Pituitary transcription factor Prop-1 stimulates porcine follicle-stimulating hormone β subunit gene expression

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Abstract

Molecular cloning of the transcription factor that modulates the expression of porcine follicle-stimulating hormone β subunit (FSHβ) gene was performed by the yeast one-hybrid cloning system using the −852/−746 upstream region (Fd2) as a bait sequence. We eventually cloned a pituitary transcription factor, Prop-1, which has been identified as an upstream transcription factor of Pit-1 gene. Binding ability of Prop-1 to the bait sequence was confirmed using recombinant Prop-1, and the binding property was investigated by DNase I footprinting, revealing that Prop-1 certainly bound to the large AT-rich region throughout the Fd2. Co-transfection of Prop-1 expression vector together with a reporter gene fused with Fd2 in CHO cells demonstrated an attractive stimulation of reporter gene expression. Immunohistochemistry of adult porcine pituitary confirmed the colocalization of the Prop-1 and FSHβ subunit. This study is the first to report that Prop-1 participates in the regulation of FSHβ gene. The present finding will provide new insights into the development of pituitary cell lineage and combined pituitary hormone deficiency (CPHD), since why the defect of Prop-1 causes CPHD including gonadotropins (FSH and LH) has yet to be clarified.

Keywords: Pituitary transcription factor; Prop-1; FSHβ; FSH; Transcription; Gene regulation; CPHD; LH; Pit-1; Pig

FSH is a pituitary glycoprotein hormone, including luteinizing hormone (LH) and thyroid-stimulating hormone (TSH). Each of these hormones shares a common α-glycoprotein subunit (α-GSU) and contains a unique β-subunit that confers physiological specificity on the respective hormone. The synthesis and secretion of FSH as well as LH are restricted to pituitary gonadotroph cells. The regulatory mechanism of gene expression of three subunits forming gonadotropin hormones is an interesting issue.

Several approaches have succeeded in providing an understanding of the molecular mechanisms and the transcription factors governing basal and cell-specific expression of the α-GSU and LHβ subunit genes [1–6]. However, for the FSHβ subunit gene, only a few lines of evidence for the basal expression have accumulated. We previously observed that GnRH significantly stimulates the expression of both c-Jun and c-Fos genes that form transcription factor, AP1 [7]. Later, it was revealed that the GnRH stimulation of FSH is mediated by the protein kinase C signaling cascade through AP-1 sites in the region of −120/−83 bp [8–10] and the distal region between −4152/−2878 and −2550/−1089 bp of the ovine FSHβ subunit gene [11]. Several steroid hormones, such as progesterone and estrogen, are known...
to suppress the expression of FSHβ subunit gene [12], but our previous analysis of porcine FSHβ subunit gene failed to identify typical responsive sequences for progesterone (PRE) and estrogen (ERE) [13]. Thereafter, multiple PREs are identified in the proximal region of the ovine [14] and rat FSHβ subunit gene [15]. Similarly, ERE was identified in −105/−72 of the ovine FSHβ subunit gene [16]. Interestingly, it was reported that the −270/−248 region of the rat FSHβ subunit gene is responsible for activin-dependent activation mediated by the transcription factors, Smad and Pitx2 [17]. A similar region is also responsible for Pitx1, the homologue of Pitx2 [18]. Nevertheless, the mechanism of cell/tissue-specific expression of FSHβ subunit gene remains unclear.

In our previous study [19], we found that multiple porcine nuclear proteins bind to the region (Fd2) of around 100 bp in length located between −852 and −746 bp upstream of the porcine FSHβ subunit gene. Hence, we have attempted to find the binding proteins by the yeast one-hybrid cloning system. The transcription factor eventually cloned was a paired-like homeodomain factor eventually cloned was a paired-like homeodomains domain factor (Pitx2) [20]. Our finding may provide novel clues to understand the development of gonadotroph cells and why a Prop-1 defect ablates gonadotroph cells as well as the Pit-1-dependent cell-lineage of GH/PRL/TSH-producing pituitary cells.

