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Cloning and expression of a novel mouse somatostatin receptor (SSTR2B)

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A mouse somatostatin (SS) receptor cDNA was cloned from neuroblastoma × glioma (NG108-15) cells. The sequence is almost identical to that of the mouse SSTR2 receptor [(1992) Proc. Natl. Acad. Sci. USA 89, 251)] but lacks about 300 nucleotides between transmembrane domain VII and the C-terminus. This spliced variant of SSTR2 (designated SSTR2B) encodes a protein which is 23 residues shorter than that predicted from the SSTR2 sequence, and differs in 15 amino acids at the C-terminus. mRNA corresponding to SSTR2B occurs in mouse tissues in higher abundance than that of SSTR2. SSTR2B binds SS peptides with high affinity when expressed in mammalian cells.

Somatostatin; Receptor; Mouse; Alternate splicing; Expression; Polymerase chain reaction

1. INTRODUCTION

Somatostatin (SS)-14, and its N-terminal extended form SS-28, exert a wide variety of actions in the pituitary, the central nervous system, as well as in various peripheral tissues [1]. These functions are thought to be mediated through G-protein-coupled receptors, which results in inhibition of adenylate cyclase, opening of potassium channels and closing of calcium channels [2,3]. Inhibition of adenylate cyclase by SS peptides has been reported in many cell lines. For instance, in the mouse neuroblastoma × rat glioma hybrid cell line (NG108-15) SS-14 was found to inhibit prostaglandin E1-stimulated adenylate cyclase [4]. SS peptides also decrease CRH (corticotropin releasing hormone)-induced cAMP levels in pituitary-derived mouse AtT-20 cells. In this cell line SS receptors couple to adenylate cyclase via $G_{i\alpha 1}$ [5].

Receptor binding studies provided evidence for the existence of at least two SS receptor subtypes (SRIF1, SRIF2) [6]. Recently, two different SS receptors (SSTR1 and SSTR2) have been cloned from human and mouse genomic libraries [7]. In addition, the rat homologue of the SSTR2 receptor was isolated from a brain cDNA library [8]. The pharmacological characteristics of the cloned SSTR1 receptor were similar to those of the SRIF2 receptor, whereas the properties of the other cloned receptor (SSTR2) were similar to those of the SRIF1 receptor [9]. Southern blot analysis suggests that the SS receptor family may comprise two additional members [7].

In this study we describe the cloning of a novel SS receptor cDNA which lacks about 300 nucleotides when compared to sequences of mouse and rat SSTR2 [7,8]. This spliced variant of SSTR2 (designated SSTR2B) encodes a protein which is 23 residues shorter than that predicted from the SSTR2 sequence, and differs in 15 amino acids at the C-terminus. mRNA corresponding to SSTR2B occurs in mouse tissues in higher abundance than that of SSTR2. These results provide evidence for a further degree of diversification amongst the SS receptors at the level of mRNA splicing.

2. MATERIALS AND METHODS

2.1. *Polymerase chain reaction (PCR), cloning and sequencing*

Degenerate primers from transmembrane segments III and VI of G-protein-coupled receptors [10] were used to amplify a cDNA derived from mouse neuroblastoma × rat glioma hybrid (NG108-15) cell mRNA. Amplified DNA was cloned into vector pBluescript (Stratagene, Heidelberg, Germany). Four clones showing the motif of G-protein-coupled receptors were obtained. Clone 561-51 was used as a probe to screen a λZAP II cDNA library constructed from size-selected poly(A)$^+$ RNA from NG108-15 cells (custom synthesis by Clontech, Heidelberg, Germany). Three hybridizing plaques were obtained by screening 1.5×10^6 phages with probe 561-51 under high stringency conditions (51CF1, 51CF2, 51CF3). The plasmids were excision-rescued from the corresponding λZAP II clones by superinfection with helper phages. The clones were sequenced by the chain termination method [11]. Clone 51CF3-1 contains a 1.9 kb *Eco*RI fragment with a complete sequence of a receptor, with seven transmembrane domains, including 3′ and 5′ non-translated regions.

