Porcine sst1 can physically interact with other somatostatin receptors, and its expression is regulated by metabolic/inflammatory sensors

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Gabete MD, Durán-Prado M, Delgado-Niebla E, Garrido JJ, Rhodes SJ, García-Navarro S, Gracia-Navarro F, Malagón MM, Luque RM, Cañada JP. Porcine sst1 can physically interact with other somatostatin receptors, and its expression is regulated by metabolic/inflammatory sensors. Am J Physiol Endocrinol Metab 306: E483–E493, 2014. First published December 24, 2013; doi:10.1152/ajpendo.00587.2013.—The majority of the biological actions attributed to somatostatin (SST) are thought to be mediated by SST receptor 2 (sst2), the most ubiquitous sst, and, to a lesser extent, by sst5. However, a growing body of evidence suggests a relevant role of sst1 in mediating SST actions in (patho)physiological situations (i.e., endometriosis, type 2 diabetes). Moreover, sst1 together with sst2 and sst5 is involved in the well-known actions of SST on pituitary somatotropes in pig and primates. Here, we cloned the porcine sst1 (psst1) and performed a structural and functional characterization using both primary and heterologous models. The psst1 sequence presents the majority of signature motifs shared among G protein-coupled receptors and, specifically, among sst and exhibits a high homology with other mammalian sst1, with only minor differences in the amino-terminal domain, reinforcing the idea of an early evolutive divergence between mammalian and nonmammalian ssts. psst1 is functional in terms of decreasing cAMP levels in response to SST when transfected in heterologous models. The psst1 receptor is expressed in several tissues, and analyses of gene cis elements predict regulation by multiple transcription factors and metabolic stimuli. Finally, psst1 is coexpressed with other sst subtypes in various tissues, and in vitro data demonstrate that psst1 can interact with itself forming homodimers and with other ssts forming heterodimers. These data highlight the functional importance of sst1 on the SST-mediated effects and its functional interaction with different ssts, which point out the necessity of exploring the consequences of such interactions.

somatostatin receptor 1, promoter regulation; expression profile; heterodimerization; fluorescence resonance energy transfer; adenosine 3’,5’-cyclic monophosphate

**SOMATOSTATIN** (SST) is a peptide hormone mainly produced by neuroendocrine cells (18, 50). It was first isolated from the ovine hypothalamus (6), and soon thereafter isolated and sequenced in porcine hypothalamus (45). SST acts as an endogenous inhibitory regulator of various cellular functions, including hormone secretion, motility, and proliferation (5, 11, 26, 32, 36, 40). SST has two active forms (14 and 28 amino acids) with comparable subnanomolar affinity for a family of G protein-coupled receptors (GPCRs) widely distributed in the brain and periphery and named SST receptors (ssts) (34, 36). To date, five separate sst genes have been described (sst1-sst5), encoding six to nine different isoforms, depending on the species (10, 14, 16, 36). Although more than one sst subtype can be simultaneously expressed in certain cell types, the expression pattern and levels depend on the tissue, the age, and the physiological status (11, 29, 36).

The majority of biological actions associated with SST are thought to be mediated by sst2, the most ubiquitous sst, and, to a lesser extent, by sst5. However, a growing body of evidence suggests a relevant role of sst1 in mediating SST actions in physiological and pathophysiological situations. Indeed, it has been firmly demonstrated that sst1 acts as an autoreceptor in retina and several brain regions, such as the hypothalamus, basal ganglia, and possibly hippocampus (for review, see Ref. 48). SST has also been suggested as a therapeutic target for patients with endometriosis, endometriotic peritoneal lesions, and ovarian endometriomata since its expression is drastically elevated in these pathological situations (17). In addition, sst1 has also been linked to type 2 diabetes, since sst1 expression is lost in pancreatic α-cells of patients with type 2 diabetes, leading to a decreased inhibitory effect on glucagon secretion, which, in turn, can contribute to the hyperglycemia observed in type 2 diabetes (38).

In addition, sst1 is one of the ssts that has been involved in the paradoxical dual stimulatory-inhibitory action of SST on growth hormone (GH) secretion observed in various mammalian species such as pigs and primates, which is dependent on the dose and the pituitary cell subpopulation tested (8, 9, 28, 29, 39). Specifically, we have previously demonstrated that high doses of SST inhibit ghrelin- or GH-releasing hormone (GHRH)-stimulated GH secretion, whereas, surprisingly, low doses of SST can stimulate basal GH release from primary pituitary cell cultures of pigs and primates, being sst1 and sst2 mainly involved in the inhibitory response to SST, whereas sst5 is the major determinant of the atypical stimulatory action of this peptide on pituitary somatotropes (8, 9, 28, 29, 39). Moreover, our data indicate that the pituitary response to SST is markedly different depending on the somatotrope subpopulation studied [low-density (LD) vs. high-density (HD) subpopulations] and that this different response is, likely, due to the unique distribution and abundance of the ssts in both somatotrope subpopulations (28).

