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Specific Antibodies against Synthetic Peptides of Salmonid GTH I and II β Subunits

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ABSTRACT—Specific polyclonal antibodies against the two salmonid gonadotropins (GTHs) were developed using two synthetic oligopeptides corresponding to GTH Iβ-(96–113) and GTH IIβ-(107–119), respectively. By Western blot analysis under reducing conditions, anti-GTH Iβ-(96–113) detected one specific band corresponding to GTH Iβ (17 kDa) and anti-GTH IIβ-(107–119) detected one specific band corresponding to GTH IIβ (20 kDa). Neither antibody recognized bands under nonreducing conditions. By immunohistochemical analysis, immunoreactive (ir-) Iβ-(96–113)- and ir-IIβ-(107–119)-cells had different regional distributions among the aldehyde fuchsin positive cells and were not stained with an anti-human thyrotropin β antibody. In salmonid fishes (chum salmon, rainbow trout, brook trout, whitespotted char, dolly varden and huchen), each antibody reacted with distinctly separate hypophyseal cells. However, in nonsalmonid species, and even in the salmoniform fish, the ayu (*Plecoglossus altivelis*), no pituitary cell was stained with these antibodies. These data indicate that these antibodies are useful tools for studying the change and the function of GTHs in salmonid fishes.

INTRODUCTION

In most tetrapods, gonadal function is known to be regulated by two gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). It is generally accepted that, in females, FSH acts on small ovarian follicles to stimulate their growth, while LH acts on large follicles to induce ovulation and, in mammals, also to luteinize the follicle cells [9]. In teleosts, two types of GTH, GTH I and GTH II, from the pituitary gland of chum salmon (*Oncorhynchus keta*) have been isolated and characterized by Kawauchi and associates [10, 12, 18–21]. Evidence for a duality of GTHs has been described for salmonids [14, 23], killifish (*Fundulus heteroclitus*) [13], common carp [30], Atlantic croaker (*Micropogonias undulatus*) [2], red sea bream (*Pagrus major*) [25] and tuna (*Thunnus obesus*) [16]. Although a large number of studies have been made on FSH and LH, investigations into the function of GTH I and GTH II have been limited [9, 24]. Highly specific antibodies against GTH I and GTH II, and purified GTHs are necessary for future studies of the role of two GTHs in teleost fishes.

In addition, salmonid GTHs have been used for various purposes in fishes, such as artificial induction of gonadal development and maturation in Japanese eel (*Anguilla japonica*) [33]. The Japanese eel has immature gonads under condition of cultivation. Gonadal development can be induced only by gonadotropic factors in this species. Injection of salmon pituitary homogenate (SPH) also induced GTH synthesis in the pituitary of female silver eel [29]. It needs to be clarified whether endogenous GTH is released from activated GTH cells or not. Therefore, antibodies which have high species specificity are needed in order to distinguish between endogenous GTHs and exogenous salmonid GTHs.

In this study we attempted to generate specific antibodies which recognize the salmonid GTHs with high specificity using synthetic peptides as the antigens.

MATERIALS AND METHODS

Animals

Masu salmon (*Oncorhynchus masou*), amago salmon (*O. rhodurus*), kokanee salmon (*O. nerka*), rainbow trout (*O. mykiss*), brook trout (*Salvelinus fontinalis*), whitespotted char (*S. leucomaenis*), dolly varden (*S. malma*) and Japanese huchen (*Hucho perryi*) were reared at Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University. Chum salmon were collected from the Yurappu River in September 1993. Ayu (*Plecoglossus altivelis*, Plecoglossidae, Salmonoidei) were reared at Ueda Station, National Research Institute of Fisheries Science. Cultivated female Japanese eels were purchased from a commercial eel supplier and induced to mature by administration of SPH [16]. Barfin flounder (*Verasper moseri*) were reared at the Hokkaido Institute of Mariculture. Greenling (*Hexagrammos agrammus*) and marbled sole (*Limanda yokohamae*) were captured off the coast of Usujiri, southern Hokkaido.

