Molecular Cloning of Rat and Porcine Retina-derived POU Domain Factor 1 (POU6F2) from a Pituitary cDNA Library

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Abstract. Homeobox transcription factors are known to play crucial roles in the anterior lobe of the pituitary gland. During molecular cloning with the Yeast One-Hybrid System using a 5’-upstream region of the porcine Fshβ as a bait sequence, we have cloned a cDNA encoding a partial sequence of the retina-derived POU domain factor 1 (RPF1) from the porcine pituitary cDNA library and confirmed its specific binding to the bait sequence. In situ hybridization was performed to examine localization of Rpf1 and showed that this gene is expressed in the stem/progenitor cells of the rat pituitary primordium as well as the diencephalon and retina. In addition, real-time PCR demonstrated that Rpf1 transcripts are abundant in early embryonic periods but that this is followed by a decrease during pituitary development, indicating that this factor plays a role in differentiating cells of the pituitary. The transcriptional activity of RPF1 for genes of Prop1, Prrx2 and Prrx1, which were characterized as genes participating in the pituitary stem/progenitor cells by our group, was then examined with full-length cDNA obtained from the rat pituitary. RPF1 showed regulatory activity for Prop1 and Prrx2, but not for Prrx1. These results indicate the involvement of this retina-derived factor in pituitary development.

Key words: Differentiation, Gene regulation, Pituitary, POU6F2, Retina, RPF1

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transcription factors from uncharacterized clones screened by the Yeast One-Hybrid System using Fd2 as a bait sequence. The newly characterized gene, retina-derived POU domain factor 1 (RPF1; same as POU6F2), is a member of the POU-homeodomain transcription factors that was originally cloned from a human retina cDNA library. Specific binding of RPF1 to the AT-rich sequence and expression in the embryonic anterior pituitary were confirmed by DNase I footprinting and real-time PCR, respectively. We demonstrated using in situ hybridization that Rpf1 without a doubt plays a role by expressing in rat’s Rathke’s pouch.

Materials and Methods

Animals
Wistar-Imamichi rats were housed individually in a temperature-controlled room under a 12 h light/12 h dark cycle. The present study was approved by the Institutional Animal Care and Use Committee, Meiji University, based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Cloning of Rpf1 from porcine and rat pituitary cDNA libraries and construction of expression vectors

Cloning of porcine Rpf1 was performed by the Yeast One-Hybrid System from porcine pituitary cDNA using Fd2 (–852/-746 b) of the porcine Fshβ promoter as a bait sequence, as described previously [6]. Rat Rpf1 of the full-length clone was obtained directly by PCR amplification from a rat pituitary cDNA library using a specific primer set (forward, 5'-ATGATAGCTGGACAAGTCAGTAAGCCC-3', and reverse, 5'-TGCTTCCTTCTGATCTATGAACGGTGTG-3'). Each datum measured in the embryonic anterior pituitary were confirmed by DNase I footprinting and real-time PCR, respectively. We demonstrated using in situ hybridization that Rpf1 without a doubt plays a role by expressing in rat’s Rathke’s pouch.

Production of recombinant protein and electrophoretic mobility shift assay (EMSA)

Bacterial recombinant proteins were expressed using the PET32a vector in E. coli BL21-CodonPlus(DE3)-RIPL (Stratagene, La Jolla, CA, USA) with an Overnight Express Autoinduction System 1 (Novagen, Madison, WI, USA). The production of FAM-labeled DNA fragments from Fd2 was described previously [5]. EMSA was carried out as previously described [9], with a primary antibody for goat IgG against human SOX2 (1:500 dilution, Neuromics, Edina, MN, USA) and secondary antibody for Cy3-conjugated AffiniPure goat IgG (1:500 dilution; Jackson ImmunoResearch, West Grove, PA, USA).

Statistical analysis
All results are presented as the mean ± SEM of quadruplicate transfections in two independent experiments. The statistical analysis was assessed by Student’s t-test for independent groups and Dunnett’s test for the remaining groups. Differences between groups were considered to be statistically significant at a P value of less than 0.05.

Results

Cloning and characterization of porcine Rpf1
One-hybrid cloning performed for 4.1 x 10^6 transformants efficiently produced a 1 x 10^5 colony by forming units for each 1 μg of DNA, and ultimately yielded 11 clones by confirming a specific interaction with the bait sequence, Fd2. Confirmation of both amino
acid requirements (Fig. 1A) and expression of the reporter gene β-galactosidase (Fig. 1B) of yeast transformants was performed.

Two of the selected clones showed a very similar identity to the nucleotide sequence of mammalian Rpf1 (accession No. AB907853) but were missing a part of the N-terminal region. Subsequent re-screening and direct PCR using several newly developed cDNA libraries resulted in failure to obtain the full-length clone. Instead, a full-length Rpf1 cDNA was obtained by direct PCR for the rat pituitary cDNA library (accession No. AB907852), and the amino acid sequence was compared with those of pigs and humans (Fig. 2). Rat and human RPF1s had greatly similar sequences and were identical in the POU-specific domain and POU homeodomains (Fig. 2). Meanwhile, porcine RPF1 lacked half of the POU-specific domain in the carboxyl region, including a 36-amino acid insertion present in the human RPF1 [21], but had a conserved POU homeodomain for DNA binding (Fig. 2). Other clones selected during this procedure and described elsewhere were Prop1 [22], Prrx2 [5] and Lhx2 [6].

