Identification of Ligand Binding Determinants in the Somatostatin Receptor Subtypes 1 and 2*

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The somatostatin (SRIF) receptors (SSTRs) 1 and 2 bind SRIF and SRIF 28 with high affinity, although a number of synthetic hexapeptide and octapeptide analogs of SRIF bind selectively to SSTR2. Extracellular loop three and its adjoining trans-membrane-spanning regions contain elements essential for the binding of such analogs to murine SSTR2. In particular, a stretch of amino acids from residues 294–297 (FDFV) in murine SSTR2 in trans-membrane domain seven can determine the affinity for the SSTR2-selective analogs. Within this region, Phe294 has previously been predicted to be essential for the binding of octapeptides (Kaupmann, K., Bruns, C., Raulf, F., Weber, H., Mattes, H., and Lubbert, H. (1995) EMBO J. 14, 727–735) based on the observation that SSTR1 can bind the octapeptide SMS-201-995 with reasonable affinity after a Ser-to-Phe conversion in the analogous region of this receptor (SSTR1S305F). We find that SSTR1S305F has low affinity for a number of SSTR2-selective hexapeptides, suggesting that these analogs have different binding requirements than SMS-201-995. A correlation is seen between the ability of SSTR1S305F to bind hexapeptide analogs and the presence of a phenylalanine, but not tyrosine, at position two in these small cyclic molecules. Thus, a single hydroxyl group in hexapeptides can play a critical role in determining receptor binding to these receptor mutants. We also find that the second extracellular loop of SSTR1 is important for the selectivity of certain SRIF agonists for binding to SSTR1. Taken together, our data indicate that there are multiple elements in the somatostatin receptors that can determine the binding affinity and selectivity of peptide analogs.

Somatostatin (SRIF) is a physiological inhibitor of growth hormone secretion from the anterior pituitary, insulin, and glucagon secretion from the pancreas and gastric acid secretion from the stomach (Bloom et al., 1975; Brazeau et al., 1972; Brown et al., 1977; Gomez-Pan et al., 1975; Hellman and Lennmark, 1969; Mandarino et al., 1981; Reichlin, 1983). The peptide is also a neurotransmitter and neuromodulator involved in locomotor activity and cognitive functions in the brain (Raynor and Reisine, 1992; Reisine and Bell, 1995). SRIF induces its biological actions by interacting with membrane-associated receptors. Recently, five SRIF receptor subtypes have been cloned (Bell and Reisine, 1993; Bruno et al., 1992; O’Carroll et al., 1992, 1994; Yamada et al., 1992, 1993; Yasuda et al., 1992). They exhibit between 45 and 68% amino acid sequence identity, and all bind SRIF and SRIF 28 with high affinity (Bell and Reisine, 1993; Raynor et al., 1993a, 1993b). The physiological roles of SSTR1 have not been established. However, SSTR1 mRNA is expressed in a number of tumors (see Reisine and Bell, 1995), and stimulation of SSTR1 has been reported to inhibit tumor cell proliferation (Buscail et al., 1994), suggesting that SSTR1 may be a target for cancer therapy. It has been proposed that the receptor subtype SSTR2 mediates SRIF inhibition of growth hormone (Raynor et al., 1993a, b), glucagon (Rossowski and Coy, 1994), and gastric acid (Rossowski et al., 1994) secretion. SSTR2 may also be an important target for the therapeutic actions of SMS-201-995, which is used to inhibit tumor cell growth in humans (Brown, 1990; Buscail et al., 1994, 1995; Kubota et al., 1994; Lamberts et al., 1991, Liebow et al., 1989; Taylor et al., 1994).

SSTR1 and SSTR2 can be distinguished by their differential affinities for a series of synthetic SRIF analogs (Raynor et al., 1993a, Liapakis et al., 1996). The peptide des-AAA1,2,5, [D-Trp8, IAmp9]SRIF binds selectively to SSTR1 (Liapakis et al., 1996). SSTR2 binds hexapeptides such as MK 678 and octapeptides such as SMS-201-995 with high affinity. In contrast, SSTR1 has very low or no affinity for these compounds.

