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Development and Use of a Receptor Antibody to Characterize the Interaction between Somatostatin Receptor Subtype 1 and G Proteins

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SUMMARY

The signal transduction pathways regulated by somatostatin receptor subtype 1 (sst1) have been difficult to define because of the variability observed when this receptor is expressed in different cell types by transfection and because pharmacological approaches are inadequate to distinguish sst1 receptor function in tissues or cells that express multiple sst receptor subtypes. To study the sst1 receptor in its endogenous environment, we developed a polyclonal antibody to a 15-amino acid peptide corresponding to a unique sequence in the receptor carboxyl terminus. The peptide antibody routinely precipitated 70% of the soluble [^{125}I-Tyr^{11}]somatostatin/receptor complex prepared from Chinese hamster ovary-K1 cells expressing the sst1 receptor but precipitated <1% of the complex from cells expressing other sst receptor subtypes. Photoaffinity-labeled sst1 receptor was also specifically immunoprecipitated and migrated as a broad 60-kDa band on sodium dodecyl sulfate polyacrylamide gels. The observation that sst receptors from GH_4C_1 pituitary cells were immunoprecipitated by the antibody and that receptors from AR4–2J pancreatic acinar cells were not indicated that only the former expressed sst1 receptor protein. Because reverse transcription-polymerase chain reaction showed that GH_4C_1 cells contained both sst1 and sst2 receptor mRNA, immunoprecipitation permitted the sst1 receptor to be separated from the other receptors present. Two observations showed that G proteins were coprecipitated with sst1 receptors from GH_4C_1 cells. First, pertussis toxin pretreatment markedly decreased hormone binding in the immunoprecipitate. Second, the addition of 20 μM guanosine-5′-(γ-thio)triphosphate to the immunoprecipitated [^{125}I-Tyr^{11}]somatostatin/receptor complex stimulated the rate of dissociation of bound ligand by 10-fold. Interestingly, however, the dissociation rate of ~30% of the ligand/receptor complex was unaffected by guanosine-5′-(γ-thio)triphosphate. In summary, we have developed an sst1 receptor-specific antibody and used it to show that sst1 receptors endogenously expressed in GH_4C_1 pituitary cells couple primarily to pertussis toxin-sensitive G proteins. Furthermore, these receptors exist in two distinct high affinity states distinguished by their GTP sensitivity.

The neuropeptide SRIF is a widely distributed regulator of endocrine, exocrine, gastrointestinal, and neural functions (1–3). It is an important physiological inhibitor of growth hormone secretion from the pituitary, insulin and glucagon secretion from pancreatic islets, and gastrointestinal peptide secretion from the gut. In addition, SRIF regulates neuronal excitability and cell growth. The biological actions of SRIF are initiated by interaction with high affinity, plasma membrane receptors (4). A diagnostic indicator of G protein involvement in receptor-mediated signal transduction is a reduction in agonist binding affinity produced by agents that uncouple the receptor/G protein complex (5). Therefore, based on early studies showing that hormone binding to endogenous SRIF receptors was inhibited by both guanine nucleotides and pertussis toxin pretreatment, these receptors were proposed to act via G proteins belonging to the pertussis toxin-sensitive G_i/G_o family (6, 7). This conclusion was subsequently supported by experiments showing that pertussis toxin blocked SRIF inhibition of adenylyl cyclase and calcium channels as well as SRIF activation of potassium channels and phosphatases (7–11). However, when different biological end points were examined, less-consistent results were obtained. Although pertussis toxin blocked SRIF inhibition of hormone secretion (7), it did not affect its inhibition of either pancreatic cell growth (12) or Na^+-H^+ exchange (13). Therefore, the latter effects were proposed to occur via pertussis toxin-insensitive G proteins or independently of G

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ABBREVIATIONS: SRIF, somatostatin; SMS, SMS 201–995 (D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol); GTPγS, guanosine-5′-(γ-thio)triphosphate; DβM, dodecyl-β-D-maltoside: CHS, cholesterol hemisuccinate; SDS, sodium dodecyl sulfate; PEG, polyethylene glycol 8000; PAGE, polyacrylamide gel electrophoresis: CHO, Chinese hamster ovary; bp, basepair(s); ANB, 5′-azido-2′-nitrobenzoyl.

protein coupling. Together, these findings suggested that sst receptors were coupled to different effector systems by distinct signaling pathways.

