Truncated variants of pig somatostatin receptor subtype 5 (sst5) act as dominant-negative modulators for sst2-mediated signaling

Mario Durán-Prado,* Manuel D. Gahete,* Elena Delgado-Niebla, Antonio J. Martínez-Fuentes, Rafael Vázquez-Martínez, Socorro García-Navarro, Francisco Gracia-Navarro, María M. Malagon, Raúl M. Luque, and Justo P. Caño
to

Department of Cell Biology, Physiology, and Immunology, University of Córdoba, Reina Sofía University Hospital, Instituto Maimónides de Investigación Biomédica de Córdoba, and CIBER Fisiopatología de la Obesidad y Nutrición, Córdoba, Spain

Submitted 5 September 2012; accepted in final form 27 September 2012

Truncated variants of pig somatostatin receptor subtype 5 (sst5) act as dominant-negative modulators for sst2-mediated signaling. Am J Physiol Endocrinol Metab 303: E1325–E1334, 2012. First published October 2, 2012; doi:10.1152/ajpendo.00445.2012.—Somatostatin (SST) and its related peptide cortistatin (CORT) exert their multiple actions through binding to the SST receptor (sst) family, generally considered to comprise five G protein-coupled receptors with seven transmembrane domains (TMD), named sst1–sst5, plus a splice sst2B variant. However, we recently discovered that human and rodent sst5 gene expression also generates, through noncanonical alternative splicing, novel truncated albeit functional sst5 variants with less than seven TMD. Here, we cloned and characterized for the first time the porcine wild-type sst5 (psst5, full-length) and identified two novel truncated psst5 variants with six and three TMD, termed psst5TMD6 and psst5TMD3, respectively. In line with that observed in human and rodent truncated sst5 variants, psst5TMD6 and psst5TMD3 are functional (e.g., activate calcium signaling), selectively respond to SST and CORT, respectively, and exhibit specific tissue expression profiles that differ from full-length psst5 and often overlap with psst2 expression. Moreover, fluorescence resonance energy transfer analysis shows that psst5 truncated variants physically interact with psst2, thereby altering their localization at the plasma membrane and specifically disrupting the cellular response to SST and/or CORT. These results represent the first characterization of a key porcine SST receptor, psst5, and, together with our previous results, provide strong evidence that alternative splicing-derived, truncated sst5 variants with distinct functional capacities exist in the mammalian lineage, where they can act as dominant-negative receptors, by interacting directly with long, seven TMD variants, potentially contributing to modulate normal and pathological SST and CORT signaling.

somatostatin; cortistatin; somatostatin receptor; truncated receptor; dominant-negative receptor

SOMATOSTATIN (SST) is a neuropeptide that exerts its multiple actions, including inhibition of neuroendocrine secretions, neurotransmission, and regulation of gastrointestinal functions, through a family of receptors (sst) belonging to the superfamily of G protein-coupled receptors (GPCR) with seven transmembrane domains (TMDs) (24, 27). In mammals, the sst family comprises five distinct receptors (sst1–5) encoded by different, nonallelic, and intronless genes (24, 27, 28, 38) and, in some species, an alternatively COOH-terminal spliced isoform of the sst2, the sst2B, originated by processing of a cryptic intron located in the 3′-untranslated region (UTR) (28, 36). These receptors also mediate the effects of cortistatin (CORT), a peptide that shows a remarkable sequential and structural homology with SST (10). In fact, SST and CORT share many actions, especially their ability to regulate the secretion of several hormones (2, 16), which seems to be determined by the capacity of both peptides to indistinguishably bind and activate all five ssts (34). However, CORT also exerts unique functions, not shared with SST, at the level of the central nervous, the immune, and even at the endocrine system, which cannot be explained by the existence of the currently known ssts (8, 17, 18).

These and other unsolved questions associated with the SST/CORT/receptor pathophysiology have led several authors to propose the existence of additional receptors and/or mechanisms, either related or not to the sst family to explain their findings (3, 31, 35). In this context, our group recently identified the existence of novel truncated but functional sst5 variants in humans (13, 14) and rodents (7). These novel variants display less than seven TMDs and have been termed accordingly: sst5TMD5 and sst5TMD4 in humans; sst5TMD4, sst5TMD2, and sst5TMD1 in mice; and sst5TMD1 in rats. In spite of their truncated nature, these sst5 variants exhibit unique functional properties that contribute to mediate SST and CORT signaling in normal and tumoral cells. Specifically, some truncated sst5 variants (human sst5TMD4 and mouse sst5TMD2 and sst5TMD1) are present in pituitary tumors (7, 13, 14) and in a subset of poorly differentiated human breast tumors (12). Most importantly, we have demonstrated that overexpression of human sst5TMD4 in pituitary tumors confers resistance to treatment with SST analogs (14), which, as has been recently reviewed (6), provides novel insights on SST/CORT/receptor pathophysiology and suggests new research avenues for the therapeutic potential of this system.

