Identification and Characterization of Two Novel Truncated but Functional Isoforms of the Somatostatin Receptor Subtype 5 Differentially Present in Pituitary Tumors

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Context: Somatostatin and its related peptide cortistatin exert multiple actions on normal and tumoral tissue targets through a family of receptors termed somatostatin receptor (sst)1-5. Despite the considerable advances in the knowledge on these receptors and their (patho)physiological roles, there is still evidence that additional receptors for these peptides should exist to fully explain their actions.

Objective: The growing number of spliced variants found in similar receptor families, often present in tumors, and results from our group obtained on sst5 from other species (pig) led us to explore the existence of new human sst5 isoforms.

Design and Results: A rapid amplification of cDNA ends PCR approach on samples from a human pituitary tumor and a cell line enabled identification of two novel alternatively spliced sst5 receptor variants. The sequences obtained encode putative proteins that correspond to truncated isoforms of five and four transmembrane domains (TMDs), accordingly named sst5TMD5 and sst5TMD4, respectively. Both novel receptors show a differential expression pattern in normal tissues and are also present in pituitary tumors of diverse etiology including nonfunctioning adenomas, corticotropinomas, somatotropinomas, and a prolactinoma. In contrast to the predominant plasma membrane localization of full-length sst5, both sst5TMD5 and sst5TMD4 show a preferentially intracellular localization. Despite their truncated nature, both receptors are functional, as shown by their ability to mediate selective, ligand-induced rises in free cytosolic calcium concentration. Specifically, whereas sst5TMD5 is selectivity activated by somatostatin compared with cortistatin, cells transfected with sst5TMD4 almost exclusively respond to cortistatin and not to somatostatin.

Conclusions: Our results demonstrate the existence of two previously unidentified sst5 spliced variants with distinct distribution in normal tissues and pituitary tumors, unique ligand-selective signaling properties, and subcellular distribution, which could contribute to somatostatin and cortistatin signaling in normal and tumoral cells. **(*J Clin Endocrinol Metab* 94: 2634–2643, 2009)**

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Abbreviations: AC, Somatotropinoma; AS, antisense; $[Ca^{2+}]_i$, intracellular free calcium concentration; CDS, coding sequence; CFP, cyan fluorescent protein; CST, cortistatin; CU, corticotropinoma; E1, exon 1; E2, exon 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; NF, normalization factor; NFPA, nonfunctioning pituitary adenoma; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SRIF, somatostatin; sst, somatostatin receptor; TMD, transmembrane domain.

The importance of somatostatin (SRIF) in the regulation of a number of physiological processes and their related pathologies is nowadays unequivocally established (1–3). This tetradecapeptide exerts multiple biological actions through activation of a family G protein-coupled receptors (GPCRs), termed somatostatin receptors (ssts) (2–4). To date, five isoforms (sst1-sst5) and the alternative splice variant sst2B (5, 6) have been isolated and characterized (2–4). These receptors seem also to mediate most actions of cortistatin (CST), a peptide highly similar to SRIF (7), which shows a wide distribution in normal and tumoral tissues (8). CST essentially mimics the endocrine actions of SRIF (9), although its precise physiological role and relevance is still to be elucidated. However, CST also exerts unique functions, particularly within the central nervous system (7) and in inflammatory processes (10, 11), and the receptors involved in these processes are still unknown.

The wide expression pattern of ssts in normal and pathological conditions, coupled to their inhibitory actions upon SRIF and CST binding, has served as basis for development and application of SRIF analogs with agonistic properties to identify and treat different tumor pathologies (1) specially in endocrine-related diseases such as pituitary tumors (12). However, success of this therapeutic approach has been often hampered by the fact that some patients respond to treatment with SRIF analogs, whereas some are partial responders, and others do not respond at all, suggesting the potential importance of the expression pattern of the ssts in each pathology (1–3, 12). Actually, studies on samples from patients suffering of pituitary tumors, especially GH-secreting adenomas causing acromegaly, with divergent responses to SRIF agonist therapy, has helped to establish that the degree of response to the treatment is strongly correlated to the type and abundance of ssts present in the target tissue (13).

Despite the wealth of knowledge gathered in recent years on the pathophysiology of SRIF/CST/ssts, there are still a number of unsolved questions, which have led several authors to propose the existence of additional receptors and/or signaling mechanisms, either related or not to the sst family, to explain their findings (14–17). Thus, for example, there is still a number of tumors that escape to the control of treatment with potent sst agonists that cannot be explained based on a specific sst profile (15–17).

Recent evidence indicates that within the GPCR superfamily, there are mechanisms to increase receptor variability involving generation of splicing variants with less than seven transmembrane domains (TMDs) (18–22). Such truncated receptors, which may possess their own function or regulate the function of their respective long, canonical receptor isoforms (18), are frequently associated with tumor pathologies, as GH-releasing factor receptor (19, 20), cholecystokinin receptor (22), or adrenoceptors (21).

The family of ssts is known to be uniquely comprised by intronless genes, thereby rendering them as good candidates for uncommon splicing mechanisms, such as the one that generates the only sst subtype variants known to date, the long (sst2A) and short (sst2B) isoforms of the sst2 gene, generated due to the presence of a cryptic splice site (5, 6). However, and despite strong evidence that additional, unidentified receptor variants may exist, particularly in the case of sst5 (23), no data have been reported in this regard. In this scenario, while investigating the unique stimulatory effect caused by SRIF on GH release from cultured porcine pituitary cells (24), we recently identified novel, truncated variants of the pig sst5 (Durán-Prado, M. and Castaño, J. P., unpublished results). This led us to question whether there are analogous truncated receptor variants in the human, and if so, what are their characteristics and potential relevance. In this work, we present the first evidence for the existence of two functional human sst5 truncated isoforms of five and four TMDs termed sst5TMD5 and sst5TMD4, which show a unique expression pattern in normal tissues as well as different pituitary tumor types and display distinct functional responses to SRIF and CST.

Patients and Methods

Patients and samples

Pituitary tumor specimens were obtained during transsphenoidal surgery from 34 patients and corresponded to: 22 nonfunctioning pituitary adenomas (NFPAs), six GH adenomas, four corticotropinomas, one prolactinoma, and one TSHoma. After surgery, tumors were immediately frozen until RNA extraction. A commercial panel of total RNA from various human tissues was obtained from CLONTECH (Human Total Master Panel II and human pituitary gland poly-A RNA; Palo Alto, CA). This study was approved by the Ethics Committee of the University of Cordoba and hospitals Reina Sofia (Cordoba), Virgen del Rocío (Sevilla), and Sant Pau (Barcelona). Informed consent was obtained from each patient before study entry.