EMSA was carried out for FAM-labeled Fd2 with RPF1 in the presence and absence of unlabeled Fd2. Binding between a labeled probe and RPF1 gave remarkably large complexes at the top of the well (Fig. 3A). Those complexes were mostly dissociated by the addition of unlabeled probes in excess molar amounts. DNase I footprinting was then performed to examine the binding region of porcine RPF1. As shown in Fig. 3B, signals between –848/–800 were apparently protected by DNase I action, confirming the specific binding of RPF1 to Fd2. Notably, the region protected was different from those of PROP1, PRRX2 and LHX2 [5, 6, 22], which were cloned as Fd2-binding proteins showing different binding characteristics for each homeobox factor.

**Rpf1-expressions during rat pituitary development**

Real-time PCR during rat pituitary development demonstrated that Rpf1 expression was observed at a level of 0.024 against the TATA box binding protein (Tbp) early on E12.5, and gradually decreased by about 0.01-fold at P0 and 0.002-fold at P60, respectively, in the anterior lobe (Fig. 4). In the postnatal intermediate/posterior lobes, there was also a low level of Rpf1 expression.

**In situ hybridization**

In situ hybridization of Rpf1 was performed for the rat embryo’s cephalic portion at E16.5 and E13.5. While a sense probe did not show any positive signals, an antisense probe gave apparent positive signals in the retina at E16.5 (Fig. 5A), and this was where the presence of cells immunopositive for anti-human RPF1 antiserum in the mouse at E13-16 was reported previously [21], verifying the specificity of this probe. Under the same conditions as for the sections at E13.5, an anti-sense probe gave specific signals in most of the cells that composed Rathke’s pouch, i.e., a primordium of the anterior pituitary with low-level signals in those of the rostral tip, a prospective area of medial eminence at the caudal area, in addition to the surrounding cells of the diencephalon, the prospective posterior lobe (Fig. 5B). In addition, the immunohistochemistry of SOX2, a pituitary stem/progenitor marker, showed that SOX2-positive cells occupy all of the primordium cells except for those of the rostral tip (Fig. 5C).

**Promoter assay of Prop1, Prrx1 and Prrx2 with RPF1**

Rat Rpf1 full-length cDNA was cloned from a rat pituitary cDNA library by PCR using a specific primer set designed to accomplish
Fig. 2. Diagram of RPF1 and amino sequence comparison. In diagram (A), Gln-rich, proline, serine and POU-specific domains and POU homeodomains are indicated. The sequence identity (%) of the domain between the rat and human is shown for each domain (B). Amino acid sequences were compared. The POU-specific domain and homeodomain are boxed.

Fig. 3. Electrophoresis gel mobility shift assay and DNase I footprinting analysis of complexes with recombinant RPF1 and FAM-labeled Fd2. (A) Electrophoresis gel mobility shift was analyzed on 4% polyacrylamide gel followed by visualization with a fluorescence viewer. The composition of each binding mixture is indicated under the electrogram. The number indicates the molar excess amount. (B) DNase I footprinting analysis for a complex the same as the above. In the comparison between DNase I digests with (upper panel) and without (lower panel) RPF1, the protected region of Fd2 is indicated with a thick horizontal line together with the nucleotide sequence.
transcriptional activity. The clone showed conservation in the POU-specific and homeobox domains very similar to those of humans. A transfection assay in CHO cells was performed to examine whether the rat RPF1 regulates genes; Prop1, Prrx1, and Prrx2 were chosen, and we sought to determine if they might play important roles in the pituitary stem/progenitor cells [9, 15, 23, 24]. Rat RPF1 showed significant stimulation of Prop1 (−2997/+21), by 3.6-fold, and significant repression of Prrx2 (−372/+21), by 0.61-fold (Fig. 6), respectively, indicating that RPF1 has the potential ability to modulate expression of Prop1 and Prrx2, which are important transcription factors in pituitary stem/progenitor cells.

Discussion

In the present study, we first cloned the Rpf1 cDNA, which is known to express specifically in retina cells, from the porcine anterior lobe of the pituitary and confirmed specific binding to the bait DNA fragment. Ontogenic Rpf1 expression in the rat pituitary was high in an early embryonic period. In situ hybridization revealed that Rpf1 was expressed in most progenitor cells of the rat embryonic pituitary at E13.5. Using a full-length rat Rpf1 clone, we demonstrated that RPF1 has the potential ability to modulate expression of Prop1 and Prrx2, which are important transcription factors in pituitary stem/progenitor cells.