Expanding the knowledge on these novel sst5 receptor variants would require exploring their possible existence and functional features in species other than the human, mouse, and rat. In this context, previous studies from our group showing that sst5- or sst5-related mechanisms may also be involved in mediating unique actions of SST and CORT in these species prompted us to investigate in more detail pig sst5 (5, 8, 16). Accordingly, in the present study, we describe for the first time the cloning, isolation, and characterization of the porcine sst5 gene (psst5), which, upon processing of two cryptic introns, originates three sst5 variants, the wild-type receptor with seven TMDs, and two truncated variants with six and four
TMDs, named psst5TMD6 and psst5TMD4, respectively. We have studied their functionality, tissue distribution, and the ability of the wild-type and truncated psst5 variants to physically interact and regulate the function of the main and more abundantly and ubiquitous psst, the psst2 (11).

MATERIALS AND METHODS

Cloning of the porcine sst5 isoforms. The complete coding sequence and the 5'- and 3'-UTR regions of the wild-type psst5 and truncated psst5 variants were obtained by random amplification of cDNA ends (RACE) PCR using the GeneRacer kit (Invitrogen, Barcelona, Spain) and specific primers designed on a partial psst5 sequence cloned in an earlier study (23). Briefly, total RNA was isolated from swine Large-White/Landrace pituitaries using Trizol (Invitrogen, Carlsbad, CA); mRNA was purified with the PolyATtract mRNA Isolation System II (Invitrogen) and specifically retrotranscribed for 5'- or 3'-RACE PCR following the manufacturer’s instructions. The 5'-UTR region was amplified using the Advantage II Taq Polymerase (BD Biosciences, San Jose, CA) in two nested PCR reactions. The first amplification was done with the primers pair AS_5'SST5 (Table 1) and GeneRacer 5'-Nested primer. The 3'-UTR region was amplified with the Certamp Complex Subsatrutes amplification kit (BioTools, Madrid, Spain). As the 5'-UTR, the 3' region was obtained in two nested PCR reactions using the S_3'SST5 (Table 1) primer combined with the GeneRacer 3'-primer for the first reaction and the S_3'SST5_NESTED (Table 1) and GeneRacer 3' Nested primer for the nested reaction. The resulting PCR fragments were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. Next, sequences were analyzed and assembled.

Table 1. Primers used to clone the porcine wild-type sst5 and truncated psst5 variants by RACE PCR

