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Presence of an 85 kDa Pituitary Protein (Pit-G) in Porcine GH-Producing Cells

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Abstract. In order to understand the molecular mechanisms responsible for hormone production by the anterior pituitary gland, information about novel molecules and their functions is required. We subjected proteins extracted from porcine pituitary crude nuclei to column chromatography and obtained a protein, Pit-G, with a molecular mass of about 85 kDa. An antibody against Pit-G was raised in a rabbit, and Western blot analysis using this antibody showed that Pit-G was present exclusively in the anterior pituitary gland, double immunocytochemical staining of which revealed that Pit-G-positive cells were localized only in growth hormone (GH)-producing cells. The result also indicated the presence of Pit-G in the cytoplasm. Interestingly, Pit-G was present in a subpopulation of GH-producing cells. We searched the literature for references describing molecular masses and localization of pituitary proteins, but found no information about proteins resembling Pit-G, which suggests that Pit-G is an 85 kDa pituitary protein that plays a specific role in some GH-producing cells.

Key words: GH, Pituitary, Protein purification

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The pituitary gland plays an essential role in vertebrates, controlling important aspects of development, reproduction, adaptation and endocrine homeostasis. Its anterior lobe is composed of distinct glandular cell types, which are defined according to the hormones that they synthesize and release. Hormone production requires the actions of several regulatory factors during pituitary development [1]. Hormones in definite cells are synthesized under the control of signals from the hypothalamus and the target

organs of the hormones [2]. External signals send their information by interacting with specific membrane or non-membrane receptors. The biochemical reactions of known and unidentified molecules induce the secretion of hormones, as well as the gene expression of hormones, by activating of intracellular signal cascades and/or bioactive molecules. Many proteins must be involved in the synthesis and secretion of pituitary hormones. Although numerous proteins, such as the hormones themselves, receptors, transcription factors and kinases, have been identified, our knowledge and understanding of their functions at

the molecular levels are incompleated.

Currently, we are performing experiments to separate nuclear proteins of the porcine anterior pituitary gland that are unique and/or DNA-binding proteins. We have obtained fractions some of which is consisting of protein in a homogeneous form. We have raised polyclonal antibodies against those fractions or proteins and characterized the antigen. By chance in one experiment, we found that one of the purified proteins with a molecular mass of 85 kDa showed a characteristic presence in the growth hormone (GH) producing cells, and the antigen of this 85 kDa protein was definitely found in the GH-producing cells. Accordingly, the 85 kDa protein was designated pituitary GH-producing cell-localized protein (Pit-G). In this paper, we describe the preparation and cellular and tissue localizations of Pit-G.

Materials and Methods

Materials

Porcine anterior pituitaries of Landrace-Yorkshire were obtained from a local slaughterhouse within 1–4 h after slaughter, frozen quickly with dry ice and stored at -80°C until required for use. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), antipain, aprotinin, chymostatin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and p-amidinophenyl methanesulfonyl fluoride hydrochloride (p-APMSF) was obtained from Wako Pure Chemical Industries Ltd. (Osaka). All the other chemicals used were of analytical grade. The antibodies against anterior pituitary hormones used for immunocytochemical analysis were anti-rat (r)-prolactin (PRL) (HAC-RT26-01RBP85), anti-r-GH (HAC-RT25-02RBP85), anti-r-thyroid stimulating hormone (TSH) (HAC-RT29-01RBP85) without cross-reactivity with other glycoprotein hormones at the dilution used in this study, anti-ovine-luteinizing hormone β subunit (LH β) (NIDDK, AFP-2-11-27), and anti-r-ACTH [3].