Materials and methods

Construction of reporter vectors and integration into chromosome of yeast YM4271. The reporter vectors, pHIS1-1 and pLacZi (Clontech, Palo Alto, CA), were digested with EcoRI and XhoI, and SmoI, respectively. The 5′ upstream region of the porcine FSHβ subunit gene, Fd2 (−852 to −746 bp), was ligated to each vector, resulting in Fd2pHIS1-1 and Fd2pLacZi, respectively. Fd2pHIS1-1 was linearized by XhoI and integrated into the chromosome of YM4271 (Clontech) generating YM4271::Fd2pHIS1-1. Fd2pLacZi was then linearized with SmaI and integrated into YM4271::Fd2pHIS1-1 generating YM4271::Fd2pHIS1-I(Fd2pLacZi). Stable genomic integrants were selected according to the manufacturer’s instructions.

One-hybrid screening of pituitary transcription factors binding to Fd2 region. The adult porcine pituitary cDNA library was constructed in pADGAL4 with the HybriZAP Two-Hybrid cDNA Gigapack Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions, as described previously [21]. The plasmid library was introduced into the yeast reporter cells, YM4271::[Fd2pHIS1-1[Fd2pLacZi]], by LiAcetate method, and yeast transformants were selected on synthetic medium lacking leucine, uracil, and histidine, but containing 3-amino-1,2,4-triazole (3-AT), to inhibit leaky expression of HIS3 integrated in pHIS1-1.

β-Galactosidase assay. The colony-lift β-galactosidase filter assay was performed for yeast cells which were grown at 30°C for 2 days on a Whatman No. 50 filter paper (Whatman International, Maidstone, England), followed by visualization as described previously [21].

Sequence analysis. DNA samples were prepared from Escherichia coli by the alkaline mini-preparation method and were employed in the fluorescence-labeled dye-terminator reaction using the Big Dye terminator system (Applied Biosystems, Foster City, CA), followed by analyzing on the ABI PRISM 310 (Perkin-Elmer Cetus, Norwalk, CT).

Production of recombinant protein. Porcine Prop-1 cDNA was cloned in-frame into the pET32a vector (Novagen, Darmstadt, Germany) and introduced into E. coli BL21 (DE3) Codon Plus RP (Stratagene). Trx-Tag and His-Tag-fused porcine Prop-1 protein was isolated after induction by 1 mM isopropyl thiogalactoside and purified by the His-Tag Mag beads (Toyobo, Tokyo, Japan).

Electrophoretic gel mobility shift assay. FAM-labeled DNA fragment was produced by PCR using FAM-labeled oligonucleotide 5′-primer. The binding reaction mixture included 10 fmol FAM-labeled probe DNA (1 ng) and 100 ng of porcine recombinant Prop-1 with 250 ng poly(dI-dC) in 10 μl of 10 mM Hepes buffer, pH 7.9, containing 0.4 mM MgCl2, 0.4 mM DT, and 50 mM NaCl, and 4% glycerol was incubated at 30°C for 30 min. Then samples were subjected to electrophoresis on a 4% polyacrylamide gel as described in the previous paper [21]. Trx-Tag and His-Tag-fused porcine Msx-1 protein (unpublished data) was used for the negative binding control.

DNase I footprinting assay. The 5′-labeled DNA was incubated with recombinant porcine Prop-1 protein in binding buffer under the same conditions used for electrophoretic gel mobility shift assay (EMSA). After a 20-min incubation at 30°C, 0.1 or 0.2 U DNase I (RQ1 RNase-Free DNase, Promega, Madison, WI) was added, and the mixture was incubated for 15 min at 25°C. The reaction was stopped by the addition of EDTA to a final concentration of 65 mM, and then proteins were removed by phenol-chloroform extraction. DNA fragments were precipitated, dissolved in 10 μl formamide containing 1 μl ROX-labeled GS-500 (Applied Biosystems) as a molecular size marker, and resolved in a Gene Scan analyzer equipped in ABI PRISM 310.

Expression vectors and secreted alkaline phosphatase (SEAP) reporter gene constructs. Porcine Prop-1 cDNA was excised by digestion with EcoRI and XhoI from cloned Prop-1-pADGAL4 plasmid and ligated into the EcoRI and XhoI site of mammalian expression vector, pcDNA3.Zeo+ (Clontech). The reporter gene constructs were generated by ligation of a upstream region of porcine FSHβ subunit gene into pSEAP2-Basic (Clontech) (F93-basic: −985/+10, AF3-basic: deletion mutant of F93-basic from −745 to −104 bp, and F95-basic: −238/+10) and pSEAP2-Promoter vectors (Clontech) (Fd2-promoter: Fd2, −852/−746).

