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Glycoconjugate Profiles of Adrenocorticotropic and Melanotropic Cells in the Pituitary of Adult Sea Lampreys (*Petromyzon marinus*): A Lectin Histochemical Study

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ABSTRACT—In the lamprey, adrenocorticotropin (ACTH) and melanotropins (MSHs) are produced from two distinct precursors, proopiocortin (POC) and proopiomelanotropin (POM). Both POC and POM have been suggested to be glycoproteins. The present study aimed to demonstrate glycoconjugates in ACTH and MSH cells in the pituitary of adult sea lampreys (*Petromyzon marinus*) by means of a lectin histochemistry. A total of 19 kinds of lectins were tested. ACTH cells were distributed in both the rostral pars distalis and the proximal pars distalis, and were stained positively with N-acetylglucosamine binding lectins (i.e., succinylated wheat germ agglutinin), N-acetylgalactosamine binding lectins (i.e., soybean agglutinin), D-mannose binding lectins (i.e., *Lens culinaris* agglutinin), and D-galactose binding lectins (i.e., *Erythrina cristagall* lectin). MSH cells were distributed in the pars intermedia, and were stained with N-acetylgalactosamine binding lectins (i.e., *Dolichos biflorus* agglutinin), D-mannose binding lectin (*Pisum sativum* agglutinin) and D-galactose binding lectins (i.e., peanut agglutinin).

These results suggested that ACTH and MSH cells produce different types of glycoconjugates which may be attributed to the difference in glycoconjugate moieties between the precursor proteins, POC and POM.

Key words: lamprey pituitary, adrenocorticotropin, melanotropin, lectin histochemistry, glycoconjugate

INTRODUCTION

Adrenocorticotropin (ACTH) and melanotropins (MSHs) are produced from a common precursor protein, proopiomelanocortin (POMC), by posttranslational proteolytic cleavage (Nakanishi *et al*., 1979; Dores *et al*., 1993). Tetrapod, dogfish and sturgeon POMCs have one N-glycosylation site within a region of γ-MSH or γ-MSH-like (Amemiya *et al*., 1997). Furthermore, an N-glycosylation site has been observed in a CLIP in mice and rats, whereas human and bovine POMC molecules contain an O-glycosylation site within the N-terminal peptide (Smith and Funder, 1988). Thus, there is a possibility that POMCs of these animals are synthesized as a glycoprotein. On the other hand, salmon POMC has no γ-MSH and no glycosylation site within the molecule (Kawauchi *et al*., 1981; Kitahara *et al*., 1988; Salbert *et al*., 1992). In support of above evidence, tetrapod ACTH cells have been shown to contain glycoprotein. For example, rat ACTH cells are weakly Periodic-acid-Schiff (PAS)-positive (Bowie *et al*., 1974), and their secretory granules are reactive with concanavalin A (Inoue and Kurosumi, 1980). On the other hand, salmon ACTH cells are virtually chromophobic and PAS-negative (Ball and Baker, 1969).

Lampreys and hagfish are the only two extant representatives of the oldest class of vertebrates, Agnatha. Among adenohypophysial hormones, ACTH and MSHs have been isolated from the lamprey pituitary (Takahashi *et al*., 1995a). Unlike gnathostome vertebrates, they were found to be encoded in two distinct genes in the lamprey (Heinig *et al*., 1995; Takahashi *et al*., 1995b): One gene encoding nasohypophysial factor (NHF; Sower *et al*., 1995), ACTH and β-endorphin in turn from the N-terminus (Fig. 1e), and the other encoding MSH-B, MSH-A and another β-endorphin (Fig. 3e). Nevertheless, ACTH and MSH cells were found in the pars distalis and the pars intermedia, respectively, as well as those of gnathostome vertebrates (Nozaki *et al*., 1995).