Preparation of pituitary proteins

Protein extracts from crude preparations of porcine anterior pituitary nuclei were prepared as described by Miskimins *et al.* [4]. Three-hundred porcine anterior pituitaries were homogenized in 15 ml buffer comprising 10 mM HEPES, pH 7.9, 500

mM sucrose, 100 μM EDTA, 5 mM MgCl_2 , 0.5% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 50 mM NaCl, 5 $\mu\text{g}/\text{ml}$ antipain, 2 TIU/ml aprotinin, 2 $\mu\text{g}/\text{ml}$ chymostatin, 5 $\mu\text{g}/\text{ml}$ leupeptin and 200 μM p-APMSF. The homogenate was centrifuged at $25,000 \times g$ for 10 min at 4°C and the resulting pellet was suspended in an equal volume of 10 mM HEPES buffer, pH 7.9, containing 600 mM NaCl, 5 mM spermidine and 200 μM p-APMSF, followed by stirring for 1 h and centrifugation at $25,000 \times g$ for 30 min at 4°C . The supernatant was dialyzed against 10 mM HEPES, pH 7.9, containing 1 mM MgCl_2 , 10% (v/v) glycerol, 50 mM NaCl and 500 μM EDTA. Solid ammonium sulfate was added to the solution to produce 20% saturation and any precipitate was removed by centrifugation. More ammonium sulfate was added to the supernatant to produce 50% saturation, and the precipitate (about 45% of the extracted proteins) was recovered and dialyzed against heparin buffer (10 mM HEPES, pH 7.9, 1 mM DTT, 10% (v/v) glycerol). Protein preparations from crude nuclei of several porcine tissues were also prepared using the above procedure.

Separation of pituitary proteins

Pituitary protein extracts were loaded on to a HiTrap heparin sepharose CL-6B column (18 mm ϕ $\times$ 26 mm long, Pharmacia LKB, Uppsala, Sweden) equilibrated with 10 mM HEPES buffer, pH 7.9, containing 50 mM NaCl, 1 mM EDTA and 500 μM p-APMSF and then washed with this buffer. The adsorbed protein was eluted with a linear gradient of 50 mM to 500 mM NaCl in the same buffer followed by one from 500 mM to 1.0 M. The fractions (500 μl each) eluted between 370 mM and 450 mM NaCl were collected, pooled and dialyzed against the above HEPES buffer. The dialysate was loaded on to a Protein pak G-DEAE column (82 mm ϕ $\times$ 75 mm long, Millipore, Bedford, MA, U.S.A.) and eluted with linear gradients of NaCl, as described above for heparin column chromatography. The fractions (500 μl each) eluted between 230 and 320 mM NaCl were collected, pooled, diluted with 10 mM HEPES buffer, pH 7.9, to 50 mM NaCl and re-chromatographed on the same column under the similar conditions.

Antiserum

The lymph node immunization method of Goudie *et al.* [5], which requires a very small

quantity of protein, was used for immunization with Pit-G. First, normal saline containing 2% (v/v) Evans blue dye was injected into the popliteal lymph-nodes (both legs, 200 μ l) of a 2-kg female rabbit to visualize a portion of the node and 1 h later, Pit-G (about 10 μ g) emulsified in Freund's complete adjuvant was injected into the popliteal lymph-nodes. Four weeks later, the rabbit was given Pit-G (about 5 μ g) by intradermal injection in the back. This was repeated three times every three weeks. Ten days after the final injection, the rabbit was bled from the common carotid artery. The serum was separated by centrifugation, and the anti-serum was prepared, enriched by ammonium precipitation of the immunoglobulin (Ig) G fraction and stored at -70°C until required for use.

Western blot analysis

Protein extracts (50 μ g protein/lane) were separated by 8% SDS-PAGE [6] and transferred to a nitrocellulose membrane filters (Advantec, Toyo Roshi Kaisha Ltd., Tokyo). The filters were blocked for 1 h with TBS (20 mM Tris, pH 7.5, 500 mM NaCl) containing 5% (w/v) nonfat dried milk (Difco Lab., Detroit, MI) and then reacted with the anti-Pit-G antiserum (diluted 1:400 or 1:4000) overnight with gentle agitation. After washing for 5 min with TBS containing 0.5% (w/v) Tween 20 and for 5 min twice with TBS, the filters were incubated for 1 to 2 h at room temperature with peroxidase-conjugated goat anti-rabbit Ig-G (Bio-Rad Laboratories, Richmond, CA) in TBS (diluted 1:1000). The filters were washed again, as described above, and the colorimetric peroxidase reaction was carried out using a Konica Immunostaining HRP Kit (Konica, Tokyo), according to the manufacturer's instructions. All the above steps were performed at 4°C .