Cell culture, DNA transfections, and reporter gene assays. Chinese hamster ovary cell (CHO) cells were cultured in F12 medium supplemented with 10% fetal bovine serum (CELLCept Gold FBS, ICN, Aurora, OH). Transfections of CHO cells were performed after 24 h from seeding approximately 1×10⁶ cells/100 μl/well in 96-well plates using FuGENE 6 (Roche Diagnostic, Indianapolis, IN). With 20 ng DNA/0.3 μl FuGENE 6/well. Then cells were incubated in 5% CO2/95% air for 24 h at 37°C. Each 5 μl of cultured medium was assayed for secreted alkaline phosphatase activity using the Phospha-Light Reporter Gene Assay System (Applied Biosystems).

Double immunocytochemical staining of porcine anterior pituitary cells. Fresh porcine pituitaries (7 months, male and female) were kindly supplied by Dr. M. Kikuchi of Kitasato University. Fixation, preparation of section, and staining were essentially performed as described previously [22]. Anti-FSH monoclonal antibody (Nichirei, Tokyo, Japan) diluted with 4 volume of PBS and anti-Prop-1 polyclonal antibody (5 ng/μl in PBS) originally raised by synthetic peptide were used. FSH was visualized by goat Cy3-labeled anti-mouse IgG (1:500), and Prop-1 was reacted with goat biotin-labeled anti-rabbit IgG (1:1000) followed by visualization with the TSA system (Perkin-Elmer Life Science, Boston, MA).
Results

Screening of porcine pituitary cDNA library

We carried out one-hybrid screening, in which transfectants of the yeast YM4271::[Fd2pHISi-1][Fd2pLacZi] harboring a porcine pituitary cDNA library in pAD-GAL4 (pAD-GAL4/cDNA library) were selected on SD-Ura-Leu-His agar plates containing 3-AT. Screening of $4.1 \times 10^6$ transformants at the efficiency of $2 \times 10^5$ colony forming units per 1 µg DNA yielded several presumptive positive clones. Eleven clones exhibited both specific amino acid requirement and β-galactosidase activity. DNAs of the clones were amplified in E. coli cells and re-transformed into YM4271::[Fd2pHISi-1][Fd2pLacZi] cells. The transformants exhibited the ability of growth on SD-agar plates lacking histidine, leucine, and uracil (Fig. 1 A) as well as the β-galactosidase activity (Fig. 1 B), indicating an interaction between the products selected by one-hybrid cloning and the Fd2 sequence of [Fd2pHISi-1] and [Fd2pLacZi] integrated into the yeast gene.

Nucleotide sequence of Prop-1

Three of the selected clones were almost completely identical to the porcine Prop-1 [23]. The nucleotide sequence of the cloned Prop-1 revealed the entire sequence of 967 bp with a poly(A) tract (DDBJ Accession No. AB187272). Open reading frame of 678 bp codes 226 amino acids with 50 and 3′ untranslated regions of 43 and 246 bp, respectively. There is no typical polyadenylation signal sequence, AATAAA. As described previously [20,23], the amino acid sequence of the central DNA-binding homeo-domain and the major trans-activation domain at the carboxyl terminus are well conserved, although the amino terminus is considerably changed among porcine, human, rat, and mouse Prop-1 as described in Sloop et al. [23].

In vitro binding assay by EMSA and DNase I footprinting

In the Fd2 region, there is one putative Prop-1-binding site, TAATTTA [20], between −836 and −827. To confirm the binding site of Prop-1 in Fd2, in vitro binding assay was performed by EMSA as well as DNase I footprinting.

EMSA demonstrated that the shift band generated by the addition of Trx-Tag and His-Tag-fused Prop-1 (Fig. 2, lane 2) migrated slightly faster by removal of the non-specific binding (lane 3) under the excessive poly(dI–dC) conditions (250-fold excess amount). Then, the binding was decomposed by the addition of an excess amount of unlabeled Fd2 (50-fold molar excess), forming multiple fast migrating bands (lane 4). On the other hand, the addition of Trx-Tag and His-Tag-fused Msx1 showed no shift band (lanes 6 and 7), indicating that not only Msx1 but also the Tag peptide has no binding affinity to Fd2 sequence.