<table>
<thead>
<tr>
<th>Oligo Name</th>
<th>Oligo Sequence (5' to 3')</th>
<th>Reference Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RACE-PCR oligos</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS_5'SST5</td>
<td>754-GTTGCAGGTGTTCCAGGCTCCTC-732</td>
<td>AY156052</td>
</tr>
<tr>
<td>AS_5'SST5_NESTED</td>
<td>722-AAAAACAGCAGACGCACGGACACACG-699</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_3'SST5</td>
<td>681-GGACACCGTCATGCTCGA-704</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_3'SST5_NESTED</td>
<td>715-GGTTGTTGCGACACGTCAAGG-737</td>
<td>AY156052</td>
</tr>
<tr>
<td><strong>Cloning oligos</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_sst5_ATG</td>
<td>166-AGCCCCGTAGTGGCAGCTTTCGC-189</td>
<td>AY156052</td>
</tr>
<tr>
<td>AS_sst5_TGA</td>
<td>1272-CCTGCAGGAGGCTGGTTGC-1253</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_sst5_ATG_HindIII</td>
<td>AAAGTTGCGCAACGCGTGTCGAGA-CTC-1503</td>
<td>AY156052</td>
</tr>
<tr>
<td>AS_sst5_TGA_EcoRI</td>
<td>GAATTCGCGAGGCTGGTTGC-1494</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_sst5_polyA</td>
<td>1774-TTCAGGAGGCTGGTTGCACACAGA-1748</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_sst5_polyA_NEST</td>
<td>1758-AAAAGGGCAGCTTACACAGGCAAGA-1737</td>
<td>AY156052</td>
</tr>
<tr>
<td>AS_sst5_TAA_BamHI</td>
<td>AAGGATCCGTTAGCCGCGTTG-1662</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_p-5B_E1_blunt</td>
<td>1003-GGCAAGACAGAACGCGTGG-982</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_p-5C_E1_blunt</td>
<td>689-GGAGAAGAATGAGGCAAGGG-661</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_p-5B_C_E2_blunt</td>
<td>1711-CTGTTTCCGCGGCCGACCAG-1729</td>
<td>AY156052</td>
</tr>
<tr>
<td><strong>RT-PCR oligos</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_sst2</td>
<td>443-CTGCAGCCCCATCAAGTGGCGCAAG-456</td>
<td>D21338</td>
</tr>
<tr>
<td>AS_sst2</td>
<td>574-TGGCTCTTCCCCACAGGATTGCTGTCG-550</td>
<td>D21338</td>
</tr>
<tr>
<td>S_sst5</td>
<td>988-CATGCGCAAGCTTCGCAACCTG-1009</td>
<td>AY156052</td>
</tr>
<tr>
<td>AS_sst5</td>
<td>1140-CGGAGAGGCTGGTTGCACACAGA-1119</td>
<td>AY156052</td>
</tr>
<tr>
<td>S_sst5TMD6</td>
<td>254-CCTGCAGGCTGGTTCAGACAGG-295</td>
<td>DQ234797</td>
</tr>
<tr>
<td>AS_sst5TMD6</td>
<td>382-GAAAGAAGGCTTCGCAACCTG-360</td>
<td>DQ234797</td>
</tr>
<tr>
<td>S_sst5TMD3</td>
<td>523-GCTGCAGGCTGGTTCAGACAGG-543</td>
<td>DQ234797</td>
</tr>
<tr>
<td>AS_sst5TMD3</td>
<td>609-CAAGCACGAGGCTTCGCAACCTG-587</td>
<td>DQ234797</td>
</tr>
<tr>
<td>S_18S</td>
<td>340-CCTGCCAGAGCAGCGATGTC-362</td>
<td>G13619</td>
</tr>
<tr>
<td>AS_18S</td>
<td>454-TGGCTCTTCCCCACAGGATTGCTGTCG-476</td>
<td>G13619</td>
</tr>
</tbody>
</table>

To ensure high fidelity, the full sequences of psst5, psst5TMD6, and psst5TMD3 were amplified from genomic pig DNA using a Pfu Ultra polymerase (Stratagene, La Jolla, CA). Wild-type psst5 was directly amplified with specific sense and antisense primers designed flanking the ATG (S_sst5_ATG) and TGA (AS_sst5_TGA) (Table 1) and then subcloned into the HindIII and EcoRI sites of the pCDNA3+ vector (Invitrogen). For the truncated receptors, each exon (E1 and E2) was separately amplified using specific primers. Specifically, the E1 was amplified with a sense primer S_sst5_ATG_HindIII, common for both variants, and different antisense primers, AS_p-5B_E1_blunt and AS_p-5C_E1_blunt, specific for each truncated variant (Table 1). The E2 was amplified with common sense and antisense primers, S_p-5B_C_E2_blunt and AS_sst5_TAA_BamHI (Table 1). The E1 and E2 were ligated by their respective blunted ends and subcloned into the HindIII and BamHI restriction sites of the pCDNA3+ vector (Invitrogen).

Subcloning into the E-YFP/F46L-N1 and E-CFP-N1 vectors. The psst5 and both truncated variants, psst5TMD6 and psst5TMD3, were PCR amplified with primers lacking the TGA or TAA and then subcloned into the HindIII and BamHI restriction sites of the previously generated (11) E-YFP/F46L-N1 and E-CFP-N1 vectors, where the COOH-terminal domain of the receptor is fused in frame with the NH2-terminal domain of the fluorescent protein. Wild-type psst5 and psst2 were additionally inserted into the C1 vectors, where the COOH-terminal domain of the fluorescent protein is fused in frame with the NH2-terminal domain of the receptor. Constructs were sequenced to ensure high fidelity.

Screening of psst2, psst5, psst5TMD6, and psst5TMD3 expression in a porcine cDNA panel. The expression of wild-type psst2, psst5, and truncated psst5 variants was screened by RT-PCR using specific primers (Table 1) and cDNAs generated from 10 different porcine tissues (intestine, liver, heart, lymph node, smooth muscle, skin, etc.).
kidney, bone marrow, testis, and spleen) kindly provided by Dr. J. J. Garrido (Department of Genetics, University of Córdoba, Córdoba, Spain) as previously reported (20). The 18S ribosomal RNA was used as the internal standard gene.