Peptide synthesis and immunization

A computer analysis of the amino acid sequence of the masu salmon GTH β subunits [10, 11] was employed for prediction of the secondary structure and the areas of hydrophilicity. One possible antigenic and specific segment was chosen to each hormone, corresponding to the following amino acid residues: GTH Iβ-(96–113) and GTH IIβ-(107–119). They were synthesized using a solid phase peptide synthesizer model 431A (Applied Biosystems, Forest City,

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CA) using Fmoc chemistry. Peptides were cleaved from HMP-resin with 6% phenol, 2% 1,2-ethanedithiol, 4% thioanisole in trifluoroacetic acid. After lyophilization, oligopeptides were coupled to bovine serum albumin (BSA) using N-(ε-maleimidocaproyloxy) succimide (Dojindo, Japan). One male rabbit was injected subcutaneously at multiple sites along the back with 1 ml of PBS (pH 7.5) containing 1 mg of each peptide mixed with an equal volume of Freund's complete adjuvant. The rabbits received four immunizations at about 7 day intervals. Sera were collected after the fourth injection and absorbed with an equal volume of 1% BSA-PBS. The absorbed antisera were used as the antipeptide antibodies. The specificity of the resulting antipeptide antibodies was tested by Western blot and immunohistochemical analysis.

Western blot analysis

A glycoprotein fraction (GF) was obtained from masu salmon pituitaries according to the method of Suzuki *et al.* [18]. Frozen pituitary glands were homogenized with 35% ethanol-10% ammonium acetate (pH 9.0) containing 5 mM EDTA and 1.5 mM phenylmethylsulfonyl fluoride for 14 hr at 4°C. The extract was mixed with 3 volumes of cold ethanol and stored for 24 hr at 4°C. The resulting precipitate was centrifuged at 10,000 rpm for 15 min and the resultant pellet, GF, was dissolved in 0.05 M ammonium acetate. This fraction was pretreated at 100°C for 2 min in 3% sodium dodecyl sulfate (SDS) with or without reduction with 10% 2-mercaptoethanol (2ME). The sample was separated using 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, pH 8.8). A stacking gel was not prepared in order to avoid dissociation of the subunits. Separated proteins were stained with Coomassie brilliant blue R-250 (CBB) or transferred to a polyvinylidine difluoride membrane (Millipore, Bedford, MA) with a Bio-rad semidry trans-blot SD. The membranes were sequentially incubated with: (1) 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl (TBS), containing 5% skim milk to block nonspecific protein binding sites for 30 min; (2) antibodies at 1:2,000 dilution overnight; (3) horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (Bio-rad) at 1:1,000 dilution for 3 hr; (4) 0.06% 4-chloro-1-naphthol in TBS containing 0.06% hydrogen peroxide.

Immunohistochemistry

Pituitary glands of teleosts were fixed with Bouin-Hollande sublimate for two days. After elimination of the sublimate with iodine alchohol, the fixed samples were dehydrated in ethanol, embedded in paraffin and sectioned at 4 μm. Deparaffinized sections were sequentially incubated with: (1) iodine-potassium iodine alcohol to remove the sublimate completely; (2) 1% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase activity; (3) 10% normal goat serum in PBS for 15 min to block nonspecific binding of antibodies; (4) antibodies at 1:2,000 dilution overnight at 4°C; (5) biotinylated goat anti-rabbit IgG at 1:400 dilution (Dako, Glostrup, Denmark) for 3 hr; (6) streptavidin-biotin-HRP complex (Dako) for 1 hr; (7) 0.01% diaminobenzidine-0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6). To confirm the specificity of the immunohistochemical staining, the following control stains were done: (1) normal rabbit serum (NRS) and anti-BSA were substituted for the primary antibody, (2) primary antibodies were absorbed with purified masu salmon GTH Iβ or GTH IIβ (50 μg/0.1 ml working dilution of the antibody) which were isolated according to the method of Suzuki *et al.* [18]. Adjacent serial sections were stained with Gomori's aldehyde fuchsin (AF) or immunostained with anti-human thyrotropin (hTSH) β (UCB bioproducts) at 1:500 dilution.