Recently, five SRIF receptor subtypes have been identified by molecular cloning and numbered sst1 through sst5 (14). Sequence analysis shows that these receptors are members of the seven-transmembrane domain receptor superfamily. However, it is not known which receptor normally mediates each of the previously characterized biological effects of SRIF. Moreover, the transduction mechanisms activated by sst receptor subtypes have differed in the various cell types in which these receptors have been expressed by transfection. Particularly for the sst1 receptor, G protein coupling has been atypical and confusing. Neither GTP analogues nor pertussis toxin treatment affected hormone binding to the sst1 receptor expressed in CHO-DG44 or COS-7 cells (15, 16). Furthermore, SRIF did not inhibit adenylyl cyclase in sst1 receptor expressing COS-1 cells or NIH 3T3 cells (16, 17). Therefore, it was suggested that signal transduction by the sst1 receptor was not mediated by G proteins. In contrast, other groups concluded that the sst1 receptor was coupled to pertussis toxin-sensitive G proteins. In sst1 receptor-expressing CHO-K1 cells (18–20), HEK 293 cells (21, 22), and Ltk^- cells (23), SRIF inhibited adenylyl cyclase in a pertussis toxin-sensitive manner, and hormone binding was regulated by guanine nucleotides and pertussis toxin. Coupling to both pertussis toxin-sensitive and -insensitive G proteins has been observed in one expression system (23). These results indicate that the cellular environment is important in determining the signal transduction machinery used by sst1 receptors, as has been documented for other seven-transmembrane domain receptors (24–26). However, the mechanism of action of endogenous sst1 receptors has not been characterized in any cell type and, as a consequence, there has been no way to evaluate the validity of any of the expression systems established to date.

In the present study, we developed a specific, immunoprecipitating antibody for the sst1 receptor and used this antibody to characterize G protein coupling by endogenous sst1 receptors in the GH_4C_1 pituitary cell line as well as by sst1 receptors stably expressed in CHO-K1 cells by transfection. A preliminary report of some of these results has been presented (27).

Experimental Procedures

Materials. Reagents were obtained from the following sources: synthetic SRIF and [Tyr^{11}]SRIF from Bachem (Torrance, CA); SMS 201–995 from Sandoz Pharmaceuticals (Basel, Switzerland); GTPγS and guanosine-5′(β,γ-imido)triphosphate from Boehringer Mannheim (Indianapolis, IN); pertussis toxin from List Biological Laboratories (Campbell, CA); *N*-5′-azido-2′-nitrobenzoyl-*N*-oxysuccinimide and bicinchroninic acid protein assay reagent from Pierce Chemical Co. (Rockford, IL); carrier-free $Na^{125}I$ from Amersham Corp. (Arlington Heights, IL); CNBr-activated Sepharose 4B from Pharmacia-LKB Biotechnology (Piscataway, NJ); DβM from Calbiochem (San Diego, CA); protein A, bacitracin, bovine serum albumin, and CHS from Sigma Chemical Co. (St. Louis, MO); goat anti-rabbit antibody conjugated with horseradish peroxidase, peroxidase-(2,2′-azino-di-[3-ethylbezthiazoline-6-sulfonic acid]) substrate kit, SDS, and PAGE reagents from Bio-Rad (Hercules, CA); [γ-^{32}P]ATP from ICN (Irvine, CA); Moloney murine leukemia virus reverse transcriptase and nitrocellulose membranes (Duralon-UV) from Stratagene (San Diego, CA); AmpliTaq DNA polymerase from Perkin-Elmer Cetus (Norwalk, CT); and tissue culture media and geneticin from GIBCO-BRL (Grand Island, NY).

