The EMBO Journal vol.14 no.4 pp.727-735, 1995

Two amino acids, located in transmembrane domains VI and VII, determine the selectivity of the peptide agonist SMS 201-995 for the SSTR2 somatostatin receptor

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Communicated by P.H.Seeburg

Human somatostatin receptor subtypes (SSTR1–5) bind their natural ligands SRIF-14 and SRIF-28 with high affinity. By contrast, short synthetic SRIF analogues such as SMS 201-995, a peptide agonist used for the treatment of various endocrine and malignant disorders, display sub-nanomolar affinity only for the receptor subtype SSTR2. To understand the molecular nature of selective peptide agonist binding to somatostatin receptors we have now, by site-directed mutagenesis, identified amino acids mediating SMS 201-995 specificity for SSTR2. Sequentially, amino acids in SSTR1, a receptor subtype exhibiting low affinity for SMS 201-995, were exchanged for the corresponding SSTR2 residues. After three consecutive steps, in which eight amino acids were exchanged, a SSTR1 mutant receptor with high affinity for SMS 201-995 was obtained. Receptor mutants with different combinations of these eight amino acids were then constructed. A single Ser305 to Phe mutation in TM VII increased the affinity of SSTR1 for SMS 201-995 nearly 100-fold. When this mutation was combined with an exchange of Gln291 to Asn in TM VI, almost full susceptibility to SMS 201-995 was obtained. Thus, it is concluded that the specificity of SMS 201-995 for SSTR2 is mainly defined by these two amino acids in transmembrane domains VI and VII. Using the conjugate gradient method we have, by analogy to the well established structure of bacteriorhodopsin, built a model for SRIF receptor–ligand interactions that explains the importance of Gln291 and Ser305 for the selectivity of agonists.

Key words: G protein-coupled receptor/octreotide/receptor modelling/site-directed mutagenesis/SRIF

Introduction

Somatostatin (somatotropin release inhibiting factor, SRIF) is a cyclic peptide originally isolated from ovine hypothalamus. It occurs in two major physiologically active forms, somatostatin-28 (SRIF-28) and somatostatin-14 (SRIF-14; Figure 1). They are expressed in a tissue-specific manner in several organs including brain, stomach, small intestine and D-cells of the pancreas. Somatostatin inhibits the release of many hormones such as growth hormone, insulin, gastrin or glucagon (Reichlin, 1983a,b). In the brain, SRIF acts as a neurotransmitter and neuromodulator. It facilitates for instance monoamine release and, consequently, affects motor activity (Tanaka and Tsujimoto, 1981; Chesselet and Reisine, 1983; Beal and Martin, 1984; Lee *et al.*, 1988).

In vivo, the effects of somatostatin are limited by its rapid proteolytic degradation (plasma half-life <3 min). For therapeutic applications in man, several short synthetic somatostatin analogues with increased metabolic stability, for example SMS 201-995 (octreotide), have been synthesized (Bauer *et al.*, 1982; Figure 1). SMS 201-995 is highly effective in inhibiting hormone secretion and therefore used for the treatment of various endocrine and malignant disorders such as growth hormone secreting pituitary adenomas. It allows the symptomatic control of gastroenteropancreatic (GEP) tumours by inhibiting hormone as well as neurotransmitter release from tumour tissue (Lamberts *et al.*, 1991; Weckbecker *et al.*, 1993).

The actions of somatostatin are mediated by specific, high affinity membrane receptors located on the target tissues. During the last few years a total of five SRIF receptor subtypes, denoted SSTR1-5, have been identified in various species by molecular cloning (Kluxen *et al.*, 1992; Li *et al.*, 1992; Meyerhof *et al.*, 1992; Yamada *et al.*, 1992a,b; Bruno *et al.*, 1992; O'Carroll *et al.*, 1993; Panetta *et al.*, 1993; Rohrer *et al.*, 1993). While all five receptor subtypes inhibit cAMP formation after expression in HEK 293 or COS-7 cells (Kaupmann *et al.*, 1993; O'Carroll *et al.*, 1993), coupling to additional signalling systems may functionally distinguish these receptors (Schweitzer *et al.*, 1993; Hou *et al.*, 1994). SSTR1-5 share at least 45% overall amino acid identity and all five receptor subtypes display high affinity binding of the natural somatostatins SRIF-14 and SRIF-28. However, remarkable differences were observed in their binding profiles for short synthetic SRIF analogues such as SMS 201-995, BIM 23014, MK 678 and RC 160 (Bruns *et al.*, 1994). High affinity binding was observed for SSTR2, low affinity for SSTR1 and SSTR4 (Kluxen *et al.*, 1992; Bell and Reisine, 1993; Rohrer *et al.*, 1993). Interestingly, these SRIF analogues bind with high, sub-nanomolar affinities also to rat SSTR5, whereas the human homologue of this subtype as well as SSTR3 from both species display intermediate (10–100 nM) affinities (Panetta *et al.*, 1993; O'Carroll *et al.*, 1993; Yamada *et al.*, 1992b).

Somatostatin receptors belong to the family of G protein-coupled receptors with the typical seven hydrophobic membrane-spanning regions (7TM receptors). For some of these receptors, in particular the α- and β-adrenergic, muscarinic and biogenic amine receptors, the ligand binding site was demonstrated to be located in a pocket formed by the hydrophobic regions (for reviews see Dohlman

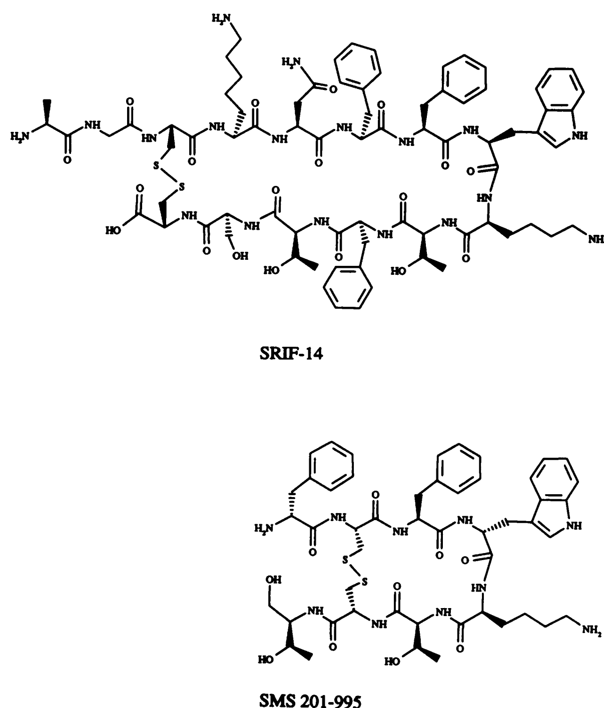


Fig. 1. Chemical structures of SRIF-14 (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys) and SMS 201-995 [D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)].