RESULTS

Western blot analysis

Electrophoretic patterns and Western blot analysis of the GF by anti-GTH Iβ-(96–113) and anti-GTH IIβ-(107–119) are shown in Figure 1. In the nonreduced GF, many protein bands were stained on the gel with CBB. Under reducing conditions, broad bands corresponding to the subunits were revealed at around 20 kDa. Two specific bands corresponding to GTH Iβ (17 kDa) and GTH IIβ (20 kDa) were detected by anti-GTH Iβ-(96–113) and anti-GTH IIβ-(107–119), respectively, after reduction with 2ME. However, no band was detected by either antibody under nonreducing conditions.

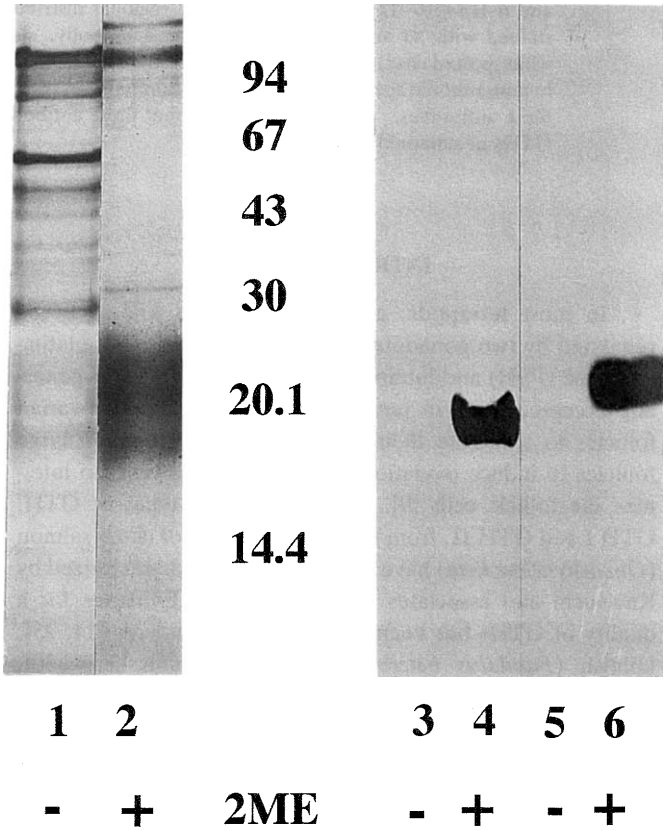

FIG. 1. Electrophoretic pattern of mature masu salmon pituitary glycoprotein fraction after reduction with 2-mercaptoethanol (lanes 2, 4 and 6) and without reduction (lanes 1, 3 and 5). Lanes 1 and 2 were stained with Coomassie brilliant blue. Lanes 3 and 4 were immunostained with anti-GTH Iβ. Lanes 5 and 6 were immunostained with anti-GTH IIβ. Positions of molecular weight markers, expressed in kilodaltons, are indicated in the center of the figure.

Immunohistochemistry

The identification of cell types in the pars distalis was based on histophysiological and immunohistochemical criteria described previously [12, 14, 15, 31].