Nucleic acids isolation and reverse transcription (RT)

Nucleic acids were isolated with Trizol (Invitrogen, Barcelona, Spain) following the manufacturer's instructions. Human lymphocytes were used as an accessible source of genomic DNA using routine techniques. Human sst5 variants were identified by rapid amplification of cDNA ends (RACE) technology using total RNA from pituitary tumors and HeLa cells. For RT-PCR studies, 5 μg of RNA were reverse transcribed using the PowerScript reverse transcriptase kit (BD Bioscience, Erembodegem, Belgium). When the RNA was used specifically for 3′ RACE cloning studies, 5 μg of total RNA were retrotranscribed using the GeneRacer oligo deoxythymidine oligonucleotide kit (Invitrogen) following the manufacturer's instructions.

Isolation of human sst5 variants, sst5TMD5 and sst5TMD4, by RACE method

Identification, isolation, and cloning of the two new human sst5 variants were carried out using RT-PCR and RACE (GeneRacer kit; Invitrogen). Standard and nested PCRs were performed using the iCycler IQ system (Bio-Rad, Madrid, Spain) and the CERTAMP complex amplifications kit chemistry (Biotools, Madrid, Spain). For the first PCR, 100 ng of cDNA were amplified with the oligonucleotide Ra_hum_sst5_3′ (Table 1) combined with the GeneRacer 3′ oligonucleotide (Invitrogen; Table 1). A nested PCR was carried out with 1 μl of the first PCR product, which was reamplified with primer pairs (Ra_hum_sst5_3′N and GeneRacer 3′ nested oligonucleotide; Invitrogen; Table 1). PCR products were electrophoresed and extracted using QuiaQuick gel extraction kit (QIAGEN, Hilden, Germany). The DNA fragments obtained were cloned into the pBluescript KSII+ plasmid (Stratagene, Madrid, Spain) and sequenced at the Servicio Centralizado de Apoyo a la Investigación of the University of Córdoba (SCAI).

TABLE 1. Primers used in this study

Name	Sequence	Reference sequence
	RACE PCR primers	
Hum_sst5_ATG	ATGGAGCCCCTGTTCCCAGCCT	GI13937340
Ra_hum_sst5_3′	TGGGTCCTGTCTCTGTGCATGTC	GI13937340
Ra_hum_sst5_3′N	CTGGTGTTCGCGGACGTGCAG	GI13937340
GeneRacer 3′	GCTGTCAACGATACGCTACGTAACG	
GeneRacer 3′ Nested	CGCTACGTAACGGCATGACAGTG	
	Cloning primers	
sst5TMD5-C_E1_S_HindIII	TCAAGCTTCGATGGAGCCCCTGTTCCCAGC	GI13937340
sst5TMD5-E1_AS_blunt	CGGCGCGAAGAAGCCCAGCAC	GI13937340
sst5TMD5-E2-S_blunt	CTGCTGAGAGGCAGCGGCC	GI13937340
sst5TMD5-E2-AS_BamHI	TTAGGATCCTCAGAGCAAGGCCAAGTTGCC	GI13937340
sst5TMD4-E1_AS_blunt	GTTGCAGGTACCGCCCTCCTG	GI13937340
sst5TMD4-E3_S_blunt	CGTCTGCCCAGAGCAGGACCTC	GI13937340
sst5TMD4-E3_AS_BamHI	ACTGGATCCTCAGCCTGGGCCTTTCTCCTG	GI13937340
	Quantitative RT-PCR primers	
sst5TMD5_S	GCGCCGTCTTCATCATCTAC	DQ448303
sst5TMD5_AS	CAGGAAAAGCTGGTGTTTGG	DQ448303
sst5TMD4_S	TACCTGCAACCGTCTGCC	DQ448304
sst5TMD4_AS	AGCCTGGGCCTTTCTCCT	DQ448304

Each group show the primers used in RACE-PCR (*top*), cloning primers (*middle*), and primers used in quantitative real-time PCR studies (*bottom*). The reference sequence at the National Center for Biotechnology Information database is indicated for each primer.

Cloning of sst5, sst5TMD5, and sst5TMD4 into pCDNA3 and E-CFP-N1 expression vectors

The sst5 cDNA was purchased from the UMR cDNA Resource Center (www.cdna.org) and its coding sequence (CDS) was amplified by PCR with *Hin*dIII and *Eco*RI adaptor primers. The complete CDSs for sst5TMD5 and sst5TMD4 were amplified using a high-fidelity *Pwo* polymerase (Roche, Barcelona, Spain) and genomic DNA as template. Then both sst5 variants were assembled by triple ligation. Each of the two novel sst5 variants, sst5TMD5 and sst5TMD4, consists of two atypical exons. Exon (E1) of sst5TMD5 fully overlaps with that of sst5TMD4, and both share 645 and 561 nucleotides of the 5′ end (NH_2 terminus of the receptor) of the long, canonical sst5, respectively. Exon 2 (E2) of sst5TMD5 only overlaps partially with E2 of sst5TMD4, being both E2 located at the 3′ untranslated region of the sst5 gene (Fig. 1A). E1 of sst5TMD5 and sst5TMD4 was amplified using the sense primer (sst5TMD5-C_E1_S_*Hin*dIII), which incorporates a *Hin*dIII restriction site, and different antisense (AS) primers (sst5TMD5-E1_AS_blunt and sst5TMD4-E1_AS_blunt, respectively; Table 1). E2 of sst5TMD5 and sst5TMD4 was amplified separately using the following primer sets: sst5TMD5-E2-S_blunt and sst5TMD5-E2-AS_*Bam*HI for the sst5TMD5, and sst5TMD4-E2_S_blunt and sst5TMD4-E2_AS_*Bam*HI in the case of the sst5TMD4 isoform, all of which incorporate a *Bam*HI restriction site. Restriction enzymes (*Hin*dIII and *Bam*HI) were used to digest both sst5TMD5 and sst5TMD4 constructs obtaining DNA fragments consisting on their respective E1 and E2. Then E1 and E2 were ligated and inserted into a pCDNA3.1 expression vector (Invitrogen) and subsequently into the E-CFP-N1 vector as previously described (25). All recombinant vectors were sequenced.