Both precursors of lamprey ACTH and MSHs, proopiocortin (POC) and proopiomelanotropin (POM), have been shown to be glycoproteins: POC contains N-glycosylation sites within both NHF and ACTH, whereas POM contains an N-glycosylation site within β-endorphin (Takahashi *et al*.,

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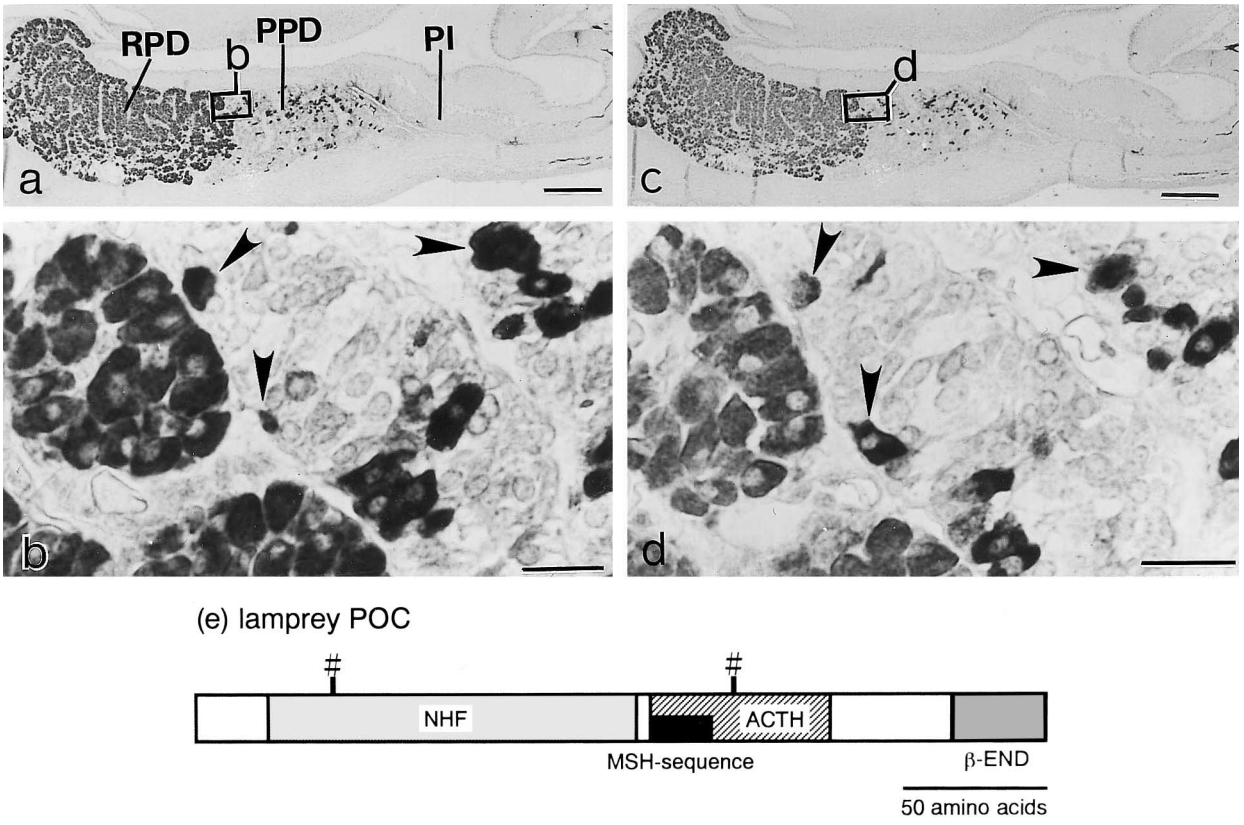

Fig. 1. Two successive sagittal sections (a and c) through the pituitary of an adult sea lamprey, stained with (a) anti-lamprey NHF, and (c) biotin conjugated-succinylated wheat germ agglutinin (s-WGA), respectively. The areas outlined by rectangles in a and c are enlarged and shown in b and d. (e) Schematic diagram of lamprey proopiocortin (POC) showing the glycosylation site (#)(Sower *et al*., 1995; Takahashi *et al*., 1995b). Arrowheads in b and d indicate same cells. PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis. Scale bars: a and c, 200 μm; b and d, 20 μm.

1995b; Figs. 1e, 3e). In agreement with this biochemical evidence, lamprey ACTH cells appear to correspond to aldehyde fuchsin (AF)-positive and PAS-positive basophils of the rostral and proximal pars distalis (Nozaki *et al*., 1995). The lamprey MSH cells appear to correspond to chromophobes or PAS-positive cells of the pars intermedia (Ball and Baker, 1969; Hardisty and Baker, 1982).