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For all the reasons mentioned above, in the present study we have used the porcine sst1 (psst1) as a model to perform the structural and functional characterization of this receptor. Specifically, we have cloned its entire coding sequence and studied its gene regulatory elements, its tissular and cellular distribution, and its functionality and interactions with other ssts in a heterologous system.

MATERIALS AND METHODS

Animals. All experimental procedures for animal care and experimentation were approved by the Ethical Committee of the Cordoba University. Pituitaries of 5- to 6-mo-old prepubertal female pigs (Large-White × Landrace) were obtained from a local abattoir. According to European Regulations for Animal Care, animals were killed by exsanguination after electrical stunning, and immediately decapitated. After extraction, the pituitaries were immediately placed in sterile cold medium (MEM; Sigma, London, UK) supplemented with 0.3% bovine serum albumin (Sigma) and stored at 4°C until cellular dispersion and culture.

Cell dispersion and subpopulation separation. Pituitary glands were dispersed to obtain a suspension of monodispersed cells following an enzymatic-mechanic process as previously reported (49). Part of the dispersed cells obtained from the whole pituitary was used in a Percoll density gradient centrifugation protocol (Amersham Pharmacia Biotech, Uppsala, Sweden) to separate both somatotrope subpopulations, LD cells and HD cells, as previously reported by our group (7, 49).

Cloning of the psst1 genomic sequence. Nucleic acids were extracted from swine pituitaries, using the Tripure Isolation Reagent (Roche Diagnostics Inc., Indianapolis, IN) to separate the somatotrope subpopulations, LD cells and HD cells, as previously reported by our group (7, 49).

Stable transfection and selection of monoclonal lines. CHO-K1 cells were cultured to semiconfluence on 12-well plates using F-12 medium, 1% of FCS, and 0.1% of antibiotic-antimycotic (Sigma) and transfected with 1.5 μg of the psst1-pCDNA3 recombinant vector, using Lipofectamine 2000 (GIBCO) according to the manufacturer’s instructions. After 24 h with the transfection solution, the medium was replaced by fresh F-12 containing 1 mg/ml of geneticin (G418; Gibco). One week later, surviving cells were detached and plated on 96-wells plates at a 0.7 cells/well density. Monoclonal cell lines expressing the psst1 were daily followed by phase-contrast microscopy.

Transient transfection for imaging studies and bioluminescence measurements. For transient transfection, HEK 293AD cells (kindly provided by Dr. E. Muñoz-Blanco, University of Cordoba, Spain) were used. Transient transfections were performed using Lipofectamine 2000 (GIBCO) following the manufacturer’s instructions. Transfected cells were used for experiments 24 h later. For cellular localization studies, HEK-293 AD cells growing on cover slips were transiently transfected with psst1 coupled to yellow fluorescent protein (YFP), psst2 coupled to cyan fluorescent protein (CFP), psst5 coupled to CFP, or combinations of psst1-YFP/psst2-CFP or psst1-YFP/psst2-CFP as described above and then fixed with 4% paraformaldehyde for 1 h after transfection. Images of transfected cells were acquired with a Leica Spectral TCС-SP2-408 confocal scanning microscope. For bioluminescence studies, cells were plated on six-well plates at a density of 104 cells/ml during 48 h and then transfected using 1 μg of each plasmid. Cells were used for assay 24 h after transfection. Specifically, cells were detached, rinsed twice in PBS, and centrifuged. The supernatant was removed, and cells were lysed by adding 50 μl of luciferase lysis buffer (25 mM Tris-HCl, 1 mM MgCl2, 1 mM DTT, 1% Triton X-100, and 15% glycerol). The measurement was carried out by mixing 50 μl of cell suspension with 50 μl of commercial luciferin (Promega) and measuring bioluminescence during 30 s in a bioluminometer Autolumat LB 9510 (Berthold, Germany).

TGTCGAGATGTG-3' and antisense: 5'-TTATAACGCCGTT-GACTGGTC-3') with no significant differences among tissues.