et al., 1991; Hibert *et al.*, 1993). Some receptors for larger glycoprotein ligands, e.g. luteinizing hormone (LH), follicle stimulating hormone (FSH) or thyroid stimulating hormone (TSH), contain large extracellular N-terminal extensions that have been demonstrated to be involved in the binding of these spacious ligands (Xie *et al.*, 1990; Braun *et al.*, 1991; Ji and Ji, 1991; Nagayama *et al.*, 1991). Much less is known about the binding domains of G protein-coupled receptors that interact with smaller peptides. Recent studies on tachykinin and bombesin receptors demonstrated the importance of both the extracellular loops and the transmembrane domains for high affinity ligand binding (Fong *et al.*, 1992; Yokota *et al.*, 1992; Fathi *et al.*, 1993; Gether *et al.*, 1993).

To understand the molecular interactions of peptide agonists with somatostatin receptor subtypes we have now, by site-directed mutagenesis, identified the amino acid residues in SSTR2 that are mainly responsible for the selectivity of SMS 201-995. To define these residues unambiguously, human SSTR1, normally showing very low affinity for SMS 201-995 (pK_i <7), was converted into a high affinity receptor for this ligand. In line with these findings and by analogy to other well established receptor models, we propose a model for SRIF receptors and their binding of peptide agonists. The models explain the differences in affinity of peptide agonists for somatostatin receptors.

Results

Strategy to identify structural components in SSTR2 mediating SMS 201-995 specificity

SRIF-14 binds with high affinity to all five cloned human somatostatin receptors, SSTR1–5 (Figure 2B). By contrast, short synthetic analogues such as SMS 201-995 bind with high affinity to receptor subtype SSTR2, show low affinities for SSTR1 and SSTR4, and intermediate affinities for SSTR3 and the human SSTR5 (Figure 2B). The goal of the present study was to characterize the structural components of somatostatin receptor subtypes that determine the specificity of SMS 201-995 for the somatostatin receptor subtype SSTR2. A sequence alignment of all human somatostatin receptor subtypes revealed that the transmembrane domains are highly conserved whereas the N- and C-termini as well as the extracellular and intracellular loops are more divergent (Figure 2A). Based on the sequence alignment, candidate amino acids with potential importance for high affinity SMS 201-995 binding were selected using the following criteria. First, they should be conserved or conservatively exchanged in SSTR2, SSTR5 and SSTR3, but different in SSTR1 and SSTR4. Second, the location should be either extracellular or within the transmembrane domains. About 30 amino acids fulfilled these criteria (Figure 2A). Most of them were located within the transmembrane domains.

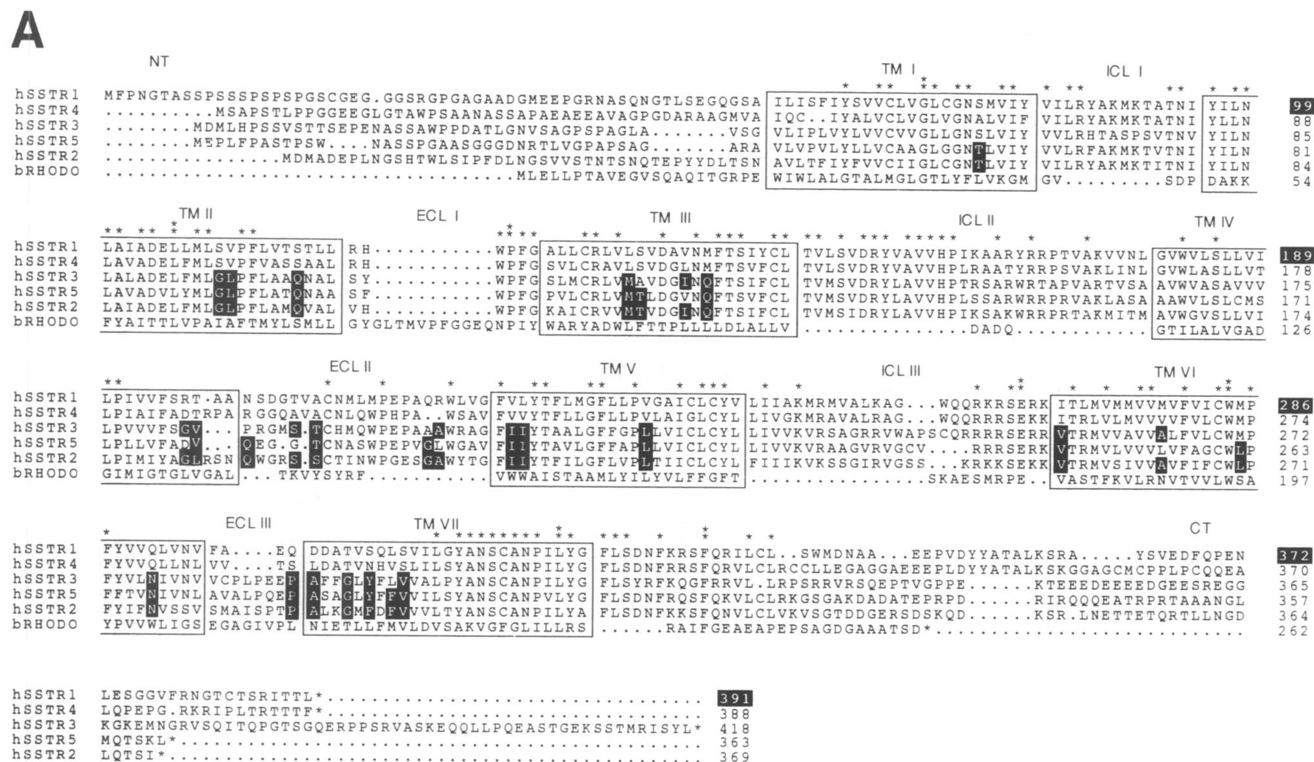
Construction of a SSTR1 receptor mutant displaying nanomolar affinity for SMS 201-995