Antisera preparation and assay. A 15-amino acid peptide corresponding to a unique carboxyl-terminal sequence in the rat sst1 receptor was identified (Fig. 1) and synthesized with an amino-terminal cysteine using an Applied Biosystems ABI synthesizer (model 430A) and the Merrifield solid-phase methodology. Peptide structure was confirmed by fast atom bombardment mass spectrometry, and purity was determined by reverse-phase high-performance liquid chromatography. The peptide was conjugated to keyhole limpet hemocyanin through the amino-terminal cysteine using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (28). New Zealand White male rabbits (Ray Nichols, Lumberton, TX) were initially immunized in complete Freund's adjuvant, boosted at monthly intervals in incomplete Freund's adjuvant, and bled 7–10 days after each boost. Antisera were screened by enzyme-linked immunosorbent assay. To measure the apparent binding affinity of the antiserum for peptide antigen, 50 ng peptide was adsorbed to each well of a 96-well plate at 4°. After saturating binding sites with 1% gelatin for 2 hr, plates were incubated for 2 hr at room temperature with 100 μl of 1:10,000 dilution of R1–201 antiserum and varying concentrations of peptide antigen. After washing, the wells were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) for 1 hr. The plate was then washed and incubated for 30 min with hydrogen peroxide and 2,2′-azino-di-[3-ethylbezthiazoline-6-sulfonic acid], diluted according to manufacturer's instructions. The reaction was stopped with 100 μl of 2% oxalic acid, and the antibody bound in each well was determined by measuring absorbance at 405 nm.

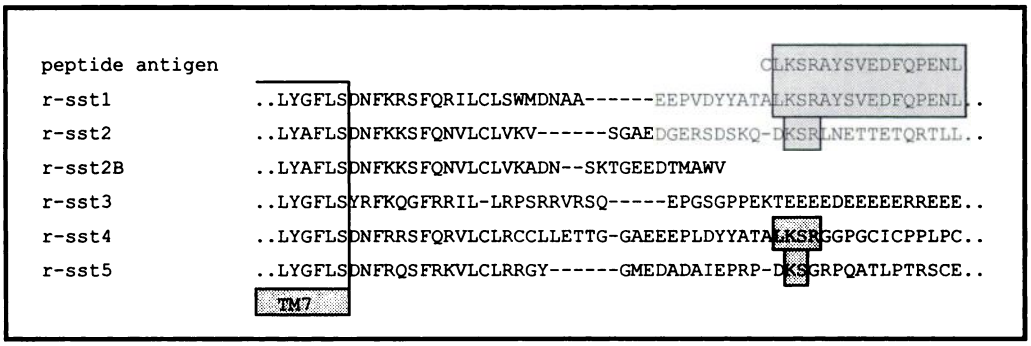

Fig. 1. Alignment of the peptide antigen with the carboxyl-terminal region of six rat sst receptor subtypes. *Single-letter code* is used to denote amino acids, and *TM7* indicates the putative seventh transmembrane domain. Gaps (*dashes*), have been introduced to maximize alignment. *Boxed and shaded areas* indicate conserved amino acid residues between the peptide antigen and the sst receptor subtypes. Nucleotide sequences for rat sst receptors were obtained from Genbank using the following accession numbers: rsst1, M97656; rsst2, M93273; rsst3, X63574; rsst4, M96544; and rsst5, L04535, X74828. *sst2B* represents a splice variant of the sst2 receptor (66).

Cell culture and membrane preparation. The CHO-R1, -R2, and -R4 cell lines were generated after stable transfection of rat sst1, rat sst2, and rat sst4 receptor plasmids into CHO-K1 cells as described (18, 29). Untransfected CHO-K1 cells do not express detectable levels of sst receptors. CHO cells were grown in F12 medium supplemented with 1 mM glutamine and 10% fetal bovine serum. After isolation of individual clones, CHO cells were selected once a month with 250 μg/ml geneticin in serum-supplemented F12 medium (18). In experiments with pertussis toxin, CHO-R1 cells were incubated with fresh complete medium containing 50–100 ng/ml pertussis toxin for 16–24 hr before membrane preparation. GH_4C_1, AtT20, and AR4–2J cells were grown and subcultured as previously described (30–32).

