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Autoradiographic Localization of Growth Hormone Binding Sites in *Sparus aurata* Tissues Using a Recombinant Gilthead Seabream Growth Hormone

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ABSTRACT. Growth hormone (GH) binding sites in tissues of gilthead seabream, *Sparus aurata,* were localized by using an *in vitro* autoradiographic analysis. Cryomicrotome sections of liver, spleen, kidney, heart and skeletal muscle were mounted on gelatin-coated slides and incubated with a radioiodinated recombinant gilthead seabream GH (0.54 ng/100 μl buffer) for 24 h at 4°C. Slides were coated with autoradiographic emulsion and exposed for 1–9 weeks. The highest density of GH binding sites was found in liver. This specific binding was evident on hepatocytes. Blood cells in hepatic vascular system also showed GH binding while intrahepatic exocrine pancreas did not. In spleen, a high density of GH binding sites was found surrounding melanomacrophage centers. Splenic ellipsoid cells also showed a strong GH binding while splenic pulp exhibited a weak GH binding. Kidney GH binding sites were present in renal tubules and renal haemopoietic tissue. Cardiac muscle exhibited an intense GH binding. However, GH binding sites were not detected in body skeletal muscle. This study confirms and extends, by using autoradiographic techniques, previous results obtained by radioreceptor assays. COMP BIOCHEM PHYSIOL 114C, 17–22, 1996

KEY WORDS. Autoradiography, growth hormone binding sites, gilthead seabream tissues, teleost, fish

INTRODUCTION

In fish, as in other vertebrates, growth hormone (GH) plays an important role on development and somatic growth (8). The major postulated mechanism of GH action is via production of hepatic and extrahepatic insulin-like growth factors (IGFs), which mediate local growth in a paracrine/autocrine fashion (22). Furthermore, there is strong evidence suggesting that GH is an important factor regulating interrenal (47) and thyroid (7) activities, gonadal function (27) and osmoregulation (42).

The first step in the action of polypeptide hormones is binding to specific receptors in target cells. In mammals, direct GH effects and/or GH binding sites have been identified and characterized in liver (24), proximal convoluted tubules of kidney (6), peritoneal mast cells and macrophages (6), lymphocytes (2), fat (17), skin (35), cartilage (9), gonads (30), brain (12) and muscle (5).

Mammalian GHs are able to bind fish receptors but with a much lower affinity than homologous hormones (13,46). Recently, a number of fish GH polypeptides and/or GH cDNAs have been isolated and characterized in various teleost species (1,28,38,44), including *Sparus aurata* (15,32). In fish, specific GH receptors have been characterized in liver of several species (13,18,20,28,36,42,46). Specific and saturable GH binding sites have also been demonstrated in central nervous system and testis of rainbow trout (29,39). In salmonids and other teleosts, saturable GH binding has been detected in ovary, testis, fat, skin, cartilage, gill, blood, brain, spleen, kidney, intestine and muscle (13,25,42,46). In gilthead seabream, both GH and GH receptors are present during early development and growth stages (34,41) and a prominent increase of GH gene expression takes place in larva within the first week after eclosion (16). GH binding sites with general characteristics of hormone receptors have been described in liver, muscle, fat and brain of adult gilthead seabream (40).

Gilthead seabream is a characteristic fish of Atlantic and mediterranean coasts and represents one of the most important species in aquaculture. Recently, our group has published the cloning and expression of a recombinant gilthead seabream GH which has been demonstrated to be very similar if not identical to the native seabream GH when analyzed by radioimmunoassay and radioreceptor assays (32,33). In this paper, we report the autoradiographic local-

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ization and quantification of GH binding sites in putative target tissues of *Sparus aurata* using this ^{125}I-labeled recombinant gilthead seabream GH.

MATERIALS AND METHODS

Animals

Four 7–9 month-old immature male specimens of gilthead seabream weighing around 150 g were purchased from a local fishery (PEMARES, Puerto de Santa María, Spain). The fish were anesthetized with benzocaine chlorhydrate, killed by decapitation and samples from liver, spleen, kidney, skeletal muscle and heart were rapidly excised, frozen into liquid nitrogen, and kept at −80°C until further processing.