We used a site-directed mutagenesis approach to investigate the potential importance of these selected residues for determining binding affinity for SMS 201-995. Since it is difficult to interpret mutations that cause a loss of function, we aimed at mutations in SSTR1 that increase the affinity for SMS 201-995. Since wild-type SSTR1 has a very low affinity for SMS 201-995 (pK_i <7; Figure 3A), we decided to sequentially exchange amino acids in SSTR1 by their corresponding SSTR2 residues, thereby shifting the amino acid sequence stepwise towards SSTR2, the receptor subtype which displays high affinity for SMS 201-995 (Figure 3B). Point mutations were introduced into SSTR1 and the affinities for SRIF-14 and SMS 201-995 were determined in competition binding experiments after transient expression of the mutant receptors in COS-1 cells. [^{125}I]SRIF-14 was chosen as radioligand because of its high affinity for both SSTR1 and SSTR2 receptors (Figure 3A and B). After three consecutive steps (Table I, clones #1–3), eight amino acids in transmembrane domains (TM) III (L134M, S135T, V139I, M141Q), TM VI (Q291N) and TM VII (S305F, L307F, S308V) were exchanged that were given high or medium priority based on theoretical considerations described above. The exchange of these eight amino acids resulted in a SSTR1 receptor mutant that displayed a 100-fold increase in SMS 201-995 affinity whereas the affinity for SRIF-14 was not significantly altered compared with wild-type SSTR1 (Table I, clone #3). The SMS 201-995 affinity was only slightly lower than that of SRIF-14, indicating that the major determinants for high affinity binding of SMS 201-995 were present in this clone (Figure 3C). Consistent with this conclusion, the exchange of further amino acids in this clone did not result in an additional increase in binding affinity for SMS 201-995 (Table I, clone #4).

Identification of individual amino acids mediating SMS 201-995 specificity for SSTR2

To identify the minimal number of amino acid exchanges required to switch the receptor from a SSTR1 to a SSTR2

A



B

pIC_{50}

Receptor	SRIF-14	SMS 201-995
hSSTR1	8.8 ±0.2	6.7 ±0.2
hSSTR2	9.6 ±0.3	9.2 ±0.2
hSSTR3	9.2 ±0.1	7.9 ±0.3
hSSTR4	8.7 ±0.2	6.0 ±0.2
hSSTR5	9.2 ±0.2	8.1 ±0.2

$\bar{x}$ ±SD (n = 3-5)

Fig. 2. (**A**) Amino acid sequence alignment of the five human somatostatin receptor subtypes and bacteriorhodopsin. Membrane-spanning regions (TMs, boxed) were defined according to the structure of bacteriorhodopsin (Henderson *et al.*, 1990). Amino acid residues conserved or conservatively exchanged in SSTR2 and, respectively, SSTR3 and/or SSTR5, but not in SSTR1 or SSTR4 are highlighted in the seven transmembrane domains and the extracellular loops (ECLs). Conservative exchanges were defined according to Gribskov and Burgess (1986). Residues identical in all five somatostatin receptors are marked by an asterisk. Two asterisks indicate residues which are also conserved in bacteriorhodopsin. References: hSSTR1 and hSSTR2 (Yamada *et al.*, 1992a); hSSTR3 (Yamada *et al.*, 1992b); hSSTR4 (Rohrer *et al.*, 1993); hSSTR5 (Panetta *et al.*, 1993); bacteriorhodopsin (Dunn *et al.*, 1981). The numbering of amino acids is indicated on the right, that of SSTR1 is highlighted since, for clarity, it is used throughout the text for both SSTR1 and SSTR2. (**B**) The affinities of SRIF-14 and SMS 201-995 were determined on membranes from stably transfected CHO cells expressing the cloned receptors. The numbers indicate the mean pIC_{50} values ±SD determined in three independent experiments. Slight variations between these affinities and those shown in Table I are due to differences in the methodology (here binding on cell membranes, in Table I binding on transiently transfected intact COS-1 cells).

phenotype, mutant receptors with different combinations of the eight amino acids exchanged in clone #3 were prepared. The dramatic shift in affinity was obtained when three mutations were added to the transmembrane domain VII of clone #2. Therefore, this domain was likely to contain important determinants for high affinity binding of SMS 201-995. When these three amino acids were investigated individually (clones #5–7), the mutation of Ser305 to Phe (clone #5) was found to have the most profound effect on SMS 201-995 affinity. This single amino acid exchange increased the affinity for SMS 201-995 nearly 100-fold (pIC_{50} 8.0, Table I). The L307F or S308V mutations (clones #6 and 7) resulted in only marginal increases in SMS 201-995 affinity and are likely to be of minor importance.

When the S305F exchange in transmembrane region VII was combined with a Q291N mutation in transmembrane region VI (clone #13), the affinity for SMS 201-995 increased further by ~3-fold. The value obtained was even higher than that found for clone #3 with eight simultaneous point mutations. In contrast, the Q291N mutation alone or in combination with the S308V mutation had only minor effects (clones #10, 11 and 12). In agreement with the data obtained for SMS 201-995, the binding affinities for two other short SRIF analogues, BIM 23014 and RC 160, were increased from pIC_{50} values of 6.3 to 7.8 and 6.8 to 8.6, respectively (n = 2). Thus, the mutation of Gln291 and Ser305 in SSTR1 to Asn and Phe, respectively, are both necessary and together sufficient to confer almost full susceptibility for these short SRIF analogues (Figures 3D and 4).

Molecular modelling of the interaction of peptides with somatostatin receptors

To explain the results of the site-directed mutagenesis study, we independently took a receptor modelling approach. By using the three-dimensional (3D) structure of bacteriorhodopsin (Henderson *et al.*, 1990) as a template