The immunoreactivities for GTH Iβ-(96–113)- and GTH

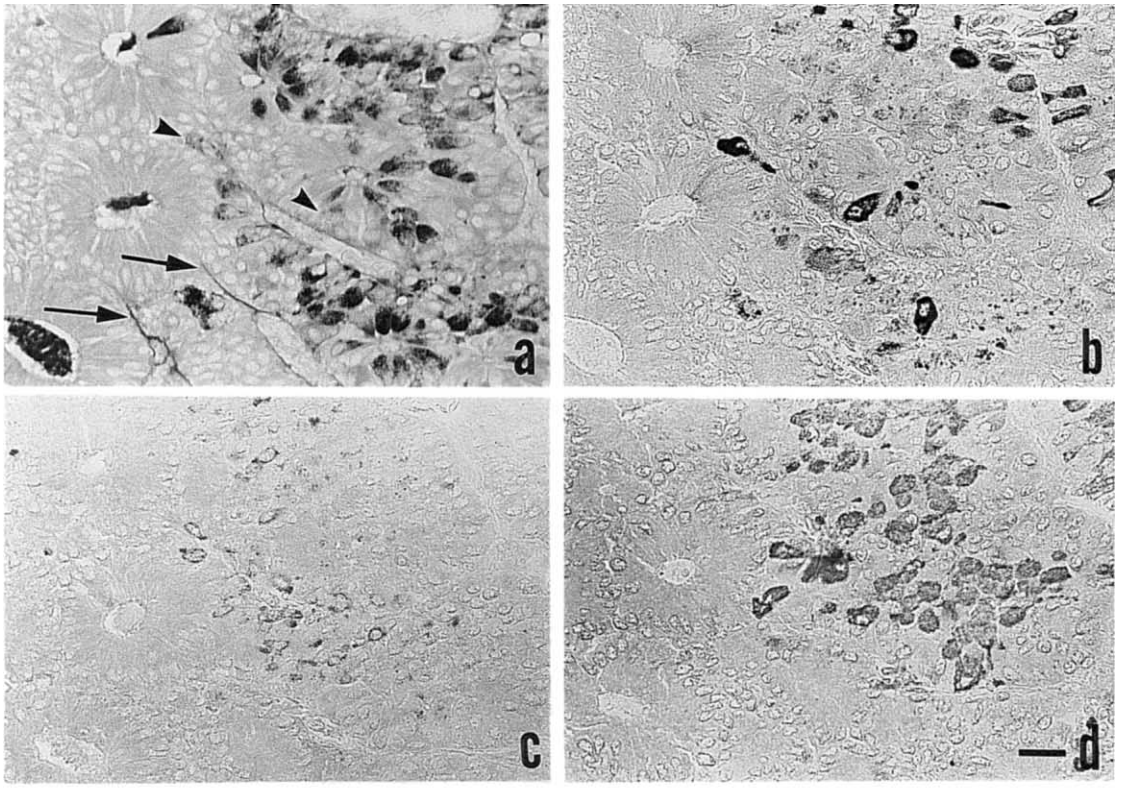

FIG. 2. Four consecutive sections of the mature masu salmon pituitary gland stained with aldehyde fuchsin (a), anti-GTH Iβ (b), anti-hTSHβ (c) and anti-GTH IIβ (d). Ir-GTH Iβ- and ir-GTH IIβ-cells had different regional distributions in the PPD and did not stain with anti-hTSHβ antiserum. Ir-GTH Iβ-cells stained faintly (arrow head) and ir-GTH IIβ-cells were stained intensely with aldehyde fuchsin. Aldehyde fuchsin-positive neurohypophysial fibers (arrow) did not stain with either antibody. Bar 25 μm.