Hormones

Recombinant gilthead seabream growth hormone (rsbGH) was obtained as described previously (32). Briefly, the gilthead seabream GH cDNA was isolated from a pituitary expression library using a flounder GH cDNA as a probe and further expressed in *E. Coli* by using the pET-3a expression system. The rsbGH purified from *E. Coli* extracts was radioiodinated to a specific activity of 55 μCi/μg by the Chloramine-T method (33).

Autoradiography

Tissue blocks were embedded in Einbett medium (Jung GmbH, Heidelberg) and placed on a section holder. Tissue sections 6–10 μm thick were obtained on a cryocut (Reichert-Jung, Heidelberg) and mounted on gelatin-coated slides (43). On each glass slide, a square was drawn around the tissue section with a PAP pen so that radioactive incubation medium would not flow off the slide. Sections were preincubated horizontally in a moist chamber with 100 μl of 50 mM Tris-HCl (pH 7.4) buffer containing 0.2% BSA for 30 min at 4°C. Subsequently, each section was incubated with 100 μl of the same buffer containing 5 mM $MgCl_2$, 0.02 mM bacitracin and 0.54 ng of radioiodinated rsbGH for 24 h at 4°C. In order to determine the unspecific binding of radiolabeled rsbGH, control sections of all tested tissues were also incubated with an excess of nonlabeled rsbGH (0.54 μg of cold rsbGH/100 μl buffer) and analyzed. After the incubation, the medium was aspirated, sections were washed with 100 μl of cold buffer (3 × 5 min) and fixed in 4% parafolmaldehyde for 30 min, at room temperature. Then the slides were rinsed, coated with Kodak NTB2 autoradiographic emulsion (Eastman Kodak, Rochester, NY) by dipping in liquid emulsion (diluted 1:1 in distilled water) and dried at room temperature. The coated slides were stored at 4°C in light-proof boxes and exposed for 1–9 weeks. Finally, they were developed with Kodak D19 for 4 min at 15°C, fixed, and washed with running tap water. Sections were counterstained with nuclear red and observed under the microscope (Olympus BH-2). Quantitative analysis was carried out at high magnification (×1560) in a microscope Olympus BH-2 provided with camera lucida. Numerical density of autoradiographic grains in liver, spleen, kidney, heart and skeletal muscle was determined by counting in 40–60 tissue areas selected at random with the help of a superimposed 1000 μm^2 grid. Counting on hepatocytes, blood cells, intrahepatic pancreas, melanomacrophage centers, ellipsoids, splenic pulp, renal tubules and haemopoietic kidney was performed in 50–70 tissue areas selected at random with the help of a superimposed 50 μm^2grid. Because the difficulty to determine the cell borders of hepatocytes in tissue sections, studies of GH binding sites were conducted in isolated hepatocytes that appeared dispersed at the border of the hepatic sections after rinsing. GH binding in blood cells was determined in cells contained in the hepatic vascular system. Tissue sections used for quantitative analysis were exposed for 54 days.

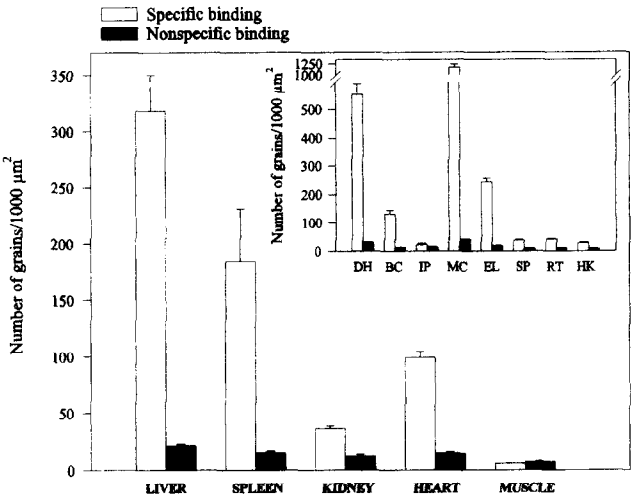

FIG. 1. Numerical density of autoradiographic grains in *Sparus aurata* tissue sections. Counting was made in sections exposed for 54 days and is expressed as number of grains (means ± standard error)/1000 μm^2 of tissue. White and black bars represent specific and nonspecific binding, respectively. DH, dispersed hepatocytes; BC, hepatic blood cells; IP, intrahepatic pancreas; MC, splenic melanomacrophage centers; EL, splenic ellipsoid cells; SP, splenic pulp; RT, renal tubules; HK, haemopoietic kidney.