2.2. *Expresion and binding*

A 1.7 *Bam*HI–*Eco*RV fragment of the cloned 51CF3-1 cDNA was

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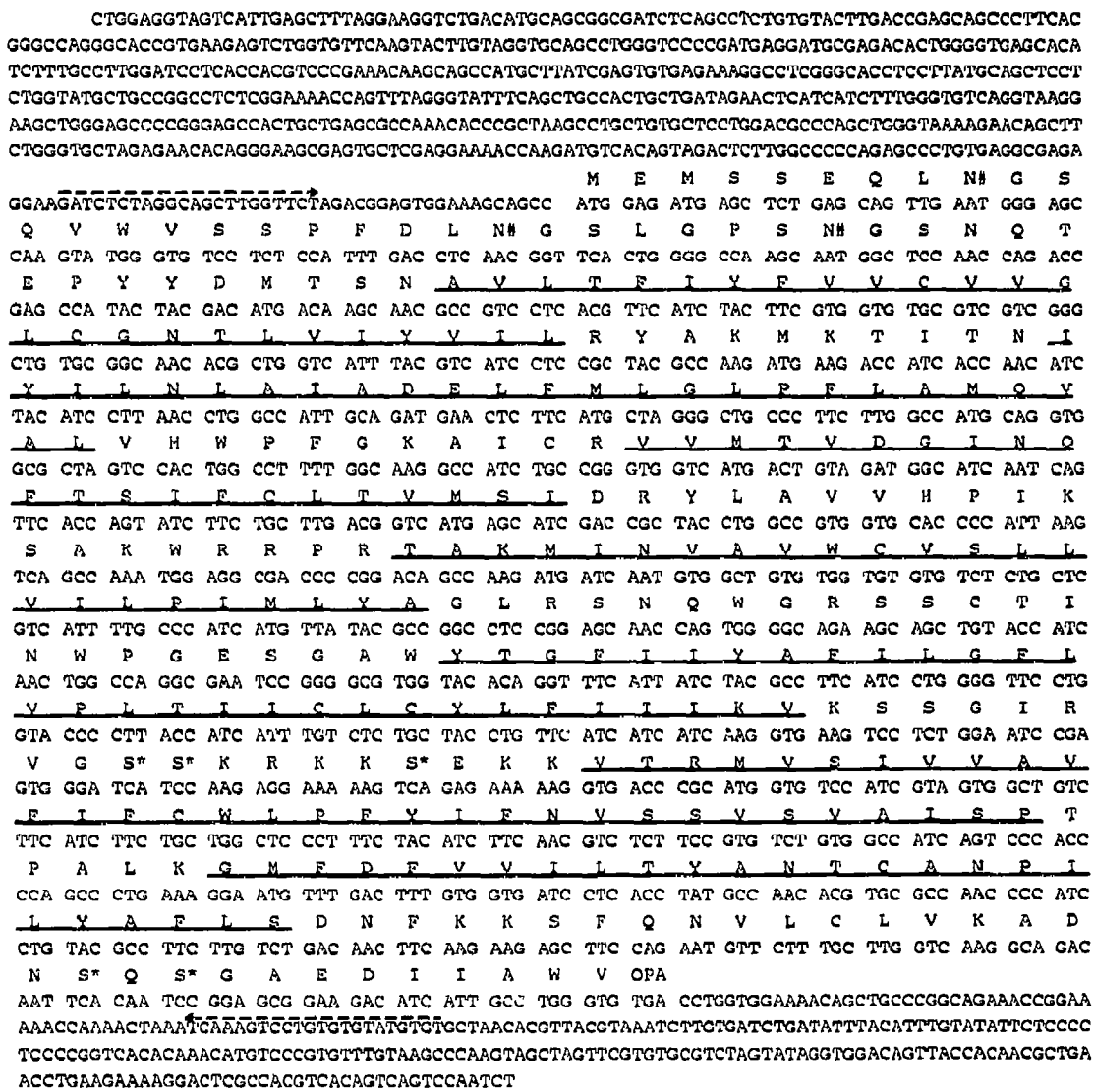

Fig. 1. Nucleotide and predicted amino acid sequence of the cloned mouse somatostatin receptor cDNA (51CF3-1). The presumptive transmembrane domains (I–VII) are underlined and are assigned on the basis of a Kyte and Doolittle hydrophobicity plot [17]. Potential *N*-linked glycosylation sites in the N-terminal region (#) and phosphorylation sites in the C-terminal region and third cytoplasmic loop are indicated (*). Primers used for PCR (SPL4, SPL5) are marked by dashed lines with arrows.