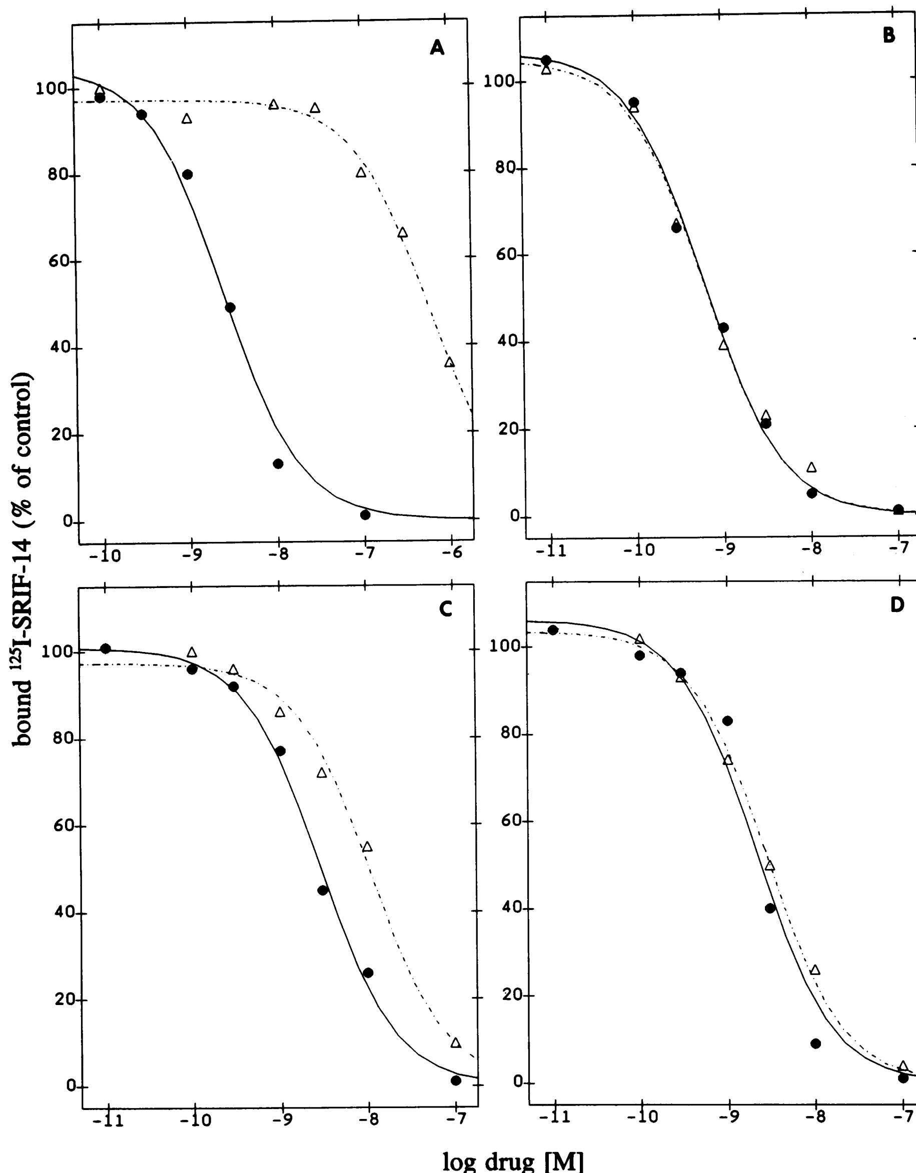


Fig. 3. Comparison of SRIF-14 and SMS 201-995 binding to somatostatin receptors: (**A**) wild-type SSTR1, (**B**) wild-type SSTR2, (**C**) SSTR1 with eight point mutations (clone #3; L134M, S135T, V139I, M141Q in TM III, Q291N in TM VI, S305F, L307F, S308V in TMVII), (**D**) SSTR1 with two point mutations (clone #13; Q291N in TMVI and S305F in TM VII). The receptors were transiently expressed in COS-1 cells. Competition binding experiments were performed with [^{125}I]SRIF-14 and replacement by unlabelled SRIF-14 (closed symbols) or SMS 201–995 (open symbols).

for the folding of G protein-coupled receptors, 3D models of the human SSTR1 and SSTR2 receptor subtypes were prepared. Energy minimization was performed using the conjugate gradient method. SRIF-14 and SMS 201-995 were then docked into the putative receptor binding pockets. Models of the SRIF-14–SSTR1 and SMS 201-995–SSTR2 complexes were prepared and energy minimized (Figure 5). The models for these complexes are based on the assumption that a conserved aspartate in TM III (Asp137 in SSTR1) anchors the ligands to the receptors by an electrostatic interaction with Nζ of Lys9 of SRIF-14 or Lys5 of SMS 201-995, respectively. This aspartate is also conserved in all monoamine receptors where it has previously been demonstrated to bind the cationic amine group of the receptor ligands (Strader *et al.*, 1988; Kurtenbach *et al.*, 1990). We found that mutating this residue in somatostatin receptors to glutamate resulted in a drastic loss of SRIF-14 binding (data not shown), indicating the importance of this residue.

Figure 5 presents computer-generated lateral views of the SRIF14/SSTR1 (Figure 5A) and SMS 201-995/SSTR2 (Figure 5B and C) complexes as well as a close-up view of the critical residues involved in SMS 201-995 binding to the SSTR2 receptor (Figure 5D). The models indicate that for both receptor types residues Phe232, Trp284 and Tyr288 are involved in ligand binding (numbering according to hSSTR1 as shown in Figure 2). This is illustrated schematically in Figure 5E and F. These residues define a lipophilic cavity for Trp8 of SRIF-14 or Trp4 of SMS 201-995, similar to the binding site proposed for the aromatic nucleus of serotonin, dopamine and noradrenaline in their respective receptors (Strader *et al.*, 1989; Trumpp-Kallmeyer *et al.*, 1992). Another important aromatic residue is Phe195 in SSTR1, respectively Tyr195 in

Table I. SRIF-14 and SMS 201-995 binding affinities of wild-type and mutant receptor clones

Clone#	Mutation TM II	TM III	TM IV	TM VI	TM VII	pIC_{50} SRIF-14	pIC_{50} SMS 201-995
SSTR1						8.6 ± 0.1 (3)	6.2 ± 0.1 (3)
SSTR2						9.4 ± 0.2 (3)	9.3 ± 0.2 (3)
1		M141Q		Q291N		9.0 ± 0.2 (3)	6.3 ± 0.3 (3)
2		L134M S135T V139I M141Q		Q291N		9.3 ± 0.1 (2)	<6.0 (2)
3		L134M S135T V139I M141Q		Q291N	S305F L307F S308V	8.7 ± 0.2 (5)	8.1 ± 0.3 (3)
4	S117Q	L134M S135T V139I M141Q	V193M F195Y	M285L V289I Q291N	S305F L307F S308V	8.7 ± 0.2 (5)	8.2 ± 0.3 (4)
5					S305F	8.9 ± 0.1 (2)	8.0 ± 0.1 (2)
6					L307F	9.1 ± 0.1 (2)	6.6 ± 0.2 (2)
7					S308V	8.7 ± 0.1 (2)	6.6 ± 0.1 (2)
8					S305F L307F S308V	8.5 ± 0.2 (4)	7.4 ± 0.1 (4)
9					S305F S308V	8.8 ± 0.1 (2)	7.9 ± 0.1 (2)
10				Q291N		9.1 ± 0.1 (2)	6.4 ± 0.1 (2)
11				Q291N	S308V	9.0 ± 0.1 (2)	5.9 ± 0.1 (2)
12				Q291N	S305F S308V	9.0 ± 0.2 (4)	8.6 ± 0.1 (3)
13				Q291N	S305F	8.9 ± 0.3 (5)	8.5 ± 0.3 (3)