RESULTS AND DISCUSSION

In this autoradiographic study, a radioiodinated rsbGH was used to evidence the cellular localization of GH binding sites in tissues of gilthead seabream, *Sparus aurata*. Two types of autoradiographic grains, small and large, were observed in sections although large autoradiographic grains seems to represent the apposition of small grains. The quantification of autoradiographic labeling in tested tissues is shown in Fig. 1. The highest density of rsbGH binding was

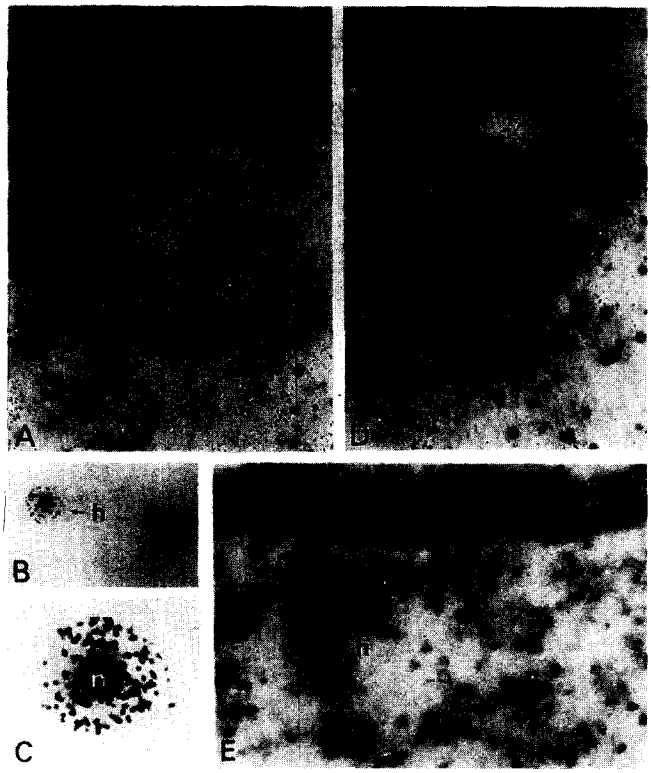

FIG. 2. Autoradiographic photomicrographs of gilthead seabream liver. Sections were incubated with ^{125}I-rsbGH and exposed for 32 days. (A) Binding in hepatocytes (h) and blood cells (bc). Intrahepatic exocrine pancreas (p) was negative; n: nucleus of hepatocyte (×430). (B) Binding in hepatocytes (h); autoradiographic grains are evident around the nucleus of the right hepatocyte (×450). (C) High magnification of a positive hepatocyte; nucleus (n) was unlabeled (×1560). (D) Binding in blood cells (bc) and hepatocytes (h) surrounding the intrahepatic pancreas (p), where GH binding was not evident (×430). (E) Control section of liver incubated with an excess cold rsbGH; h: hepatocytes (×430).

found in the liver of gilthead seabream (Fig. 2A). This specific binding was evident on hepatocytes (Fig. 2B and 2C). However, intrahepatic exocrine pancreas did not show rsbGH binding (Fig. 2A and 2D). Control hepatic sections only exhibited a few autoradiographic grains (Fig. 2E). The highest hepatic autoradiographic labeling found in *Sparus aurata* is consistent with the highest GH binding capacity described for the liver in gilthead seabream (40), rainbow trout (42,46), coho salmon (18) and higher vertebrates (23). High levels of GH binding sites in the liver of gilthead seabream support direct and/or IGF-I-mediated actions of GH. In fact, a GH-induced increase in serum IGF-I levels and a coordinated regulation of hepatic GH binding and plasma somatomedin-like activity have been described in gilthead seabream (14,40). The presence of ^{125}I-rsbGH binding scattered throughout the cytoplasm and around the nucleus of the gilthead seabream hepatocytes (Fig. 2B and 2C) agrees with the results of Lobie et al. (31), who reported that GH receptor-binding protein immunoreactivity can be demonstrated in the cytoplasm and nuclear envelope of hepatocytes and other GH-responsive tissues from rat and rabbit. In mammals, high levels of GH receptors are associated to the endoplasmic reticulum of hepatocytes (21). This fact seems to reflect an intense synthesis of GH receptors due to its short half-life (4). On the other hand, tissues of origin of GH binding proteins (GHBPs) correspond to the distribution of GH receptors and it has been suggested that the liver is a predominant source of GHBPs (3). Thus, it is possible that cytoplasmic rsbGH binding in hepatocytes represents the binding to newly synthesized GH receptors and/or GHBPs. At least in part, this binding could also represent the existence of internalized receptors which have not already been degraded.