inserted into expression vector pcDNAI (Invitrogen, San Diego, USA). The resulting vector pcDNAI-51 was transiently expressed in COS-7 monkey kidney cells using the DEAE–dextran method [12]. For stable expression, 2 μg of the plasmid pcDNAI-51 were co-transfected with 0.1 μg plasmid pcDNAIneo in Chinese hamster ovary cells (CHO-K1) with lipofectin (Gibco-BRL, Eggenstein, Germany) according to the instructions of the manufacturers. Stable transfectants were selected in DMEM medium containing 600 μg G418 per ml and checked for expression by Northern blot analysis.

For binding measurements, COS-7 cells were grown to 50% confluence in 35 mm six-cluster wells and transfected. After 48 h, the cells were washed with 10 mM HEPES buffer (pH 7.5) and incubated with buffer containing 10 mM HEPES (pH 7.5), 5 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl and 1% (w/v) bovine serum albumin, 5 mg/ml bacitracin, 20,000 cpm [^{125}I]Tyr^{11}-SS (Amersham, Braunschweig, Germany) and the indicated concentrations of SS-14, SS-28 (Saxon Biochemicals, Hannover, Germany) or SMS 201-995 (Sandoz, Basle, Switzerland) for 60 min at room temperature. Thereafter the cells were washed twice with ice-cold 10 mM HEPES buffer and dissolved in 1.5 ml of 8 M urea/3 M acetic acid. The radioactivity in the dissolved cells was measured in a γ-counter.

2.3. *RNA extraction and Northern blotting*

Male NMRI mice weighing 25–30 g were killed by decapitation and the tissues immediately removed, dissected and placed on dry ice. Total RNA was extracted from the tissues by using the acid guanidinium isothiocyanate/phenol chloroform method [13]. Poly(A) RNA was isolated from total RNA using the QuickPrep mRNA purification kit from Pharmacia (Freiburg, Germany). In addition, RNA was extracted from AtT-20/D-16v cells (Dr. Kelly, San Francisco, USA), NG108-15 cells (Dr. Hamprecht, Tübingen, Germany) and GH3 cells (Dr. Baxter, San Francisco, USA). Aliquots of 3 μg poly(A) RNA were denatured by glyoxal, separated by electrophoresis, transferred to nylon supports and hybridized with radiolabelled single-stranded

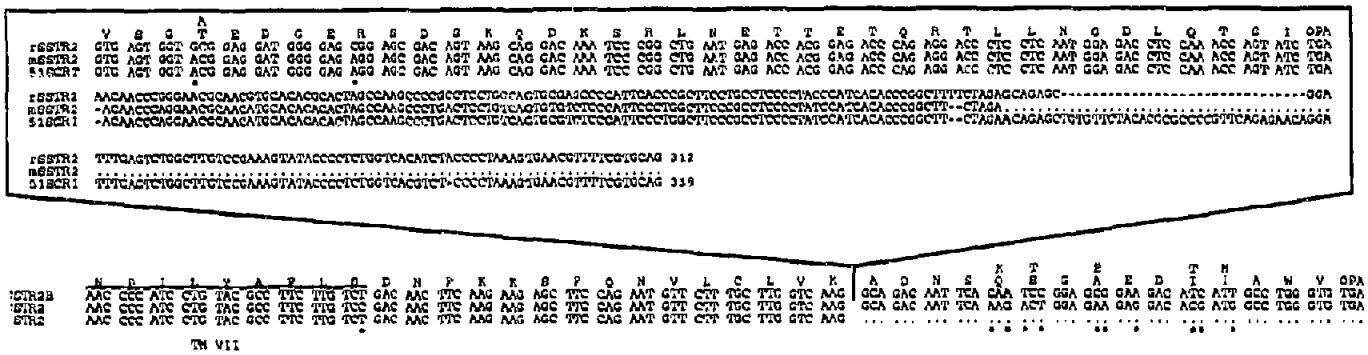

Fig. 2. Alignment of nucleotide and predicted amino acid sequence of the C-terminal portion of 51CF3-1 cDNA with the mouse SSTR2 genomic sequences and the sequences of rat SSTR2 cDNA. The position of nucleotide differences within the coding regions are indicated by asterisks. The genomic sequence of mouse SSTR2 has not been completely published [7]. The missing nucleotides are indicated by dots.

cRNA probe. The probe was synthesized with T3 polymerase and [^{32}P]UTP using clone 561-51 as a template and hybridized at conditions previously described [14].

