The Role of Subtype-specific Ligand Binding and the C-tail Domain in Dimer Formation of Human Somatostatin Receptors*

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G-protein-coupled receptors (GPCRs) represent the largest and most diverse family of cell surface receptors. Several GPCRs have been documented to dimerize with resulting changes in pharmacology. We have previously reported by means of photobleaching fluorescence resonance energy transfer (pbFRET) microscopy and fluorescence correlation spectroscopic (FCS) analysis in live cells, that human somatostatin receptor (hSSTR) 5 could both homodimerize and heterodimerize with hSSTR1 in the presence of the agonist SST-14. In contrast, hSSTR1 remained monomeric when expressed alone regardless of agonist exposure in live cells. In an effort to elucidate the role of ligand and receptor subtypes in heterodimerization, we have employed both pbFRET microscopy and Western blot on cells stably co-expressing hSSTR1 and hSSTR5 treated with subtype-specific agonists. Here we provide evidence that activation of hSSTR5 but not hSSTR1 is necessary for heterodimeric assembly. This property was also reflected in signaling as shown by increases in adenyl cyclase coupling efficiencies. Furthermore, receptor C-tail chimeras allowed for the identification of the C-tail as a determinant for dimerization. Finally, we demonstrate that heterodimerization is subtype-selective involving ligand-induced conformational changes in hSSTR5 but not hSSTR1 and could be attributed to molecular events occurring at the C-tail, Understanding the mechanisms by which GPCRs dimerize holds promise for improvements in drug design and efficacy.

In recent years, G-protein-coupled receptors (GPCRs), once believed to exist at the plasma membrane as monomers, have been shown to assemble on the membrane as functional homo- and heterodimers (1, 2). Dimerization of GPCRs has been shown to affect a multitude of receptor functions including ligand binding, signaling, receptor desensitization, and receptor trafficking (1, 2). The influence of GPCR dimerization was shown to include cellular immunity, neurotransmission (1), taste (3–5), and disease (6). Although the mechanism by which GPCR dimerization occurs remains obscure, one model suggests that ligand binding of cell surface receptors induces a conformational change that favors dimer formation; while the other suggests that dimerization is an exclusive event occurring early on during receptor biogenesis most probably in the ER and is a necessary event for proper receptor trafficking and function.

This latter model has been suggested for members of the class C subfamily of GPCRs, which include the GABAergic receptors (7–9), calcium-sensing receptor (10, 11), the metabotropic glutamate receptor (12), and the sweet taste receptors (3–5). However, this paradigm of GPCR assembly is not consistent among the class A/rhodopsin-like family of GPCRs. Several reports have shown that agonist plays an active role in GPCR dimerization at the plasma membrane, suggesting an equilibrium between GPCR dimers/monomers that can be regulated by ligand occupancy. These receptors include the human somatostatin receptors (hSSTRs) (13, 14), dopamine D2 receptor (15), gonadotrophin-releasing hormone receptor (16, 17), luteinizing hormone/chorionic gonadotrophin hormone receptor (18), bradykinin B2 receptor (19), thyrotropin-releasing hormone receptor (20), cholecystokinin receptor (21), thyrotropin receptor (22), and the chemokine receptors (23–26).

We have previously reported that hSSTRs, known to modulate neurotransmission, cell secretion, and cell proliferation (27, 28) are capable of undergoing both homo- and heterodimerization at the cell membrane (13, 14, 29). Recently, we have demonstrated ligand-dependent homo- and heterodimers on the plasma membrane in live cells in both a homogeneous and heterogeneous receptor expressing cell line, using both single and two photon dual color fluorescence correlation spectroscopy (FCS) with cross-correlation analysis (a method that discriminates based upon molecular size, number density, and average brightness/particle in femtoliter confocal volumes) (14). One of the receptor subtypes, hSSTR1, did not form homodimers in either the absence or presence of ligand. In contrast, hSSTR5 showed robust dimerization upon agonist exposure. When both receptors were co-expressed in the same cell, we were able to observe two populations of dimers, hSSTR5 homodimers and hSSTR1/hSSTR5 heterodimers (14). However, it remains unclear as to whether one or both receptor subtypes are capable of promoting heterodimerization, and which receptor motifs may be attributed to this behavior.