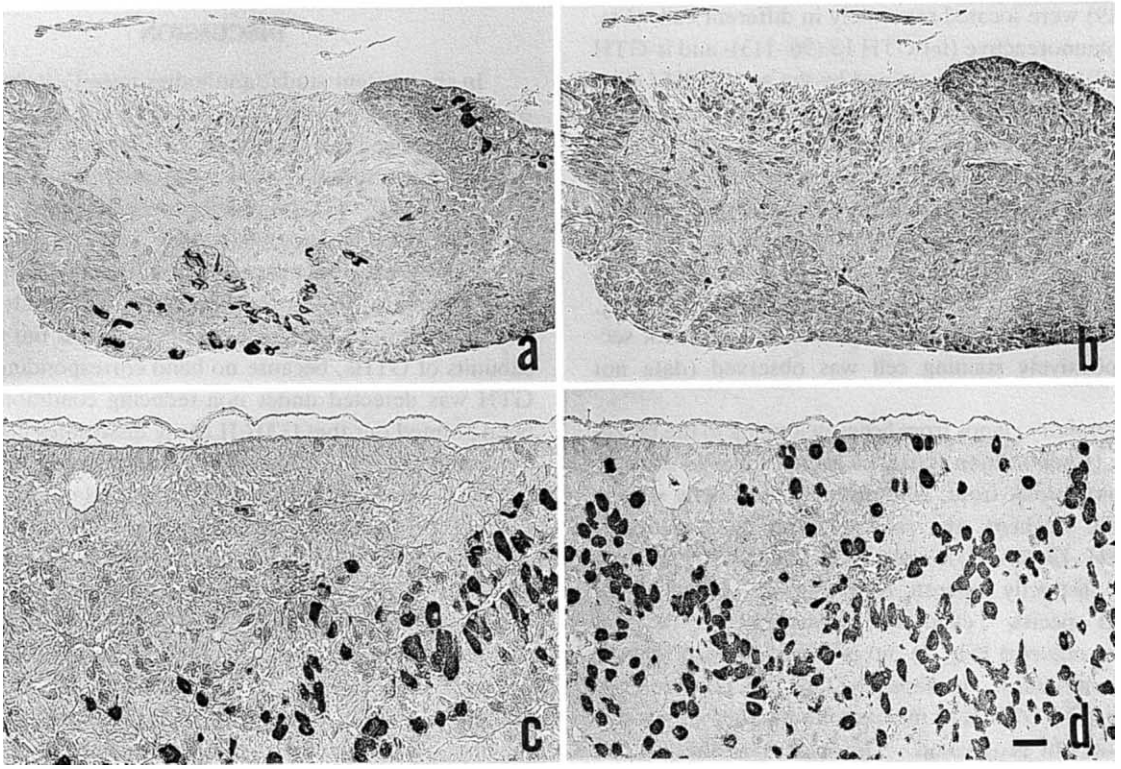

FIG. 3. Adjacent sections of immature masu salmon pituitary stained with anti-GTH Iβ (a) and anti-GTH IIβ (b). Only ir-GTH Iβ-cells were present in this stage. Adjacent sections of mature masu salmon pituitary stained with anti-GTH Iβ (c) and anti-GTH IIβ (d). Bar 25 μm.

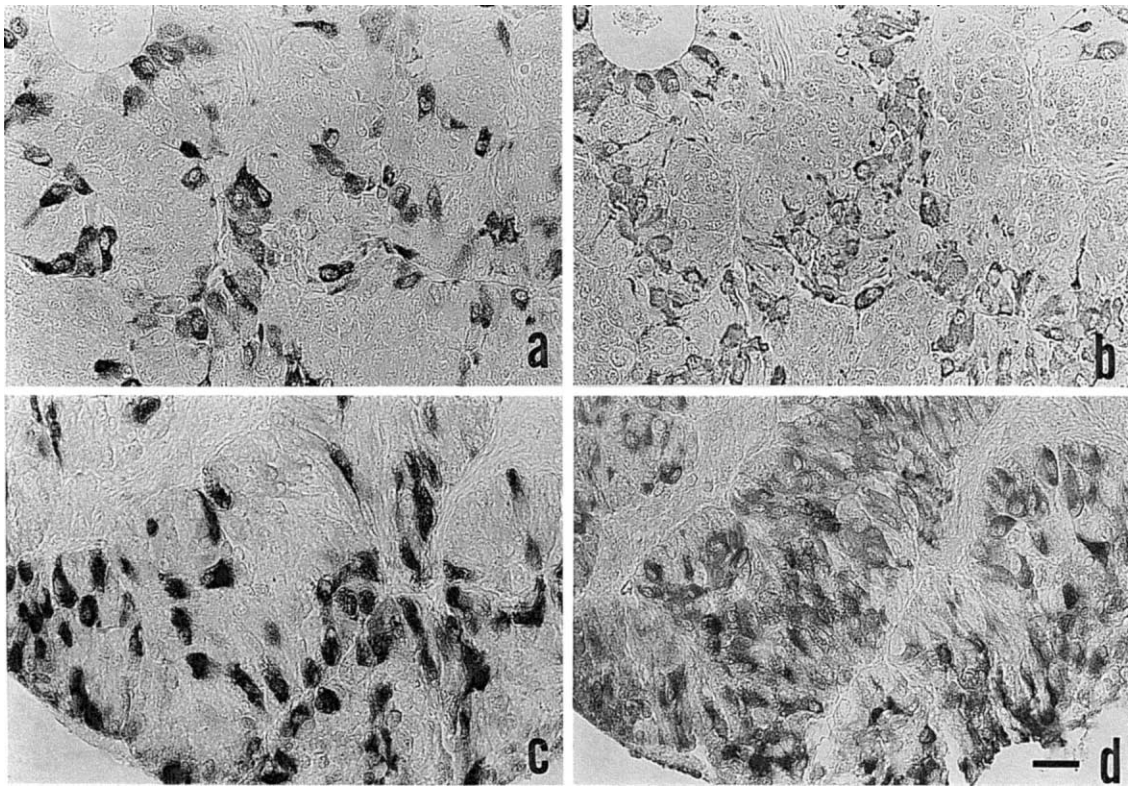

FIG. 4. Adjacent sections of brook trout at the vitellogenic stage (a and b) and Japanese huchen at the vitellogenic stage (c and d) in the PPD stained with anti-GTH Iβ (a and c) and anti-GTH IIβ (b and d). Ir-GTH Iβ- and ir-GTH IIβ-cells had different regional distributions in the PPD of both animals. Bar 25 μm.