Wild-type human SSTR1 and SSTR2 and mutant receptor clones (constructed from hSSTR1) were transiently expressed in COS-1 cells. Their affinity profiles were determined in competition binding experiments with $[^{125}I]$SRIF-14 and unlabelled SRIF-14 or SMS 201-995. Data are given as mean values ± SD with the number of independent experiments indicated in brackets. Mutations are described by the respective amino acid in wild-type SSTR1 in single letter code, followed by the position of the amino acid in SSTR1 and by the amino acid introduced at that position.

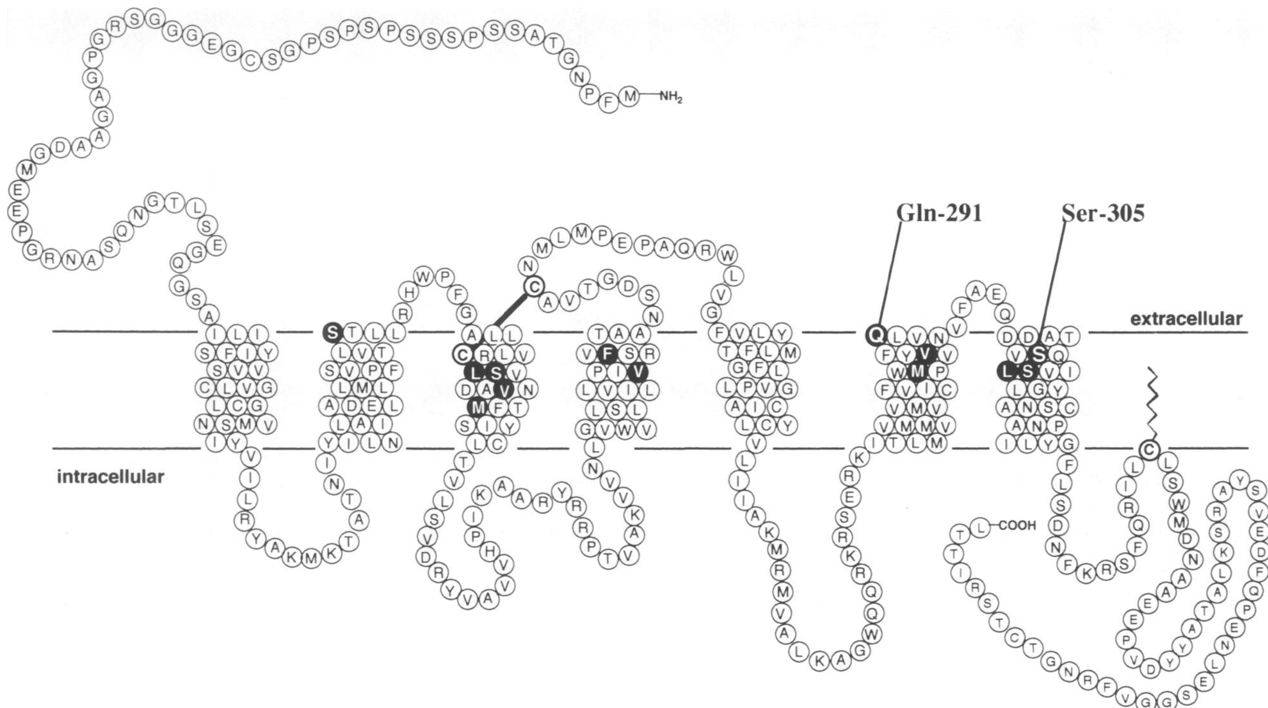


Fig. 4. Schematic diagram of human SSTR1 showing putative transmembrane helices and the relative locations of the amino acids mutated in this study (highlighted residues). The mutations Gln291 to Asn (Q291N) and Ser305 to Phe (S305F) were found to be both necessary and sufficient to confer high affinity for SMS 201-995.