In the present study, using subtype-specific agonists and both photobleaching fluorescence resonance energy transfer (pbFRET) and Western blot analysis, we demonstrate that ligand-bound hSSTR5 but not hSSTR1 can promote the het-
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erdimerization of hSSTR1/hSSTR5. Moreover, using receptor C-tail chimeras, we were able to abrogate the homodimerization of hSSTR5 and induce the formation of hSSTR1 homodimers. The hSSTR5 subtype-specific analog of somatostatin, SMS 201-955, displayed a relatively poor signaling profile for hSSTR5 expressed alone despite having nanomolar binding affinity. Accordingly, co-expression with hSSTR1 resulted in a robust increase in its signaling efficiency by SMS 201-955 that remained in part with its ability to induce heterodimerization. Finally, we demonstrate that not all agonists can induce heterodimerization, which was dependent on ligand occupancy of a specific receptor subtype that can lead to alterations in pharmacology.

EXPERIMENTAL PROCEDURES

Materials and Antisera—The peptides SST-14, d-Trp-SST-14, SST-28, and [Leu (8)-d-Trp-22, Tyr-25]-SST-28 (LTT-SST-28) were purchased from Bachem, Torrance, CA; Octet Octet (SMS 201-955) was given by Sandoz, Basel, Switzerland and des-t-A,D,L-isoLeu-Trp-Tyr-Leu-SST-28 (SC-275) was a gift from Dr. J. Rivier, Salk Institute. Fluorescein- and rhodamine-conjugated and unconjugated mouse monoclonal antibodies against hemagglutinin (HA) (12CA5) were purchased from Roche Applied Science. Anti-c-Myc monoclonal antibody was purchased from Sigma-Aldrich, Inc. Rabbit polyclonal antibodies directed against the N-terminus fragment of hSSTR1 was generated and characterized as described (30). Protein A/G-agarose beads were purchased from Oncogene Research Products, La Jolla, CA.

SSTR Constructs and Expressing Cell Lines—Stable transfections of CHO-K1 cells expressing hSSTR5, hSSTR1, and both HA-tagged hSSTR5 and hSSTR1 and c-Myc-tagged hSSTR5 were prepared by Lipofectamin transfection reagent as previously described (13). Chimeric receptors R1CR5 and R5CR1 were constructed by interchanging the C-tail of each receptor with another. R1CR5 was created by adding the C-tail of hSSTR5, the last 46 residues, to hSSTR1 after residue 331. Similarly, R5CR1 includes the remaining 60 residues of hSSTR1 joined to hSSTR5 following residue 318 (31). Clones were selected and maintained in CHO-K1 medium containing Ham’s F12 medium receptors R1CR5 and R5CR1 were constructed by interchanging the receptor constructs, and 125I-labeled LTT-SST-28 radioligand (28, and [Leu (8)-D-Trp-22, Tyr-25]-SST-28 (LTT-SST-28) were purchased from Bachem, Torrance, CA; Octet Octet (SMS 201-955) was given by Sandoz, Basel, Switzerland and des-t-A,D,L-isoLeu-Trp-Tyr-Leu-SST-28 (SC-275) was a gift from Dr. J. Rivier, Salk Institute. Fluorescein- and rhodamine-conjugated and unconjugated mouse monoclonal antibodies against hemagglutinin (HA) (12CA5) were purchased from Roche Applied Science. Anti-c-Myc monoclonal antibody was purchased from Sigma-Aldrich, Inc. Rabbit polyclonal antibodies directed against the N-terminus fragment of hSSTR1 was generated and characterized as described (30). Protein A/G-agarose beads were purchased from Oncogene Research Products, La Jolla, CA.