Expression of ssts in human tissues and pituitary tumors

Development, validation, and application of quantitative real-time RT-PCR to assess mRNA levels have been previously reported (13). To control for variations in the amount of RNA and the efficiency of the RT reaction, the expression level (copy number) of two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, was determined for each sample as previously reported (13). GeNorm 3.3 visual basic application for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/) (26) was used to assess the stability of the housekeeping genes. The geometric means of the copy numbers for GAPDH and β-actin within each sample were used as a normalization factor (NF). Results were then reported as sst copy number minus background divided by NF. The sensitivity of the quantitative real-time RT-PCR was 10 copies per 0.05 μg total RNA. To estimate the starting copy number of cDNA, sample signal was compared with that of the standard curve run on the same plate. In addition, total RNA samples not reverse transcribed and a no-cDNA control were routinely run in each plate to control for genomic DNA contamination and to monitor potential exogenous contamination (background), respectively. Primer sets for the new truncated sstr5 variants (Table 1) were selected using Primer 3 software (http://frodo.wi.mit.edu/) with human mRNA sequences as templates. Primer set selection and validation for the sst1-5 subtypes and housekeeping genes were previously reported (13).

Antibodies and immunostaining

Immunostaining of human sst5 variants was carried out using two different rabbit polyclonal antisera, which were generated through a commercial source (Genosphere Biotechnologies, Paris, France). A polyclonal antiserum (sst5 antibody) was generated against the sequence MEPLFPASTPSWNAC, which is located at the common NH_2 domain, and therefore shared by the three sst5 isoforms. A second antiserum (anti-sst5TMD4) was generated against the sequence CRERLSGHKSWQEKG, which is unique for the sst5TMD4 isoform, and identified only this receptor variant.

For immunocytochemistry, CHO-K1 cells growing onto coverslips were transiently transfected with sst5, sst5TMD5, and sst5TMD4 receptors and 24 h later were fixed for 5 min in 4% paraformaldehyde, rinsed twice in PBS, and then immunolabeled for sst5 using the polyclonal anti-sst5 antibody. Cells transfected with sst5TMD4 were also immunolabeled with the anti-sst5TMD4, alone or preadsorbed with 10^{-5} M of its blocking peptide. In addition, the specificity of the antiserum was checked in mock-transfected cells. Primary antibodies were immunostained with an antirabbit Alexa594 antibody (1:500; Molecular Probes, Barcelona, Spain). Coverslips were mounted onto slides with 4′,6′-diamino-2-phenylindole (Sigma, Madrid, Spain), sealed, and stored at −20 C until their analysis.

Immunohistochemistry

For immunohistochemical staining, 7-μm paraffin sections from human pituitary adenomas were used. Samples were boiled for 20 min in 10 mM citric acid (pH 6.0) to retrieve antigen. To assess sst5TMD4 presence, slides were incubated overnight at 4 C with rabbit polyclonal anti-sst5TMD4 antibody. Staining of primary antibody was performed

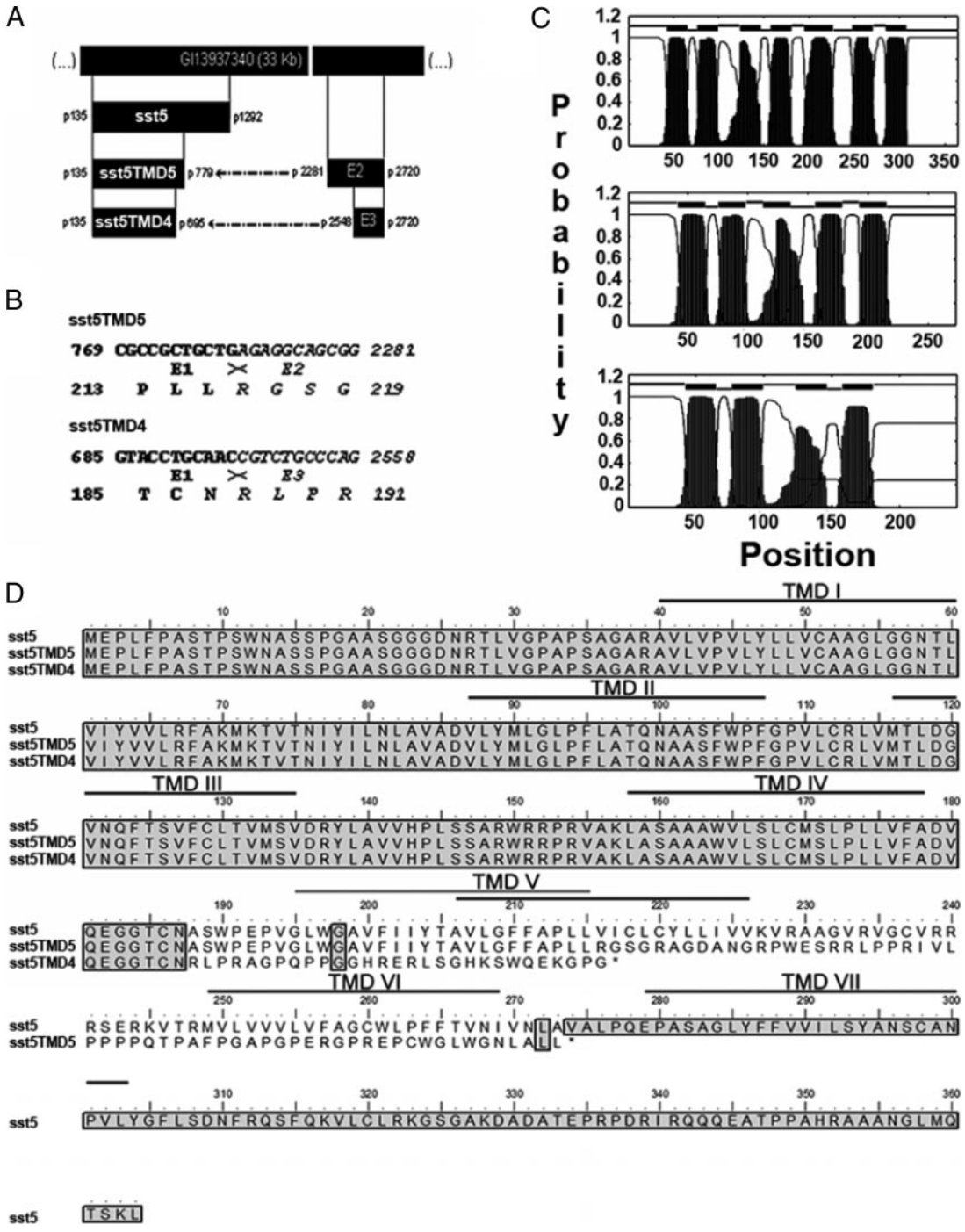

FIG. 1. Molecular identification and characterization of the novel sst5 truncated variants. A, Genomic structure of the human sst5 gene containing the truncated sst5 isoforms. B, Sequence of the junction between the cryptic exons that give rise to the CDS of the truncated sst5TMD5 and sst5TMD4. C, Hydrophobicity profiles of full-length sst5 (*top panel*), sst5TMD5 (*middle panel*), and sst5TMD4 (*bottom panel*) obtained with the program TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). D, Sequence alignment of the three sst5 isoforms performed with ClustalW (http://ebi.ac.uk/Tools/clustalw2/index.html). Identity is *shadowed* and *boxed*. Their predicted TMDs are indicated above.

following the protocol described in the Vectastain ABC-AP kit (Vector Laboratories, Inc., Burlingame, CA) and then counterstained with hematoxylin. Parallel samples were immunolabeled with the same antiserum preadsorbed with molar excess of the immunizing peptide to ensure specificity of signal.