Bioinformatic analysis and sequential deletions of psst1 promoter. FASTA sequence of psst1 receptor promoter was analyzed using the AliBaba2 software to predict putative transcription factor binding sites, according to the TRANSFAC 6.0-Public website (a database of eukaryotic transcription factors) and default parameters (pairisms to known sites: 36; matrix width in bp: 10; minimum number of sites: 3; minimum matrix conservation: 70%; similarity sequence to matrix: 1%; factor class level: 5). The data obtained from the bioinformatics analysis served to design a set of seven upper primers and one lower primer used to generate the sequentially deleted fragments of the psst1 promoter. Specifically, full-length psst1 promoter was PCR amplified with the primers Prom sense: 5'-ATAGAGCTCGGT- TACGAGAGTTGGGGCGA-3' (position -1522 from the ATG no. AY138806) and Prom antisense: 5'-ATTTCGAGGGGAAACTCT-CAGTGTAGGG-3' (position +8 from the ATG no. AY138806) that contain the target sequences for the restriction enzymes SacI and Xhol, respectively. The rest of the upper primers were located between two predicted binding sites [forward (FW) 1 after GR + Pti-1: 5'- ATAGAATCTCCGAGAGAGGCTTT-3'; Fw2 after Gacac: 5'- ATAGAATCTCCGAGAGAGGCTTT-3']; FW3 after ETS: 5'-ATAAGGTCATGGGAGTGTACAGG-3'; FW4 after Oct-1: 5'-ATAAGGTCATGGGAGTGTACAGG-3'; FW5 after cAMP-response element (CRE)-binding protein (CRE-BP1): 5'-ATAGAATCTCCGAGAGAGGCTTT-3'; FW6 after Fos-related antigen 2 (Fra-2): 5'-ATAAGGTCATGGGAGTGTACAGG-3'. These primers were used in PCR-amplified sequentially deleted fragments of the psst1 promoter. These upper primers also contain the target sequences for the restriction enzyme SacI. The PCR fragments were gel purified and subcloned into the SacI and Xhol sites of the pGL3-Basic Vector (Promega, Madison, WI), a luciferase reporter vector, and subsequently used for transcriptional activity studies.

Tissue distribution of the psst1 gene sequence. In swine tissues was analyzed by RT-PCR using a cDNA panel (product length: 258 bp). Hypoxanthine phosphoribosyl transferase (Hprt) was used as an endogenous control. The PCR amplification of the Hprt gene was performed using the primers sense Hprt-3': 5'-CAGCTGATGGG-3' and antisense: 5'-AAAGTGTACAA-3'. The PCR products were gel purified and subcloned into the pGEM Vector, and subsequently used for transcriptional activity studies.
Bad Wildbad, Germany). All of the experiments were performed in triplicate, using the empty pGL3-Basic Vector as control.

cAMP measurements. To measure the intracellular accumulation of cAMP, transfected CHO-K1 cells were plated in six-well plates at a density of 10^6 cells/well in F-12 plus 10% FCS. After 2 h preincubation in FCS-free medium, cells were incubated during 30 min with F-12 containing 1 mM phosphodiesterase inhibitor, the 3-isobutyl-1-methylxanthine (IBMX; Sigma), to prevent the enzymatic degradation of cAMP. Next, cells were incubated for an additional 30 min in 1 mM IBMX medium with the respective treatments: SST at five doses (10^{-13}, 10^{-11}, 10^{-9}, 10^{-7}, and 10^{-6} M) in the presence or absence of 10^{-5} M forskolin (FK; an adenylyl cyclase activator) to study the effect on FK-stimulated cAMP level. The amount of cAMP was measured with a [^3]HjcAMP assay kit (Amersham Pharmacia Bio-

Fig. 1. Schematic representation of porcine somatostatin receptor type 1 (sst1) cloning process. Nucleotide sequence corresponding to the cloned porcine sst1 (psst1) gene and deduced amino acids sequence are represented. Predicted transmembrane domains (TMD) are shown in boxes. BAC, bacterial artificial chromosome.
The data are plotted as decrease of percentage of cAMP vs. control (being the control, the basal, or FK-stimulated level).

**Protein extraction and Western blot analysis.** Proteins from the whole pituitary cell culture, or from LD and HD cells (40,000 cells), were extracted after PBS washing and centrifugation of dispersed cells. The pellet was boiled for 5 min in water with proteases inhibitor (CLAP) and resuspended in Laemmli buffer. The protein concentration was determined with the Bradford method. Proteins were separated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Biorad NT; Pall, Madrid, Spain). Membranes were blocked with 5% nonfat dry milk and incubated with the primary sst1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and, subsequently, the secondary antibody (rabbit IgG against goat IgG conjugated with horseradish peroxidase; Santa Cruz Biotechnology). Immunoreactive bands were developed with ECL Plus (Amersham Biosciences) and exposed to an autoradiographic film (Kodak X-OMAT-XR; Kodak).