Blood cells in hepatic vascular system also showed rsbGH binding (Fig. 2A and 2D). The localization of ^{125}I-rsbGH binding in blood cells of gilthead seabream is in agreement with the saturable binding of GH found in trout blood cells (46). Although blood plasma proteins do not bind specifically salmonid GH (46) this possibility cannot be excluded because specific serum GH binding proteins have been identified in mammals (3). In higher vertebrates, it has been demonstrated that GH interacts with the immune system, intensifying the respiratory burst of macrophages and the sensitivity of lymphocytes to mitogens (26). Therefore, GH binding in blood cells could also be explained by the presence of lymphocytes, macrophages and/or mast cells in which, at least in mammals, GH binding has been described (2,6).

In the spleen, specific binding of rsbGH was also detected while autoradiographic labeling in control splenic sections was almost absent (Figs. 1 and 3). A very high number of autoradiographic grains was found surrounding melanomacrophage centers (Figs. 3A and 3B). Splenic ellipsoid cells also showed an intense GH binding (Fig. 3D) while splenic pulp exhibited a weak and variable number of GH binding sites (Figs. 3A, 3B and 3D). The presence of ^{125}I-rsbGH binding in spleen of gilthead seabream agrees with the existence of saturable binding in rainbow trout spleen (46). However, significant specific binding was not found in the spleen of the same species by Sakamoto and Hirano (42). The marked localization of GH binding around the splenic melanomacrophage centers could be due to the presence of a crown of lymphocytes surrounding these centers, as it has been reported in spleen of flounder (10). The wall of splenic ellipsoids also showed ^{125}I-rsbGH binding. Ellipsoids represent a network of thick-walled filter capillaries which result from the division of the splenic arterioles and association of phagocytic and blood cells, including lymphocytes, to the wall of ellipsoids has been described in fish (11). Splenic pulp also showed GH binding; it should be noted that splenic pulp consists of sinusoidal phagocytic and haemopoietic (mainly lymphopoietic) tissues, in which large numbers of blood cells may be held (10). Furthermore, it has