Subcellular localization of sst5 variants

Subcellular localization of each sst5 isoform coupled in frame to E-CFP or immunolabeled as described above was assayed by confocal microscopy as described previously (25). Images were acquired with an Espectral TCS-SP2-AOBS confocal microscope (Leica Corp., Heidelberg, Germany) with the cyan fluorescent protein (CFP) settings. Alternatively, colocalization in CHO-K1 living cells of each CFP-tagged receptor with the lipophilic membrane marker FM5-95 (Molecular Probes) was evaluated using a Eclipse TE2000-E (Nikon, Tokyo, Japan). Briefly, FM5-95 signal was used to define a region at the plasma membrane and measure the CFP signal contained in it. Membrane fluorescence was subtracted to whole-cell CFP signal. Analysis was done with ImageJ (Bethesda, MD).

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

CHO-K1 cells stably transfected with sst5, sst5TMD5, or sst5TMD4 were grown onto glass coverslips for 24 h and incubated for 30 min at 37 C with 2.5 μM fura-2 AM (Molecular Probes, Eugene, OR) in phenol red-free DMEM containing 20 mM $NaHCO_3$ (pH 7.4). Coverslips were washed with DMEM and cells imaged as described previously (25). Changes in $[Ca^{2+}]_i$ were recorded as ratio of the corresponding excitation wavelengths (F340/F380) after background subtraction using MetaFluor software (Imaging Corporation, West Chester, PA).

Results

Cloning of human sst5 spliced isoforms

Two bands corresponding to novel variants of sst5 were obtained by RACE PCR. The first one, 650 bp, was initially obtained from HeLa cells, whereas the second band, 300 bp, was amplified from a pituitary corticotropinoma (not included in Table 2). Bioinformatic analysis of their sequences revealed a complete identity with discontinuous segments of a genomic sequence containing the sst5 gene (GenInfo Identifier: 13937340) (Fig. 1A). Specifically, the sequence of 650 bp, which subsequently led to identify the sst5TMD5 variant, was found to have full identity against two different and distant areas of the human genomic sequence: the first one containing nucleotides 135-779 (E1), which corresponds to the middle area of the fifth TMD of the sst5, and the second one containing nucleotides 2281-2720 (E2), which encodes a unique COOH-terminal tail in the truncated sst5TMD5 not shared by the canonical, full-length sst5 isoform. Similarly, the sequence of 300 bp, which subsequently led to clone the sst5TMD4 variant, was found to have full identity against two different areas of the human genomic sequence: the first one containing nucleotides 135-695 (E1), which corresponds to the area between the fourth and fifth TMD of the sst5, and a second one containing nucleotides 2548-2720 (E2), which would also encode a unique COOH-terminal tail. It should be noted that, despite the partial overlapping between E2 of the sst5TMD5 and sst5TMD4 variants, the predicted amino acid sequence of this truncated isoforms differ in the COOH terminal tail due to a frame shift on the starting nucleotide sequence of E2. In both cases, the exons junction differs from a consensus sequence (Fig. 1B).

The complete CDS of 822 and 651 nucleotides for the sst5TMD5 and sst5TMD4, respectively, which were subsequently cloned as described in *Materials and Methods*, would encode proteins of 273 and 216 amino acids. Analysis of their corresponding secondary structures (hydrophobicity profiles) indicated that these sequences would encode truncated sst5 variants with five and four putative TMDs, and thus the receptor variants were termed sst5TMD5 and sst5TMD4, respectively (Fig. 1, C and D). Both newly identified sst5 variants posses the conserved DRY motif at the end of the third TMD (3), which is

TABLE 2. Absolute quantification of ssts and mRNA copy number in pituitary tumors

Identifier	Tumor type	sst1/NF	AVG	sst2/NF	AVG	sst3/NF	AVG	sst5/NF	AVG	sst5TMD5/NF	AVG	sst5TMD4/NF	AVG
NF1	NFPA	46	62	8,825	1989	3,206	4363	134	1152	0	95.5	95	1,410
NF2	NFPA	65		321		136		0		1473		18,389	
NF3	NFPA	0		3,123		12,198		786		0		318	
NF4	NFPA	0		1,836		11,321		221		0		1,110	
NF5	NFPA	339		131		2,607		102		0		210	
NF6	NFPA	53		4,575		4,709		22		0		356	
NF7	NFPA	59		352		3,756		4		0		337	
NF8	NFPA	0		3,583		12,333		685		0		139	
NF9	NFPA	55		173		3,508		0		0		171	
NF10	NFPA	0		34		1		8		0		14	
NF11	NFPA	424		459		998		10		0		2,367	
NF12	NFPA	0		10,205		12,367		40		0		1,752	
NF13	NFPA	0		54		8,283		15		0		366	
NF14	NFPA	258		1,944		47		19,624		0		777	
NF15	NFPA	0		201		1,379		0		131		132	
NF16	NFPA	0		520		1,212		11		90		83	
NF17	NFPA	0		122		4,289		8		45		59	
NF18	NFPA	0		535		2,736		17		362		509	
NF19	NFPA	0		125		5,617		0		0		38	
NF20	NFPA	71		308		2,449		5		0		3,501	
NF21	NFPA	0		6,188		12		3,653		0		82	
NF22	NFPA	0		160		2,829		17		0		232	
AC1	Somatotropinoma	896	1016	12,638	8842	430	411	1,959	9825	0	133	10,354	2,776
AC2	Somatotropinoma	233		16,822	[a,b]	350	[a]	19,024	[a]	549		0	
AC3	Somatotropinoma	0		13,531		669		9,758		0		218	
AC4	Somatotropinoma	0		4,719		959		6,638		0		103	
AC5	Somatotropinoma	4970		4,440		0		6,626		251		424	
AC6	Somatotropinoma	0		905		63		14,947		0		5,559	
CU1	Corticotropinoma	465	641	153	458	21	*255*	9,650	6408	0		48,132	13,030
CU2	Corticotropinoma	674		1,535		921	[a]	7,210	[a]	0		3,244	
CU3	Corticotropinoma	267		103		47		8,775		0		565	
CU4	Corticotropinoma	1159		44		31		0		0		182	
PRL	Prolactinoma	4006		339		6		52		0		1,315	
TSH	TSHoma	0		44,652		22		791		0		0	
NP	Normal pituitary	210		1,075		354		3,708		0		0	

Expression is shown as copy number normalized against a NF, obtained from the level of GAPDH and β -actin, using the GeNorm 3.3 visual basic application for Microsoft Excel. PRL, Prolactinoma. The averaged value (AVG), obtained for each sst in each tumor group is shown. *a*, $P < 0.05$ *vs.* NFPA; *b*, $P < 0.005$ *vs.* CU.