Fitzpatrick and Vanden (1994) used the selective SSTR2 agonist MK 678 to map the ligand binding domains of mSSTR2 using site-directed mutagenesis. They reported that regions encompassing the second and third extracellular loops of mSSTR2 were critical for the high affinity binding of MK 678. These findings indicate that extracellular domains of SSTR2 may be most essential for agonist binding, as has already been reported for other peptide receptors, such as the tachykinins (Fong et al., 1991) and opioid receptors (Kong et al., 1994; Xue et al., 1994; Wang et al., 1994). Furthermore, Kaumann et al. (1995) have reported that a phenylalanine in trans-membrane 7 and an asparagine in trans-membrane 6 of human SSTR2 (corresponding to Phe294 and Asn276 of mSSTR2), near the third extracellular loop, are necessary for the binding of the octapeptide SMS-201-995.

Identification of the precise determinants for ligand binding to SSTR1 and SSTR2 may be critical for the rational design of nonpeptide ligands, which may be more clinically useful than the peptide agonists presently available. No information is presently available on the ligand binding determinants of SSTR1. Therefore, we have used a series of mSSTR1 and...
FIG. 1. Diagrams of the mutant receptors. Predicted amino acid sequences are taken from Reisine and Bell (1995). Shaded regions, portions of mSSTR1; open regions, portions of mSSTR2. The receptors presented are mSSTR1, mSSTR2A, chimera 258 (CHIM.258), chimera 282 (CHIM.282), chimera 281 (CHIM.281), chimera 72 (CHIM.72), chimera 394 (CHIM.394), mutant receptor 409 (MUT.409), mutant receptor 410 (MUT.410), and mutant receptor 416 (MUT.416).
mSSTR2 chimeras and the SSTR1-selective ligand des-AA^{1,2,5}-[D-Trp^{8},IAmp^{9}]SRIF to identify a critical ligand binding domain of mSSTR1. Our results reveal that the second extracellular loop of mSSTR1 is necessary for the binding of selective agonists. To more precisely define amino acid residues in mSSTR2 involved in the binding of selective agonists and to determine constituents in hexapeptides and octapeptides needed for binding to mSSTR2, a series of analogs of the hexapeptide MK 678 and the octapeptide SMS-201-995 were tested for their interaction with a series of mSSTR1 and mSSTR2 chimeras. We found that a 4-amino acid sequence in trans-membrane7 of mSSTR2 is needed for high affinity binding of octapeptides. Phe^{294} of SSTR2 is essential for high affinity binding of octapeptides but not for hexapeptides such as MK 678. Interestingly, in the SSTR1 mutant SSTR1_{S305F}, the binding of hexapeptides can be distinguished depending on whether they have a Phe or Tyr at position 2. Thus, a single hydroxyl group hinders hexapeptide binding to SSTR1_{S305F}. These data further delineate which residues in the receptor are important for ligand binding.

MATERIALS AND METHODS

Peptides—SRIF and SRIF28 were purchased from Bachem (Torrance, CA). MK 678, L363,301 (c-[Pro-Phe-o-Trp-Lys-Thr-Phe]) and L363,398 (c-[Pro-Phe-o-Trp-Lys-Abu-Phe]) (where c refers to cyclic and Abu refers to aminobutyric acid), L363,377 (c-[Pro-Tyr-o-Trp-Lys-Thr-Phe]), L363,398 (c-[Pro-Phe-o-Trp-Lys-Abu-Phe]), L363,409 (c-[N-methylalanine-Phe-o-Trp-Lys-Thr-Phe]) and L363,447 (c-[Pro-Phe-o-Trp-Lys-Val-Thr-Phe]) were obtained from Roger Friedinger (Merck). BIM23027, NC8-12, and NC4-28B were obtained from Biomeasures (Watford, MA). SMS-201-995 was obtained from Sandoz (Basel, Switzerland). The peptide des-AA^{1,2,5}-[D-Trp^{8},IAmp^{9}]SRIF was synthesized by Drs. C. Hoeger and J. Rivier at the Salk Institute as described previously (Liapakis et al., 1996).