Double immunocytochemical staining of anterior pituitary cells

The anterior pituitaries were fixed by immersion in Bouin-Hollande solution, dehydrated and embedded in paraplast. Five-micron thick sections were cut and mounted on gelatin-coated glass slides. The sections were deparaffinized and then incubated in a solution of 500 mM sodium metaperiodate in distilled water for 30 min to inhibit endogenous peroxidase activity. They were then rinsed with distilled water and then with phosphate-buffered saline (PBS), pretreated with

1% (v/v) normal horse serum in PBS followed by incubation with rabbit anti-Pit-G (1:2000 in PBS) and then with goat anti-rabbit Ig (1:5000 in PBS). All immunocytochemistry procedures were carried out at room temperature. The peroxidase anti-peroxidase complex (PAP) method [7] with different dilutions (1:400 and 1:4,000) of anti-Pit-G antiserum was employed. In order to detect other pituitary hormones, each section was regenerated with glycine buffer (100 mM glycine-HCl, pH 2.3, 100 mM NaCl) for 1 to 3 h at room temperature, neutralized by immersion in PBS and reacted with a series of different antisera, anti-o-LH β s (1:2000), anti-r-GH (1:2000), anti-r-PRL (1:2000), anti-r-ACTH (1:2000) and anti-r-TSH (1:5000) and finally, incubated with FITC-labeled rabbit anti-rabbit immunoglobulin (1:50). The regeneration with glycine buffer gave sufficient removal of the reacted anti-Pit-G antibodies from the sections (data not shown).

Results

Separation of nuclear proteins

This study was aimed to purify DNA binding proteins from the porcine pituitary nuclear extract and characterize the molecular properties of the purified proteins. We obtained several fractions which contained DNA binding protein(s) and other proteins. Some fractions consisted of a homogeneous protein, though they did not have DNA binding activity. In the following data, we describe one of the purified proteins with a molecular mass of 85 kDa. Fig. 1 shows a representative elution pattern of proteins isolated from porcine anterior pituitary crude nuclei. About 3% of the loaded proteins were recovered in the fractions eluted between 350 and 450 mM NaCl, as indicated in Fig. 1A. The fractions were separated further by G-DEAE column chromatography (Fig. 1B) and the fractions eluted between 230 and 320 mM NaCl were collected. Pit-G was obtained by re-chromatography on the same G-DEAE column with a slow NaCl gradient. As shown in the inset of Fig. 1C, a protein with a molecular mass of about 85 kDa was eluted mostly in a single form in the fraction numbers between 82 and 86.

Preparation of antiserum

An antiserum against the purified protein was

Fig. 1. Purification of Pit-G. A. Heparin-sepharose chromatography. B. G-DEAE sepharose chromatography. C. G-DEAE sepharose re-chromatography. Inset: SDS-polyacrylamide gel electrophoresis of Pit-G fractions (fraction number 80-86). The pooled fractions in each chromatogram are shaded.

raised by injection of the protein into the popliteal lymph nodes of a rabbit. The IgG fraction was obtained by ammonium sulfate precipitation and the molecular species of the antigen was analyzed by Western blotting. As shown in Fig. 2, the antibody reacted with a single protein of the crude nuclear extract as well as with the fraction loaded

on to the second G-DEAE column, indicating that the antigen was a single species.