2.4 *PCR amplification of cDNAs*

cDNAs were reverse transcribed from RNAs extracted from AtT-20 cells, treated with RNAse-free DNase and subjected to 50 cycles of PCR with *Taq* polymerase [15]. The following primers were used: SLP4 (5'-GATCTCTAGGCAGCTTGGTTCT-3', matching at nucleotides 554–575) and SLP5 (5'-ACACATACACACAGGACTTTGA-3', matching at nucleotides 1,707–1,686). Cycle conditions were 1 min at 96°C, 1 min at 55°C and 1 min at 72°C.

3. RESULTS AND DISCUSSION

A cDNA was prepared from poly(A) RNA of NG108-15 cells and amplified by PCR using degenerated primers corresponding to transmembrane domain III and VI according to a protocol previously described [10].

Nucleotide sequence determination of cloned amplified sequences revealed that fragments of the rat endothelin 1A receptor [16] had been amplified. In addition, three novel putative receptor-encoding fragments were identified (561-8; 561-39; 561-51). Clone 561-51 was used to identify full-length cDNAs in a NG108-15 cDNA library (λZAP). Three phage λ clones were obtained. The largest clone (51CF3-1) contains a 1,901 bp *Eco*RI fragment with an open reading frame of 346 amino acids (Fig. 1). Hydrophobicity analysis of the amino acid sequence indicates seven hydrophobic regions characteristic of G-protein-coupled receptors. The protein has potential *N*-linked glycosylation sites and several serine residues that may provide potential phosphorylation sites. Sequence comparison of 51CF3-1 with the mouse genomic sequence of a somatostatin receptor (SSTR2) published recently [7] shows an almost complete identity up to amino acid 332 (only isoleucine at position 180 is replaced by a leucine and serine 305 by a threonine). On the other hand, the protein predicted from the 51CF3-1 sequence was 23 amino acids shorter and differed in the last 15 C-terminal amino acids from mouse SSTR2 sequence (Figs. 1 and 2). Sequence alignment of 51CF3-1 with a recently published cDNA encoding the rat homologue of SSTR2 [8] revealed that the mouse cDNA clone 51CF3-1 lacks 312 nucleotides between the bases coding for lysine (332) and alanine (333). It is likely that the missing nucleotides represent an intronic sequence since (i) the junctions of the putative intronic sequences follow the GT-AG rule, (ii) there is a high degree of homology between the rat and the mouse cDNA sequences downstream of the intron, and (iii) there is a high homology within the

Fig. 3. Northern blot analysis of SSTR2 mRNAs in mouse cell lines and tissues, as well as in rat GH3 cells. 3.5 μg aliquots of poly(A) RNA were subjected to electrophoresis, blotted on nylon membranes and hybridized with ^{32}P-labelled 561-51 cRNA probe. *Hind*III-digested fragments of λ phage were used as size markers. (a) NG108-15; (b) liver; (c) testis; (d) striatum; (e) midbrain; (f) cerebellum; (g) hippocampus; (h) hypothalamus; (i) cortex; (k) AtT-20 cells; (l) GH3 cells.