Tissue Culture—COS-7 cells were transfected with either the mouse SSTR1 or SSTR2 or the chimeric receptor cDNAs as described (Yasuda et al., 1992). The cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics for 2 days and then tested for receptor binding.

Radioligand Binding Assay—Receptor binding assays on the cloned SRIF receptors were performed using membranes from COS-7 cells transfected with the different receptors. For the binding assay, cells were harvested in 50 mM Tris-HCl (pH 7.8) containing 1 mM EGTA, 5 mM MgCl₂, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 200 mg/ml bacitracin, and 0.5 mg/ml aprotinin (Buffer 1) and were centrifuged at 24,000 × g for 7 min at 4 °C. The pellet was homogenized in Buffer 1 using a Brinkmann Instruments Polytron (setting 2, 30 s). The homogenate was then centrifuged at 48,000 × g for 20 min at 4 °C. The pellet was homogenized in Buffer 1 and this membrane preparation was used for the radioligand binding studies. Cell membranes were incubated with [¹²⁵I-Tyr^{11}]SRIF (0.1 nM; specific activity, 2000 Ci/mmol) for 30 min at 25 °C in the presence and absence of competing peptide in a final volume of 250 µl. Nonspecific binding was defined as the radioactivity remaining bound in the presence of 1 µM SRIF. For the saturation studies, increasing concentrations of [¹²⁵I-Tyr^{11}]SRIF were incubated in
the presence or absence of 1 μM SRIF. The binding reaction was terminated by the addition of ice-cold 50 mΜ Tris-ClHCl (pH 7.8) and rapid filtration over Whatman GF/C glass fiber filters pre-soaked in 0.3% polyethyleneimine and 0.1% bovine serum albumin for 1 h. The filters were then washed with 12 ml of ice-cold 50 mΜ Tris-ClHCl (pH 7.8) buffer, and the bound radioactivity was counted in a γ counter (80% efficiency). Data from radioligand binding studies were used to generate inhibition curves. IC50 values were obtained from curve-fitting performed by the mathematical modeling program FITCOMP available on the National Institutes of Health-sponsored PROPHET system. The computer analysis allows for determination of an apparent affinity and an affinity based on the binding to a single site (one-site analysis). Where appropriate, we have indicated IC50 values based on the different statistical analyses, which use different criteria.

Mutagenesis—All expression plasmids used in this article used pCDNA3 (Invitrogen) as the vector backbone. Clones 72, 258, 261, and 282 are the pCDNA3 equivalents of clones TM4a, F, B, and C in the paper by Fitzpatrick and Vandlen (1994). Clone 394, which encodes a SSTR2 derivative with FDFV replaced by SQLS, was made using tailed oligos in a polymerase chain reaction mutagenesis procedure based on the procedure described by Imai et al. (1991). SSTR3[FDFV] (clone 410), SSTR2[SQLS] (clone 409), and SSTR1[SDFV] (clone 416) were made using the mutagenesis procedure of Kunkel (1985).