**Cell transfection.** CHO-K1 cells were cultured to semiconfluence in 12-well plates using F-12 medium supplemented with 1% FBS and 0.1% antibiotic-antimycotic and transfected with 1.5 μg of the corresponding plasmids, using Lipofectamine 2000TM (Gibco, Barcelona, Spain). After transfection (24 h), media was replaced by fresh F-12 containing 1 mg/ml of geneticin (G418; Gibco). Media was replaced every 48 h for 1 wk, and surviving cells were detached, plated onto a flask, and grown until confluence, replacing the selective media every 4 days.

For transient transfection, 10⁵ cells were plated onto round cover slips, transfected with 1 μg of plasmid 48 h after plating, and used for imaging (see below) at least 24 h after transfection. Where appropriate, cells were fixed for 5 min in 4% paraformaldehyde, rinsed two times in PBS, and mounted onto a slide using Fluoromount (Molecular Probes, Eugene, OR).

**Measurement of free cytosolic calcium concentration in single cells.** CHO-K1 transfected cells were incubated for 30 min at 37°C with 2.5 μM fura 2-AM (Molecular Probes) in phenol red-free DMEM containing 20 mM NaHCO₃, pH 7.4. Cover slips were washed with phenol red-free DMEM and mounted on the stage of a Nikon Eclipse TE2000 E microscope (Nikon, Tokyo, Japan) with an attached back thinned-CCD cooled digital camera (ORCA II BT; Hamamatsu Photonics, Hamamatsu, Japan). Cells were examined under a ×40 oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 505 nm was measured every 5 s. Changes in free cytosolic calcium

---

**Fig. 1. Genomic and protein structure of the pig somatostatin receptor type 5 (sst5). A:** genomic structure of the porcine sst5 gene containing the truncated porcine wild-type sst5 (psst5) variants. Sequence of the junction between the cryptic exons (E1/2) to originate the CDS of the truncated psst5 variants with 6 (psst5TMD6) and 3 (psst5TMD3) transmembrane domains (TMD) is indicated. Accession nos. submitted to Genbank are indicated for each of the psst5 variants. UTR, untranslated region; CDS, coding DNA sequence. **B:** hydrophobicity profiles of the full-length psst5 and of the truncated psst5TMD6 and psst5TMD3 transmembrane domains (TMD) is indicated. Accession nos. submitted to Genbank are indicated for each of the psst5 variants. UTR, untranslated region; CDS, coding DNA sequence. **C:** predicted transmembrane domain structure of full-length and truncated psst5 variants is also represented (right). N, NH₂ terminus; C, COOH terminus.
concentration ([Ca^{2+}]_i) after 10^{-7} M SST or CORT administration were recorded as background-subtracted ratios of the corresponding excitation wavelengths (F340/F380) using MetaFluor Software (Imaging, West Chester, PA), as previously reported (7, 12). To determine the effect of psst5 variants on psst2-mediated signaling, CHO-K1 cells stably expressing psst2 were cotransfected with each psst5 variant tagged with yellow fluorescent protein (YFP). Calcium responses in single cells were related to the whole cell YFP fluorescence, and results were fitted to one-phase exponential decay curves using GraphPad Prism. Values for the percent calcium response at a low level of YFP-tagged receptors (R0), the percent calcium response at a high level of YFP-tagged receptors (R90), and the amount of YFP-tagged receptor to decrease the calcium response in 50% (R1/2) were calculated from each curve.

cAMP measurements. To measure intracellular cAMP accumulation, CHO-K1 cells stably transfected with each psst5 variant were plated in six-well plates at a density of 10^6 cells/well, in 2 ml of F-12-FBS. After 2 h of preincubation in serum-free media, cells were incubated for 30 min with F-12 containing 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Sigma) to prevent enzymatic degradation of cAMP. Next, cells were stimulated for an additional 30-min period with 10 μM forskolin alone or combined with 10^{-7} M SST or CORT. The amount of cAMP was measured with a [3H]cAMP assay kit (Amersham Pharmacia Biotech, Aylesbury, UK). Data are presented as percent of picomoles of cAMP per milligram protein vs. forskolin-stimulated cAMP levels.

Cellular localization and colocalization. Cells growing on glass cover slips were transiently transfected with each psst5 variant alone tagged with E-CFP or were cotransfected with the sst pairs psst2-CFP/psst5-YFP, psst2-CFP/psst5TMD6-YFP, psst2-CFP/psst5TMD3-YFP, psst5-CFP/psst5TMD6-YFP, and psst5-CFP/psst5TMD3-YFP. Transfected cells were fixed for 5 min in 4% paraformaldehyde, rinsed twice in PBS, and mounted on a slide using Fluoromount (Molecular Probes). Images were acquired with a Leica Spectral TCS-SP2-AOBS confocal scanning microscope (Leica, Heidelberg, Germany) with the cyan fluorescent protein (CFP) settings for single transfected cells or with CFP and YFP channels for cotransfected cells. Images of CFP and YFP channels were analyzed and merged with ImageJ (NIH, Bethesda, MD).