**Fluorescence resonance energy transfer measurements.** The interaction of the psst1 with itself or with other pssts was evaluated using the fluorescence resonance energy transfer (FRET) method, as described previously (13–15). Briefly, images of HEK-293 AD-transfected cells were acquired with an inverted Nikon Eclipse TE2000 E scope equipped with a 400 DCLP dichroic filter (Chroma) and recorded with an ORCA II BT digital camera, both controlled with the MetaMorph software (Imaging, West Chester, PA). Net FRET was calculated using the three filters method with the methodology developed previously (13–15). FRET efficiency was calculated in relation to the positive control consisting in a vector with enhanced CFP and showed previously (13–15). FRET efficiency was calculated in relation to the positive control consisting in a vector with enhanced CFP and enhanced YFP coupled in frame, which provided the upper FRET efficiency limit (100%).

**Statistical analysis.** Data are expressed as means ± SE obtained from at least three separate, independent experiments carried out in different days and with different cell preparations. Statistical analysis was carried out using Student’s t-test or nonparametric one-way ANOVA (Kruskal-Wallis test) followed by Dunn’s test for multiple comparisons. Differences were considered to be significant when P < 0.05.

**RESULTS**

**Psst1 shares the typical seven transmembrane domain GPCR structure.** The CDS of the psst1 gene (Gene Bank accession no. AY138806) was cloned and sequenced employing the techniques described above. An 1,173-bp DNA sequence encoding a putative protein of 390 amino acids was obtained (Fig. 1). The hydrophobicity profile of its amino acid sequence confirmed the presence of seven transmembrane domains (TMDs) spanned by short hydrophilic domains, which is the typical structure found in the GPCR family (Fig. 1).

A detailed analysis of the protein sequence revealed three putative N-glycosylation sites (Asp^5^, Asp^42^, and Asp^149^), located in the amino-terminal region of the protein. Two PKA-phosphorylation consensus sequences were found in the second and third intracellular loop (Thr^130^ and Ser^265^) and a PKC-phosphorylation consensus sequence in the third intracellular loop. Moreover, we identified some conserved GPCRs domains, in particular, GNorm in TMD1, NLAXAD in TMD2, TSX^1^LX^3^SX^8^SAX^7^DRY in DTM3, W^2^X^3^SX^7^P in TMD4, and FX^3^P in TMD5 (where X could be any amino acid) as well as two cysteine residues (Cys^1^28^ in the first extracellular loop and Cys^3^06^ in the second extracellular loop).

**The sst1 sequence is conserved.** Comparison of the nucleotide sequence obtained herein for psst1 with other previously published sst1 sequences (human, rat, mouse, sheep, dog, bull, and goldfish) showed a high degree of identity (data not shown). Importantly, amino acid sequence comparison showed a high level of identity between psst1 and the human, mouse, and rat sst1 (99, 98, and 97%, respectively), with some minor differences located mainly in the amino-terminus (data not shown). Alignment of psst1 protein sequence with that of other species (sheep, dog, bull, and goldfish) confirmed a highly conserved homology, especially among mammals (data not shown). Indeed, phylogenetic analysis using CLUSTAL W (MegAlign; DNA Star) showed an initial evolutionary divergence between mammalian and nonmammalian sst1 genes (data not shown). These data, therefore, suggest that psst1 is a good candidate to study the properties of mammalian sst1. Compared with other pssts, psst1 presents a higher homology with psst4 (data not shown), which is consistent with the fact that sst1 and sst4 comprise a subfamily within ssts.

**Multiple regulatory binding sites are predicted in the psst1 promoter.** The promoters of sst genes, including the psst1 cloned here, are characterized by the lack of TATA and CAAT boxes (2, 34). Alternatively, a binding site for the embryonic TEA domain-containing factor (ETF factor), which is known to exert similar functions (23), was found in the psst1 promoter (Fig. 2). As described previously in other species (3), bioinformatic analysis of psst1 promoter revealed the presence of putative binding sites for POU domain proteins such as the pituitary-specific transcription factor Pit-1 and the ubiquitous factor Oct-1. In addition, a putative proximal Pit-1-binding site was predicted by homology with the psst1 promoter of other species (Fig. 2). Our analysis also revealed the presence of a potential CRE-BP1 site, as well as putative glucocorticoid (GR) and thyroid (T3R) hormone-response elements in the psst1 promoter (Fig. 2). Finally, putative binding sites for NF-κB (REL family), PU.1 (ETFS family), and Fra-2 (AP-1 family, which overlaps with the proximal Pit-1 site) were also identified (Fig. 2).