RESULTS

Several different SRIF receptor chimeras were investigated for their ability to bind different classes of peptides. The chimeras investigated are depicted in Fig. 1. The receptors were expressed in COS-7 cells and detected with [125I]-Tyr11-SRIF. The binding of [125I]-Tyr11]SRIF to each receptor was saturable and of high affinity, with Kd values varying from 0.4 to 1.9 nM (Table I). Expression levels of each chimera, as determined by Scatchard analysis, were relatively consistent between experiments, but the absolute levels varied among the various chimeras (Table I).

| Table I | Binding affinities of [125I-Tyr11]SRIF to the chimeras |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Receptor       | Kd(nM) | Bmax(fmol/mg protein) |
|------------------|------------------|------------------|------------------|------------------|------------------|
| SSTR1           | 0.6              | 1388             |
| SSTR2           | 0.7              | 3369             |
| 258             | 1.9              | 574              |
| 282             | 0.4              | 170              |
| 394             | 0.4              | 734              |
| 409             | 1.4              | 564              |
| 410             | 0.6              | 797              |

The affinities of SSTR1 and SSTR2 for the native peptide SRIF were similar (Table II). In contrast, the affinities of the various hexapeptides and octapeptides are over 1000-fold higher for SSTR2 compared with SSTR1 (Table II). The structures of the various analogs used in this study are presented in Table III.

Binding Determinants for SSTR2—The first two chimeras examined were 282 and 258 (see Fig. 1). Chimera 282 consisted of SSTR2 with the putative second and third extracellular loops of the receptor derived from SSTR1. Chimera 258 consisted of SSTR1 with the region from the beginning of the second extracellular loop to the end of the third extracellular loop replaced by the comparable residues of SSTR2. These chimeras were generated to test the hypothesis that the second and third extracellular loops of mSSTR2 are essential for the binding of constrained synthetic analogs of SRIF as proposed by Fitzpatrick and Vandlen (1994).

SRIF and SRIF 28 had similar binding affinities to these two chimeras. Hexapeptides and octapeptides had relatively high affinities for chimera 258 (Table II and Fig. 2), with their affinities being more similar to their binding to SSTR2 than to SSTR1. Hexapeptides and octapeptides had much lower affinities for chimera 282 than their binding to wild-type SSTR2 (Table II and Figs. 3 and 4). These findings indicate that the second and third extracellular loops of SSTR2 are essential for high affinity binding of hexapeptide and octapeptide analogs of SRIF.

Chimera 281 consists of SSTR2 with the second extracellular loop of SSTR1 (see Fig. 1). Both hexapeptide and octapeptide binding to chimera 281 and SSTR2 were similar, indicating that the second extracellular loop of SSTR2 is not essential for the binding of these peptides (Table II and Figs. 3 and 4). Comparison of the affinities for the hexapeptides and octapeptides for chimeras 281 and 282 indicate that extracellular loop 3 and the adjacent trans-membrane-spanning regions may be the most essential portion of SSTR2, which determines binding by the reduced ring analogs of SRIF.

Analyzing an alignment of all SSTR subtypes (see Reisine and Bell, 1995), we noted a correlation between the presence of aromatic residues in trans-membrane domain 7 and the ability of a given SRIF receptor subtype to bind MK 678. Based on this observation, mutant 394 was generated (see Fig. 1). This mutant consists of mSSTR2 with 4-amino acid sequence FDFV (residues 294–297, according to the amino acid sequence alignment of Reisine and Bell, 1995) replaced by the corresponding
sequence of mSSTR1 (SQLS, residues 305–308).

The affinities of octapeptides for mutant 394 were reduced over 100-fold compared with their binding to SSTR2 (Table II and Fig. 4). Hexapeptides also had reduced affinity for mutant 394. These findings indicate an essential role of the FDFV sequence in high affinity binding of SSTR2-selective agonists.

In addition to the phenylalanine in trans-membrane 7 of human SSTR2, Kaufmann et al. (1995) have also proposed that an FDFV sequence in high affinity binding of SSTR1-selective agonists. This residue corresponds to the first Phe of the FDFV sequence we mutated. To determine if this residue had a similar effect in our studies on mouse SRIF receptor subtypes, the point mutants SSTR2F294S and SSTR2F294T were generated and tested. Their synthesis and ability to bind to SRIF receptors requires a specific orientation for interaction with SSTR1S305F. In contrast, the isomer with a wild-type parent that is insensitive to such analogs (see Table IV and Fig. 4).