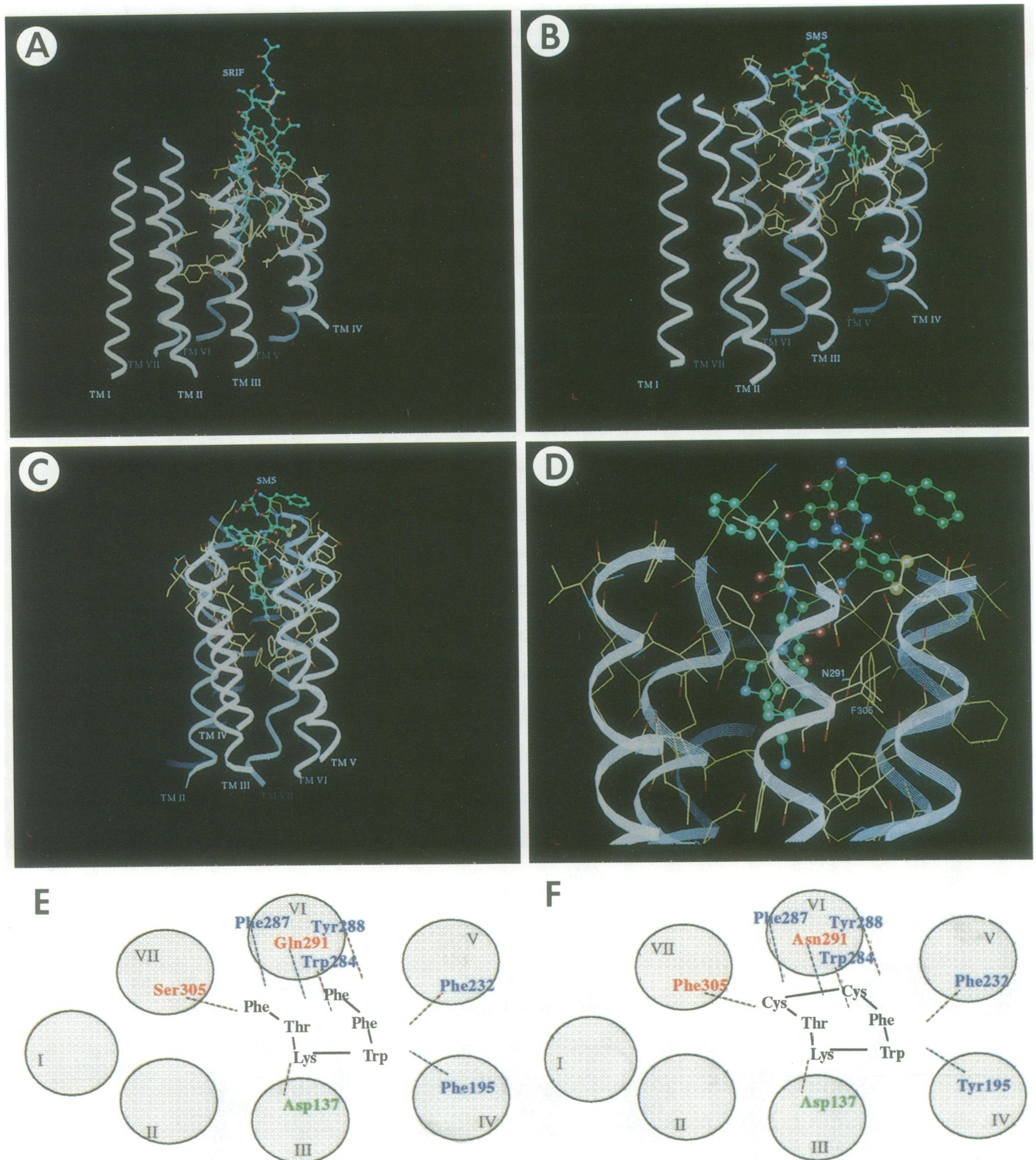


Fig. 5. Models of SRIF-14 in the putative binding pocket of SSTR1 (A and E) and SMS 201-995 in the binding pocket of SSTR2 (B, C, D and F). (**A**) and (**B**) present lateral views, (**C**) and (**D**) the view after turning the model shown in (B) by 90°. (D) is a close-up view of (C). Helices are represented by their α-traces. SMS and SRIF are coloured in green. N, O and S are shown in blue, red and yellow, respectively. All receptor amino acid residues within a distance of 5 Å from the ligands are illustrated in yellow. In (D), the two residues relevant for selective SMS 201-995 binding are indicated. (**E**) and (**F**) are schematic representations of the SSTR1–SRIF-14 and SSTR2–SMS 201-995 interactions, respectively, viewed from the surface of the plasma membrane. The circles represent the seven transmembrane helices. The conserved aspartates which are thought to form ionic interactions with Lys9 of SRIF-14 or Lys5 of SMS 201-995 are marked in green. Residues found to be critical for high affinity SMS 201-995 binding are coloured in red. Additional aromatic and hydrophilic receptor residues which represent potential binding sites are indicated in blue. To facilitate the comparison, the amino acid numbering for both receptors is according to the SSTR1 sequence.

SSTR2, which forms a stabilizing π–π interaction with Phe7 of SRIF-14 or Phe3 of SMS 201-995.

In SSTR1, the ligand binding pocket is lined by residues Phe287, Gln291 and Ser305 which make lipophilic interactions with the Phe6–Phe11 assembly of SRIF-14 (Figure 5E). An attempt to dock SMS 201-995 into the SSTR1

binding pocket indicated that the location of the disulfide bridge of SMS 201-995 still allows stabilizing lipophilic interactions with Gln291. However, due to its smaller size compared with the Phe6–Phe11 assembly of SRIF-14, interactions with Phe287 and Ser305 are not possible, resulting in low affinity of SMS 201-995 for SSTR1.

In SSTR2, the SSTR1 residues Gln291 and Ser305 are replaced by Asn and Phe, respectively. Phe287 is present in both SSTR1 and SSTR2. The Phe6–Phe11 cluster of SRIF-14 interacts with residues Phe287, Asn291 and Phe305 of SSTR2. Binding of SMS 201-995 to SSTR2 is stabilized by lipophilic interactions between the disulfide bridge of SMS 201-995 and residues Asn291 and Phe305 (Figure 5F). In comparison with Gln291 of SSTR1, the shorter side-chain of Asn291 in SSTR2 slightly modifies the ligand orientation within the receptor, thereby pulling the disulfide bridge of SMS 201-995 into close contact with Phe305 (numbering according to SSTR1 as shown in Figure 2).

Thus, in agreement with the results obtained with receptor mutants, the difference in the binding affinity of SMS 201-995 to receptor subtypes SSTR1 and SSTR2 can be attributed to two amino acids located close to the extracellular rims of helices VI and VII, Gln291 and Ser305 in SSTR1 or Asn and Phe in SSTR2, respectively.

Discussion

The aim of the present study was to identify structural domains that determine the specificity of somatostatin receptors for a subtype-selective peptide agonist, SMS 201-995 (octreotide). This metabolically stabilized somatostatin analogue displays antiproliferative properties and is used medically to treat acromegaly and gastroentero-pancreatic tumours (Lamberts *et al.*, 1991; Weckbecker *et al.*, 1993). It is a representative of a group of small peptides, including BIM 23014, MK678 and RC 160, which display sub-nanomolar affinities for receptor type SSTR2 but only micromolar affinities for SSTR1 and SSTR4. Moderate affinities are found for SSTR3 and human SSTR5. To unravel the molecular basis of this selectivity, we used a site-directed mutagenesis approach in which point mutations were sequentially added to SSTR1. The aim was to shift the low affinity binding of SMS 201-995 to SSTR1 by site-directed mutagenesis to a high affinity binding as observed for SSTR2.