IIβ-(107–119) were located separately in different cells (Fig. 2b, d). Immunoreactive (ir)-GTH Iβ-(96–113)- and ir-GTH IIβ-(107–119)-cells were not stained by the anti-hTSHβ (Fig. 2c). Ir-hTSHβ-cells and AF-positive neurohypophysial fibers were not stained with either antibody (Fig. 2). Ir-GTH Iβ-(96–113)-cells stained faintly with AF (Fig. 2a,b). These cells were observed in underyearling and postspawning masu salmon (Fig. 3a,c). On the other hand, ir-GTH IIβ-(107–119)-cells which were not observed in underyearling masu salmon (Fig. 3b), but were present in mature fish (Fig. 3d), stained intensely with AF (Fig. 2a,c). In control sections, no positively staining cell was observed (data not shown).

In the pituitary glands from fishes belonging to the family Salmonidae (chum salmon, kokanee salmon, amago salmon, rainbow trout, brook trout, whitespotted char, dolly varden and Japanese huchen), the reactions with each antibody against GTH Iβ-(96–113) and GTH IIβ-(107–119) were observed in distinctly separate cells (Fig. 4). However, in nonsalmonid species - even in a salmoniform , the ayu (*Plecoglossus altivelis*) (Fig. 5)- no pituitary cell was stained with these antibodies. In Japanese eel, AF positive cells which seem to be GTH cells increased in number and in size as SPH treatment progressed. However, no pituitary cell was stained with these antibodies (Fig. 5).

DISCUSSION

In the present study, antibodies raised against synthetic oligopeptides of masu salmon GTH Iβ and GTH IIβ specifically recognized their β subunits in salmonids. The specificity of the antibodies was evaluated by Western blot analysis of GF and immunohistochemistry of pituitaries. Although the GF contained many kinds of peptides, our antibodies each recognized only one band corresponding to the β subunit by Western blot analysis. It is likely that these antibodies do not recognize the intact forms but only the β subunits of GTHs, because no band corresponding to native GTH was detected under non-reducing conditions. It has been pointed out that GTH II easily dissociates into its α and β subunits [16, 19, 23, 25]. It is possible therefore that there was no intact molecule of GTH II because of the complete dissociation during sample preparation. In order to confirm whether these antibodies recognize intact hormone, dissociation into the subunits needs to be avoided. Because the spacer gel is acidic (pH 6.8), GTH I and GTH II dissociated and these antibodies recognized their corresponding β subunit under non-reducing conditions (data not shown). On account of keeping the gel basic (pH 8.8) without spacer gel, the dissociation was suppressed and these antibodies did not recognize the band corresponding to intact hormone.