**ETF, cAMP, NF-κB, and glucocorticoids are crucial regulators of psst1 expression.** To analyze the potential role that the regulatory-binding sites identified in silico play on basal psst1 expression, sequential deletions of the promoter coupled to transcriptional activity studies were performed as described above. Results revealed that deletion of the putative T3R, GR, and distal Pit-1a sites did not alter basal expression of psst1 promoter (Fig. 3A). In contrast, additional deletion of fragment containing NF-κB and PU.1 sites caused a 2.5-fold increase in promoter transcriptional activity that was reverted by the subsequent elimination of the ETF-binding site (Fig. 3A). Cells transfected with the mutant promoter in which the Oct-1 recognition site was also absent exhibited a twofold increase in the transcriptional activity. Finally, the subsequent deletion of the CRE-BP1 site caused an inhibition of the bioluminescent signal to basal level, whereas the additional elimination of Fra-2/Pit-1a-binding sites had no effect on promoter activity (Fig. 3A).

To specifically study the role of Pit-1, a pituitary-specific transcription factor with two putative binding sites in the psst1 promoter, on psst1 expression, HEK-293 AD cells were cotransfected with the psst1-luciferase construct plus a CMV-rat-Pit-1 vector. The porcine GH promoter (pGH) was used as a positive control since GH expression is known to be stimulated by Pit-1 (19). In every instance, a vector carrying only the luciferase gene (pGL3) was used as a negative control for basal bioluminescence measurements. Interestingly, Pit-1 did not
alter psst1 activity when both psst1 and Pit-1 were cotransfected in HEK-293 AD cells (Fig. 3B); however, as expected, Pit-1 increased pGH activity threefold under the same experimental conditions (Fig. 3B).

To analyze the effect of exogenous factors on psst1 promoter regulation, full-length psst1-luc-transfected HEK-293 AD cells were exposed, during 5 h, to glucocorticoids (dexamethasone, \(10^{-7}\)M), FK (\(10^{-7}\)M), tumor necrosis factor (TNF), and dexamethasone/TNF (Fig. 3C). Cells treated with dexamethasone exhibited a significant reduction of promoter activity, whereas cells treated with FK, an adenylate cyclase activator that stimulates cAMP-mediated gene expression by acting indirectly on the CRE-BP1-binding site (30), exhibit a clear increase of the bioluminescence signal. In contrast, TNF did not modify the observed activity but blocked dexamethasone-induced reduction.

Psst1 expression is widely distributed. RT-PCR analysis of a cDNA panel containing porcine endocrine and nonendocrine tissues showed that the ss1 gene is expressed mainly in intestine, lung, kidney, and testis, whereas it is absent in bone marrow and spleen (data not shown).

Psst1 signals through cAMP in response to SST. To confirm the functionality of the cloned psst1 receptor, CHO-K1 cells stably transfected with the psst1 were used to measure the inhibition of basal or FK-stimulated cAMP levels in response to SST treatment. Basal cAMP levels were significantly reduced by 40% at all the SST concentrations tested, from \(10^{-11}\) to \(10^{-6}\)M (Fig. 4A). In addition, SST was able to decrease the FK-stimulated cAMP levels in a dose-dependent manner (at concentrations ranging \(10^{-13}\) to \(10^{-6}\)M), showing a 25% maximal inhibition at the \(10^{-6}\)M dose (Fig. 4B). Dose-response results were fitted to a sigmoid dose-response curve showing an EC50 value of 9.13 pM (Fig. 4B).

Psst1 expression is spatially colocalized with other pssts at the cellular level. To determine the subcellular localization of the psst1, its CDS was coupled in frame to the amino-terminal domain of the YFP protein, and the recombinant construct was then transiently transfected into HEK-293 AD cells. The same proce-
dure was followed with the psst2 and psst5 coupled in frame to CFP. Confocal microscopy analysis of cells independently transfected with each recombinant psst showed a differential distribution of these receptors (Fig. 5A). Specifically, the psst1 shows a predominant intracellular localization (Fig. 5A, left), with a minor proportion of psst1 pool located at the plasma membrane, whereas both psst2 and psst5 are mainly located at the plasma membrane (Fig. 5A, middle and left, respectively).