Immunocytochemical analysis of the porcine anterior pituitary

Immunocytochemical staining using the anti-Pit-G antibody and other anti-hormone antisera was

Fig. 2. Western blot analysis using the anti-Pit-G antibody. A. Pituitary nuclear extracts. B. Pooled fraction after G-DEAE sepharose re-chromatography.

carried out to investigate the localization of the Pit-G antigen in the anterior pituitary. The results (Fig. 3) obtained after detection by the PAP (Pit-G) and fluorescence (antisera against 5 major pituitary hormones) methods revealed distinctly different localizations of Pit-G and the other hormones, except for the GH-producing cells. As shown in the top panels of Fig. 3, cells that immunoreacted with anti-Pit-G also reacted with the anti-GH antiserum. Interestingly, Pit-G immunoreactivity was not observed in all the cells that were anti-GH immunoreactive (Figs. 3 and 4), which indicates Pit-G was present in a subpopulation of the GH-producing cells.

Western blotting analysis of various porcine tissues using anti-Pit-G antiserum

Western blot analysis of protein preparations from porcine crude nuclei of intermediate and posterior pituitary lobes, anterior pituitary,

hypothalamus, cerebellum, cerebrum, liver, pancreas, heart, testis, spleen and kidney was carried out using the anti-Pit-G antibody diluted 1:4,000 (Fig. 5A) and 1:400 (Fig. 5B). Only the anterior pituitary extract incubated with the anti-Pit-G antibody at a dilution of 1:4,000 yielded a positive band. When the antiserum was used at a dilution of 1:400, some positive bands with different molecular masses also appeared in the preparations of several tissues, suggesting that they were non-specific or similar proteins that cross-reacted with the anti-Pit-G antibody.

When we examined the DNA binding properties using appropriately labeled gene fragments there was no evidence that Pit-G bound to DNA (data not shown). In addition, we have not succeeded in cloning Pit-G cDNA using our antibody.

Discussion

In this study, we examined the biochemical and cytological properties of a novel pituitary protein, Pit-G, the molecular mass of which is about 85 kDa. Immunocytochemical staining revealed an interesting localization pattern of Pit-G in the anterior pituitary, that is, only the GH-producing cells expressed this protein. Furthermore, although localization of GH and Pit-G corresponded, the Pit-G reactivity indicated that the population of GH-producing cells was heterogeneous. Western blot analysis indicated that Pit-G is present primarily in the pituitary, although it is possible that proteins with similar antigenicities and/or different molecular masses are present in other tissues. We could not find any information in the literature about proteins with similar biochemical properties and cellular and tissue localizations to Pit-G. Therefore, Pit-G is almost certainly a novel protein.

We obtained Pit-G from a porcine anterior pituitary extract prepared using crude nuclei. However, our preliminary Western blot analysis revealed this protein is in abundance in the cytoplasmic fractions of cell extracts (data not shown). Immunocytochemical staining (Figs. 3 and 4) also showed that Pit-G was present in the cytoplasm. Taken together, we might have obtained Pit-G present in the cytoplasm during the preparation of nuclear extracts, although the possibility that Pit-G located in nuclei does not react with this antibody still remains.

Fig. 3. Immunocytochemical staining of pituitary hormones and Pit-G in the porcine anterior pituitary. The regenerated sections were neutralized by immersion in PBS and reacted with a series of different antisera against rat (r)-GH (1:2000), o-LH β (1:2000), r-ACTH (1:2000), r-TSH (1:5000) and r-PRL (1:2000). The arrowheads indicate representative cells that reacted with the anti-Pit-G antibody.

Fig. 4. Immunocytochemical staining of GH and Pit-G in the porcine anterior pituitary. The arrowheads indicate GH-producing cells that did not react with the anti-Pit-G antibody.

Fig. 5. Western blot analysis using the anti-Pit-G antibody of porcine tissues. Nuclear proteins (50 μ g protein/lane) were separated by 8% SDS-PAGE, transferred to a nitrocellulose membrane, and Western blot analyses using the anti-Pit-G antibody diluted 1:4,000 (A) and 1:400 (B) were carried out. 1. Porcine intermediate and posterior lobes, 2. anterior pituitary, 3. hypothalamus, 4. cerebellum, 5. cerebrum, 6. liver, 7. pancreas, 8. heart, 9. testis, 10. spleen, 11. kidney.

We have carried out immunocytochemical analysis analyses of human and rat pituitaries (unpublished data), but, unfortunately, did not obtain positive results for Pit-G. This may be because the antigenicity of Pit-G is species-specific,

rather than because Pit-G is not present in other species.