Phe294 appears to play a less significant role in binding of the hexapeptides MK 678 and BIM23027, as the mutant SSTR2F294S demonstrated low affinity for these SRIF analogs (Table IV and Fig. 3). Furthermore, the substitution of the Phe to Ser in SSTR2 (SSTR2F294S) only had a small effect on the affinity for these analogs.

Other hexapeptides (see Table III for sequences) were then analyzed to determine if the selective binding of SSTR2F294S for octapeptides but not hexapeptides was a general rule. Following analysis of seven hexapeptide analogs (see Table IV and Fig. 4), we found that the analogs could be grouped into two classes: those with moderate affinity for SSTR2F294S (MK 678, BIM23027, and L363,409), and those with weak or no affinity for SSTR2F294S (L363,301, L363,398, and L363,447) and those with weak or no affinity for SSTR1S305F (MK 678, BIM23027, and L363,377). Despite several amino acid differences between the various analogs, the most apparent characteristic that distinguished the two hexapeptide groups was the residue at position 2 in the cyclic peptide structure: for the analogs with moderate affinity, this residue was a Phe; for the low affinity analogs, this residue was a Tyr. This is most clearly seen when comparing L363,301 with L363,377, which are identical molecules except for the single Phe-to-Tyr substitution at position two. This single residue exchange produces a 70-fold reduction in affinity for SSTR1S305F. Since a phenylalanine and tyrosine only differ by a hydroxyl group, these results suggest that the hydroxyl group hinders interaction of the peptide with the SSTR1S305F receptor.

To further investigate the conformation of L363,301 needed for binding to SSTR1S305F, two isomers of L363,301 were tested. Their synthesis and ability to bind to SRIF receptors endogenously expressed in the cell line AtT-20 are described by Huang et al. (1992). Neither isomer bound to SSTR1 (data not shown). The isomer c-[Pro-(25,35)-a-MePhe-o-Trp-Lys-Thr-Phe], with a constrained side chain group around the phenylalanine in position 2, bound to SSTR1S305F with moderate affinity (192 ± 38 nM; n = 3), suggesting that this side chain orientation of the phenylalanine in the cyclic peptide is important for interaction with SSTR1S305F. In contrast, the isomer c-[Pro-(S)-a-MePhe-o-Trp-Lys-Thr-Phe], which creates a "flat" conformation of the hexapeptide (Huang et al., 1992) displayed no affinity for SSTR1S305F (IC50 > 1000 nM; n = 2). These findings suggest that the phenylalanine in position 2 of L363,301 requires a specific orientation for interaction with SSTR1S305F.