Co-immunoprecipitation and Western Blot—Membranes from HA-

SSTR1, HA-SSTR5, and HA-SSTR1/c-Myc-hSSTR5 stably trans- 

fected in HEK-293 cells were prepared using a glass homogenizer in 20 mM Tris-HCl, 2.5 mM dithiorethiol, pH 7.5 as previously described (13). The membrane pellet was washed and resuspended in 20 mM Tris-HCl, pH 7.5 in the absence of dithiorethiol. Membrane protein (300 μg) was treated with SST-14 (10 and 100 nM) in binding buffer (50 mM Heps, 2 mM CaCl2, 5 mM MgCl2, pH 7.5) for 30 min at 37 °C. Following treatment membrane protein was solubilized in 1 ml of radioimmune precipita- 

tion assay buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0) for 1 h at 4 °C. Samples were centrifuged, and lysate was collected and incubated with 1 μg of anti-HA antibody overnight at 4 °C. Antibody was immunopre- 

cipitated with 20 μl of protein A/G-agarose beads for 2 h at 4 °C. Beads were then washed three times in radioimmune precipitation assay buffer before being solubilized in Laemmli sample buffer containing 65 mM Tris-HCl (pH 6.8), 25% glycerol, 0.01% bromphenol blue, and 7 mg/ml β-mercaptoethanol (Bio-Rad). The membrane was heated at 85 °C for 5 min before being fractionated by electrophoresis on a 7% SDS-polyacrylamide gel. The fractionated proteins were transferred by electrophoresis to a 0.2 μm nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad) in transfer buffer consisting of 25 mM Tris, 192 mM glycine, and 20% methanol. Membrane was blotted with anti-HA antibody (dilution 1:5000) for detection of HA-hSSTR1 and HA-hSSTR5 from single expressions, and anti-c-Myc antibody (1:5000) for detection of c-Myc-hSSTR5 from co-expressions. Blocking of membrane, incubation of primary antibodies, incubation of secondary antibodies, and detec- 

tion by chemiluminescence were performed following WesternBreeze® (Invitrogen Life Technologies) according to manufacturer’s instructions. Images were captured using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, CA) gel box imager and densitometry was carried out using FluorChem software (Alpha Innotech Co.).

RESULTS

Ligand-dependent Heterodimerization of hSSTR1 and hSSTR5 by pBFRET—To study the heterodimerization of hSSTRs, we stably expressed hSSTR5 with an N-terminal HA tag and wild-type hSSTR1 in CHO-K1 cells (Bmax 395 ± 12 fmol/mg of protein; Kp, 2.3 ± 0.1 nM). Cells were treated with various concentrations of the agonists: SST-14, SST-28, endog- 

enous agonists for both the receptors, SCH-275 (subtype-agonist for hSSTR1) and SMS 201-955 (subtype-agonist for hSSTR5) for 15 min. Treatment was terminated by putting the cells on ice, washing once with phosphate-buffered saline followed by fixing in 4% paraformaldehyde for 20 min. To determine the physical association between the two receptors, we performed pBFRET microscopy on the cells by using a primary antibody followed by a secondary antibody conjugated with fluorescein (donor) to hSSTR1 and an anti-HA monoclonal an- 

tibody conjugated with rhodamine (acceptor) to hSSTR5. A panel of images depicting the co-expression of both receptor subtypes within the same cell is shown in Fig. 1. The decrease in donor fluorescence intensity due to photobleaching during prolonged exposure to excitation light was monitored in the
absence and presence of acceptor fluorophore. Delays in the photobleaching decay of the donor in the presence of the acceptor related to an increase in FRET efficiency. Because FRET occurs at distances between $10^{-10}$–$10^{-2}$ Å, it is a direct measure of protein-protein interaction. By taking a series of digital photographs, we analyzed the photobleaching decay of the donor on the surface of cell membranes on a pixel-by-pixel basis (Fig. 1, B and C). Cells were treated with different concentrations of four agonists, which displayed differences in their ability to induce heterodimerization. As shown in Fig. 2, in absence of agonist, a low relative FRET efficiency ($<3\%$) was present in each condition. Treatment of SST-14 resulted in a concentration-dependent increase in heterodimer formation as indicated by increases in FRET efficiency. A maximum of $13.0 \pm 1.1\%$ at $10^{-6}$ M was achieved possibly suggesting a saturation in the response (EC$_{50}$ of $3.4 \pm 2.1$ nM) (Fig. 2A). A similar phenomenon was observed for SST-28, which also induced a concentration-dependent increase in FRET efficiency however with greater efficacy (EC$_{50}$ $0.14 \pm 0.04$ nM) (Fig. 2B). This may indicate that SST-28 is a more potent agonist at inducing heterodimerization than is SST-14. The hSSTR5 subtype agonist SMS 201-995, although capable of promoting heterodimerization, did so at
much higher concentrations as determined by its EC\textsubscript{50} value (EC\textsubscript{50} 119 ± 16 nM) (Fig. 2C). One possible explanation for this event could be that SMS 201-995 favors the formation of hSSTR5 homodimers than heterodimers; however, further studies are required. In contrast, treatment with the hSSTR1 subtype agonist SCH-275, did not result in significant increases in FRET efficiency (Fig. 2D). These results demonstrate that hSSTR1 is unable to promote heterodimerization.