Lectins are naturally occurring glycoproteins, which selectively bind non-covalently to carbohydrate residues. It is for this reason that they are of interest and are used in cytochemistry. However, only a few studies have been conducted on the lectin cytochemistry in the pituitary even in higher vertebrates (Inoue and Kurosumi, 1980; Komuro and Shioda, 1981; Kurosaki *et al*., 1995). In the lamprey pituitary, although basophils in both the rostral and the proximal pars distalis have been identified to be ACTH cells (Nozaki *et al*., 1995), they were described as different cell types in earlier studies, since they exhibited different affinity to AF and PAS (Hardisty and Baker, 1982). Such difference in basophils between the rostral and the proximal pars distalis may be attributed to the difference in the glycoconjugate moieties of POC.

The aim of this study was to demonstrate glycoconjugates in ACTH and MSH cells in the pituitary of adult sea lampreys by means of a lectin histochemistry. Special attention was paid to detect any difference in ACTH cells between the rostral and the proximal pars distalis.

MATERIALS AND METHODS

Animals

Twenty adult sea lampreys (*Petromyzon marinus*) of both sexes were used. They were collected in May and June 1997 during their upstream spawning migration from the sea, from traps located at the upstream end of a salmon ladder on the Cocheco River in Dover, New Hampshire, U.S.A. The animals were transported to the Fish Hatchery of University of New Hampshire, and were kept in a fresh water flow through system supplied with ambient local reservoir water, under natural photoperiod up to one month. They were approximately 65 cm in total length and weighed approximately 900 g at the time sacrifice.

Tissue preparations

They were killed by decapitation after being anesthetized by immersion in ethyl m-aminobenzonate methane-sulfonate (MS222). After rapid removal of the dorsal fibrocranium and exposure of the dorsal surface of the brain, the dissected brain and the attached pituitary were immersed in Bouin-Hollande sublimate solution (Romeis, 1948) for about 24 hr. The fixed tissues were put in 70% ethanol, and were supplied to us by Prof. Stacia A. Sower, Univer-

sity of New Hampshire. The tissues were dehydrated through a series of solutions with increasing concentrations of ethanol. Deposited mercuric chloride was removed by treatment with iodine-potassium iodine in 90% ethanol for 1-2 day. Tissues were embedded in Paraplast, and serial sagittal sections of 6 μm were mounted on glass slides.

Antisera and Lectins

Antisera used were anti-lamprey NHF (lot No. 9207-09-08, optimal working dilution, 1:20,000; Sower *et al*., 1995) and anti-lamprey MSH-B (lot No. 9311, optimal working dilution, 1:8,000; Nozaki *et al*., 1995). These antisera have been validated for localization of ACTH and MSH cells, respectively, in the sea lamprey pituitary (Sower *et al*., 1995; Nozaki *et al*., 1995). The biotinylated lectins used were listed in Table 1, together with applied concentrations and inhibitory sugar. They were purchased from Vector Laboratories, Inc.

Histochemistry

Immunohistochemical staining was performed by use of a Vectastain avidin-biotin peroxidase complex (Elite ABC) kit. In brief, sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in phosphate-buffered saline (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.5; PBS). All procedures were performed at room temperature, and incubations were performed in close humid chambers. First, the tissue sections were incubated for 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activities, and washed in PBS. To reduce nonspecific staining, sections were treated with normal goat serum for 30 min. Primary antisera were applied to the sections for about 12 hr, and the biotinylated secondary antibody solution and ABC reagent were each applied for 1 hr. The final reaction product was visualized with 3,3'-diaminobenzidine tetrahydrochloride in 10 mM Tris-HCl containing 0.003% hydrogen peroxide. The sections were then counterstained with hematoxylin, washed in running water, dehydrated through an increased ethanol series, and mounted in Entellan (Merck).

Lectin cytochemistry was also performed by use of a Vectastain avidin-biotin peroxidase complex (Elite ABC) kit. The staining procedures were followed as those of above-mentioned immunostaining, but in the case of Con A staining, 0.05 M Tris-HCl buffered saline (pH 7.6, TBS) was used as a substitute for PBS. Thus, biotin-conjugated lectins were applied for sections for about 12 hr, instead of primary antibodies and biotinylated secondary antibody. In control experiments of lectin cytochemistry, biotinylated lectins were preincubated with 0.1 M of the specific inhibitory sugar (see Table 1) for 1 hr before cytochemistry.