The results demonstrate the important role of individual amino acids in determining the specificity of subtype-selective peptide agonists for somatostatin receptor subtypes. Only two amino acid exchanges, Gln291 and Ser305 from SSTR1 to the corresponding SSTR2 residues Asn and Phe, respectively, were found to be both necessary and sufficient to confer high affinity binding of SMS 201-995. In addition, the mutated receptor displayed high affinity binding also for two other short SRIF analogues, RC 160 and BIM 23014, indicating a common mechanism for the interaction of these SRIF analogues with the receptor. The two amino acids determining high affinity binding are located in transmembrane domains VI and VII (Figure 4), regions which have been shown previously to contribute to ligand binding in several other receptor systems. For example, aromatic residues on helix VI are involved in ligand binding of cholinergic receptors (Strader *et al.*, 1989; Trumpp-Kallmeyer *et al.*, 1992). Studies with human α_2- and β_2-adrenergic receptors have shown that the differences in subtype-specific ligand binding are partially determined by the seventh hydrophobic domain (Kobilka *et al.*, 1988). A single point mutation in the α_2 receptor, Phe412 to Asn, substantially altered the subtype-specific binding of agonists and, even more, antagonists (Suryanarayana *et al.*, 1991). Furthermore, major pharmacological differences between human and rodent 5-hydroxytryptamine receptors ($5HT_{1B}$) have been attributed to a single amino acid difference in TM VII, Thr355 in the human receptor to the corresponding Asn found in the rodent receptor (Metcalf *et al.*, 1992; Oksenberg *et al.*, 1992). In both receptors, the residues in transmembrane segment VII which contribute to ligand binding are located in similar positions (Hibert *et al.*, 1993). A sequence comparison of these receptors with somatostatin receptors showed that the Phe305 in hSSTR2 which we found to be relevant for selective agonist binding is located four amino acids or one helix turn higher up in the membrane compared with the other two receptors (data not shown). In bacteriorhodopsin, retinal is covalently attached to Lys216 located one turn of the helix further down in the seventh membrane-spanning region (Henderson *et al.*, 1990; Trumpp-Kallmeyer *et al.*, 1992). At a similar position (one amino acid deeper in the membrane), the substitution of Trp299 in the thromboxane A_2 receptor with leucine resulted in a receptor that discriminated between agonist and antagonist binding (Funk *et al.*, 1993). Furthermore, Thr277 in the human A_1 adenosine receptor was demonstrated to contribute to agonist binding (Townsend-Nicholson and Schofield, 1994). This residue is located at the position corresponding to the amino acid in front of Lys216 in bacteriorhodopsin. Therefore, it is concluded that important determinants for ligand binding to 7TM receptors are present in the seventh membrane-spanning regions at positions which vary in height by two turns of the helix.

To explain the molecular interactions between the peptide ligand and the receptor residues identified by the *in vitro* mutagenesis study, we have applied a molecular modelling approach. The method used was similar to that described by Trumpp-Kallmeyer *et al.* (1992). The most likely positions of the membrane-spanning regions were determined by sequence alignments of 40 sequences for G protein-coupled receptors. The results of our alignments are in agreement with those of Trumpp-Kallmeyer *et al.* (1992). The membrane-spanning regions were individually modelled as α-helices and superimposed onto the experimentally determined bacteriorhodopsin model (Henderson *et al.*, 1990). Although the value of bacteriorhodopsin as a template for G protein-coupled receptors has been questioned since the relative positions of the helices in a receptor may be slightly different (Baldwin, 1993), the same approach has been applied before successfully to various monoamine receptors (Trumpp-Kallmeyer *et al.*, 1992; Wess *et al.*, 1993). The resulting models have been shown to explain the results of labelling experiments, *in vitro* mutagenesis studies and ligand structure–activity relationship studies (Hibert *et al.*, 1993). Our somatostatin receptor models can explain the results obtained in the mutational analysis, indicating that they are at least partly reliable for analysis of ligand–receptor interactions. In

somatostatin receptors, a disulfide bridge may link Cys130 to Cys208. According to the models, Cys130 is located at the upper rim of helix III and contact with Cys208 is sterically possible. We have used these models to predict additional points of interaction between somatostatin and its receptors that have not been verified experimentally. Since SRIF-14 and SMS 201-995 are closely related agonists, it has been assumed that they share overlapping binding sites. In fact, residues that we postulate to be involved in somatostatin binding are mostly equivalent to those found previously to be involved in the binding of monoamines to their receptors. A basis for our models is the assumption that the conserved aspartate in helix III forms an ion pair with the Lys9 of SRIF-14 or Lys5 of SMS 201-995. This assumption seems reasonable given the clear evidence for an involvement of the corresponding aspartate in ligand binding of monoaminergic receptors (Kurtenbach *et al.*, 1990; Hibert *et al.*, 1993). Similar to our somatostatin receptor models, in monoamine receptors, highly conserved hydrophobic pockets have been defined in the direct vicinity of the aspartate which bind the aromatic nucleus of the ligands (Strader *et al.*, 1989; Hibert *et al.*, 1993; Wess *et al.*, 1993). Finally, Phe232 in transmembrane domain IV is located in a similar position in space to His197 from helix V of the NK1 receptor, which has previously been shown to be involved in the binding of the non-peptide antagonist CP 96345 (Fong *et al.*, 1993).

Our results suggest that the major determinants for the selectivity of the peptide agonist SMS 201-995 to somatostatin receptor subtype 2 (SSTR2) are located in the TM VI and VII. Studies on bombesin and tachykinin receptors demonstrated the importance of both the extracellular and the transmembrane domains for peptide agonist binding. In the case of the neuromedin receptor the fifth transmembrane domain has been shown to be critical for high affinity neuromedin binding (Fathi *et al.*, 1993) whereas tachykinin peptides interact with multiple domains scattered throughout the receptor structure (Fong *et al.*, 1992; Gether *et al.*, 1993). In this study, we have concentrated on ligand binding determinants within the membrane-spanning domains of somatostatin receptors. Although contributions from the extracellular loops cannot be excluded, we demonstrate that the peptide agonist binding site is located within a pocket formed by the membrane-spanning regions, very similar to the binding sites of small molecules in their respective receptors.

A prerequisite to establish the physiological roles of the different somatostatin receptor subtypes is the identification of selective, high affinity receptor ligands. With the present study we determined the structural basis for the subtype 2 (SSTR2) selectivity of the SRIF analogue SMS 201-995, a peptide agonist important for cancer therapy. Characterizing the interactions of this agonist with its receptor will prove to be very helpful for the design of further receptor subtype-selective analogues that are needed to explore the physiological and functional characteristics of the SRIF receptor subtypes, SSTR1–5.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco, BRL. COS-1 cells were obtained from the American Type Culture Collection. Oligonucleotides were synthetized on an Applied Biosystems 380A synthesizer and purified by gel filtration through Sephadex G 25 columns (NAP-10 columns, Pharmacia). Dideoxy sequencing was performed using a T7 Sequencing™ kit (Pharmacia) or an automated sequencer (Applied Biosystems 373A). Somatostatin-14 (SRIF-14) and SMS 201-995 were synthesized at Sandoz, Basel. All other chemicals were obtained from commercial sources.