The cotransfection of the psst1/YFP construct with psst2/CFP and psst5/CFP did not alter the localization of any of the receptors (Fig. 5B) but in both instances revealed a certain degree of colocalization of YFP and CFP signals at the plasma membrane level and also at intracellular compartments (Fig. 5B), indicating that, although psst1 and psst2/psst5 display a dissimilar preferential subcellular location pattern, they can colocalize at certain regions.

psst1 interacts with itself and other ssts forming homo- and heterodimers. To determine if the spatial colocalization between psst1 and other pssts (specifically, psst2 and psst5) translates into a physical interaction, we investigated whether psst1 can form dimers with itself (homodimers) and with other pssts (heterodimers) by transfecting HEK-293 AD cells with constructs containing two different ssts labeled with CFP or YFP and using FRET. As shown in Fig. 5C, a significant FRET signal was observed for psst1 homodimers (**P < 0.01 vs. pGH alone). In addition, psst1 was able to interact constitutively with psst2, psst3, native psst5, as well as with the truncated psst5 variants (psst5TMD6 and psst5TMD3) recently identified by our group (**P < 0.01, 0.05, and 0.001, respectively) (14), suggesting that psst1, under nonstimulated basal conditions, also interacts with other pssts (Fig. 5C).

Somatotrope cells are enriched with psst1. Previous results obtained by our group showed that psst1 mRNA is differentially expressed in somatotropes of LD and HD, which display distinct functional responses to SST (28). To verify whether mRNA expression levels are translated into a different psst1

Fig. 3. Analysis of the transcriptional activity of the psst1 gene promoter. A: relative expression of pGL3, full-length psst1-luc, and six 5'-deletion mutants [after Pit1a-psst1, after PU.1-psst1, after ETF-psst1, after Oct-1-psst1, after cAMP-response element-binding protein (CRE-BP1)-psst1, and after Fra2/Pit1a-psst1] was calculated by measuring the bioluminescence signal of transfected HEK-293 AD cell extracts. The luciferase activity of the full-length psst1-luc construct was set as 100%. *P < 0.05 vs. full-length psst1-luc. B: transactivation of the full-length psst1 promoter in response to Pit-1. HEK-293 AD cells were cotransfected with sst1-luc alone or combined with CMV-rat-Pit-1. As a positive control, growth hormone (GH)-luc was used, which is known to respond to CMV-rat-Pit-1. **P < 0.01 vs. pGH alone. C: transactivation of the full-length psst1 promoter in response to various transcription factors. HEK-293 AD cells were transfected with full-length sst1-luc and treated during 5 h with forskolin (10^-5 M), dexamethasone (DEX; 10^-8 M), tumor necrosis factor (TNF; 10 ng/ml), and dexamethasone plus TNF. *P < 0.05 and **P < 0.01 vs. control. In all cases, bioluminescence was measured during 30 s in a bioluminometer. Data are expressed as averages ± SE of 3–4 independent experiments.
protein distribution in porcine somatotrope subpopulations, we employed two methodological approaches. First, immunostaining of pGH and psst1 was carried out in the total pituitary population, as well as in both LD and HD subpopulations (Fig. 6A), using protocols previously validated (15) in which control experiments (i.e., no primary or secondary antibodies) exhibited the expected results (data not shown). This approach demonstrated that the psst1 protein was present in the majority (>80%) of GH-producing cells (Fig. 6B). However, a small proportion of psst1 immunostaining was found in other pituitary cell types (Table 1). Interestingly, the percentages of psst1-expressing somatotrope cells within the total population and the subpopulations of LD and HD were very similar (Fig. 6B). In addition, Western blot analysis revealed the existence of a unique band of 94 kDa, with the same migration characteristics in the whole pituitary cell culture than in LD and HD subpopulations (Fig. 6C). As expected, densitometric quantification of the bands showed that psst1 protein content was lower in the whole pituitary cell culture compared with LD or HD somatotrope subpopulations. Interestingly, psst1 levels were higher in the LD compared with the HD subpopulation (Fig. 6D).

DISCUSSION

In the present study, we cloned psst1 and demonstrated, by analyzing its full sequence and through phylogenetic analysis, that psst1 provides a valuable resource to understand the functional regulation of mammalian sst1. Specifically, psst1 shares the typical 7TMD GPCR structure, encompassing several structural/functional motifs within the psst1 TMDs that are similar to those found on numerous GPCRs (32, 36, 40) and have been suggested to be necessary to maintain the appropriate tertiary structure and confer the correct functionality to the receptors. For instance, two GPCR-characteristic cysteine residues located in the extracellular domains 1 and 2 were found in the psst1, which are essential for establishing the disulfide bridge necessary to maintain the tertiary structure on these receptors (12, 24, 46). In addition, the presence of putative glycosylation sites in the identified psst1 sequence, together with the fact that Western blots of native psst1 from pituitary cells revealed a protein size higher than that expected for this receptor according to deduced amino acid sequence, strongly suggests that psst1 could be constitutively glycosylated.