Table 1. Biotinylated lectins used in the present study

Acronym of lectins	Concentration (μg/ml)		Inhibitory sugar
	Tested range	Optimum	
BSL-I*	1	1	D-galactose
BSL-II	1	1	D-galactose
Con A	0.08–1	0.1	D-mannose
DBA	0.3 –1	0.5	GalNAc
DSL	1	1	GlcNAc
ECL	0.5–1	0.5	D-galactose
Jacalin	1	1	GalNAc
LCA	0.1–1	0.2	D-mannose
LEL	0.5–1	0.5	GlcNAc
PNA	0.07–1	0.1	D-galactose
PSA	0.2–1	0.25	D-mannose
RCA-I	0.5–1	0.5	D-galactose
SBA	0.25–1	0.5	GalNAc
SJA	1	1	GalNAc
STL	0.5–1	0.5	GlcNAc
UEA-I	1	1	L-fucose
VVA	0.3–1	0.5	GalNAc
WGA	0.1–1	0.16	GlcNAc
s-WGA	0.12–1	0.16	GlcNAc

*BSL-I, *Bandeiraea simplicifolia* lectin I; BSL-II, *Bandeiraea simplicifolia* lectin II; Con A, concanavalin A; DBA, *Dolichos biflorus* agglutinin; DSL, *Datura stramoniu* lectin; ECL, *Erythrina cristagall* lectin; LCA, *Lens culinaris* agglutinin; LEL, *Lycopersicon esculentum* (tomato) lectin; PNA, peanut agglutinin; PSA, *Pisum sativum* agglutinin; RCA-I, *Ricinus communis* agglutinin I; SBA, soybean agglutinin; SJA, *Sophora japonica* agglutinin; STL, *Solanum tuberosum* (potato) lectin; UEA-I, *Ulex europaeus* agglutinin I; VVA, *Vicia villosa* agglutinin; WGA, wheat germ agglutinin; s-WGA, succinylated wheat germ agglutinin.

Enzyme treatment of the paraffin sections

Some dewaxed sections were treated with trypsin before lectin cytochemistry (Brooks *et al*., 1997). For trypsinization, sections were incubated with 12.5 mg of trypsin (from porcine pancreas, 12,000–15,000 BAEE units/ mg, Wako Inc., Japan) and 100 mg of calcium chloride in 100 ml of TBS for 10 min at room temperature.

Evaluation of lectin cytochemistry in ACTH and MSH cells

Although many lectins gave positive reaction in the lamprey pituitary, only those, which gave positive reaction in the cytoplasm of most NHF-positive cells, were considered to be ACTH-positive lectins. Similarly, only lectins, which gave positive reaction in the cytoplasm of most MSH-B-positive cells, were considered to be MSH-positive lectins.

RESULTS

Three different types of glycoconjugate-positive cells were revealed by lectin cytochemistry. The first and second types of cells corresponded with ACTH and MSH cells, respectively. The third type of cells was stained positively by Con A and VVA, and was found in the ventral half of the proximal pars distalis (data not shown, see Nozaki *et al*., 1999). The last type of cells was also stained by several anti-LH-related gonadotropin (GTH) antibodies, and was considered to be GTH cells.

ACTH cells

Positive reaction to anti-lamprey NHF was found in most cells of the rostral pars distalis and a few cells scattering in the proximal pars distalis (Fig. 1a–b). These results agreed well with those reported by Sower *et al*. (1995) in the same species using the same antibody.

Glycoconjugate-positive reaction to WGA and s-WGA was found in most cells of the rostral pars distalis and a few cells dispersed in the proximal pars distalis (Fig. 1c–d).