To further illustrate the active contribution of hSSTR5 in heterodimerization, we performed Western blot and co-immunoprecipitation on membranes prepared from cells either individually or co-expressing the two receptors.

**Western Blot on Ligand-activated hSSTRs**—To verify the receptor subtype actively involved in the heteromeric assembly of hSSTR1 and hSSTR5, we performed co-immunoprecipitation and Western blot on membranes from HEK-293 cells mono- and co-expressing the two receptors. In the absence of SST-14, hSSTR5 was found mainly as a monomer (~55 kDa) (Fig. 3). Treatment with SST-14 resulted in the formation of dimers (~110 kDa) including higher order oligomers (Fig. 3). A similar phenomenon was reported for hSSTR5 transfected in CHO-K1 cells, whereby agonist induced the dimerization of the receptor (13). Unlike hSSTR5, hSSTR1 did not form dimers in response to agonist nor was it self-associated under basal conditions (Fig. 3).

This is in agreement with a previous report on hSSTR1 showing that it remained monomeric even in the presence of agonist in live cells using FCS (14). Co-immunoprecipitation of membranes expressing both receptor subtypes resulted in the detection of a weak band in the absence of agonist, however, upon agonist stimulation a strong signal was detected (~115 kDa) indicating heterodimeric interaction (Fig. 3). Taken together these results and those obtained by pbFRET (Fig. 2), suggest that hSSTR1 is not actively involved in heterodimeric assembly.

**Membrane Binding Analysis of the hSSTR1 and hSSTR5 Heterodimer**—To determine whether heterodimerization altered the binding properties of the receptors, we compared the binding constants for each agonist. Membranes were collected from CHO-K1 cells stably expressing hSSTR1, hSSTR5, and

![Fig. 2](http://www.jbc.org/)

**Fig. 2. Concentration-dependent increase in effective FRET efficiencies from CHO-K1 cells stably expressing HA-hSSTR5 and hSSTR1 by different agonists.** Cells were treated with the indicated concentrations of each agonist and analyzed by pbFRET microscopy. The calculated FRET efficiencies (%) and EC\textsubscript{50} values for each agonist (A) SST-14 (3.4 ± 2.1 nM), (B) SST-28 (0.14 ± 0.04 nM), (C) SMS 201-995 (119 ± 16 nM) were plotted and analyzed by a sigmoidal dose-response equation using Graph Pad Prism 3.0. D, treatment with SCH-275 did not result in a significant increase in FRET efficiency. Data were analyzed by ANOVA, posthoc Dunnett’s and compared with basal conditions without treatment. Means ± S.E. are representative of three independent experiments performed in triplicate; *, p < 0.05; **, p < 0.01.

![Fig. 3](http://www.jbc.org/)