It is known that stable and unstable types of salmonid

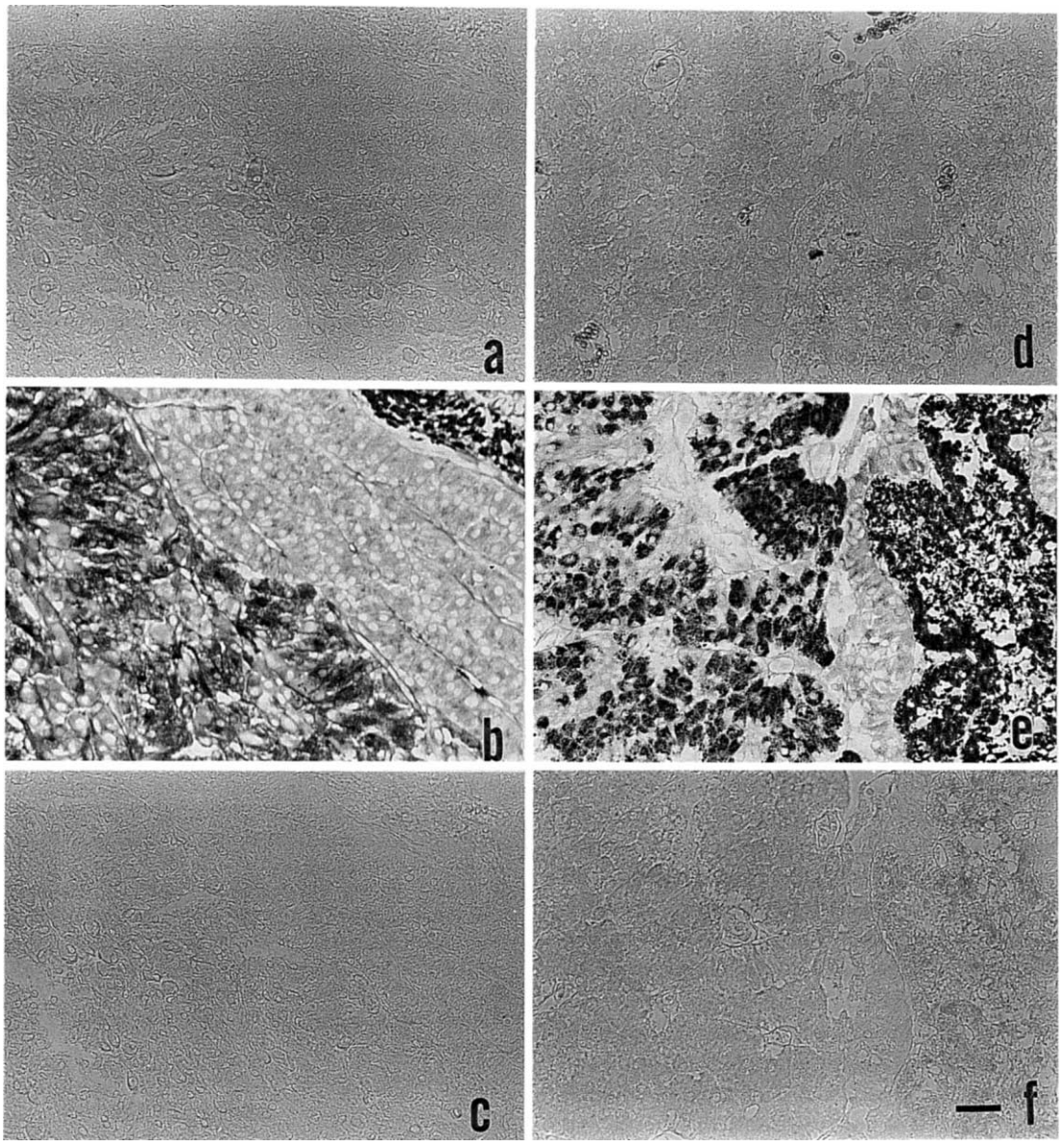

FIG. 5. PPD of the mature ayu stained with anti-GTH Iβ (a), aldehyde fuchsin (AF) (b), and anti-GTH IIβ (c). PPD of the Japanese eel injected with chum salmon pituitary homogenate, stained with anti-GTH Iβ (d), AF (e), and anti-GTH IIβ (f). The eel was injected 23 times and its GSI was 38.2%. Although there were many AF-positive cells, they showed no immunoreactivity with anti-GTH Iβ and anti-GTH IIβ in both animals. Bar 25 μm.

GTH I exist [1, 19, 23]. The intact molecules of both types probably existed under non-reducing condition. It is likely that the antibodies do not recognize the intact forms of either GTH type. Anti- GTH Iβ-(96–113) recognized the β subunit of unstable type under non-reducing condition with spacer gel (data not shown). However, because the β subunit of both types migrated as single band under reducing conditions, it is difficult to show whether the β subunit of the stable type can be also recognized by anti- GTH Iβ-(96–113). This subject will be discussed in a forthcoming paper concerned with immunoassay of salmonid GTHs, because quantitative analysis may be needed after dissociation of stable forms owing to the characteristic of these antibodies.