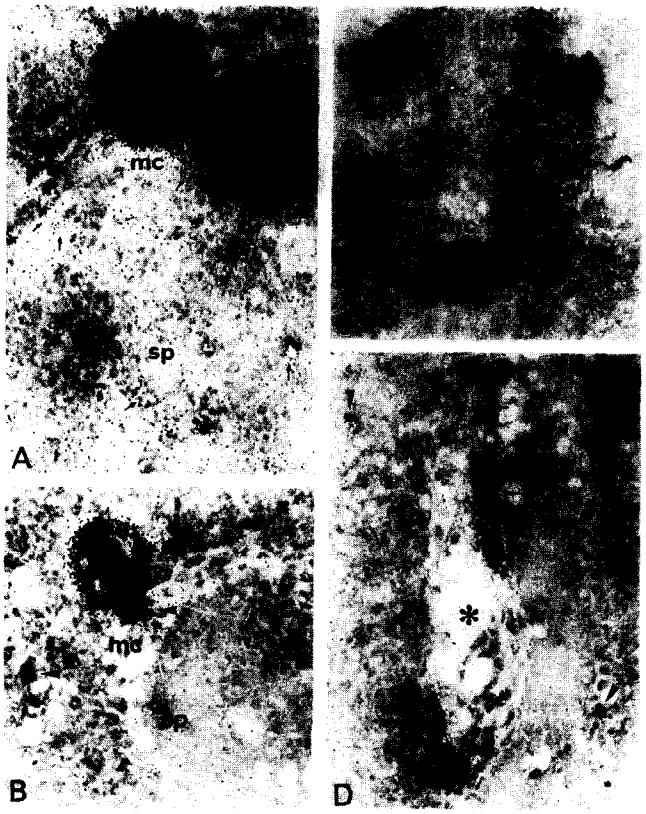

FIG. 3. Autoradiographic photomicrographs of gilthead seabream spleen. Sections were incubated with ^{125}I-rsbGH and exposed for 62 days. (A) Binding in melanomacrophage centers (mc) and splenic pulp (sp) cells (arrows) (×460). (B) Intense binding of GH in melanomacrophage centers (mc). Binding in splenic pulp (sp) is weaker than in Fig. 2(A). Three presumptive macrophages (arrowheads) migrating towards the melanomacrophage centers are labeled in the splenic pulp (×460). (C) Splenic control section incubated with an excess cold rsbGH. Note the absence of GH binding in melanomacrophage centers (mc) (×460). (D) Splenic section showing GH binding (arrows) in cells of an ellipsoid (*). It should be noted the presence of GH binding in two presumptive macrophages (arrow heads) migrating from ellipsoids through the splenic pulp (sp) (×460).

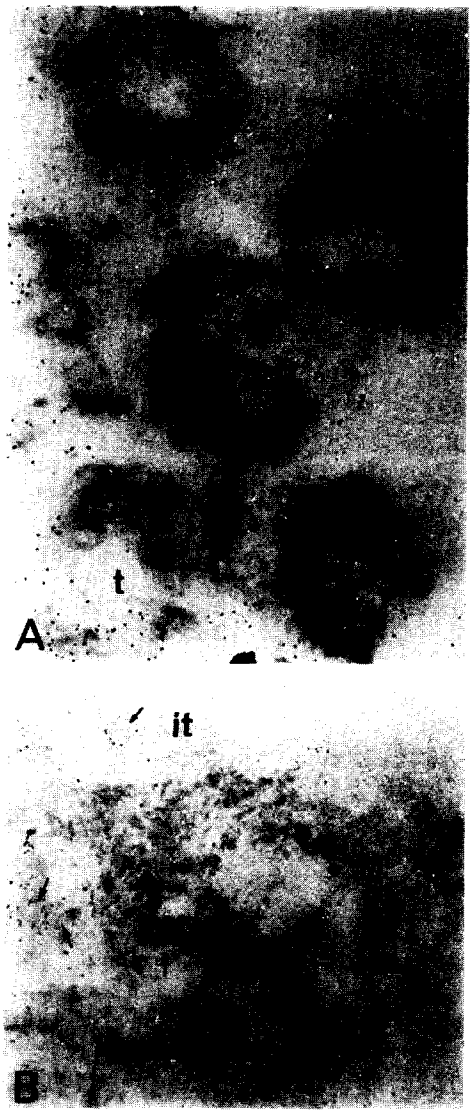

FIG. 4. Autoradiographic photomicrographs of gilthead seabream kidney. Sections were incubated with ^{125}I-rsbGH and exposed for 54 days. (A) Binding in renal tubules (t) (×450). (B) Interstitial haemopoietic tissue of the kidney (it) showing GH binding in cells (arrows) (×400).

been reported in turbot that replete macrophages migrate from ellipsoids to melanomacrophage centers through the splenic pulp (11). In this way, some labeled cells observed in the splenic pulp could represent this type of migrating macrophages (Fig. 3B and 3D, arrowheads).

Kidney GH binding sites were present in renal tubules (Figs. 1 and 4A). The presence of ^{125}I-rsbGH binding in renal tubules of an eurihaline fish such as gilthead seabream is consistent with the existence of GH receptors in proximal convoluted tubules of mammalian kidney (6), the presence of saturable GH binding in kidney of rainbow trout, masu salmon and tilapia (13,25,46), and the proposed osmoregulatory role of GH in fish (42). A weak specific GH binding was also evident in renal interstitial tissue (Figs. 1 and 4B). This binding could be related with the proposed haemopoietic and temporary blood bank functions of this tissue in fish (10). However, specific binding in head kidney and anterior body kidney, composed mostly of haemopoietic tissue, was not significant in rainbow trout (42). The localization of rsbGH binding could also indicate the uptake of GH into clearance tissues. In this way, the presence of GH binding in liver, kidney and immune tissues may support this possibility.