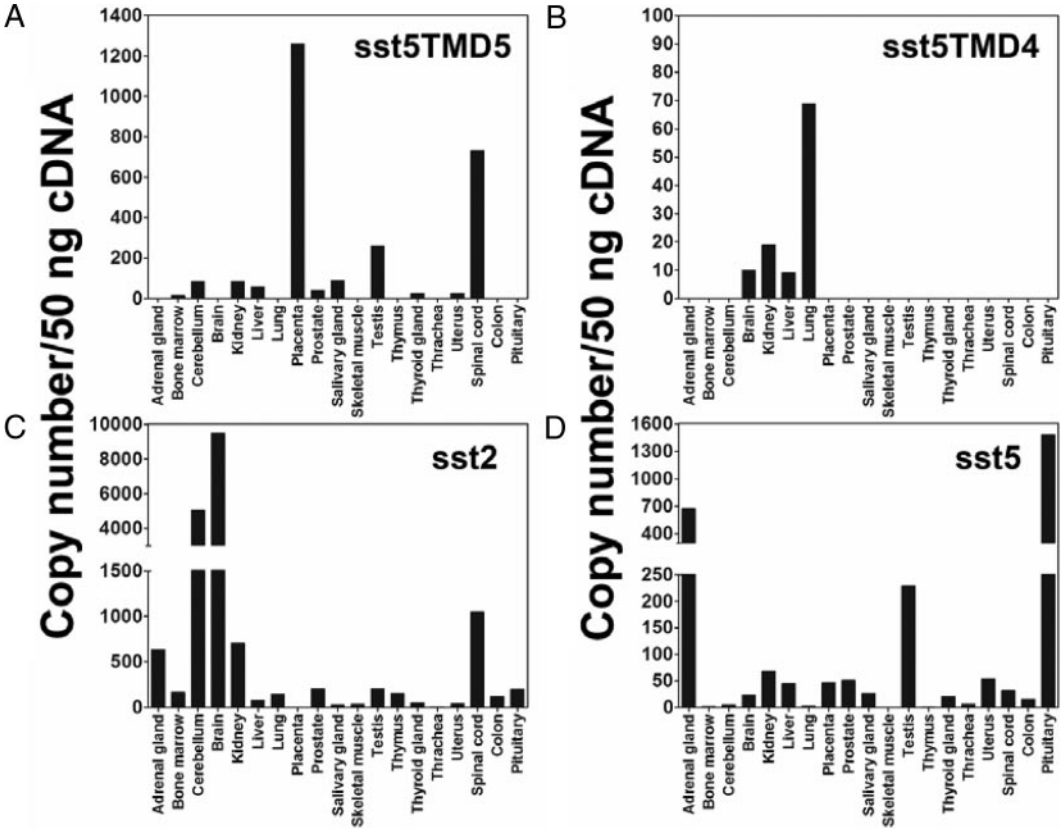

FIG. 2. Quantification of ssts in a cDNA panel (CLONTECH). The expression level of sst5TMD5 (A), sst5TMD4 (B), sst2 (C), and sst5 (D), was evaluated by real-time quantitative PCR and is shown as absolute copy number in 50 ng of cDNA.

involved in Gα protein coupling and selectivity (27, 28) but are truncated before the YANSCANPVLY signature motif that defines the family of mammalian sst receptors (2). Moreover, the resulting COOH-terminal domain of sst5TMD5 (intracellular) and sst5TMD4 (extracellular) do not present significant similarity with other previously known sst domains of any species nor with any known protein. The new sequences for both sst5 variants have been deposited at the National Center for Biotechnology Information database (Bethesda, MD) with accession no. DQ448303 (sst5TMD5) and DQ448304 (sst5TMD4).

Distribution of sst isoforms in normal tissues

sst5TMD5 is expressed in the 11 of 19 tissues analyzed (Fig. 2A), showing appreciable levels in placenta, spinal cord, and testis (1260, 730, and 261 copies per 50 ng cDNA, respectively). Conversely, sst5TMD4 was barely expressed in normal tissues, showing highest levels in lung (69 copies) and detectable but low levels (<20 copies) in liver, brain, and kidney (Fig. 2B). Both sst2 and sst5 were found to be widely expressed (Fig. 2, C and D, respectively) and coexpressed with sst5TMD5 in 11 of 19 samples, although without quantitative correlation.

Expression of ssts in pituitary tumors

ssts expression was screened in 34 pituitary tumors of diverse etiology (Table 2 and Fig. 3). sst1 was found to be expressed in 50% tumors analyzed (Table 2). sst3 was the predominant receptor found in NFPA (Fig. 3A and Table 2, $P < 0.05$ *vs.* somatotropinoma and corticotropinoma). Somatotropinoma showed high levels of sst2 (Fig. 3B and Table 2, $P < 0.05$ and $P < 0.005$ *vs.* NFPA and corticotropinoma, respectively) and sst5 (Fig. 3B and Table 2, $P < 0.05$ *vs.* NFPA). Corticotropinoma were characterized by high levels of sst5 (Fig. 3C and Table 2, $P < 0.05$ *vs.* NFPA). Only one prolactinoma (Fig. 3D and Table 2) and one THSoma (Fig. 3E and Table 2) were analyzed, showing predominance of sst1 and sst2, respectively. Expression of sst4 was not examined in this study, given its reported negligible expression in normal and adenomatous pituitary tissue. Expression of sst5TMD5 was detected in seven of 34 samples, specifically five of 22 NFPA and two of six somatotropinomas. Neither quantitative nor qualitative correlation was found between sst5TMD5 expression and tumor type (Table 2). sst5TMD4, infrequently and slightly expressed in normal tissues, was detected in 32 of 34 tumors (Table 2). Expression levels were variable, with some tumors showing high expression [*e.g.* NF2, NF4, NF11, NF12, NF20, somatotropinoma (AC)-1, AC6, corticotropinoma (CU)-1, CU2]. As for sst5TMD5, neither quantitative nor qualitative correlation was found between sst5TMD4 expression and tumor type (Table 2). Nevertheless, in NFPAs and somatotropinomas, the expression level of this receptor is close to that of other ssts. Both truncated receptors were absent in normal pituitary (Table 2 and Fig. 3F).