Fluorescence resonance energy transfer measurements. The interaction between the truncated psst5 variants, psst2 and psst5, as well as the interaction between psst2 and each psst5 variant (wild-type and truncated psst5) was evaluated by fluorescence resonance energy transfer (FRET), as described previously (11, 12). Images of cells transfected with each pair of receptors of interest 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 MetaMorph software (Imaging). Net FRET was calculated using the three filters method with the methodology developed previously (15). FRET efficiency was calculated in relation to the positive control consisting in a vector with E-CFP and E-YFP coupled in frame, which provided the upper FRET efficiency limit (100%). Images were acquired with the MetaMorph software and analyzed with ImageJ and Microsoft Excel.
analysis and coefficient calculation, background was always subtracted in each picture. A 1:1 E-YFP-to-E-CFP ratio and equal E-YFP and E-CFP intensities between all samples were used for FRET measurements. Representative analyzed net FRET images were generated with MetaMorph, using the coefficients calculated previously for the analysis.

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. For single cell analysis, a minimum of 14 total cells were analyzed. Mann-Whitney U-test analysis was used for nonparametric variables, whereas Student’s t-test was used for normally distributed variables. P values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism.

RESULTS

Isolation of psst5 variants using RACE-PCR. The full-length psst5 and both psst5 truncated variants, psst5TMD6 and psst5TMD3, were obtained using a two-round RACE PCR-based strategy with the primers listed in Table 1. With this approach, we obtained a single 5'-RACE PCR fragment, common for the three psst5 variants, containing the beginning of the coding sequence (CDS) and the 5'-UTR. In addition, we obtained three different 3'-RACE PCR fragments, one corresponding to the full-length and two smaller fragments matching to truncated variants. The full psst5 sequence (AY156052) comprises 1,845 bp; the first 172 bp corresponds to the 5'-UTR, from 173 to 1,288 to the CDS, and from that position to the end corresponds to the 3'-UTR (Fig. 1A). The psst5 CDS is a GC-enriched region (up to 70%) and encodes a protein of 371 amino acids (aa) that matches to a predicted structure of seven TMDs (Fig. 1B, top). Bioinformatic analysis of the sequences obtained by RACE PCR (using BioEdit Software and the GENSCAN Web Server) revealed that, like humans and rodents, truncated psst5 variants are generated by a nonclassical alternative splicing process. Specifically, psst5TMD6 is generated by a splicing event between cryptic donor (gctgcc) and acceptor (tggtcc) sequences located at positions 1,023 and 1,275 of the wild-type psst5 sequence, respectively. Similarly, psst5TMD3 is generated by a splicing event between a cryptic donor site (gctgcc) located at the position 708 of the wild-type psst5 and the same acceptor site in psst5TMD6. Thus, both truncated variants share the last region of their CDSs and 3'-UTR, which has been called exon 2 (E2; Fig. 1A). The CDS of psst5TMD6 (DQ234797) and psst5TMD3 (DQ234799) encode receptors of 301 and 196 aa, respectively. Tertiary structure analysis performed with the TMHMM Server version 2.0, which predicts the existence of TMDs based on their hydrophobicity profiles, revealed that truncated receptors present less than seven TMDs. Specifically, bioinformatic analysis of psst5TMD6 predicts a 6-TMD receptor with an extracellular 17 aa COOH-terminal domain (Fig. 1B, middle). In contrast, psst5TMD3 has three putative TMDs and a 51 aa intracellular COOH-terminal tail (Fig. 1B, bottom). Similar to the human sst5 variants, both psst5TMD6 and psst5TMD3 share the conserved DRY motif in the second intracellular loop but are truncated before the YANSCANPI/VLY motif that defines the family of mammalian sst receptors (27). Both porcine truncated receptors also lack the PDZ domain and the SRL motif, responsible of the interaction with scaffolding proteins (37). However, both truncated receptors share a putative SH3 binding domain, PRPRP motif, absent in the wild-type psst5.