DNase I footprinting demonstrated that many fragments covering the entire region of the Fd2 were generated by DNase I without recombinant Prop-1 (Fig. 3 upper panel). In contrast, the digestion in the presence of Prop-1 produced a marked change in the profile of the fragment. Apparently the signals of fragments between −846 and −778 had disappeared or decreased, except for the position at −797 bp. Downstream of the binding region, the signals of the sequence AGTT between −777 and −774 increased, but the other 3′ part did not change, presumably providing DNA-bending at this site to increase the nuclease sensitivity. Thus, Prop-1 protects the Fd2 fragment over the 60-bp region of −844/−780 which has 80% AT-content including a putative Prop-1-binding site.

Fig. 1. Amino acid requirements and β-galactosidase activity of yeast transformants. The amino acid requirements using SD-Ura-Leu-His agar plates (A), and β-galactosidase activity of transformants grown on SD-Ura-Leu agar plates (B) were examined. The transformants contained cDNA/pADGAL4 in YM4271::[Fd2pHISi-1][Fd2pLacZi] (a), pADGAL4 in YM4271::[Fd2pHISi-1][Fd2pLacZi] (b), cDNA/pADGAL4 in YM4271 (c), and YM4271::[Fd2pHISi-1][Fd2pLacZi] (d).

Fig. 2. Electrophoretic gel mobility shift assay of Prop-1. Complex with recombinant Prop-1 and FAM-labeled Fd2 was analyzed on 4% polyacrylamide gel followed by visualization with a fluorescence viewer. The composition of each binding mixture is indicated under the electrogram. Recombinant Msx-1, which does not bind to Fd2, was used as a negative control.
Transcriptional activation of the porcine FSHβ subunit gene by Prop-1 in CHO cells

To ascertain whether Prop-1 serves as a transcription factor for the FSHβ subunit gene, we investigated the transcriptional activity by co-transfection of Prop-1 expression vector and reporter vector fused with the upstream region of the FSHβ subunit gene into CHO cells.

Transfection of pSEAP2-Basic and pSEAP2-Promoter (SV40 promoter) with or without expression vector of porcine Prop-1 gave almost the same expression level of SEAP gene in CHO cells (Fig. 4), indicating the absence of endogenous activation by unexpected regulatory elements present in the vector construct. By transfection of the reporter vector fused with the −985/+10 region (Fβ3-basic), the expression level of the SEAP gene increased significantly by about 2.5-fold in the presence of the Prop-1 expression vector. The deletion of −745/−104 leaving Fd2 and the endogenous proximal promoter region (−103/+10 bp, ΔFβ3-basic) did not alter the activation by Prop-1. In addition, the −238/+10 region (Fβ5-basic) does not have any effect. In contrast, when the reporter vector fused with pSEAP2-Promoter with Fd2 was co-transfected with Prop-1 expression vector, transcriptional activity of Prop-1 was not observed, indicating the promoter-specific activation of Prop-1.

Fig. 4. Transcriptional activation of FSHβ gene in CHO cells. Porcine Prop-1 cDNA ligated in pcDNA3.1Zeo+ vector or pcDNA3.1Zeo+ vector alone was cotransfected into CHO cells with pSEAP2-Basic or reporter vector fused with region of −985/+10 (Fβ3-basic), −745/−104 bp deleted from Fβ3-basic (ΔFβ3-basic), and −238/+10 (Fβ5-basic). pSEAP2-Promoter fused with Fd2 (Fd2-promoter) was also co-transfected. After 48 h incubation, an aliquot of cultured medium was assayed for the alkaline phosphatase activity. Each activity was normalized using that of transfection with pcDNA3.1+ vector alone.
Double immunohistochemical staining

Double immunohistochemical staining using a porcine adult pituitary was performed for Prop-1 (Fig. 5, left) and FSH (Fig. 5, right). More cells were positive for anti-Prop-1 antibody than those for anti-FSH antibody. The cells positive for both Prop-1 and FSH staining are indicated by arrows. The cells positive only for anti-Prop-1 antibody are indicated by arrowheads.