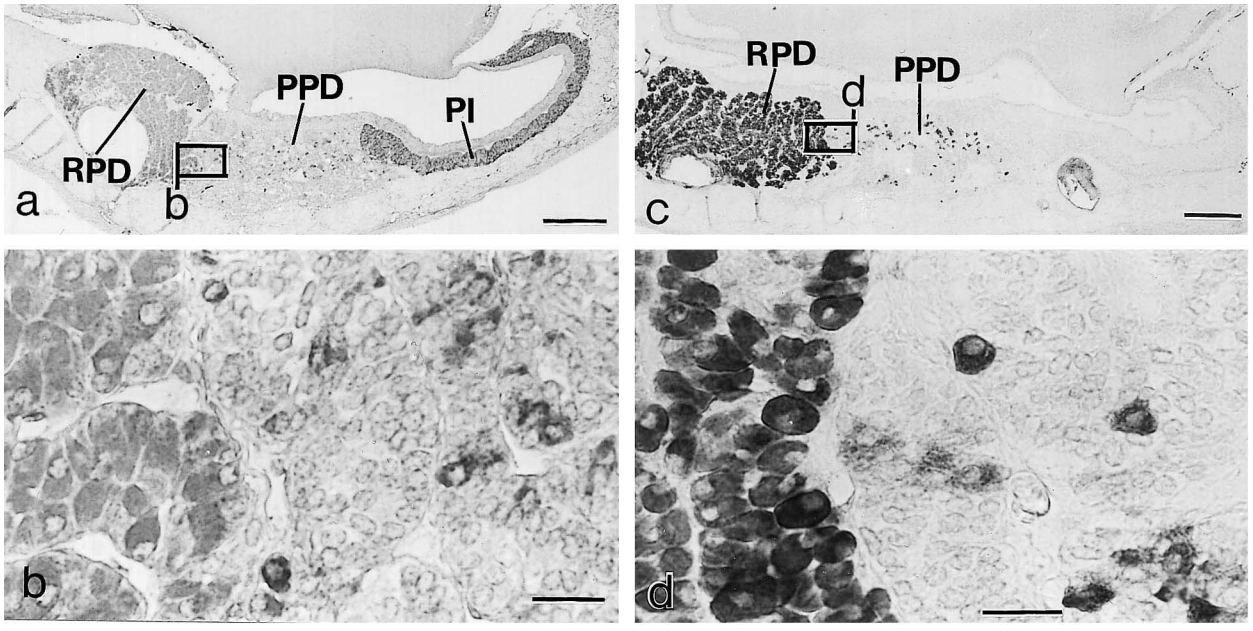

Fig. 2. Two sagittal sections (a and c) through the pituitary of adult sea lampreys, stained with (a) biotin conjugated-*Pisum sativum* agglutinin (PSA), and (c) biotin conjugated-*Sophora japonica* agglutinin (SJA), respectively. The areas outlined by rectangles in a and c are enlarged and shown in b and d. In a and b, note also PSA-positive reaction in almost all PI cells. PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis. Scale bars: a and c, 200 μm; b and d, 20 μm.

Table 2. Results on lectin staining in lamprey ACTH cells.

Lectins*	Inhibitory sugar	Reactivity	
		RPD**	PPD
WGA	GlcNAc	++***	++
S-WGA	GlcNAc	++	++
LEL	GlcNAc	+t)	+t)
SBA	GalNAc	++	++
SJA	GalNAc	++t)	++t)
DBA	GalNAc	+	+
VVA	GalNAc	+	+
LCA	D-mannose	++	++
PSA	D-mannose	+	++
ECL	D-galactose	+	+
BSL-I	D-galactose	+t)	+t)

*The remaining lectins listed in Table 1 gave no positive reaction in ACTH cells.
**RPD, Rostral pars distalis; PPD, Proximal pars distalis.
***++, Moderately intense reaction; +, Weak reaction.
t)Positive reaction after trypsin treatment.

These two lectins-positive cells were identical with those stained by anti-lamprey NHF (Fig. 1a–d). A faint to moderately intense glycoconjugate-positive reaction was found in NHF-positive cells in both the rostral and proximal pars distalis by SBA, DBA, VVA, LCA, PSA and ECL (Table 2 and Fig. 2a–b). After trypsinization of sections, NHF-positive cells in both the rostral and proximal pars distalis were also stained by LEL, SJA and BSL-I (Table 2 and Fig. 2c–d). Thus, no difference in lectin reactivity of ACTH cells was detected between the rostral and the proximal pars distalis.