In vitro mutagenesis

Oligonucleotide-directed mutagenesis was performed using an *in vitro* mutagenesis kit (Transformer™ site-directed mutagenesis kit, Clontech Laboratories) which is based on the method described by Deng and Nickoloff (1992). Point mutations were introduced into the human SSTR1 template cloned into the cytomegalovirus-promotor-based expression vector pKS1. Two selection primer sequences were designed from the pKS1 vector sequence:

5′-TCGTTGTGTAGATATCGCTGTATTCC-3′ and
5′-TCGTTGTGTAGGTACCGCTGTATTCC-3′.

These primers mutate a *Kpn*I site to an *Eco*RV site and vice versa. In subsequent mutagenesis steps mutated plasmids were selected by digestion with these two enzymes. Mutagenic primers were designed based on the published human SSTR1 sequence (Yamada *et al.*, 1992a). Mutagenic and selection primers were annealed to the denatured template and the DNA strands were completed with T4 DNA polymerase essentially as described by the manufacturer. For the introduction of multiple substitutions, combinations of oligonucleotides were added to the *in vitro* reaction. The heteroduplex DNA was then used to transform the repair-deficient *Escherichia coli* strain BMH 71-18 mutS. Plasmids prepared from the pool of transformants were subjected to selective restriction digestion to enrich for those carrying the selection primer sequence. After the final transformation into the *E.coli* strain TOP 10, plasmids were isolated from individual colonies and analysed for the presence of the selection and mutagenic primer sequence. All mutations were confirmed by sequencing.

Cell culture and expression of receptor clones

COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics (10 μg/ml streptomycin, 10 IU/ml penicillin) at 37°C in a humidified atmosphere with 5% CO_2. DEAE–dextran-mediated DNA transfection of COS-1 cells was performed as decribed by Kluxen *et al.* (1992) with some modifications. The cells were seeded at a density of ~15 000 cells/cm^2. One day later, after two washes with DMEM, the medium was replaced with DMEM containing 10% (v/v) Nu-serum (Collaborative Research), 0.1 mg/ml (w/v) DEAE–dextran (Pharmacia) and 100 μM chloroquine (10 ml per 15-cm dish). DNA was added to a concentration of 1.25 μg/ml and the cells incubated for 4 h at 37°C and 5% CO_2. After incubation, the medium was removed and the cells were incubated for 2 min in 10% dimethylsulfoxide (v/v) in phosphate-buffered saline (PBS). After a rinse with PBS complete DMEM medium was added and the cells were incubated for 2 additional days.

Receptor binding assays

Binding assays with SRIF-14 were performed on intact cells grown in multiwell dishes as decribed (Kaupmann *et al.*, 1993). The cells were trypsinized ~48 h after transfection and seeded into 24-well plates. One day later, the cells were washed twice with 10 mM HEPES, pH 7.6, containing 5 mM $MgCl_2$, 20 μg/ml bacitracin, 0.5% (w/v) bovine serum albumin and incubated with 30 000 c.p.m. of [^{125}I]Tyr11-SRIF-14 (2000 Ci/mmol) in 300 μl HEPES buffer for 1 h at room temperature. The cells were then washed, detached with 10% (w/v) SDS and the radioactivity bound to the cells was determined in a gamma counter (LKB Instruments).

Non-specific binding was defined as binding in the presence of 1 μM SRIF-14. For competition binding experiments, the cells were incubated with increasing concentrations of unlabelled SRIF-14 and SMS 201-995. Binding curves were generated from triplicate determinations using the computer fitting program of De Lean (1979).

Molecular modelling

The sequences of the human SSTR1, SSTR2, SSTR3, SSTR4 and rat SSTR5 somatostatin receptors were aligned and the hydrophobic transmembrane stretches identified by hydropathicity analysis using the Kyte–Doolittle parameters (Kyte and Doolittle, 1982). The precise localization of the transmembrane regions was defined by an alignment of ~40 different G protein-coupled receptors and opsins (H.Mattes,

unpublished results) using a combination of hydropathy analyses (Kyte and Doolittle, 1982) with variability analyses (Donnelly *et al.*, 1989; Hulme *et al.*, 1991) and the identification of highly conserved residues.

The identified seven transmembrane helices of SSTR1 were then individually built up and each was energy minimized using the cvff-forcefield of DISCOVER (Biosym Ltd, San Diego, CA). The Cαs of the optimized helices were then superimposed onto the Cαs of the corresponding helices of the experimental bacteriorhodopsin model of Henderson (Henderson *et al.*, 1990; '1brd' in the Brookhaven PDB). After removing steric conflicts between side-chains of neighbouring helices 'by hand', an energy minimization of the seven-helix model was performed. Initially, 2000 conjugate gradient optimizations were iterated with all Cαs fixed at original position. This was followed by another 200 iterations (conjugate gradient method) without any constraints. A model of SRIF-14 forming an ideal two-stranded β-sheet with a type II' β turn at Trp8 −Lys9 was then constructed and energy optimized without constraints. This model was manually docked into the putative binding site between the helices III, IV, V, VI and VII with Nζ of Lys9 in H-bond distance from Asp137 (helix III, SSTR1 numbering). An energy minimization of the SSTR1−SRIF-14 complex was then performed. First, the Cαs of SSTR1 were fixed at a distance where Nζ of Lys9 of SRIF-14 was within 3.0 Å of the OD2 of Asp137 (2000 iterations of conjugate gradient). This was followed by an unconstrained refinement of the complex for another 2000 iterations. An identical approach was used to build 3D models of the SSTR2−SMS 201-995 and SSTR2−SRIF-14 complexes. In addition, to explain the low affinity of SMS 201-995 for SSTR1, it was attempted to energy-minimize the complex of SMS 201-995 docked into SSTR1.

Acknowledgements

We thank Beatrice Urban and Désirée Eckert for expert technical assistance and Armand Wanner for help with the automated sequencing of mutant receptor clones. Plasmids containing human SSTR1, 2 and 3 were provided by Dr Graeme I.Bell, University of Chicago. Human SSTR5 was a generous gift of Dr S.Seino, Chiba University.

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Received on September 29, 1994; revised on November 9, 1994