Discussion

The present study demonstrated that a binding factor cloned by the yeast one-hybrid system using the 5′-upstream region of the porcine FSHβ gene (Fd2 region: −852/−746 bp) as a bait sequence is the pituitary-specific transcription factor, Prop-1, and that porcine Prop-1 certainly participates in the regulation of the FSHβ gene. Recombinant porcine Prop-1 binds to Fd2 fragment at multiple binding sites presented in the AT-rich sequence extensively expanded from −846 to −778 bp, including a consensus Prop-1-binding sequence of TAATtaATTA [20] (Figs. 3 and 4). In CHO cells, the expression of reporter gene fused with 5′-upstream region of the porcine FSHβ gene (−985/+10 bp) was significantly activated (2.5-fold) with Prop-1 (Fig. 4). This transcriptional activation caused by Prop-1 is specific to the set of Fd2 sequence and endogenous FSHβ-promoter without relation to the intervening sequences (Fig. 4). Immunohistochemical analysis using a porcine adult pituitary shows the co-localization of Prop-1 and FSH, suggesting that Prop-1 participates in the regulation of FSHβ gene expression in adult gonadotroph cells.

Here we have indicated for the first time that Prop-1 directly regulates the FSHβ subunit gene expression. Originally, Prop-1 had been positionally cloned as a gene apparently responsible for a heritable form of murine pituitary-dependent dwarfism (Ames dwarf, df). The gene was confirmed as a novel, tissue-specific, paired-like homeodomain transcription factor, termed Prophet of Pit-1 (Prop-1) [20]. Prop-1 heterodimerizes with Rpx/Hesx1, which expresses in the early pituitary development, and activates the Pit1 gene expression to result in the terminal differentiation of the Pit1-lineage of the thyrotrhop, somatotroph, and lactotroph cell types [20]. Both Ames df mice and human CPHD patients exhibit a reduction of Pit1-lineage cells and reduced gonadotropin levels [24–26]. This loss of Pit1-lineage cells is explained by a failure of initial proliferation of three Pit1-dependent cell types caused by the mutation of Prop-1 gene, but the reason for the amination of the gonadotroph cells remains obscure [25]. The present finding that Prop-1 participates directly in the FSHβ subunit gene expression may well provide a breakthrough for the understanding of the CPHD mechanism.

The ontogeny of the murine Prop-1 gene expression could first be detected at about embryonic day 10 (e10) preceding the formation of Rathke's pouch. The Prop-1 gene expression reached the maximum level by e12, followed by a decrease to an extremely low level by e14.5 with the distribution of the dorsal/ventral gradient [20]. During anterior pituitary development, the differentiation of hormone-producing cell types takes place with a temporal order in distinct regions. In the murine anterior pituitary, POMC is first detected at e12.5 on the dorsal side of the lobe towards the rostral tip when and where the expression level of the Prop-1 is high. TSH is detected at e14.5 in the central region, GH and PRL at e15.5 in the dorsal region, and finally LH and FSH at e16.5 and e17.5, respectively, in the ventral region where the expression level of Prop-1 is low [27]. Thus, Prop-1 plays its role for the determination and differentiation of pituitary hormone-producing cells by a spatially and temporally unique expression.

In this study we demonstrated for the first time that Prop-1 directly regulates the FSHβ subunit gene expression. However, the above observation that LH and FSH are detected in the region of low Prop-1 expression may indicate the importance for the differentiation of gonadotroph. Actually, the transgenic mouse that overexpresses Prop-1 gene shows the interference of anterior pituitary cell differentiation and the susceptibility for pituitary tumors, followed by a delay in the terminal differentiation of pituitary gonadotroph and transient hypogonadism [28]. On the other hand, it would be contradictory to hold that the mutation of Prop-1 gene found in Ames df mice [20] and human CPHD patients [25,26] triggers hypogonadism as well as the deficiency of GH, PRL, and TSH. These observations indicate that Prop-1 participates in the development of gonadotroph cells and gene expression of gonadotropins by altering the expression level spatially and temporally. At least in part the mechanism of hypogonadism in CPHD may be accounted for by the loss of Prop-1 function that regulates the FSHβ subunit gene expression as demonstrated by the present study. Interestingly, Sornson et al. [20] found that Prop-1 forms a dimer with Hesx1/Rpx, which inhibits the expression of Pit-1 gene.
More recently, a comparative functional analysis of Prop-1 protein revealed that two domains for transcriptional activation and repression are located separately in the carboxyl termini, and the amino termini and homeodomain, respectively [24]. The ability of Prop-1 to interact with other pituitary factors and the presence of multifunctional domains in its internal structure may explain the complexity and diversity of its biological functions in pituitary development and gene regulation.

The present study demonstrated a novel aspect of Prop-1 function that directly regulates porcine FSHβ subunit gene. Hence, we now seek to understand whether Prop-1 modulates other gonadotropin subunit genes, common α, and LHβ.

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References