MSH cells

As we have previously demonstrated (Nozaki *et al*., 1995), positive reaction to anti-lamprey MSH-B was found in almost all cells of the pars intermedia (Fig. 3a–b). DBA-, VVA-, PSA- and PNA-positive reaction was found in MSH-B-positive cells (Table 3, Fig. 2a–b, 3c–d, 4a–b). After trypsinization of sections, cells in the pars intermedia were stained positively by LEL, SBA and BSL-I (Table 3, Fig. 4c–d).

DISCUSSION

In the present study, ACTH cells, which were stained by anti-lamprey NHF (Sower *et al*., 1995; Nozaki *et al*., 1995), were also positive for N-acetylglucosamine binding lectins (WGA and s-WGA), N-acetylgalactosamine binding lectins (SBA, SJA, DBA, and VVA), D-mannose binding lectins (LCA and PSA) and D-galactose binding lectins (ECL). On the other hand, MSH cells immunopositive for anti-lamprey MSH-B (Nozaki *et al*., 1995) were stained by N-acetylgalactosamine binding lectins (DBA, SBA and VVA), D-mannose binding lectin (PSA) and D-galactose binding lectins (PNA and BSL- I). These results suggested that ACTH and MSH cells produce different types of glycoconjugates which may be attributed to the difference in glycoconjugate moieties between the precursor proteins, POC and POM.

The present study confirmed that both precursors of lamprey ACTH and MSHs, POC and POM, have been