Cell membranes were prepared and stored according to published procedures (33). Briefly, cells from either monolayer or suspension cultures were collected, washed with phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4), and homogenized at 4° in Tris buffer (10 mM Tris·HCl, 2 mM $MgCl_2$, 2 mM EDTA, 0.5 mM freshly prepared phenylmethylsulfonyl fluoride, pH 7.6). After centrifugation at 500 × *g* for 10 min, the supernatant was collected and centrifuged again at 10,000 × *g* for 30 min. The membrane pellet was then resuspended in gly-gly buffer (20 mM glycyl-glycine, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2) and stored at −70°.

Ligand binding and receptor photoaffinity labeling. [Tyr^{11}]SRIF and the photoactive SRIF analogue [Tyr^{11},ANB-Lys^4]SRIF were iodinated with Chloramine-T, and the radiolabeled peptides were purified by reverse-phase high-performance liquid chromatography to a specific activity of 2200 Ci/mmol (33, 34).

Binding reactions were performed according to our published procedure (33). Briefly, membranes were incubated at 30° for 2 hr in HEPES binding buffer (50 mM HEPES, pH 7.6, 7 mM $MgCl_2$, 2 mM EDTA, and 2 units/ml of bacitracin) containing radiolabeled peptide (0.05–0.15 nM) with or without 100 nM unlabeled SRIF. After dilution with cold binding buffer, samples were centrifuged at 40,000 × *g* for 15 min, and the radioactivity associated with the pellets was measured in an LKB CliniGamma Counter. Specific binding was calculated as the difference between the amount of radioactivity bound in the absence and the presence of 100 nM unlabeled SRIF. Curve-fitting and data analysis was carried out as described previously (33). All experiments were repeated at least twice.

Receptor photoaffinity labeling was performed with either membranes or solubilized receptors. For membrane labeling, the binding reaction was carried out in the dark with [^{125}I-Tyr^{11},ANB-Lys^4]SRIF (33). After centrifugation, membranes were resuspended in cold HEPES binding buffer to a final concentration of 0.1 mg/ml and irradiated at 254 nm on ice for 10 min (Mineralight model R-52, Ultraviolet Products, San Gabriel, CA) (33). The reaction was stopped by the addition of 1 M Tris·Cl, pH 7.6. Membranes were then pelleted, solubilized in sample buffer (62.5 mM Tris·Cl, pH 6.8, 2% SDS, 20% glycerol, and 50 mM dithiothreitol), and analyzed by SDS-PAGE on 10% acrylamide gels according to the method of Laemmli (35). The gels were dried onto filter paper and exposed to Amersham Hyperfilm at −70°. For immunoprecipitation experiments, [^{125}I-Tyr^{11},ANB-Lys^4]SRIF binding was carried out in the dark as described above, but irradiation was performed after solubilization of the ligand/receptor complex with DβM/CHS. The photoaffinity-labeled receptor was then subjected to immunoprecipitation and analysis by SDS-PAGE and autoradiography.

Immunoprecipitation. Unless otherwise stated, cell membranes were preincubated with a radiolabeled SRIF analogue in the presence and absence of 100 nM unlabeled SRIF, centrifuged, and then solubilized for 1 hr at 4° in HEPES binding buffer containing 1 mg/ml DβM, 0.2 μg/ml CHS, 10 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin, and 10 μg/ml leupeptin (32). The detergent/protein ratio was usually 4:1. After centrifugation at 100,000 × *g*, the solubilized ligand/receptor complex was either quantified by PEG precipitation or subjected to immunoprecipitation.