Subcellular localization of sst5 isoforms

Study of subcellular localization of sst5 isoforms revealed that whereas full-length sst5 is preferentially located at the plasma membrane (Fig. 4, A and E), both truncated isoforms sst5TMD5

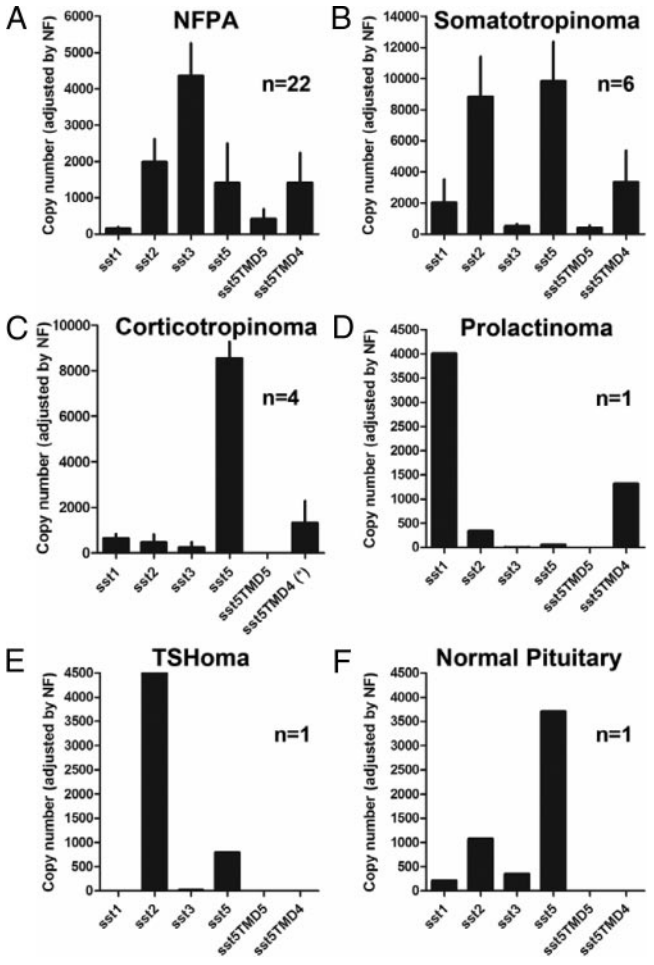

FIG. 3. sst expression profile in a set of pituitary tumors and normal pituitary. The absolute copy number of sst1, sst2, sst3, sst5, sst5TMD5, and sst5TMD4 was analyzed by quantitative real-time PCR in 34 tumor samples of diverse etiology, including nonfunctioning pituitary adenomas, NFPA (A), GHomas producing acromegaly, somatotropinoma (B), ACTH-secreting adenomas causing Cushing's disease, corticotropinoma (C), prolactinoma (D), TSHoma (E), and normal pituitary as control (F). Expression is shown as copy number normalized against a NF obtained from the level of GAPDH and β-actin, using the GeNorm 3.3 visual basic application for Microsoft Excel. An outlier value for sst5TMD4, labeled with an *asterisk*, has been excluded from C, although it is shown in Table 2. Its presence or absence does not alter any statistical significance.

(Fig. 4, B and G) and sst5TMD4 (Fig. 4, C and I) are mainly located at intracellular compartments. Nonetheless, signal in plasma membrane was detectable for sst5TMD5 and sst5TMD4. Specifically, *in vivo* colocalization of these CFP tagged receptors with the lipophilic membrane marker FM5-95 revealed that fluorescent signal located at plasma membrane for sst5TMD5 and sst5TMD4 was half that for full-length sst5 (Fig. 4D, $P < 0.001$). In the case of sst5TMD4, we also developed and validated a specific antiserum, which recognizes exclusively this isoform (Fig. 4H). As shown in Fig. 4F, there was no immunostaining in mock-transfected cells or cells transfected with sst5TMD4 incubated with the preadsorbed antibody (Fig. 4J). Using this antibody, we observed expression of sst5TMD4 in two pituitary adenomas (Fig. 4, K and M). In these samples, specific immunostaining of sst5TMD4 shows a predominant intracellular localization. Specificity of the signal is supported by the absence of immunostaining after incubating the sample with preadsorbed antibody (Fig. 4, L and N).

Functional test for truncated sst5 isoforms measuring free cytosolic calcium level ($[Ca^{2+}]_i$)

The ability of each sst5 isoform to modify the $[Ca^{2+}]_i$ kinetics in response to their natural ligands, SRIF and CST, was evaluated in CHO-K1 cells stably transfected with sst5 (positive control), sst5TMD5, or sst5TMD4 (Fig. 5). This approach demonstrated that cells transfected with full-length sst5 responded to SRIF and CST in a similar manner. Thus, both the proportion of responsive cells (41 and 54% for SRIF and CST, respectively) and the profile of the responses evoked showed comparable temporal and quantitative features (Fig. 5A). In striking contrast, most of the cells transfected with sst5TMD5 (60%) responded preferentially to SRIF, showing marked $[Ca^{2+}]_i$ increases, whereas only 12% of those cells responded to CST, which evoked calcium rises of very low magnitude (Fig. 5B). Moreover, an opposite situation was observed for sst5TMD4, in that only 4% of the cells responded to SRIF by showing delayed, transient responses, whereas CST evoked clear $[Ca^{2+}]_i$ increases in 43% of the cells transfected with this receptor (Fig. 5C).

Discussion

The unrivaled pharmacological importance of GPCRs is demonstrated by the fact that 50–60% of approved drugs elicit their therapeutic effect by selectively targeting members of this superfamily (29). The signature feature of GPCRs are seven helices that transverse the membrane dividing the proteins into cytoplasmic, transmembrane, and extracellular domains. However, in recent years, new truncated GPCR isoforms with less than seven TMDs are being discovered, although little is known about their physiological role and functionality (18). Here we isolated and characterized, for the first time in the SRIF receptor family two novel truncated isoforms of the sst5 with five and four TMDs, accordingly termed sst5TMD5 and sst5TMD4. Moreover, we demonstrate that mRNAs for these two receptors are expressed differentially in normal tissues and are also present in diverse types of pituitary tumors. The precise implications of our results are still to be established, but they likely are of potential pathophysiological relevance. Thus, although SRIF analogs therapy has been widely used over 2 decades to treat a variety of endocrine pathologies, particularly pituitary tumors, it has been argued that not all actions (or lack thereof) of these analogs can be explained by the known properties of the ssts identified to date, suggesting the existence of new sst isoforms.