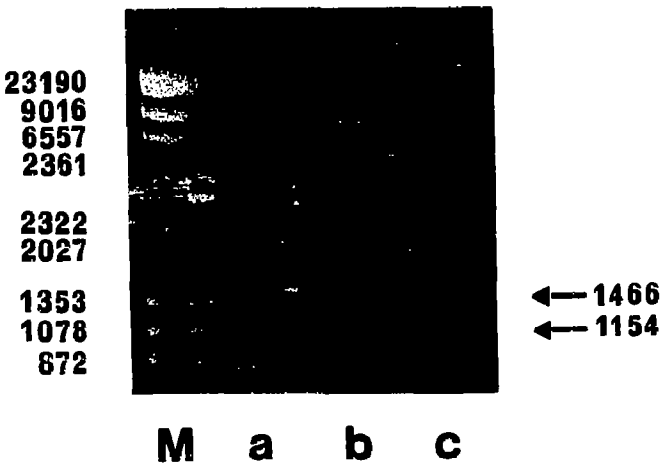

Fig. 4. PCR analysis of SSTR2 receptor transcripts in AtT-20 cells. PCR with primers corresponding to 5′ and 3′ non-translated regions of 51CF3-1 (SPL4, SPL5, Fig. 1) was carried out on cDNAs synthesized from AtT-20 RNA. Aliquots corresponding to 500 ng (a), 50 ng (b) and 5 ng (c) cDNA were amplified, electrophoresed and stained by ethidium bromide. (M) *Hin*dIII fragments of λ phage and *Hae*III-digested fragments of Φx174 phage were used as size markers.

intron between the rat cDNA and the published 228 nucleotides of the mouse gene (Fig. 2).

To investigate the existence of two forms of SSTR2 mRNAs (spliced and unspliced) in the mouse, a Northern blot analysis was performed using clone 561-51 as a probe. As seen in Fig. 3, analysis of poly(A) RNA derived from mouse brain areas, AtT-20 cells and NG108-15 cells revealed a single band corresponding to a mRNA species of 2.3 kb. In contrast, an additional mRNA species of 2.6 kb could be visualized in GH3 cells, a rat pituitary cell line.

The 2.3 kb mRNA species was found in several brain regions, such as cortex, hippocampus, striatum, hypothalamus and also in the cerebellum. No SSTR2 mRNA was found in the liver and testis. Relatively high levels of SSTR2 expression were found in the NG108-15 and particularly in the AtT-20 cells, a mouse pituitary tumor cell line. In order to investigate whether the 2.3 kb mRNA species in the mouse tissues corresponds to our cloned cDNA we performed PCR experiments. The PCR was carried out with a template cDNA constructed from poly(A) RNA derived from AtT-20 cells. A pair of oligonucleotides matching to a 5′ non-translated region (SLP4) and a 3′ non-translated region (SPL5) of 51CF3-1 served as primers (Fig. 1). The gel-resolved DNA products comprised two DNA fragments consistent with the existence of two forms of SSTR2 in the mouse tissue (Fig. 4). The shorter DNA corresponds to 51CF3-1 (1,154 bp), whereas the longer DNA corresponds to the about 300 nucleotides larger form of the SSTR2 (1,493 bp). This larger PCR product (51SCR1), corresponding to the unspliced form of SSTR2, is also seen in PCR amplification of mouse genomic DNA (data not shown). When a series dilution of cDNAs derived from AtT-20 cells was amplified by PCR, the band corresponding to the larger band disappeared at dilutions at which the band of the smaller