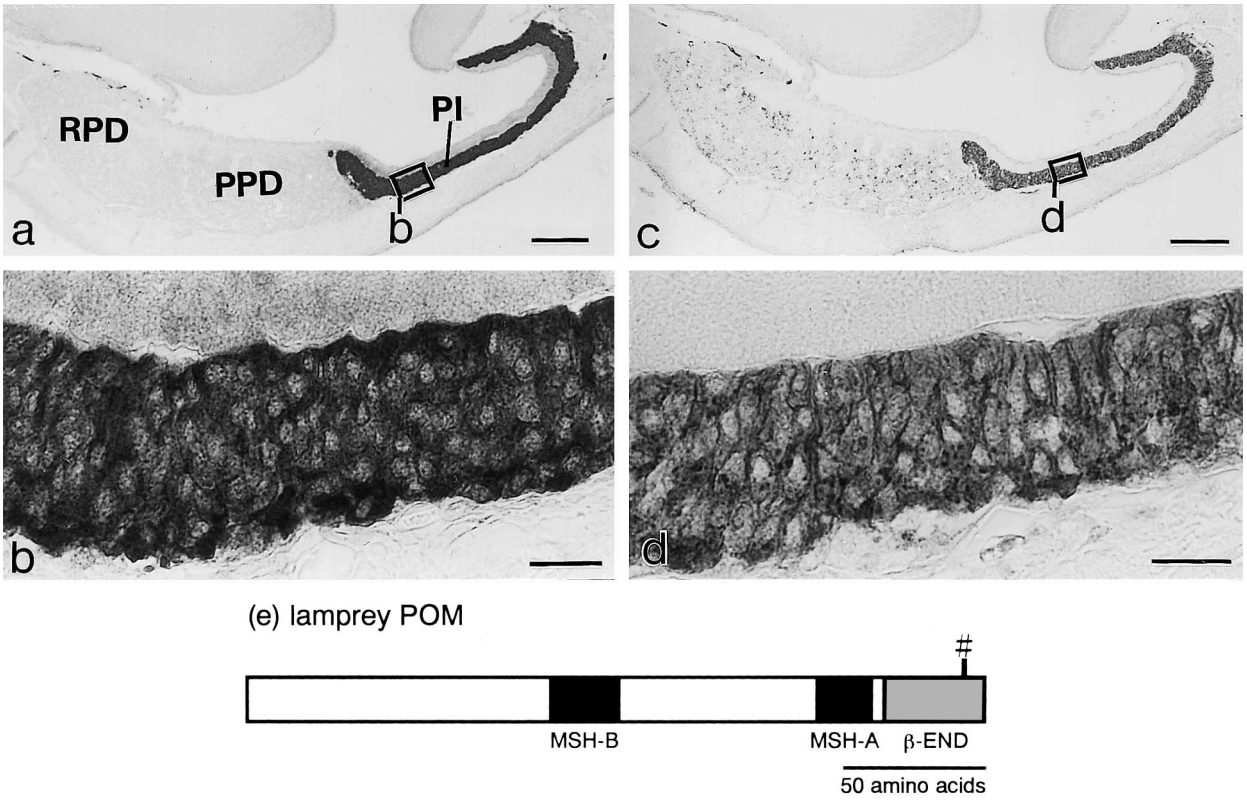

Fig. 3. Two adjacent sagittal sections (a and c) through the pituitary of an adult sea lamprey, stained with (a) anti-lamprey MSH-B, and (c) biotin conjugated-*Dolichos biflorus* agglutinin (DBA), respectively. The areas outlined by rectangles in a and c are enlarged and shown in b and d. (e), Schematic diagram of lamprey proopiomelanotropin (POM) showing the glycosylation site (#)(Takahashi *et al*., 1995b). PI, pars intermedia; PPD, proximal pars distalis; RPD, rostral pars distalis. Scale bars: a and c, 200 μm; b and d, 20 μm.

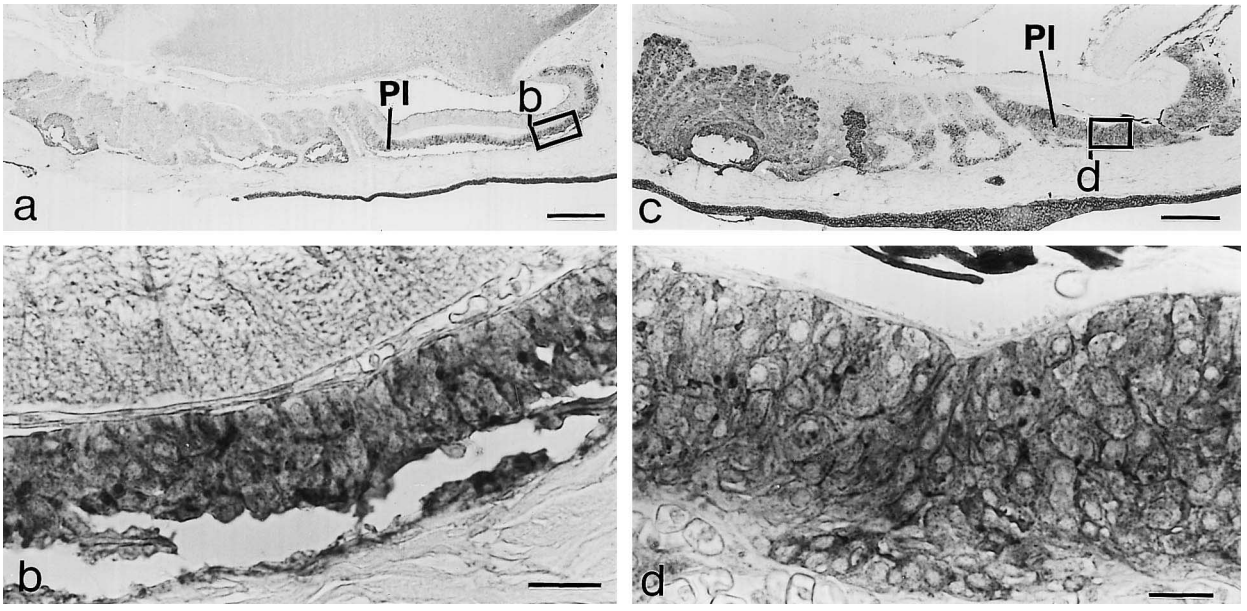

Fig. 4. Two sagittal sections (a and c) through the pituitary of adult sea lampreys, stained with (a) biotin conjugated-peanut agglutinin (PNA), and (c) biotin conjugated- *Bandeiraea simplicifolia* lectin I (BSL-I), respectively. The areas outlined by rectangles in a and c are enlarged and shown in b and d. PI, pars intermedia. Scale bars: a and c, 200 μm; b and d, 20 μm.

Table 3. Results on lectin staining in lamprey MSH cells.