It was shown by Western blotting that neither of the antibodies recognized intact hormones, but they reacted with β subunits. We therefore needed to confirm that these antibodies stained only their corresponding hormone producing cells. To identify GTH cells, AF staining was performed because basophils corresponding to GTH cells react with this stain [31]. There are three types of AF positive cells in the salmonid pituitary: the first stained faintly or remained unstained in the PPD - GTH I cells; the second stained intensely in the PPD - GTH II cells; the third stained intensely on the boundary between the PPD and the RPD - TSH cells [14]. In this study, all cells which were recognized by each anti-glycoprotein hormone β subunit antibody were AF positive

and there were no AF positive cells that did not react to the antibodies in the pars distalis. In addition, it has been reported in rainbow trout that only GTH I cells were found in immature fish, and in mature fish, GTH II cells exceeded GTH I cells in number [15]. In this study, ir-GTH I cells were the only GTH cell present in immature masu salmon while both ir-GTH I and ir-GTH II cells were present in the pituitary of mature animals. It can be concluded that these antibodies have high specificity for their corresponding β subunits, based on our observation on the immunoreactive materials whose characteristics, such as molecular weight, localization and ontogenic changes, agree with previous reports.

There are two possible reasons why antibodies that recognize only the β subunit were able to stain GTH cells. The first possiblility is that GTH cells contain free β subunits. However, it has been suggested that in the rat pituitary, the β subunit for LH associates with the α subunit in the endoplasmic reticulum [8]. Moreover, there are no reports of cells containing free β subunits, although free α subunits exist in the bullfrog [26, 27], porcine fetal [3], and human fetal [4] hypophysis and in pituitary tumor cells [5–7, 32]. More likely is the possibility that the conformations of both β subunits change during fixation. Salmonid GTHs are easily dissociated under acidic conditions [1, 19, 23]. The most satisfactory explanation for our antibodies recognizing only β subunits is that the antigenic sequence of the synthetic peptide corresponds to the α-β binding region, or that conformational changes occur upon association of the β subunit with the α subunit. It has been reported that the region in human LH and human chorionic gonadotropin corresponding to the carboxyl (C)-terminal ends of masu salmon GTHs is not the site of subunit association [17]. Therefore, it is likely that the region corresponding to synthetic peptides is not correspond to the α-β binding region, but that conformational and antigenic changes occurs when α and β subunits combine to form the dimer. Thus, it seems reasonable to suppose that the antigenic regions appeared after dissociation of the subunit with fixative.

The synthetic peptides corresponding to amino acid sequences were intentionally chosen based on their lack of homology to GTHs of other species. In fishes treated with salmonid GTH, especially the eel, exogenous salmonid GTHs and endogenous GTHs need to be separately investigated by raising antibodies against these oligopeptides which have high species specificity. Each antigenic sequence corresponding to our synthetic oligopeptide, the C-terminal end of the β subunit, is not only a hormone specific but also a species specific region [10, 13, 16, 30]. Injection of SPH induced GTH synthesis in the pituitary of female silver eel [29]. In the present study, no pituitary cell in SPH-treated Japanese eels was actually stained with these antibodies, although there were many AF positive cells which seems to be GTH cells. These AF positive cells reacted with the antibody raised against synthetic oligopeptide of Japanese eel GTH IIβ (T. Ikeuchi, M. Nagae, S. Adachi, and K. Yamauchi, unpublished data). Nevertheless, in the pituitaries of char, huchen and other salmonid fishes in this study, two types of GTH cells were recognized by these antibodies, indicating that this epitope at least has been conserved in both GTH I and GTH II of salmonids.

It is clearly important to investigate the functional differences between GTH I and GTH II. The steroidogenic properties of these hormones in salmonid reproduction have already been investigated [20, 22]. These studies suggest that both GTH I and GTH II are approximately equipotent in inducing estradiol-17β production, but that GTH II is more potent in enhancing maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one, production than GTH I. Furthermore, it has been shown that GTH I enhanced vitellogenin uptake *in vitro* and *in vivo*, whereas GTH II did not enhance uptake significantly [28]. Because the functions of GTH I and GTH II in teleosts have not been as thoroughly investigated as in tetrapods, more studies are needed to elucidate details of the biological actions of GTHs. Because of their specificity, these antibodies will be useful in the development of immunoassays and for studies on the role of GTHs in salmonids and in fishes which have been treated with salmonid GTH.

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