**Fig. 3. Western blot and co-immunoprecipitation of HEK-293 cells stably expressing HA-hSSTR1, HA-hSSTR5, and co-expressing HA-hSSTR1 and c-Myc-hSSTR5.** A, membranes from HEK-293 cells stably expressing HA epitope-tagged hSSTR5 were treated with or without 1 μM SST-14 for 30 min before being separated on a SDS-polyacrylamide gel. HSSTR5 can be seen mainly as a monomer (~55 kDa) but upon treatment with agonist self-associates into dimers (~110 kDa) and higher order oligomers. B, immunoblot of membranes expressing HA epitope-tagged hSSTR1 in the absence or presence of 1 μM SST-14 appearing at ~58 kDa. C, membranes co-expressing HA-hSSTR1 and c-Myc-hSSTR5 were incubated with or without SST-14 (1 μM) for 30 min, solubilized, and immunoprecipitated with anti-HA antibody against hSSTR1. Immunoblotting was performed with anti-c-Myc antibody for detection of hSSTR5. Note the formation of heterodimers upon agonist treatment. Immunoblots are representations of three independent experiments.
from cells co-expressing the two receptors. Saturation analysis with the radioligand 125I-LTT-SST-28 gave a $B_{\text{max}}$ of 415 ± 14 fmol/mg of protein and a $K_D$ of 0.49 ± 0.08 nM from membranes of the co-transfectants and $B_{\text{max}}$ and $K_D$ values of 284 ± 5 fmol/mg, 1.4 ± 0.05 nM and 231 ± 25 fmol/mg, 1.1 ± 0.15 nM for membranes transfected with hSSTR5 and hSSTR1 respectively. Binding constants represented as $K_I$ values for each of the four agonists from each receptor species are shown in Table I. Heteromeric assembly of hSSTR1/hSSTR5 did not result in changes in the $K_I$ values for SST-14 as determined by the lack of statistical significance when compared with the individual receptors. Although the $K_I$ value for SST-28 was lower for the heterodimer than for the individual receptors, indicating a higher affinity, the difference was ~2-fold in comparison to hSSTR5 and 4-fold to hSSTR1. For the subtype-specific agonists SMS 201-995 and SCH-275, $K_I$ values were slightly higher for the heterodimer than for the individual hSSTRs. Based on our results heterodimerization did not markedly alter the binding properties of the receptors.

**Signalining of the Heterodimer**—To determine the signaling properties of the heterodimer we measured cAMP accumulation. HSSTRs are well known to inhibit cAMP production through G$_{i/o}$ coupling (28), we monitored the dose-dependent effect of all four agonists on the inhibition of forskolin-stimulated cAMP production in CHO-K1 cells mono or co-expressing hSSTR1 and hSSTR5. Cells were treated with each of the four agonists with the indicated concentrations in the presence of forskolin (20 μM) and measured for cAMP. Treatment of cells with SST-14 or SST-28 co-expressing hSSTR1/hSSTR5 resulted in greater signaling efficiencies when compared with treatment of cells expressing either receptor separately (Fig. 4, A and B; Table I). The signaling efficiency of SMS 201-995 in cells expressing hSSTR5 was greatly enhanced upon hSSTR1 co-expression (Fig. 4C). It has been previously reported that SMS 201-995 poorly stimulates hSSTR5 when expressed in CHO-K1 cells contrary to its relatively high binding affinity to the receptor (34). To verify that our results were not dependent on the cell type, we stably expressed hSSTR5 in HEK-293 cells and performed the same signaling experiments using SMS 201-995. The results were similar to those obtained in CHO-K1 cells therefore indicating that this property was independent of cell type (data not shown). Treatment with the subtype-specific agonist SCH-275 did not demonstrate changes in signaling efficiency for hSSTR1 expressed alone or when co-expressed with hSSTR5 (Fig. 4D).

To determine if heterodimerization resulted in a synergistic effect on adenylyl cyclase coupling efficacy, we compared the total inhibition of forskolin-stimulated cAMP production achieved by saturating concentrations of ligand from both the mono- and co-expressing cell lines. The maximum inhibition achieved in cells expressing hSSTR5 was ~85% as determined by treatment with SST-14 and SST-28 (endogenous ligands) using 1 μM concentrations (Fig. 5, A and B). The subtype agonist SMS 201-995 did not reach this receptor maximum at 1 μM concentrations (Fig. 5C) despite receptor saturation. The total inhibition reached by cells expressing hSSTR1 was ~25%, in agreement with what has been previously reported (Fig. 5D) (31). When both receptors were co-expressed the total inhibition achieved was ~50% for the agonists SST-14, SST-28, and SMS 201-995 but was unchanged upon treatment with SCH-275 (~25%). These results correlate with our pbFRET and Western blot data indicating that stimulation of hSSTR1 specifically was not sufficient to promote heterodimerization and therefore did not result in changes in signaling. Although the maximum inhibition achievable was lower for the heterodimer, the efficiency for inducing maximum stimulation was higher for agonists capable of inducing heterodimerization (Fig. 4). This indicates that heterodimerization may not always result in a synergistic effect on coupling efficiency contrary to what we have previously reported for the dopamine receptor 2 and hSSTR5 heterodimer (29). This does not rule out other possible efficacies that may be altered such as MAP kinase activation.