To measure the amount of ligand/receptor complex in the soluble fraction, PEG precipitation was performed as previously described (32). Solubilized receptor (40 μl) was diluted with 860 μl of HEPES binding buffer and 100 μl of 0.6% bovine γ-globulin. After the addition of 1 ml of 40% PEG (w/v), the solution was mixed vigorously and incubated at 4° for 30 min. Samples were then centrifuged at 3300 × *g* for 25 min, and the radioactivity in the pellet was quantified.

For immunoprecipitation, antiserum R1–201 was added to the solubilized ligand/receptor complex to the final concentration indicated and incubated at 4° for 3–20 hr. Protein A/Sepharose-4B (20 μl of a 50% suspension) was then added, and the incubation was continued for another hour at 4°. After centrifugation at 10,000 × *g* for 2 min, the pellet was washed with cold HEPES binding buffer containing 0.25 mg/ml DβM and then analyzed in a gamma-counter.

To determine the effect of GTPγS on ligand dissociation from immunoprecipitated sst1 receptors, membrane binding, receptor solubilization, and immunoprecipitation were carried out essentially as described above. The immunoprecipitates were then washed, resuspended in dissociation buffer, and incubated at the indicated temperature for various times. The dissociation buffer consisted of HEPES binding buffer with 10 nM SRIF and 0.25 mg/ml DβM either with or without 20 mM GTPγS. The 10 nM unlabeled SRIF is sufficient to saturate available receptors and will prevent the rebinding of dissociated [^{125}I-Tyr^{11}]SRIF. Replicate aliquots were removed after different times of incubation, and the radiolabeled ligand/sst1 receptor complex remaining associated with the pellets after centrifugation was then quantified.

Preparation of total RNA. Cells (3–5 confluent 100-mm dishes) were washed once with phosphate-buffered saline (pH 7.4) and then dissolved in 8 μl of guanidine isothiocyanate solution (5 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% sodium *N*-lauryl sarcosine) (36). After centrifugation at 2000 × *g* for 15 min, the supernatant was layered over 2.5 ml of cesium chloride solution (5.7 M cesium chloride, 2 mM EDTA, 25 mM sodium acetate, pH 5.4) and centrifuged at 107,000 × *g* for 20 hr at 20°. After extraction of the dissolved pellet with chloroform/isoamyl alcohol, total RNA was precipitated with ethanol and stored at −70°.

Detection of sst receptor mRNAs. The reverse transcription reaction contained 10 μg total RNA, 4 μl of 5× polymerase buffer (250 mM Tris·Cl, 40 mM $MgCl_2$, 150 mM KCl, and 50 mM dithiothreitol, pH 8.3 at 37°), 2 ml of 25 mM dNTPs, 2 ml of 5 mg/ml random hexamers, and 20 units of RNasin in a total volume of 20 μl. After heating to 94° for 1 min and then cooling to room temperature, 20 units of Moloney murine leukemia virus reverse transcriptase and another 20 units of RNasin were added. After incubation at 37° for 1 hr, the reaction was terminated by heating to 94° for 5 min.

The PCR amplification reaction contained 20 μl of amplification buffer (33.5 mM $MgCl_2$, 83 mM $(NH_4)_2SO_4$, 25 mM β-mercaptoethanol, 333 mM Tris·HCl, 34 μM EDTA, and 400 μg/ml bovine serum albumin, pH 8.8, at 25°), 4 μl of 25 mM dNTPs, 1.25 μg 3′ and 5′ primers, 5 μl of the reverse transcriptase products, and 5 units of *Taq* DNA polymerase, diluted to a final volume of 100 μl. The sst receptor 5′-primer and 3′-primer sequences were 5′TGTGATCCTGCGCTACGCCAA and 5′GTAGAGTATGGGGTTGGCACA, respectively, and contained an added *Eco*R1 site at their 5′-termini. PCR was carried out for 30 cycles of denaturation (94° for 30 sec), annealing (56° for 45 sec), and polymerization (72° for 2.5 min) in a Twin Block thermocycler (Ericomp, San Diego, CA). The PCR product was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). An aliquot (15 ml) of the aqueous phase was subjected to electrophoresis on a 0.8% agarose gel. After transfer to nitrocellulose membranes, the DNA was hybridized to receptor subtype-specific oligonucleotide probes (10^6 cpm) radiolabeled with [γ-^{32}P]ATP at the 5′ end using T4 polynucleotide kinase. The sst1 receptor-specific hybridization probe was 5′TCTTGCTCGGCGAACACG. The sst2 receptor-specific probe was 5′GTCAAACATGCCTTTCAG, and this probe will not distinguish between the sst2A and sst2B receptor splice variants. Hybridization to the sst1 receptor-specific oligonucleotide was carried out overnight