Truncated receptors have a specific, distinct tissue expression pattern. The tissue expression pattern of the three psst5 variants and psst2 was studied by RT-PCR, using specific primer pairs listed in Table 1. Our results revealed that psst2 was expressed in all tissues analyzed; however, psst5 variants exhibited a distinct expression pattern, being coexpressed in some tissues (Fig. 2). Specifically, full-length psst5 is expressed in liver, bone marrow, spleen, and, to a lesser extent, in intestine, heart, kidney, and testis, whereas psst5TMD6 expression is restricted to intestine. Surprisingly, we found a widespread expression pattern of psst5TMD3, which is present in intestine, liver, heart, smooth muscle, skin, kidney, bone marrow, and, to a lesser extent, in lymph nodes and testis (Fig. 2).

Truncated receptors differentially respond to SST and CORT. To test the ability of each receptor to modulate [Ca2+]i, in response to SST and CORT, we employed CHO-K1 cells stably transfected with each receptor. SST and CORT evoked very similar [Ca2+]i rises (in terms of maximal response, time to maximal increase, and % of responsive cells) in CHO-K1 cells expressing the full-length psst5 (Fig. 3A, left). Specifically, this receptor-mediated calcium increases in response to SST or CORT of 205.9 ± 9.4 and 258.2 ± 28.1% over the basal level, with time to maximal response of 28.5 ± 3 and 38.6 ± 2.3 s in 35 of 176 cells (19.8%) and in 40 of 226 cells (17.6%), respectively (Fig. 3A, left). Cells expressing psst5TMD6 rapidly responded to SST, increasing the [Ca2+]i, in 197.2 ± 8.3% over the basal level at 22.8 ± 1 s, in 44 of 130 cells (34%) (Fig. 3A, middle). Conversely, only a small proportion of psst5TMD6 cells (6 of 92 cells, 6%) responded to CORT, producing a delayed [Ca2+]i increase of similar magnitude to that evoked by SST (212.9 ± 7.6%) (Fig. 3A, middle). Interestingly, an identical treatment with SST or CORT in
psst5TMD3-transfected cells induced the opposite response profile: CORT evoked a high and transient [Ca^{2+}]_i increase of 355 ± 88% over the basal level in 64 of 111 cells (57%) at 33 ± 2.6 s, whereas only 1% of the cells (3 of 271) responded to SST with a transient increase of 220.6 ± 23.3% at 24.8 ± 3 s (Fig. 3A, right).

The ability of SST and CORT to modulate forskolin-stimulated cAMP levels in CHO-K1 cells transfected with each receptor was also evaluated. SST decreases cAMP levels by 30% in the cells transfected with the full-length psst5, whereas CORT had no effect (Fig. 3B, left). In contrast, neither SST nor CORT affected forskolin-induced cAMP levels in cells transfected with psst5TMD6 or psst5TMD3 (Fig. 3B, middle and right, respectively).

Truncated receptors display a preferential intracellular localization. The subcellular localization of each psst5 variant coupled in frame to E-YFP was assayed by confocal microscopy. Analysis of cells transfected separately with each psst5 variant revealed that, similar to that previously observed for psst2 (11), full-length psst5 is preferentially located at the plasma membrane. In contrast, both psst5TMD6 and psst5TMD3 were preferentially located at intracellular compartments (Fig. 4).

psst5 variants colocalize and physically interact with psst2. The observation that truncated psst5 variants (specially psst5TMD3) can be coexpressed with psst2 in the tissues analyzed (Fig. 2) prompted us to ascertain their possible physical/functional interaction by using a heterologous cell model, CHO-K1 cells. Confocal microscopy using recombinant forms of these receptors tagged with CFP and YFP revealed that full-length psst5 and psst2 colocalized at the plasma membrane, similar to that observed when these receptors are expressed alone (Fig. 5A). Conversely, psst5TMD6 or psst5TMD3 significantly altered the localization of psst2 when expressed together (Fig. 5, B and C, respectively), retaining a proportion of psst2 in intracellular compartments (as indicated by the yellow color in the merged picture).

We also evaluated the ability of each one of the psst5 variants to physically interact (heterodimerize) with the most ubiquitously expressed receptor in pig tissues, the psst2, by measuring FRET between CFP- and YFP-tagged receptor pairs, using the method validated previously in our laboratory (11, 12). With this approach, we found that 51.2% of the full-length psst2 and psst5 form heterodimers when they are coexpressed in CHO-K1 cells (Fig. 6). Similarly, cotransf-

![Fig. 5. Colocalization between psst2 and psst5 variants. Images of CHO-K1 cells cotransfected with psst2/YFP (green) and each of the psst5 variants tagged with CFP (red). Colocalization is shown in yellow in the merge images. A: colocalization between psst2 and full-length psst5. CYP, cyan fluorescent protein. B: colocalization between psst2 and truncated psst5TMD6. C: colocalization between psst2 and truncated psst5TMD3. Arrowheads indicate regions where psst5TMD6 or psst5TMD3 significantly altered the localization of psst2.](E1330 TRUNCATED sst5 ISOFORMS ACT AS DOMINANT-NEGATIVE RECEPTORS)
...tion of psst2 with psst5TMD6 and psst5TMD3 resulted in 31.6 and 27.2% of heterodimers, respectively (Fig. 6).