Lectins*	Inhibitory sugar	Reactivity
LEL	GlcNAc	+**t)
DBA	GalNAc	++
SBA	GalNAc	+t)
VVA	GalNAc	+
PSA	D-mannose	+
PNA	D-galactose	+
BSL-I	D-galactose	+t)

*The remaining lectins listed in Table 1 gave no positive reaction in MSH cells.
**++, Moderately intense reaction; +, Weak reaction.
t)Positive reaction after trypsin treatment.

shown to be glycoproteins (Takahashi *et al*., 1995b). Indeed, NHF was isolated during an attempt to identify a GTH homologue in adult sea lamprey pituitaries, and was shown to be a dimeric glycoprotein (Sower *et al*., 1995). Previous immunocytochemical study in the lamprey pituitary also reported that ACTH-immunopositive cells were identical with basophils stained by AF and PAS in the rostral and proximal pars distalis (Nozaki *et al*., 1995).

In the present study, no difference in lectin stainability of ACTH cells was detected between the rostral and the proximal pars distalis. Thus, the present results contrasted to those of earlier studies using tinctorial stain, in which basophils (=ACTH cells) in the rostral and the proximal pars distalis were often described as different cell types, since they exhibited different affinity to AF and PAS (Hardisty and Baker, 1982). For example, basophils in the rostral pars distalis were found before metamorphosis, whereas those in the proximal pars distalis appeared at the end of metamorphosis (Hardisty and Baker, 1982). It was also reported that basophils in the proximal pars distalis degranulate during gonadal maturation and are relatively inactive in spent animals (Holmes and Ball, 1974; Hardisty and Baker, 1982). However, NHF-like and ACTH-like immunoreactivities are demonstrable in the younger ammocoetes, even when PAS reaction is completely absent from either cells of the rostral or the proximal pars distalis (Sower *et al*., 1995; Nozaki *et al*., 2001). Thus, affinity to PAS staining does not coincide with the NHF/ACTH-immunoreactivity in ACTH cells. It seems likely that basophilic substance reactive to PAS and/or AF may be related to the granular formation, but not to POC.

The pars intermedia cells of the lamprey pituitary were described as chromophobes or PAS-positive cells (Ball and Baker, 1969; Hardisty and Baker, 1982). The present study demonstrated that carbohydrate moiety was very similar among pars intermedia cells. Immunohistochemical study also showed that almost all cells of the pars intermedia were MSH cells (Nozaki *et al*., 1995). The difference in response to PAS reaction among MSH cells may reflect different stages in the secretory cycle of a single functional cell type.

Although POMC of most species has been proven to be a glycoprotein, the functional significance of the glycosylation of POMC has never been established. For example, there is no difference in the processing or secretion of peptides between non-glycosylated and glycosylated prohormones in mice (Jenks *et al*., 1985). Jenks *et al*. (1985) suggested that the presence of the carbohydrate on POMC might simply reflect the evolutionary history of the protein. Further studies are needed to know why POMC is evolved as a glycoprotein.

ACTH, MSHs, growth hormone (GH) and GTH appear to be major adenohypophysial hormones in the lamprey. Very recently, a cDNA coding conserved amino acid sequence in gnathostome GHs has been cloned from the sea lamprey pituitary, followed by the isolation of the mature protein from the sea lamprey pituitary (Sower and Kawauchi, 2001; Kawauchi *et al*., 2002). That protein was identified to be lamprey GH by demonstrating an increase of expression of lamprey IGF gene in the liver (Sower and Kawauchi, 2001; Kawauchi *et al*., 2002). Subsequent immunohistochemical study has revealed that GH-like cells are found in most cells dsitributed in the dorsal half of the proximal pars distalis (Nozaki *et al*., 2001; Kawauchi *et al*., 2002). Moreover, although GTH has not yet been isolated from the lamprey, GTH-like cells are found in most cells distributed in the ventral half of the proximal pars distalis of the sea lamprey pituitary (Nozaki *et al*., 1999). Those four types of cells occupy almost all parts of the sea lamprey adenohypophysis, and indeed there is little room for other cells in the pituitary. Lamprey GH is a single protein and does not contain glycoconjugate in the molecle (Kawauchi *et al*., 2002). Thus, the present lectin histochemistry demonstrated glycoconjugates in all cell types (ACTH, MSH and GTH) which contain glycoconjugates in the hormonal molecules in the sea lamprey pituitary (for GTH, see Nozaki *et al*., 1999).

Although different results were obtained among lectins, which share the same inhibitory sugar, the reason for such differences in responsiveness among lectins is not known at the present time.

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