In addition to the phenylalanine in trans-membrane 7 of SSTR2, Kaufmann et al. (1995) have also proposed that an asparagine in trans-membrane 6 has a role in the high affinity binding of SMS-201-995 to SSTR2. To test the role of this amino acid in hexapeptide and octapeptide binding to SSTR2, glutamine in position 291 of mSSTR1 was mutated to an asparagine, which corresponds to residue 276 of mSSTR2, to generate SSTR1Q291N (clone 416). SRIF bound to SSTR1Q291N with high affinity (Table IV). However, neither hexapeptides nor octapeptides bound to this mutant receptor, indicating that by itself it does not reconstitute a recognition site for these synthetic peptides (Table IV). The data of Kaufmann et al. (1994) are essentially the same; the Q291N mutation only becomes obvious when generated together as a double mutant with the S305F exchange.
Dose-response curves of hexapeptide analogs inhibiting [¹²⁵I-Tyr¹¹]SRIF binding to the chimeric receptors. Representative inhibition curves of the different hexapeptides in binding to the wild-type and mutant SRIF receptors are shown. The hexapeptides are MK 678 (A), BIM23027 (B), L363,301 (C), L363,398 (D), L363,377 (E), L363,447 (F), L363,409 (G), and L363,377 (H). Average IC₅₀ values are described in Tables II and IV, and the structures of the peptides are described in Table III.
Binding Determinants of SSTR1—The SSTR1-selective peptide des-AA^{1,2,5}-[d-Trp^{8},lAmp^{9}]SRIF has an affinity for SSTR1 of 4.9 nM (Table V and Fig. 5). It only displaces binding of [125I-Tyr^{11}]SRIF to SSTR2 at concentrations above 1 \mu M (Table V and Fig. 5). To investigate determinants in SSTR1 needed for its binding, this peptide was tested in initial studies for its interaction with chimera 72 (see Fig. 1). Chimera 72 consists of the NH₂-terminal portion of SSTR1 up to the junction of transmembrane 4 and extracellular loop 2, with the remainder consisting of SSTR2. SRIF bound to chimera 72 with high affinity (0.99 nM; \( n = 2 \)). des-AA^{1,2,5}-[d-Trp^{8},lAmp^{9}]SRIF bound with very low affinity to this chimera (Table V and Fig. 5), suggesting that regions NH₂-terminal of the second extracellular loop of SSTR1 are not essential for the SSTR1-selective binding of des-AA^{1,2,5}-[d-Trp^{8},lAmp^{9}]SRIF.

To investigate the possible role of the second and third extracellular loops in the binding of des-AA^{1,2,5}-[d-Trp^{8},lAmp^{9}]SRIF to SSTR1, the peptide was tested for its interaction with chimera 282, which consists of SSTR2 with the second and third extracellular loops of SSTR1. The peptide bound poorly to this chimera (Table V and Fig. 5). Nevertheless, when des-AA^{1,2,5}-[d-Trp^{8},lAmp^{9}]SRIF was tested for its interaction with chimera 282, it bound with very low affinity (0.1 \mu M; \( n = 5 \)).

**Table IV**

| Binding affinities of SRIF analogs to the mutants |
|-----------------------------------------------|
| \( IC_{50} \) (nM) | Mutant 409 | Mutant 410 | Mutant 416 |
|---------------------|-----------|-----------|-----------|
| SRIF 14             | 9 ± 2 (n = 3) | 0.6 (n = 2) | 0.1 ± 0.02 (n = 5) |
| SRIF 28             | 20 ± 2 (n = 3) | 0.6 (n = 2) |           |
| SMS-201-995         | 14 ± 2 (n = 3) | 8 ± 1 (n = 3) | >1000 (n = 3) |
| NC8-12              | 26 ± 16 (n = 3) | 24 ± 9 (n = 3) | >1000 (n = 3) |
| NC4-24 B            | 24 ± 5 (n = 3) | 14 ± 4 (n = 3) | ND* |
| Hexapeptides MK 678 | 4 ± 1 (n = 3) | 764 ± 118 (n = 3) | >1000 (n = 4) |
| BIM23027            | 1 ± 0.6 (n = 3) | 507 ± 159 (n = 3) | >1000 (n = 3) |
| L363,301            | 67 ± 19 (n = 3) | 112 ± 15 (n = 3) | >1000 (n = 3) |
| L363,398            | 142 ± 61 (n = 3) | 139 ± 24 (n = 3) | >1000 (n = 3) |
| L363,377            | 53 ± 10 (n = 3) | 781 ± 64 (n = 3) | >1000 (n = 3) |
| L363,409            | 12 ± 4 (n = 3) | 67 ± 8 (n = 3) | >1000 (n = 3) |
| L363,447            | 130 ± 49 (n = 3) | 64 ± 11 (n = 3) | >1000 (n = 3) |