**Homodimerization of hSSTR1, hSSTR5, hSSTR5-C-tail-R1, and hSSTR1-C-tail-R5 Using Labeled Ligands**—To determine the possible molecular determinants involved in the heterodimerization of hSSTR1 and hSSTR5, we compared the pbFRET results using labeled ligands in CHO-K1 cells individually expressing hSSTR1, hSSTR5, and two chimeric receptors: hSSTR5 possessing the C-tail of hSSTR1 (R5CR1) and hSSTR1 possessing the C-tail of hSSTR5 (R1CR5). The R5CR1 chimera was created by replacing C-tail residues 319–363 and swappimg them with residues 332–391 of the hSSTR1 C-tail. Similarly, R1CR5 was created by exchanging the same residues. The chimeric receptors were correctly targeted to the plasma membrane as determined by saturation binding analysis and forskolin-stimulated cAMP inhibition analysis (31). As previously reported, replacement of the hSSTR5 C-tail with the C-tail of hSSTR1 completely abolishes agonist-mediated internalization (31). The R5CR1 chimera mimicked the properties of hSSTR1, suggesting the presence of negative internalization signals in the C-tail of hSSTR1 sufficient to block the internalization of hSSTR5. Similarly, replacement of the hSSTR1 C-tail with that of hSSTR5 not only mimicked the signaling properties of hSSTR5 but also its internalization characteristics (31). We have previously reported that hSSTR1 does not undergo homodimerization contrary to hSSTR5, which is fully capable of dimerizing (13, 14). Therefore, using the chimeric receptors, R5CR1 and R1CR5, we proceeded to determine whether these receptors reflected their wild-type counterparts in undergoing homodimerization. Labeled ligands were generated by conjugation of fluorescein isothiocyanate to SST-14-d-Trp8 as the donor and Texas Red-succinamidyl ester to SST-14 as the acceptor. In this fashion, the labeled conjugates gave comparable binding affinities, similar to unconjugated SST-14, to be used together in our pbFRET studies (data not shown). CHO-K1 cells stably expressing any of the four receptors were grown on coverslips and then incubated with either the donor ligand alone or the donor ligand with the acceptor ligand, each at a concentration of 20 nM. As shown in Fig. 6, the FRET efficiencies of hSSTR1 and R5CR1 were comparable suggesting the absence of homodimerization, contrary to the FRET efficiencies obtained for hSSTR5 and R1CR5 (21.5 ± 1.7% and 14.9 ± 2.8%).
indicating homodimer formation. Taken together, these results suggest that the C-tail of hSSTR1 responsible for the inhibition of internalization and up-regulation may also be responsible for its inability to homodimerize.

**DISCUSSION**

There have been several reports documenting GPCR heterodimerization but the mechanism underlying such an event remains largely unknown (1, 2). To our knowledge this is the first time that heterodimerization has been shown to be modulated by subtype-specific agonists and more specifically through the occupancy of one receptor subtype over another. Using pbFRET microscopy, we were able to demonstrate that both the agonists, SST-14 and SST-28, endogenous ligands for both receptor subtypes, induced a dose-dependent increase in FRET efficiency. These agonists were able to efficiently induce heterodimerization at values corresponding to their binding constants. Co-immunoprecipitation and Western blot also demonstrated increases in heterodimeric interaction when agonist was present. However, this was not the case when specific activation of the individual receptor subtypes was involved. Selective activation of hSSTR1 by SCH-275 did not result in changes in FRET efficiency nor did it present as a dimer or higher order oligomer on immunoblots, an indication that by itself is monomeric and unable to induce heterodimerization. In contrast, selective activation of hSSTR5 resulted in increases in FRET efficiency and therefore could be the active subtype involved in this heterodimeric assembly. Immunoblots of hSSTR5 demonstrated it as being monomeric in basal conditions but displayed profound changes in receptor stoichiometry ranging from receptor dimers to higher order oligomers upon agonist activation. However, for both SST-14 and SST-28, the ligand-induced FRET based efficiency was severalfold higher (3.4 ± 99 nM and 0.14 ± 0.04 nM, respectively) when compared with SMS 201-995 (119 ± 16 nM). This difference could be the result of activation of both receptor subtypes simultaneously that allow for conformational changes to better stabilize the heterodimer. Another possibility could be that stimulation of hSSTR5 alone could preferentially form homodimers at lower concentrations of agonist followed by heterodimer formation at higher concentrations. In our previous study using live cells expressing hSSTR1 and hSSTR5 at least three populations of receptors may exist upon stimulation with SST-14; hSSTR1 monomers, hSSTR5 homodimers and hSSTR1/hSSTR5 heterodimers (14). It would be interesting if indeed stimulation of hSSTR5 alone preferentially stabilizes homodimers, then one could develop ways of tailoring such processes. A similar scenario has been described for the chemokine receptors CCR2 and CCR5 (26).