The cardiac muscle also exhibited an intense rsbGH binding (Fig. 1 and 5A), while control sections were almost devoid of signal (Fig. 5B). The physiological significance of autoradiographic ^{125}I-rsbGH binding in cardiac muscle of gilthead seabream remains unknown. Specific binding was not significant in heart of rainbow trout (42). However, GH

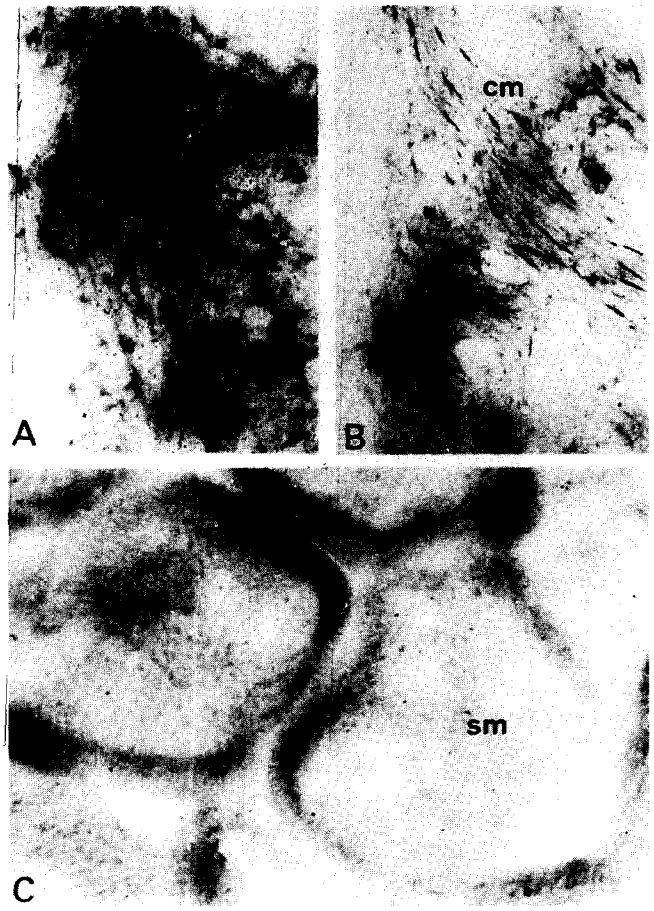

FIG. 5. Autoradiographic photomicrographs of gilthead seabream muscle. Sections were incubated with ^{125}I-rsbGH and exposed for 54 days. (A) Binding in cardiac muscle (cm) (×450). (B) Control section showing nonspecific binding in cardiac muscle (cm) (×450). (C) Absence of specific GH binding in body skeletal musculature (sm) (×635).

receptor and GH binding protein mRNAs were present in the heart of rat (45) and high levels of IGF-I receptors were found in heart muscle of salmonids (37). In higher vertebrates, it has been shown that if parts of cardiac muscle fibers are injured, the volume of the unharmed parts are increased (19). In spite of that, information about the regenerating and growth capacity of fish cardiac muscle is rather scarce.

Specific GH binding sites were not detected in body skeletal muscle by autoradiography (Figs. 1 and 5C). This result is consistent with that obtained by other authors, who failed to find specific binding sites for GH in rainbow trout, eel and tilapia skeletal muscle (20,36,42). In contrast, other studies referred an appreciable GH binding in muscle of rainbow trout, coho salmon and gilthead seabream (18,40,46). However, these authors reported that the amount of specific binding was markedly lower than in liver. So, there is the possibility that sensitive tissues to the hormone action might have a few binding sites and therefore show little evidence in autoradiography.

In conclusion, our results show that rsbGH can be used successfully to determine the cellular localization of GH binding sites in gilthead seabream tissues. Previous results using this rsbGH in homologous radioimmunoassay and radioreceptor assay indicate that, at least in liver, these GH binding sites correspond to GH receptors (32,33). A variety of biological actions of GH on many of these tissues also argues strongly for a functional significance of these GH binding sites.

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