Indeed, although several authors proposed that, besides sst1-5, additional receptors for SRIF and/or CST should exist (7, 14–16), none had been identified hitherto, and this may be related to the uncommon molecular nature of sst5TMD5 and sst5TMD4. Thus, GPCRs are commonly encoded by genes with an exon-intron structure, wherein alternative splicing originates proteins with different length and sequence, specially within the C- and N-terminal tails, and the intracellular and extracellular loops, but also generating a shorter receptor isoform with less

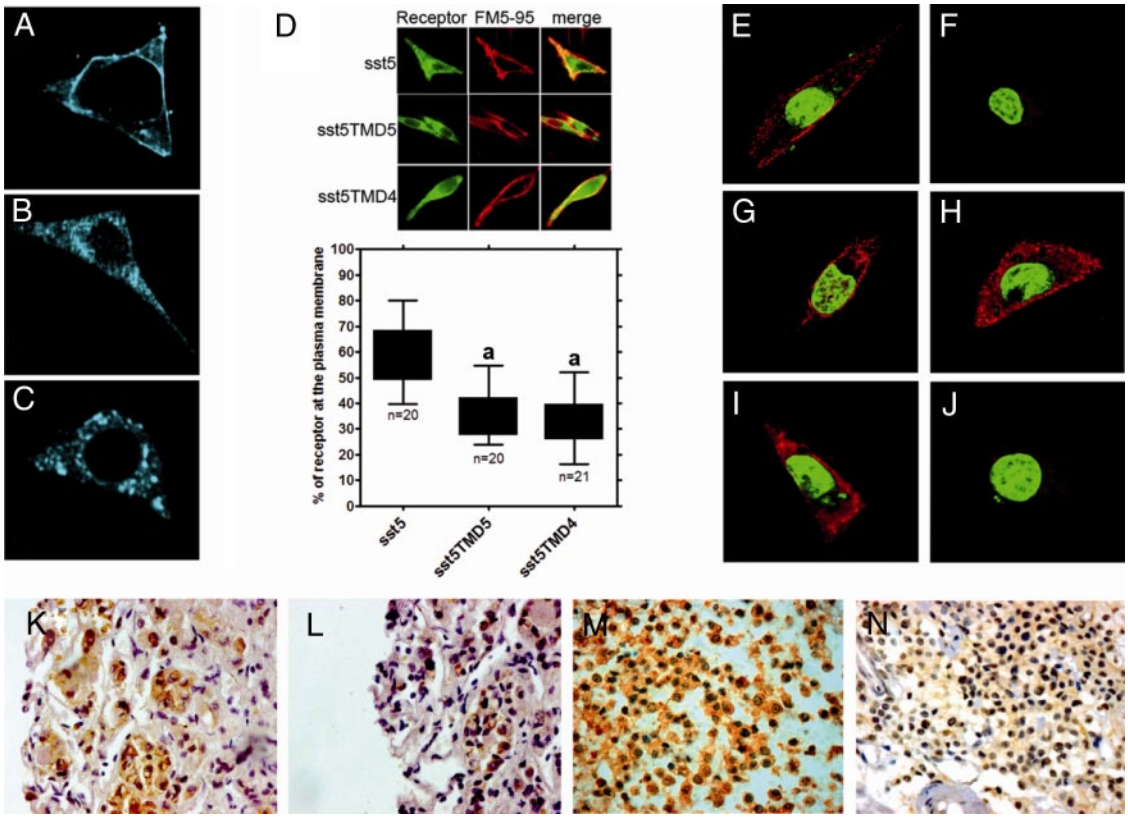

FIG. 4. Subcellular localization of the three sst5 isoforms and immunolabeling of sst5TMD4 in pituitary adenomas. Subcellular distribution was studied by confocal microscopy for full-length sst5 (A), sst5TMD5 (B), and sst5TMD4 (C) coupled in frame to the N-terminal domain of CFP and transfected in CHO-K1 cells. D, Pictures (*top*) and quantification (*bottom graph*) of colocalization, *in vivo*, of sst5-, sst5TMD5-, and sst5TMD4-CFP tagged receptors transiently transfected in CHO-K1 cells, with the membrane marker FM5-95. C shows the immunolabeling of untagged receptors with specific polyclonal antibodies. E, G, and I, Images of sst5, sst5TMD5, and sst5TMD4 transiently transfected CHO-K1 cells, respectively, immunolabeled with the polyclonal antiserum anti-sst5. An image of mock cells incubated with the same antibody is shown in F. H and J, Images of sst5TMD4-transfected cells immunolabeled with the polyclonal antiserum anti-sst5TMD4 and the preadsorbed antibody, respectively. K and M, Immunohistochemical detection of sst5TMD4 in two pituitary tumors using the anti-sst5TMD4 antibody or the preadsorbed antibody as shown in L and N. a, $P < 0.001$ *vs.* sst5.

than seven TMDs (for review see Ref. 18). Such changes may alter the normal G protein coupling and signaling of the receptor and, less often, ligand recognition (18). At variance with the above, the two truncated sst5 isoforms described herein are not originated by classical alternative splicing because the sst5 gene, like the rest of ssts, lacks canonical introns in the CDS (2). Conversely, both sst5 variants are generated presumably by a splicing of the sst5 gene involving the presence of cryptic, noncanonical donor and acceptor splice sites in the CDS and 3′ untranslated region, respectively, similar to that reported previously for sst2 (5), which generates two receptor variants that conserve seven TMDs but bear distinct C-tails (sst2A and sst2B). This atypical 3′ splicing has likely precluded earlier prediction/detection of these sst5 receptor variants from its genomic sequence (GenInfo Identifier: 13937340) using bioinformatic tools.

Interestingly, various truncated GPCRs have been recently associated with tumor pathologies because it is the case for receptors for GHRH (19, 20) and particularly the family of adrenoceptors, which includes up to 11 alternatively spliced variants (21). Their function and relation with tumor processes are still quite unknown and sometimes paradoxical. For example, in lung cancer cells, truncated receptor for the orexigenic hormone ghrelin, GH secretagogue receptor 1b, of five TMDs, does not appear to respond to ghrelin itself but seems to form a heterodimer with the neurotensin receptor 1, and this heterodimer, in turn, responds specifically to neuromedin U and induces cell proliferation in this type of cancer cells (30). Our results clearly demonstrate that the truncated sst5TMD5 and sst5TMD4 variants are differentially expressed in normal human tissues and pituitary tumors of diverse etiology, suggesting these transcripts may encode a peptide with potential physiological relevance in coordinating endocrine response to SRIF and CST in normal or pathological states.