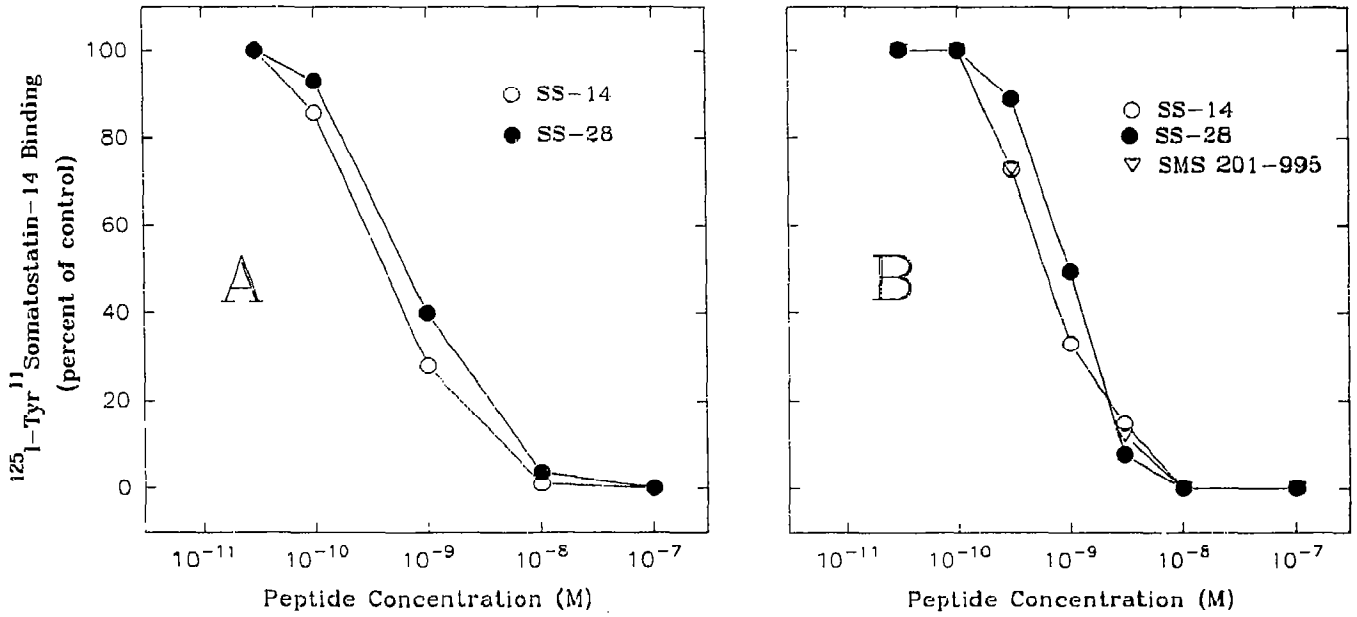

Fig. 5. Binding of [^{125}I]Tyr^{11}-SS to cells expressing 51CF3-1. (A) COS-7 cells were transiently transfected and the displacement of the labelled ligand by SS-14 and SS-28 has been measured. Values are means of triplicate determinations normalized to percent of maximum binding. (Binding in the absence of unlabelled ligand was 1900 cpm; in the presence of 100 nM SS-14 was 200 cpm; non-transfected COS-7 cells bind 300 cpm in the presence and absence of unlabelled ligand). (B) CHO-K1 cells were stably transfected. The selected clone, CHO-K1-30, binds 1,800 cpm in the absence and 300 cpm in the presence of 100 nM SS-14. The displacement of [^{125}I]Tyr^{11}-SS by SS-14, SS-28 and SMS 201-995 was measured. Values are means of triplicate determinations.

form was still visible. As judged from the relative abundance of the PCR products, the smaller form is expressed at about 30-fold higher levels in AtT-20 cells than the larger form. This low abundance of the larger form in mice may explain why only the smaller form of the SSTR2 mRNA is detected by Northern blot analysis (Fig. 3).

The situation appears to be different in rat pituitary GH3 cells where two mRNA species of 2.3 and 2.6 kb of about equal abundance are seen by Northern blot analysis (Fig. 3). In addition, similar quantities of both the 2.3 and the 2.6 kb form of SSTR2 mRNAs have been found in various tissues of the rat by Northern blot analysis [8]. It is likely that the larger form represents the unspliced form, whereas the smaller mRNA is a spliced rat analogue of the SSTR2 mRNA.

In order to investigate the binding properties of the shorter SSTR2 form we transfected the cDNA either transiently in COS-7 monkey kidney cells or stably in Chinese hamster ovary (CHO) cells. As seen in Fig. 5A and B, SS-14, SS-28 and/or the synthetic SS analogue SMS 201-995 displaced [^{125}I]Tyr^{11}-SS-14 from its newly expressed binding sites in COS-7 or CHO cells with a similar high affinity (IC_{50} 0.3–0.6 nM). The binding affinities of the short of the SSTR2 cDNA for SS-14, SS-28 and/or SMS 201-995 appear to be similar to those reported for the expressed mouse and rat SSTR2 receptors [7–9].

The two SSTR2 differ in amino acid compositions at the C-terminal end, a region shown to be important for receptor desensitization involving phosphorylation of serine and threonine residues [18]. It is interesting to note that the shorter form of the mouse SSTR2 contains three serine residues less than the larger form at the C-terminal region. Experiments are presently being carried out in our group to find out whether the two SSTR2 receptor forms differ in their desensitization characteristics.

The use of alternate splicing to generate different forms was first described for the D2 receptor. Two forms of D2 receptors have been identified which differ by a 29 residue peptide sequence located in the third cytoplasmic loop [19]. The larger form has been termed D2A and the shorter form the D2B receptor. In analogy we propose to designate the longer form of the SSTR2 receptor as SSTR2A and the shorter form as SSTR2B.

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