Rpf1 (POU6F2) was first cloned as retina-derived POU-domain factor-1 (aliases: POU6F2, RPF-1, WTS, WTL, Wilms tumor suppressor locus) [21]. Localization of RPF1 was first observed in the developing mouse retina at E11, at which time it localized to neuroblasts that migrated from the mitotic zone to the future ganglion cell layer. Hence, RPF1 is considered to be involved in the early differentiation of amacrine and ganglion cells. Since then, very few reports have been published regarding this factor. Cloning of this retina-specific transcription factor from the pituitary cDNA library was unexpected. However, several investigators postulated that the anterior pituitary and eyes have a close embryological relation. More than 25 years ago, a high-level of transient expression of δ-crystallin gene in the chicken Rathke’s pouch was reported [25], implying a close relationship of the cell’s state of development between the early lens and pituitary primordium. At the end of the last century, Kondoh et al. reported that mutations in gli-mediated hedgehog signaling in Zebrafish led to lens transdifferentiation from the adenohypophysis primordium [26]. Thereafter, it was reported that proper expansion of retinal and pituitary precursor cell populations is regulated with Six6/CKI regulatory network [27] and that Pitx3 defines an equivalence domain for the lens and anterior pituitary placodes [28]. Taken together, the lens and pituitary develop from the same cell lineage via the action of many signaling and transcription factors. RPF1 may play a role as one of the common factors, both
in the pituitary and lens.

Real-time PCR of the ontogenic expression and in situ hybridization of the rat embryo strongly suggests that RPF1 is involved in early development of the anterior pituitary, and modulates genes acting in pituitary stem/progenitor cells. We recently demonstrated that the transcription factors PROP1, PRRX1 and PRRX2 are present in pituitary stem/progenitor cells and are involved in the differentiation and development of pituitary hormone-producing cells [9, 15, 23, 24]. Moreover, transfection assays showed that RPF1 is able to modulate gene expression of Prop1 by stimulating it and that of Prrx2 by repressing it. Considering the results in the CHO cell line directly, RPF1, which is expressed in the early embryonic pituitary, may stimulate expression of Prop1 in pituitary primordium cells [29] and inversely repress that of Prrx2 appearing in a small number of postnatal pituitary cells [15]. Cloning of pituitary RPF1 thus provides a novel clue for clarifying the molecular mechanism of pituitary organogenesis. We observed that PRRX1 appears in embryonic pituitary stem/progenitor cells around day E15.5 in the rat by changing Prop1 expression [15], while RPF1 did not show any effect on the Prrxl promoter. Hence, the mechanism of Prrxl expression in the PROP1-positive cells remains to be clarified. Moreover, in the near future, it is an urgent issue to clarify the role of RPF1 during early pituitary organogenesis especially in the period of ectodermal invagination (E9-10) and PROP1 appearance (E11.5), and to examine whether SOX2 and RPF1 participate in the regulation of Prop1 in vivo. Additionally, expression of Rpf1 in the diencephalon, a presumptive posterior lobe first found in the present study, has not yet been investigated, and the role of RPF1 in this tissue remains to be clarified.

Alternative splicing variants of Rpf1 have been reported in retina transcripts [21]. In the present study, we obtained clones of the same length and sequence but a partial one from the pig pituitary cDNAs and a single PCR product from rat cDNAs. In addition, we confirmed that rat and human RPF1s are mostly of the same length and have highly homologous nucleotide and amino acid sequences. These results suggest the presence of tissue-dependent splicing in the Rpf1 gene. On the other hand, RPF1 has a bipartite DNA binding domain consisting of a POU-specific domain and a POU homeodomain, which are known to have overlapping recognition sequences but different sequence specificities [30]. The POU-specific domain of RPF1 has a 36-amino acid insertion in comparison with that of the typical POU-homeodomain factor OCT1, and thereby RPF1 may have a slightly different binding specificity. The left and right halves of the consensus sequence [a(a/t)TATGC(A/T)AA[T/a]t] are recognized by the POU-specific domain and POU homeodomain, respectively [30]. Although porcine RPF1 has a deletion of the carboxyl half of the POU-specific domain, the binding region identified by DNase I footprinting contains a very similar sequence to the OCT1 binding consensus in three segments, AAGGAGCTTAAT, AAATAGGTCTTAAT and TAATGGCTCAATT (Fig. 3B), respectively, indicating multiple bindings of RPF1 to the bait DNA fragment and an alternative explanation for the large complex formation in the EMSA (Fig. 2A). Moreover, since SOX2 is known to interact with POU domain factors [31], colocalization of RPF1 and SOX2 might indicate a possibility to cooperatively modulate expression of genes during pituitary organogenesis.

In summary, the present study identified the retina-derived POU domain factor RPF1 for the first time in the stem/progenitor cells of the anterior pituitary, indicating a close developmental relation between the pituitary and eye. Since POU domain factors including pituitary-specific transcription factor 1 (PIT1) are known to play a role in cell-type-specific differentiation, the finding of RPF1 in the pituitary might provide a clue to clarify the molecular mechanism of pituitary organogenesis.

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