Analysis of the localization of CFP-tagged receptors in CHO-K1 cells by confocal microscopy, and *in vivo* colocalization studies with the membrane marker FM5-95, demonstrated that both novel sst5 variants display a distinct subcellular distribution, *i.e.* preferentially in intracellular compartments, compared with the well-known predominant localization of the full-length sst5 at the plasma membrane. Interestingly, such preferential intracellular distribution of sst5TMD5 and sst5TMD4 is reminiscent of that atypically observed for sst5 by immunohistochemical methods in some human pancreatic tumors (31) and the human hepatocarcinoma cell line HepG2 (32) as well as for sst2 in human small-cell lung carcinomas (33) and sst1 in rat hypothalamic cells (34). Intracellular localization of GPCR is caused by heterotypic interaction of receptors (31–34). Our observation that both sst5TMD5 and sst5TMD4 are coexpressed

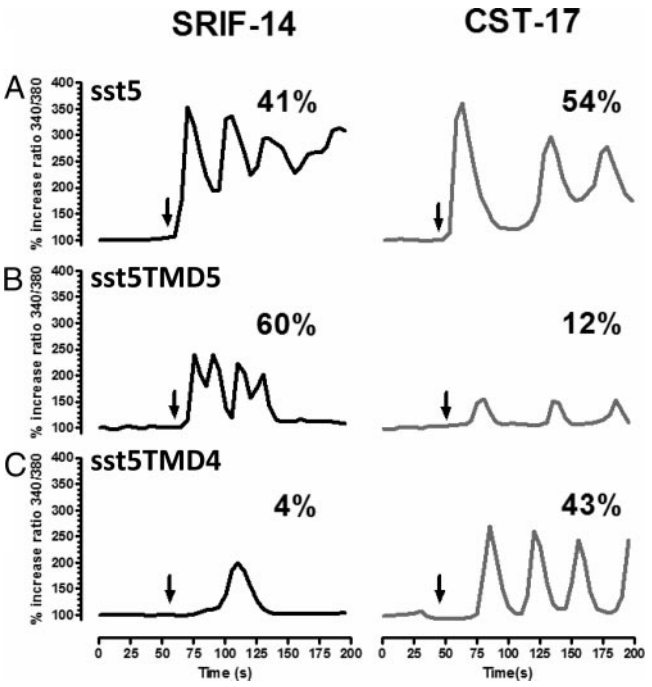

FIG. 5. Representative profiles of changes in the $[Ca^{2+}]_i$ evoked by SRIF (*left*) and CST (*right*) in CHO-K1 cells transfected with each of the sst5 isoforms. A, Typical profiles of sst5 transfected cells in response to SRIF and CST (representative of 59 of 144 and 101 of 186 cells, respectively). B, Responses of cells transfected with sst5TMD5 (representative of 81 of 135 and 16 of 124 cells for SRIF and CST, respectively). C, Representative profiles of cells transfected with sst5TMD4, similar to that observed in other nine of 228 and 127 of 294 cells for SRIF and CST, respectively. The percentage of responsive cells is indicated in each graph. The *arrow* indicates the injection time.

with other sst subtypes in normal tissues and pituitary tumors agree with this possibility.

Despite the fact that sst5TMD5 and sst5TMD4 possess less than seven TMDs and display distinct C-terminal tails from that of the canonical sst5, both receptors maintain an intact N-terminal domain and the DRY motif located at the end of the third TMD, which is commonly involved in Gα protein coupling and selectivity of GPCRs (27, 28). In marked contrast to the general ability of all ssts, including full-length sst5, we observed that both receptors, transfected in CHO-K1 cells, failed to modulate either basal or forskolin-stimulated cAMP levels in response to various doses of SRIF (data not shown), thereby suggesting that these novel sst5 isoforms might not be functional *per se*. Yet additional functional studies using $[Ca^{2+}]_i$ as a reporter signal demonstrated that this was not the case because both sst5TMD5 and sst5TMD4 were indeed able to elicit ligand-induced rises in $[Ca^{2+}]_i$.

This capacity of both receptors to induce a functional response despite lacking important structural elements for normal Gi protein coupling is uncommon but not unique. Thus, for example, the truncated calcitonin ΔE13 receptor of six TMDs lacks the ability to increase the cAMP level, whereas it is able to modulate the $[Ca^{2+}]_i$ on activation by its ligand (35). Of note, this experimental approach unveiled a differential capacity of each receptor to mediate ligand-selective responses. Specifically, whereas sst5TMD5 predominantly responded to SRIF, sst5TMD4 almost exclusively responded to CST. These differences are not apparently due to a differential binding to SRIF or CST because in pilot studies using fluorescently tagged peptides, both truncated receptors seem to exhibit a similar, reduced ability to bind fluo-SRIF and fluo-CST in comparison with sst5 but do not show any appreciable difference between them (data not shown). Nevertheless, the selective activation by SRIF and CST was unexpected because there is no evidence to date that any sst can mediate a differential response to these highly similar peptides. In fact, SRIF and CST not only show comparable affinities for each sst but also share most of their known effects, specially at endocrine targets. However, CST exerts selective actions in processes such as induction of slow-wave sleep and reduction of locomotor activity (36) and as antiinflammatory peptide (37), although the receptors that mediate those unique actions are still unknown. In this scenario, it is tempting to speculate that these new receptor variants may be involved in some of the selective functions of SRIF and CST.

In summary, we report for the first time the existence of novel mRNA splice variants of the human sst5 gene, named sst5TMD5 and sst5TMD4, with five and four TMDs, respectively, which are expressed under normal, physiological conditions in different human tissues. These results support the often proposed notion that there exist additional ssts to the known sst1-5 and that they are generated by an uncommon mechanism. These new sst5 variants may be of physiological and pharmacological relevance because they are expressed in different pituitary tumors in an isoform-dependent manner. The fact that both novel sst5 variants are found in a different cellular localization to that of the long, originally identified sst5 isoform, together with the differential ability of each variant to mediate functional responses to the endogenous ligands SRIF (sst5TMD5) and CST (sst5TMD4) suggests that these transcripts may encode peptide receptors with important roles in regulating (patho)physiological states. As such, these results pave the way for the elucidation of the specific pathophysiological role of each sst5 variant and the identification of other putative new sst isoforms, which could enhance the already complex and highly versatile functional array comprised by SRIF, CST, and the sst1-5 family.

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