Truncated receptors functionally interact with psst2. Having established that psst5 variants colocalize and physically interact with psst2, altering its subcellular localization, we sought to determine the consequences of their heterodimerization in terms of SST and CORT signaling. To this end, we quantified the ability of SST and CORT to modulate the [Ca\(^{2+}\)]

response of psst2-expressing cells to SST (being the % of calcium increase of 232 ± 10), as it is indicated by the resultant R\(0\) values equal to 174 ± 26 and 110 ± 9% for psst5 and psst5TMD6, respectively (\(P = 0.047\) and 0.042, respectively). On the other hand, low levels of psst5TMD3 did not significantly affect the response of psst2-expressing cells to SST (\(R_0 = 190 ± 12\%\)). Likewise, the response of psst2-expressing cells to CORT (being the % of calcium increase of 247 ± 18) was not affected by low levels of any of the three psst5 variants (Fig. 7, bottom).

High levels of expression of each psst5-tagged variant (\(R_\infty\)) inhibited the signaling of psst2-expressing cells in response to SST, being psst5TMD6 and psst5TMD3 stronger inhibitors than full-length psst5 (\(R_\infty = 0.3 ± 2, 2.9 ± 11,\) and 39 ± 26%, respectively; \(P < 0.001\) in all instances; Fig. 7, top). The response of psst2-expressing cells to CORT was also reduced by the presence of psst5 variants, with full-length psst5 and psst5TMD6 variant being stronger inhibitors than psst5TMD3 variant (\(R_\infty = -2.8 ± 2, 4.9 ± 5,\) and 35 ± 24%, respectively; \(P < 0.001\) in all instances; Fig. 7, bottom).

The value for R\(_{1/2}\) was also different for each psst5 variant and for each peptide. Specifically, the amount of psst5TMD3 receptor necessary to reduce by 50% the response of psst2-expressing cells to SST was double than the amount of full-length psst5 or psst5TMD6 variant (\(R_{1/2} = 301, 137,\) or 116 relative fluorescence units (RFUs), respectively; Fig. 7, top). Similarly, the amount of full-length psst5 receptor necessary to decrease in 50% the response of psst2-expressing cells to CORT was 4- or 2.5-fold higher than the amount of psst5TMD6 or psst5TMD3 variants necessary to mediate the same effect (\(R_{1/2} = 189, 24,\) or 115 RFUs, respectively; Fig. 7, bottom).

DISCUSSION

GPCRs comprise the largest protein superfamily and represent the main target for the pharmaceutical industry (33, 34). Originally, it was though that the structural signature of GPCRs was constituted by seven TMDs; however, over the last years several, different groups have identified and characterized new truncated GPCR variants with less than seven TMD displaying unique molecular/functional features that were difficult to predict using bioinformatics approaches (21). This is also the case for the sst receptor family, wherein SST analogs are frequently used to treat endocrine/tumoral pathologies. Indeed, our group has recently identified new truncated sst5 variants that contribute to increase the complexity, and likely to enhance the functional versatility, of the SST/CORT/sst signaling system. Specifically, we have isolated and characterized new truncated sst5 variants from rodents (7), humans (13, 14), and now from pigs, which exhibit overlapping tissue expression patterns with long sst isoforms while having peculiar signaling properties. Of note, human truncated receptors are involved in the resistance of pituitary tumors to the pharmacological treatment with SST analogs when coexpressed with full-length receptors (13) and...
have been found to be overexpressed in a subset of breast tumors with poor prognosis (12). In this work, we show for the first time the cloning and characterization of the psst5 and of two spliced variants with six and three TMDs, thus termed psst5TMD6 and psst5TMD3. We also demonstrate that these porcine truncated receptors are functional and have a distinct tissue-dependent expression pattern. Most importantly, this study indicates that truncated psst5 variants are able to colocalize and interact with the most ubiquitously expressed and, likely, the more relevant psst receptor from a pathophysiological point of view, psst2 (9, 39), which results in a marked regulation of the ability of psst2 to respond to its ligands.