**Psst1 gene regulation.** Our data indicate that psst1 gene transcription is likely under the control of a complex pattern, involving regulation by several transcription factors [ETF, Pit-1, other POU-domain transcription factors (Oct-1)] and metabolic stimuli (cAMP, thyroid hormones, or glucocorticoids).

Similar to other sst gene promoters, the psst1 promoter lacks the typical TATA and CAAT boxes (2, 34). However, a binding site for the ETF, a TATA-less promoter-specific transcription factor implicated in neural development (23), was identified in the psst1 promoter. The observation that deletion of ETF-binding sites significantly decreases psst1 transcription clearly suggests that ETF could be a potent inducer of psst1 transcription. Additionally, our data suggest that other transcription factors may contribute to regulate psst1 expression in specific tissues. Thus, consistent with that previously described in other species (4, 34), psst1 promoter contains Pit-1-binding sites that could be key functional components for the basal gene expression of this receptor in the pituitary (4). To be more specific, a distal Pit-1-binding site was identified in a silencer region, whereas a proximal site was identified adjacent to an enhancer region, suggesting that Pit-1 could have a dual role in the transcriptional regulation of psst1. On the other hand, because expression of the sst1 gene is not restricted to the pituitary (32), other factors must regulate its specific transcription in Pit-1-lacking cells (2). In fact, our results demonstrate that elimination of an Oct-1 (a POU domain protein)-binding site in the psst1 promoter causes a twofold increment in luciferase transcription rate, which is consistent with previous data indicating that other POU domain transcription factors (Tst-1) can regulate human sst1 expression (3), and thereby reinforces the idea that POU domain proteins may contribute to sst1 gene expression in a cell-specific manner.

Potential (canonic) binding sites for GR and CRE-BP1 were also detected in the psst1 promoter, which may confer a metabolic responsiveness to the psst1 gene as has been found in other species (25, 27, 29, 34). Indeed, the synthetic gluco-
corticoid dexamethasone caused a significant decrease in psst1 promoter transcription, consistent with previous reports showing glucocorticoid treatment can cause a reduction in sst1 mRNA levels in rat pituitary cells (35). Interestingly, NF-κH9260 stimulation by TNF treatment was able to prevent the inhibitory action of dexamethasone on the psst1 promoter, likely through a physical interaction between NF-κH9260 and glucocorticoid receptors as previously shown (31, 47). These results suggest that these transcription factors could influence the regulation of sst1 expression in immune system cells where these receptors have been demonstrated to play a crucial role during inflammatory processes (1, 20, 21).

Interestingly, deletion of a potential CRE-BP1-binding site in the psst1 promoter caused a significant reduction in transcription rate, whereas treatment with FK, a receptor-independent cAMP activator, stimulated psst1 promoter transcription rate. This is consistent with our previous data demonstrating that FK increases psst1 mRNA level in cultured pig pituitary cells (29). Moreover, the fact that SST was able to inhibit basal cAMP level at all doses tested in cells transfected with the psst1 invite to suggest the existence of a cAMP-dependent autoregulatory mechanism for psst1 gene expression. In fact, in terms of cAMP levels, psst1 seems to be the most potent inhibitory sst receptor in swine, even more than the psst2 (13).

Fig. 5. Subcellular localization of psst1, colocalization with other ssts, and dimerization of psst1 with itself and other ssts. A: the images illustrate the subcellular localization of psst1/yellow fluorescent protein (YFP), psst2/cyan fluorescent protein (CFP), and psst5A/CFP-transfected HEK-293 AD cells. B: images show the colocalization of psst1/YFP and psst2/CFP (top) and psst1/YFP and psst5A/CFP (bottom) in HEK-293 AD cells transfected with the combination of two receptors (colocalization is depicted as green signal in merge images). C: measurements of fluorescence resonance energy transfer (FRET) efficiency of the psst1 homodimers and heterodimers with other ssts in HEK-293 AD cells. Cells expressing YFP and CFP coupled in frame within the same plasmid construct were used as positive control. Cells coexpressing YFP and CFP empty vectors were used as negative control. Data are expressed as averages ± SE of three or four independent experiments. *P < 0.05 and ****P < 0.001 vs. negative control. Scale bar corresponds to 5 μM.
These findings are further supported by our previous observation indicating that only a specific sst1 synthetic analog, and not other sst-specific compounds, was able to efficiently inhibit GHRH-stimulated GH secretion at high ($10^{-7}$ M) and low ($10^{-13}$ M) doses in porcine pituitary cells, where GHRH has been demonstrated to signal primarily through the cAMP pathway (28).