As we have not succeeded in cloning Pit-G cDNA using our antibody, a substantial amount of information about Pit-G at the molecular level

remains to be discovered. Nevertheless, the presence of Pit-G in a subpopulation of GH-producing cells is quite interesting. The anterior pituitary regions are defined by the specific production of hormones by distinct cell types. Five major types of hormone-producing cells are generated from one cell lineage during pituitary development [8–12]. The cell lineage between GH-producing cells and PRL-producing cells was postulated on the basis of experiments on a transgenic animal transfected with a chimeric gene constructed by fusion with an upstream sequence of the GH gene and thymidine kinase [13]. PRL-producing cells appear after the development of GH-producing cells and originate from common stem cells. Furthermore, a GH-producing cell line, MtT/S, in an *in vitro* system retained the ability to differentiate into PRL-producing cells in response to growth factors [14]. It is noteworthy that both GH- and PRL-producing cells (somatomammotropes) are present in the anterior pituitary and several cell lines of pituitary lineage that produce both GH and PRL. These

circumstances provide interesting aspects to the study of the functions of Pit-G.

Although its biological function is unclear, Pit-G may play an interesting role in GH-producing cells. It is absolutely essential to elucidate the molecular structure of Pit-G. The antibody raised against Pit-G in this study appears to be a useful tool for investigating the biological functions of Pit-G and for obtaining its cDNA clone and protein.

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References

1. Treier M, Rosenfeld MG. The hypothalamic-pituitary axis: co-development of two organs. *Curr Opin Cell Biol* 1996; 8: 833–843.
2. Voss JW, Rosenfeld MG. Anterior pituitary development: Short tales from dwarf mice. *Cell* 1992; 70: 527–530.
3. Inoue K, Hagino N. Comparative immunocytochemical demonstration of ACTH-, LH- and FSH-containing cells in the pituitary of neonatal, immature and adult rats. *Cell Tissue Res* 1984; 235: 71–75.
4. Miskimins WK, Robert MP, McClelland A, Ruddle FH. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor gene. *Proc Natl Acad Sci USA* 1985; 82: 6741–6744.
5. Goudie RB, Horne CHW, Wilkinson PC. A simple method for producing antibody specific to a single selected diffusible antigen. *Lancet* 1966; 2: 1224–1226.
6. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680–685.
7. Sternberger LA, Hardy PH, Cuculis JJ, Mayer HG. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-horseradish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem* 1970; 18: 315–333.
8. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes Dev* 1990; 4: 695–711.
9. Japón MA, Rubinstein M, Low MJ. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J Histochem Cytochem* 1994; 42: 1117–1125.
10. Ma E, Milewski N, Grossmann R, Ivell R, Kato Y, Ellendorff F. Proopiomelanocortin gene expression during pig pituitary and brain development. *J Neuroendocrinol* 1994; 6: 201–209.
11. Ma E, Milewski N, Grossmann R, Ivell R, Kato Y, Ellendorff F. Expression of GH, TSH β , LH β and FSH β genes during fetal pituitary development in the pig. *Exp Clin Endocrinol Diabetes* 1996; 104: 464–472.
12. Granz S, Ellendorff F, Grossmann R, Kato Y, Mülbauer E, Elsaesser F. Ontogeny of growth hormone and LH β -, FSH β - and α -subunit mRNA levels in the porcine fetal and neonatal anterior pituitary. *J Neuroendocrinol* 1997; 9: 439–449.

13. **Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM.** Transgenic mice with inducible dwarfism. *Nature* 1989; 339: 538–541.
14. **Inoue K, Sakai T.** Conversion of growth hormone-secreting cells into prolactin-secreting cells and its promotion by insulin and insulin-like growth factor-1 in vitro. *Exp Cell Res* 1991; 195: 53–58.
15. **Tremblay JJ, Lanctôt C, Drouin J.** The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit-1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. *Mol Endocrinol* 1998; 12: 428–441.