*ND, not done.*

**Fig. 4.** Dose-response curves of octapeptide binding to the wild-type and mutant SRIF receptors. Representative dose-response curves of octapeptides binding to the wild-type and mutant receptors are presented. The octapeptides are SMS-201-995 (A), NC8-12 (B), and NC4-28B (C). The average \( IC_{50} \) values are described in Tables II and IV, and the peptide structures are in Table IV.
interaction with chimera 281 (which consists of SSTR2 with the second extracellular loop of SSTR1), the peptide bound with relatively high affinity to this chimera (Table V and Fig. 5). Compared with its binding to SSTR2, this compound bound with over 25-fold greater affinity to chimera 281. This suggests a role of the second extracellular loop of SSTR1 in the binding of this peptide.

**DISCUSSION**

Using site-directed mutagenesis, we have found that extracellular loop 2 of SSTR1 grafted into SSTR2 allows a SSTR1-specific peptide to bind to SSTR2. A similar role of extracellular loop 2 for the binding of the peptide dynorphin A to the κ receptor has recently been reported (Xue et al., 1994; Wang et al., 1994; Kong et al., 1994).

Although extracellular loop 2 is critical for the binding of selective ligands to SSTR1, it is not essential for the binding of selective peptides to SSTR2, since hexapeptide and octapeptide analogs of SSTR2 bound with high affinity for chimera 281. In contrast, extracellular loop 3 and its surrounding trans-membrane-spanning regions may be more essential for the binding of selective agonists to SSTR2. These findings demonstrate that the elements that define selectivity are dependent on both the SSTR1 analog being tested as well as the receptor subtype being analyzed.

Previous mutagenesis studies by Fitzpatrick and Vandlen (1994) suggested that extracellular loop 3 of mSSTR2 was critical for high affinity binding of the hexapeptide MK 678. Kaupmann et al. (1995) reported that two amino acids, an asparagine in trans-membrane 6 and a phenylalanine in trans-membrane 7, were essential for the binding of the octapeptide SMS-201-995 to human SSTR2. The studies reported here extend the previous studies of Fitzpatrick and Vandlen (1994) and Kaupmann et al. (1995), in that both hexapeptide and octapeptide SRIF agonists, with different activities and specificities, were tested on a variety of mouse SRIF receptor mutants.

Our findings reveal an essential role of the amino acid sequence FDFV (residues 294–297) in the binding of octapeptide SRIF analogs to mSSTR2. Mutation of this amino acid sequence to the corresponding SQVS sequence found in mSSTR1 (residues 305–308) abolished high affinity binding of a number of synthetic peptides to mSSTR2.

Since the phenylalanine in trans-membrane 7 of human SSTR2 described by Kaupmann et al. (1995) is part of this 4-amino acid sequence, we further explored the role of this amino acid in mSSTR2 by testing the binding of both hexapeptides and octapeptides to SSTR1S305F. Consistent with the results of Kaupmann et al. (1995), octapeptides such as SMS-201-995, NC8-12, and NC4-288 exhibited enhanced affinity for SSTR1S305F compared with their binding to SSTR1, indicating that this residue could perform the same function in both human and mouse SSTR homologs. It is interesting to note that the critical role of Phe305 in the SSTR1 mutant for octapeptide binding is not duplicated in SSTR2, in which replacement of this residue (SSTR2Phe295) has little effect on octapeptide sensitivity. This result may be interpreted two ways: it is possible that other residues in SSTR2 may also play a role in analog binding, although in the context of a SSTR1 background, these elements are not required; the second explanation may be that the SSTR1S305F receptor, being a "synthetic" receptor, does not accurately indicate the role of Phe in the "natural" receptor (i.e. SSTR2).