at 37° in 20 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, pH 6.5, containing 150 μg/ml sheared salmon sperm DNA. Membranes were then washed with 2× standard sodium citrate and 0.1% SDS for 20 min at room temperature, scanned by Betascope blot analyzer (model 603, Betagen Corp., Waltham, MA), and exposed to Amersham Hyperfilm for 10–15 min. In some experiments, the membrane was then stripped with 0.1× standard sodium citrate and 0.1% SDS at 65° for 1 hr and subsequently hybridized with sst2 receptor-specific probe under the conditions described above.

Results

Antibody preparation. The immunogen was a 15-amino acid polypeptide coupled to keyhole limpet hemocyanin through an amino-terminal cysteine. Its peptide sequence corresponds to amino acids 358–372 near the carboxyl terminus of the rat sst1 receptor, a region postulated to be intracellular (Fig. 1). This sequence is unique to sst receptor 1 (Fig. 1) and is identical in the rat, mouse, and human receptors.

Rabbits were immunized with keyhole limpet hemocyanin-coupled sst1 receptor peptide and immune sera were assayed by enzyme-linked immunosorbent assay. One antiserum (R1–201) was shown to bind the peptide antigen with very high affinity (ED_{50} = 18 ± 1 nM) (data not shown). This antiserum was used for all subsequent experiments.

Specific immunoprecipitation of sst1 receptors. To determine whether the R1–201 antiserum specifically recognized the sst1 receptor, we used CHO-K1 cells stably transfected with a rat sst1 receptor expression plasmid (CHO-R1) (18). In initial studies, we determined that the addition of antiserum to the membrane receptor binding incubation had no effect on ligand binding. Therefore, our assay was designed to monitor the immunoprecipitation of the [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex. After preincubation of CHO-R1 membranes with [^{125}I-Tyr^{11}]SRIF, the membrane receptor/ligand complex was solubilized with DβM. The soluble complex is extremely stable in this detergent ($t_{½}$ = 11 days at 4°) (32). The experiment in Fig. 2 (*top*) shows that 72% of the soluble [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex was precipitated by immune serum, whereas preimmune serum precipitated only 0.7% of the receptor. The addition of 10 μM antigen peptide during the incubation with antiserum completely inhibited immunoprecipitation of the complex (Fig. 2), whereas unrelated peptides had no effect (data not shown). In a total of nine independent experiments, 67 ± 2% (mean ± standard error) of the soluble sst1 receptor/ligand complex was precipitated by R1–201 antiserum. Therefore, the peptide antibody efficiently immunoprecipitates the sst1 receptor.

As shown in Fig. 1, the peptide antigen used for immunization contains short regions of identity to the sst2 (three amino acids), the sst4 (four amino acids), and the sst5 (two amino acids) receptors but no homology to the sst3 receptor. To assess the receptor subtype specificity of the peptide antibody, we tested the ability of the R1–201 antiserum to precipitate [^{125}I-Tyr^{11}]SRIF/receptor complexes prepared from transfected CHO-K1 cell lines stably expressing either the rat sst1 (CHO-R1), sst2 (CHO-R2), or sst4 (CHO-R4) receptors (18, 29). In Fig. 2 (*bottom*), 56% of the [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex was precipitated by R1–201 antibody. In contrast, <1% of the sst2 or sst4 receptor