**psst1 coexpression and interaction with other ssts.** Analysis of psst1 mRNA distribution revealed its presence in several extrapituitary tissues such as testsis, kidney, lung, intestine, and lymphatic nodes. These results partially correlate with those reported in rat where sst1 expression was found in major gastrointestinal tract, kidney, cerebrum, lung, heart, and liver (2) and in human where sst1 is expressed in gastrointestinal tract, kidney, and testis (51), and altogether indicate that sst1 displays a widespread tissue expression pattern, which provides the basis for this receptor to mediate the response of a markedly diverse range of cell types to its endogenous ligands. Moreover, the present data, together with previous reports from our group, indicate that psst1 is coexpressed in intestine with psst2, native psst5, and truncated psst5TMD6 and psst5TMD3 (13, 14), in lung with psst2 and psst3 (13), in kidney with psst2, native psst5, and truncated psst5TMD3 (13, 14), and in tests with psst2 and native psst5 (13, 14). These data are consistent with findings obtained in humans, wherein hsst1 is coexpressed in intestine with hsst5 (33, 51) and in kidney with hsst2 (44, 51). Interestingly, our present and previous results indicate not only that the cellular distribution of psst1 overlaps with that of other pssts but also that psst1s are capable to oligomerize, generating homo- and heterodimers/multimers, which would support a plausible physical and functional interaction of ssts in the tissues where they are coexpressed. Of note, it has been demonstrated that oligomerization modifies the functional properties of ssts, such as ligand-binding affinity, internalization, signal transduction, and upregulation, in a receptor-specific manner (37, 41, 42). Specifically, previous results have shown that human sst1 is the only sst able to exist as a monomer irrespective of agonist-induced activation (42). However, hsst1 has also been shown to heterodimerize with other ssts such as hsst5 but not with hsst4 (22, 37, 42). Here, we extend those data and demonstrate, for first time, that psst1 can physically interact with itself and with other pssts, including the recently identified truncated psst5 receptors (14), suggesting that psst1 can be also constitutively forming homo-/heteromultimers in plasma membrane and exert a key functional role in the cells where it is present.

**pss1: Its role in the pituitary.** In previous reports, we have shown that psst1, psst2, and psst5 are differentially expressed in pituitary cells (whole pituitary cell culture, LD and HD cells). In particular, we found that psst1 and psst2 are more abundant in LD cells, whereas psst5 is more abundant in HD cells and that this differential expression in both somatotrope subpopulations might be associated with the dual stimulatory-inhibitory response of somatotrope cells observed in response to high/low doses of SST (28). Here, double immunostaining of psst1 and pGH indicated that $>80\%$ of somatotropes of the whole pituitary cell population expressed psst1 and also that a similar proportion of somatotropes of the LD and HD subpopulations expressed psst1, thus suggesting that there are no major differences in the number of cells expressing psst1 between the whole pituitary cell population or the HD and LD somatotrope.

### Table 1. Analysis of psst1 and pGH in porcine total pituitary and in LD and HD somatotrope subpopulations

<table>
<thead>
<tr>
<th></th>
<th>ICS</th>
<th>LD</th>
<th>HD</th>
</tr>
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<tbody>
<tr>
<td>psst1-Positive cells, %</td>
<td>$44.9 \pm 4.0$</td>
<td>$64.5 \pm 11.3$</td>
<td>$55.3 \pm 7.0$</td>
</tr>
<tr>
<td>Somatotrope psst1-positive cells, %</td>
<td>$81.8 \pm 4.8$</td>
<td>$87.2 \pm 2.6$</td>
<td>$87.4 \pm 2.4$</td>
</tr>
<tr>
<td>Nonsomatotrope psst1-positive cells, %</td>
<td>$9.4 \pm 6.7$</td>
<td>$7.9 \pm 6.7$</td>
<td>$4.0 \pm 1.2$</td>
</tr>
</tbody>
</table>

Values represent means ± SE. psst1, Porcine somatostatin type 1; pGH, porcine growth hormone; LD, low density; HD, high density; ICS, pituitary cells of the total population.
REFERENCES
M.M.M., R.M.L., and J.P.C. approved final version of manuscript.

DISCLOSURES
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AUTHOR CONTRIBUTIONS
The authors have nothing to disclose or conflict of interest.

AUTHOR CONTRIBUTIONS

REFERENCES
CHARACTERIZATION OF PORCINE sst1