Binding constants for all four agonists for the heterodimer in

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**Table II**

Comparison of adenylyl cyclase coupling efficiencies by SST agonists

CHO-K1 cells stably expressing hSSTR1 and hSSTR5 or coexpressing both receptors (hSSTR5/R1) were treated with given concentrations of each agonist in the presence of forskolin and measured for cAMP accumulation. EC50 values represented in nM are the half-maximal inhibition of forskolin-induced cAMP production. Means ± S.E. represent three independent experiments performed in triplicate.

| Receptors   | SST-14 | SST-28 | SMS 201-995 | SCH-275 |
|-------------|--------|--------|-------------|---------|
| hSSTR1      | 1.21 ± 0.38 | 5.19 ± 0.23 | ≥1000       | 4.93 ± 1.12 |
| hSSTR5/R1   | 0.37 ± 0.05  | 0.10 ± 0.03  | 12.8 ± 4.3  | 7.56 ± 1.50 |
| hSSTR5      | 2.85 ± 1.31  | 0.32 ± 0.11  | 643 ± 99    | ≥1000    |

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**Fig. 4.** Adenylyl cyclase coupling efficiency of the hSSTR1/hSSTR5 heterodimer. CHO-K1 cells monotransfected with hSSTR1 (A) and hSSTR5 (B) or cotransfected with both receptors (C) were treated with forskolin (20 μM) alone or in combination with the indicated concentrations of the agonists (D) SST-14, (E) SST-28, (F) SMS 201-995, and (G) SCH-275 for 30 min. Treatment of cells with forskolin alone was taken as 0% inhibition and treatment of forskolin with 1 μM concentrations of agonist was taken as 100% inhibition. The data represent means ± S.E. from three independent experiments performed in triplicate.
comparison to the individual receptors did not reveal any marked differences. There was however a small but significant rightward shift in the binding curve for SMS 201-995 toward the heterodimer, indicating a decrease in binding affinity but was less apparent for SCH-275. Finally, the endogenous ligands SST-14 and SST-28 bound to all three receptor combinations (hSSTR1, hSSTR5, R1CR5, or R5CR1) were treated with 20 nM SST-FITC (donor) or with 20 nM SST-FITC and SST-TR (donor + acceptor) and processed for pbFRET microscopy (see text for “Experimental Procedures”). Note the significant changes in FRET efficiencies upon C-tail transposition. Homodimerization of hSSTR1 is promoted through C-tail replacement with hSSTR5.

Fig. 6. The characterization of the functional importance of the C-tail in the homodimerization of hSSTR1 and hSSTR5 by pbFRET microscopy. CHO-K1 cells stably expressing either hSSTR1, hSSTR5, R1CR5, or R5CR1 were treated with 20 nM SST-FITC (donor) or with 20 nM SST-FITC and SST-TR (donor + acceptor) and processed for pbFRET microscopy (see text for “Experimental Procedures”). Note the significant changes in FRET efficiencies upon C-tail transposition. Homodimerization of hSSTR1 is promoted through C-tail replacement with hSSTR5.