GPCRs are commonly encoded by genes with an exon-intron structure, wherein alternative splicing originates proteins with different length and sequence but also generating shorter receptor isoforms with less than seven TMD (21). Conversely, sst genes are intronless or, at least, do not present canonical introns in their coding sequences (24). However, sst5 exhibit cryptic introns in the coding sequence as it has been described for sst2 and for the human and rodent sst5 truncated isoforms (7, 13, 30). Indeed, and as occurs in other species, we have found that the psst5 does not have introns in its coding sequence but possesses at least two cryptic introns that, upon processing, originate spliced variants with six and three TMDs. This atypical splicing, conserved between human, rodent, and porcine sst5 variants, is still poorly understood but may probably be related to the presence of GC-rich specific motifs involved in the recognition of noncanonical introns, as it has been reported for other genes (4, 25). Of note, sst5 shows the highest GC content among all the ssts, having a 66.3, 64.4, and 55.07% averaged GC content for the human, porcine, and mouse genes, respectively (Genbank nos. NM_001053, NM_001038229, and NM_011425.1). Interestingly, generation of this type of spliced proteins involving noncanonical cryptic introns is not rare and has been shown to involve new signals and mechanisms that are still poorly understood and require further study (25). Similarly, the precise function of truncated sst5 variants is still a matter of analysis. In this context, as will be detailed below, our current results add solid evidence indicating that truncated psst5 variants act as dominant-negative receptors modulating the cellular response of the full-length receptors in response to their endogenous ligands (SST and CORT).

Fig. 7. Functional interaction between the psst5 variants and psst2. Relevance of the functional interaction of each psst5 variant with psst2 in response to SST and CORT was assessed by measuring changes in the [Ca$^{2+}$], in CHO-K1 cells stably transfected with psst2 and coexpressing different quantities of each psst5 variant tagged with YFP. Graphs show the response to each peptide (expressed as % of [Ca$^{2+}$], increase) vs. the expression level of each recombinant psst5-YFP variant [expressed as relative fluorescence units (RFUs)]. Results were fitted to one-phase exponential decay curves (lines) from which the parameters $R_0$ (psst2 response at low expression of the psst5 variant), $R_\infty$ (psst2 response at high expression of the psst5 variant), and $R_{1/2}$ (amount of psst5 variant necessary to decrease psst2 response by 50%) were calculated.
gesting that such distinct distribution could be related to the lack of specific localization signals in the absent C region and/or to differences in the structure and orientation of the resulting new COOH-terminal domains (7, 13). Perhaps also in relation to these differences, unlike some rodent sst5 variants (7), truncated psst5 variants were unable to modulate cAMP signaling, at least under the conditions assayed. However, the fraction of psst5TMD6 and psst5TMD3 located at the plasma membrane was capable to elicit ligand-induced rises in [Ca^{2+}]. This ability of both receptors to induce a functional response despite lacking important structural elements for normal G protein coupling is uncommon, but not unique, as signaling. Accordingly, it would be interesting to investigate in response despite lacking important structural elements for normal G protein coupling is uncommon, but not unique, as signaling. Accordingly, it would be interesting to investigate in

the future whether these novel truncated receptors contribute to mediate, for example, the effects of CORT not shared with SST in the central and immune systems and at the cardiovascular level (17, 18, 22). More specifically, it is well known that CORT but not SST decreases locomotor activity, showing potent sleep-promoting activities (10). Similarly, CORT but not SST potently deactivates inflammatory and autoimmune responses in various experimental models (17, 18). Likewise, CORT but not SST attenuates vascular calcification in athero-sclerotic arteries (22). As mentioned before, the human ortholog for the psst5TMD3 is expressed in pituitary tumors where its expression is linked to a decreased response to SST analogs (14) and in breast cancer cell lines, where its presence is associated with altered SST signaling (12). These and other evidences (6, 7, 12–14) suggest that truncated sst5 variants comprise a new class of receptors for SST and CORT in mammals, which may help understand more profoundly the differential effects between SST and CORT, broaden our perspective of the pathophysiological functions of this peptide-receptor family, and, hopefully could provide future tools for the development of new therapeutic targets for the pharmacological management of pathological situations linked to a lack of response to SST or its analogs.

GRANTS

This work has been funded by grants from Fundacion Caja Madrid and the “Sara Borrell” program (Grant no. CD11/00276) (to M. D. Gahete), RYC-2007-00186 (to R. M. Luque), and BFU2010-19300, CIBERobn, and CTS-5051 (J. P. Castaño); CIBER Om is an initiative of the Instituto de Salud Carlos III, Spain.

DISCLOSURES

The authors have nothing to disclose neither conflict of interest.

AUTHOR CONTRIBUTIONS


REFERENCES

TRUNCATED sst5 ISOFORMS ACT AS DOMINANT-NEGATIVE RECEPTORS


