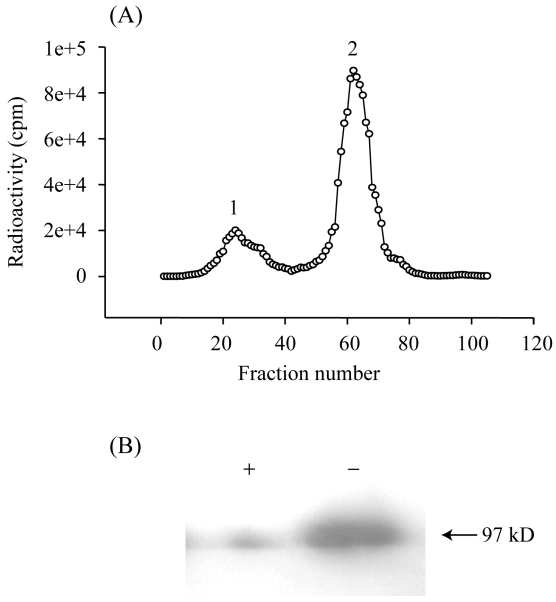

Fig. 3 Analysis of Chinese sturgeon hepatic membrane GHR cross-linking with ^{125}I-brGH

(A) Separation of cross-linking complex solubilized in Triton X-100 on Sephadex G-200 column, 0.75 ml/tube. Peak 1, ^{125}I-brGH-GHR; Peak 2, free ^{125}I-brGH. (B) Analysis of cross-linking complex by 10% SDS-PAGE. (+), (-) denotes presence or absence of excessive unlabeled brGH respectively in binding experiment.

Identification of Chinese sturgeon growth hormone-binding protein

It was reported that GHBP is completely identical to the extracellular domain of GHR [17] and binds to GH with high affinity and specificity similar to GHR. 800 μg total serum protein from Chinese sturgeon was incubated with excess ^{125}I-brGH, and fractionated by Sephadex G-75. Fig. 4 showed a clear peak of ^{125}I-brGH-GHBP which proved the existence of GHBP in Chinese sturgeon serum.

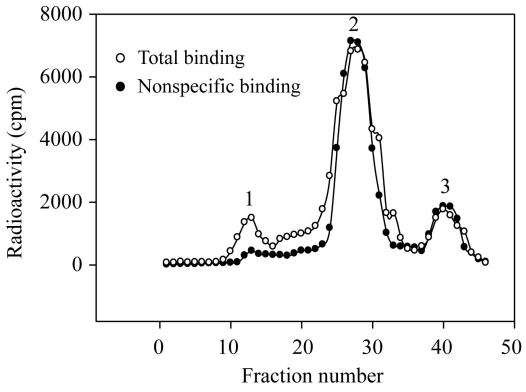

Fig. 4 Analysis of Chinese sturgeon serum ^{125}I-brGH-GHBP complex on Sephadex G-75 column (0.8 cm×30 cm)

0.5 ml/tube. Peak 1, ^{125}I-brGH-GHBP complex; Peak 2, free ^{125}I-brGH; Peak 3, small molecule containing ^{125}I.

Discussion

Studies on growth hormone receptor of lower animals provided significant information about evolution taxonomy [18]. GHBP, a soluble isoform of GHR, was first reported in rat [19,20] and then in other animals [21,22]. The mechanism how GHBP is generated still remains unclear and also quite differs among species, which might be due to the fast evolution of GHR molecule.

Our study testified that GHR was expressed in most tissues of Chinese sturgeon, especially highest in liver and relatively high in intestines and kidney, being in agreement with previous reports in other animals. However, GHR level in fat, brain and muscle tissue was too low to be undetected, which suggested the conservation of Chinese sturgeon GH's function and alternative of acting target during its evolution. Ligand binding results showed brGH bound specially to Chinese sturgeon GHR with high affinity. We obtained its binding constant of 3.1×10^9 M^{-1}, which was very similar to other fish GHRs [6,21]. This indicates that Chinese sturgeon GHR maintains its high affinity for brGH ligand. However, the human GH is only partially active in this regard, possessing less than 1% affinity. The bovine and chicken GH and ovine prolactin nearly lack activity in interacting with Chinese sturgeon GHR, showing that the evolution of GH and GHR might be a coordinate process. Western blot analysis identified a band of 90–130 kD similar to GHRs of other animals, and no band with lower molecular size was observed, indicating that GHR was not degraded in this experiment.

The Chinese sturgeon serum GHBP was detected by separating ^{125}I-brGH-GHBP complex on Sephadex G-75 column, confirming that Chinese sturgeon GHBP also well bound to brGH. This result was identical to that of goldfish and snakehead fish GHR [6,21]. These data suggested that all the GHR isoforms may exist one billion years ago or come to the existing forms gradually during evolution. To further study Chinese sturgeon GHR and GHBP and gain more insight in GHR evolution, cloning the GHR cDNA, isolating and sequencing the GHBP is necessary.

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