tered coupling efficiency by comparing EC50 values. Using the subtype-specific agonist for hSSTR1, we were unable to observe alterations in the signaling profiles for hSSTR1 expressed alone or when co-expressed with hSSTR5. In contrast, activation of hSSTR5 by SMS 201-995 resulted in a robust increase in coupling efficiency when both hSSTR5 and hSSTR1 were co-expressed. This property correlated in part with the ability of SMS 201-995 to induce heterodimerization. The alteration in maximum coupling efficacy associated with heterodimerization may have functional implications. One possible consequence may be associated with the poor response of human prolactinomas to SMS 201-995 treatment. Human prolactinomas are pituitary adenomas that hypersecrete prolactin and predominately express hSSTR1 and hSSTR5 (35, 36). In cultured studies of human excised prolactinomas, tumors that displayed increased expression of hSSTR1 fared poorly to treatment with SMS 201-995 in controlling prolactin release compared with those expressing lower levels of hSSTR1 regardless of hSSTR5 expression (36). However, a direct association between heterodimerization and treatment outcome in prolactinomas would be necessary to validate such claims.

HSSTR1 is the only member of the human SSTR family that does not internalize but up-regulates at the cell surface in response to prolonged agonist exposure (31). Knowing that the C-tail of hSSTR1 is responsible for its ability to up-regulate at the cell surface, we proceeded to determine whether it also mediates its monomeric state. Using receptor C-tail chimeras, whereby the C-tails of both receptors were interchanged, we attempted to characterize a putative interface involved in somatostatin receptor dimerization. Replacement of the C-tail for hSSTR5 with that of hSSTR1 was enough to antagonize homodimerization of hSSTR5. Moreover, when the C-tail of hSSTR5 was in place of the C-tail for hSSTR1, hSSTR1 was capable of forming homodimers. Surprisingly, the molecular determinant responsible for the up-regulation of hSSTR1 was also responsible for preventing its homodimerization. Unlike hSSTR1, hSSTR5 does internalize and homodimerize when in the presence of agonist, yet these properties were both inhibited when the C-tail of hSSTR1 was added. It is not the first time that the C-tail has been suggested to be implicated in the heterodimerization of GPCRs. The GABA<sub>B</sub> receptor is a het-
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erodimer composed of the subtypes GABA_B1R and GABA_B2R (7–9). Initial attempts in its cloning have shown that heterologous expression of the individual receptor subtypes were either found essentially retained in the ER (GABA_B1R) or expressed at the cell surface but functionally inactive (GABA_B2R). When both receptors were co-expressed a retention sequence found in the C-tail of GABA_B1R was masked similarly, the C-tail of the erodimer composed of the subtypes GABA_B1 and GABA_B2. This allowed for proper trafficking and functioning of the GABA_B1 receptor. Similarly, the C-tail of the 6-opioid receptor was found necessary for dimerization and when perturbed through C-tail deletion internalization was impaired (37). However, this does not rule out other possible domains that may also be contributing to dimerization such as the transmembrane region (38–40).

Although a general mechanism such as preformed or ligand induced dimer formation does not seem to be exclusively valid, our study highlights the importance of ligands, especially receptor specific ligands, in dimer formation. It is noteworthy that hSSTR1 homodimers cannot be formed, yet ligand bound hSSTR5 forms heterodimers with hSSTR1. Indeed, the ligand bound hSSTR1/hSSTR5 heterodimer is more stable as demonstrated by phFRET and signalning efficiencies of SST-14 and SST-28 suggesting a preferred conformational state resulting from ligand binding. These findings are consistent with earlier results with dopamine 2/hSSTR5 (29) and hSSTR1/hSSTR5 (14) heterodimers in which a dimer containing one or two ligands could be established.

In conclusion, we have demonstrated that activation of hSSTR5 but not hSSTR1 was capable of promoting hSSTR1/ hSSTR5 heterodimerization. These results also demonstrate that agonist-mediated heterodimerization may occur through ligand occupancy of only one receptor subtype. Furthermore, this process resulted in changes in maximum coupling efficacy and coupling efficiency with little changes in ligand binding. We have also demonstrated that the C-tail of hSSTR1 was responsible for preventing dimerization. However, further studies are required to define any specific residues or motifs that may account for this inhibition of interaction. Our data provide direct biophysical and functional evidence that heterodimerization is a receptor and ligand specific process. We recognize the importance of the C-tail in receptor internalization, G-protein coupling, and dimer formation. A detailed understanding of the significance on the interrelationship(s) among these events are lacking and need to be elucidated.

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