Although Phe294 has a role for the binding of octapeptides to SSTR1S305F, the role of this amino acid in the binding of the hexapeptide MK 678 is less clear, since the affinity of MK 678 for binding to SSTR1S305F is relatively weak (764 nM) and arguable comparable to MK 678 binding to SSTR1 (1000 nM). Similar results were observed with the hexapeptide BIM23027. Nevertheless, analysis of a series of hexapeptides revealed that some exhibited clearly improved binding affinities for SSTR1S305F compared with SSTR1. We find that all the hexapeptide analogs with reasonable affinity for SSTR1S305F contain a phenylalanine instead of a tyrosine at position 2 in the SRIF analog. Since phenylalanine and tyrosine only differ by a hydroxyl group, these findings suggest that the phenylalanine may facilitate interaction of the hexapeptide with some element in the SSTR1S305F receptor. Despite this clear ability to separate the hexapeptides into two groups by affinity for SSTR1S305F, the same classification does not hold true when one examines native SSTR2. The binding affinity of SSTR2 for hexapeptide analogs appears independent of the specific aromatic residue seen at position two, i.e. it may be either Tyr or Phe with no loss of affinity.

An aromatic residue, either phenylalanine or tyrosine, at position 2 of the hexapeptide, appears to be essential for their high affinity binding to SSTR2, since L363,376 (c-[Pro-Ala-D-Trp-Lys-Thr-Phe]), with an alanine substituted for the phenylalanine of L363,301, has very low affinity for SSTR2 (IC<sub>50</sub> 356 nM) (Raynor et al., 1993).

In contrast to the hexapeptides, the tyrosine adjacent to d-tryptophan in the octapeptides is not critical for their interaction with SSTR1S305F. Since both SMS-201-995 and NC4-288 (with a phenylalanine and tyrosine, respectively) exhibited enhanced binding to SSTR1S305F compared with their binding to mSSTR1. These findings suggest that hexapeptides and octapeptides may differ in some specific constituents needed for high affinity binding to SSTR2.

Our results reveal that the second extracellular loop of SSTR1 is involved in selecting out particular peptides for bind-

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**Table V**

| Peptide | IC<sub>50</sub> (nM) |
|---------|---------------------|
| SSTR1   | 5 ± 0.5 (n = 6)     |
| SSTR2   | >1000 (n = 3)       |
| Chimera 72 | 842 ± 38 (n = 3) |
| Chimera 258 | 315 ± 31 (n = 3) |
| Chimera 281 | 40 ± 16 (n = 4)  |
| Chimera 282 | 466 ± 118 (n = 6) |

**Fig. 5.** Dose-response curves for des-AA<sup>1,2,5</sup>-[D-Trp<sup>8</sup>,IAMP<sup>9</sup>]-SRIF binding to the chimeric receptors. Representative dose-response curves are presented. The averaged IC<sub>50</sub> values are described in Table V. The synthesis and complete structure of this peptide was described by Liapakis et al. (1996).
ing. The affinity of chimera 281 for des-AA\textsuperscript{1,2,5}[-Trp\textsuperscript{3}] Amp\textsuperscript{9} SRIF was over 100-fold greater that the affinity of SSTR2 for this peptide and approximately 8-fold lower than for mSSTR1, suggesting that other residues besides those in extracellular loop 2 are essential for the natural binding of this peptide to SSTR1. Modeling based on our results may help in the design of more selective SSTR1 ligands.

SSTR2-selective agonists have a number of therapeutic uses (Risine and Bell, 1995; Buscail et al., 1994, 1995; Kaupmann et al., 1995; Lamberts et al., 1991). SMS 201-995 is used clinically to inhibit cancer growth. In humans, this peptide primarily acts on SSTR2 (O’Carroll et al., 1994). However, being a peptide, its usefulness is limited by its route of administration and stability. Rational development of nonpeptide SSTR2 agonists could overcome these limitations. Similarly, SSTR1-selective agonists may be useful in treating cancer and may have advantages over SMS-201-995, since there is little evidence that SSTR1 desensitizes (Rens-Domiano et al., 1992).

Identification of ligand binding domains of SSTR1 may facilitate development of nonpeptide SSTR1 agonists with clinical uses.

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