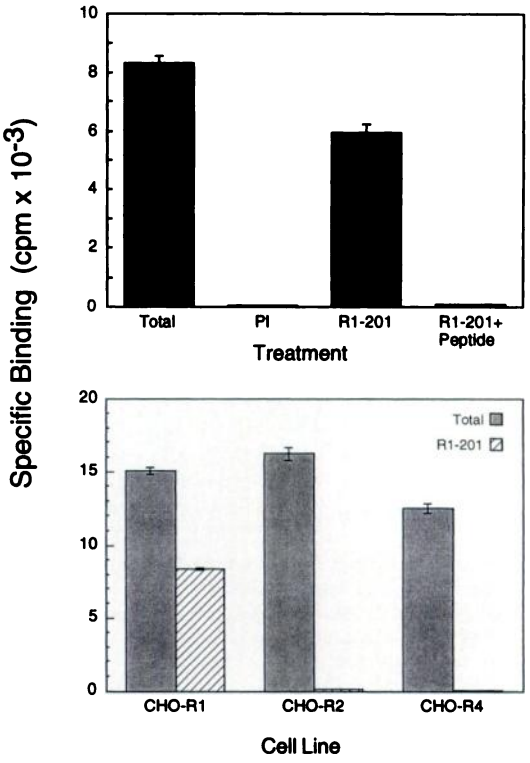

Fig. 2. Specific immunoprecipitation of sst1 receptors by antiserum R1–201. *Top*, CHO-R1 membranes (78 μg/ml) were incubated with [^{125}I-Tyr^{11}]SRIF (189,720 cpm/ml, 0.052 nM) in the absence or presence of 100 nM unlabeled SRIF for 2 hr at 30°. After binding, membranes were separated from free ligand by centrifugation and solubilized for 1 hr at 4° with 1 mg/ml DβM and 0.2 mg/ml CHS at a detergent/protein ratio of 5:1. The amount of intact ligand/receptor complex in the soluble fraction (Total) was determined by precipitating an aliquot with 40% PEG as described in Experimental Procedures. Immunoabsorption was performed by incubating solubilized receptor from 60 μg membrane protein with either preimmune serum (*PI*) or antiserum R1–201 at a final dilution of 1:400 for 3 hr at 4° followed by precipitation with Protein A-Sepharose. In replicate aliquots, the antigen peptide (10 μM) was added with antiserum R1–201. *Bottom*, Membranes from either CHO-R1 cells (100 μg/ml), CHO-R2 cells (80 μg/ml), or CHO-R4 cells (90 μg/ml) were incubated with [^{125}I-Tyr^{11}]SRIF (260,550 cpm/ml or 0.072 nM) in the absence or presence of 100 nM cold SRIF for 2 hr at 30°. Receptor solubilization and PEG precipitation were performed as described above. For immunoabsorption, R1–201 antiserum was added to solubilized membranes from CHO-R1 (50 μg), CHO-R2 (28 μg), or CHO-R4 (90 μg) cells to a final dilution of 1:400 and incubated at 4° for 3 hr. After precipitation with Protein A-Sepharose, specific binding in the pellets was measured as described in Experimental Procedures. For both panels, the data show the mean ± standard error for specific binding in triplicate samples for both the PEG precipitate and the immunoprecipitates.

complexes were precipitated. In similar experiments, the sst3 receptor was also not recognized by the antibody (data not shown). Thus, the peptide antibody is specific for the sst1 receptor.

We next characterized the nature of the receptor protein recognized by the peptide antiserum. To biochemically identify individual receptor subtypes, membranes were prepared from untransfected CHO-K1 cells and CHO-R1 and CHO-R2 cells and affinity labeled with [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF. We have previously shown that this photoaffinity analogue specifically labels sst receptors (33). The results in Fig. 3 (*left*) show that [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF covalently labeled broad bands of 60 and 85 kDa in CHO-R1 and CHO-R2 cells,

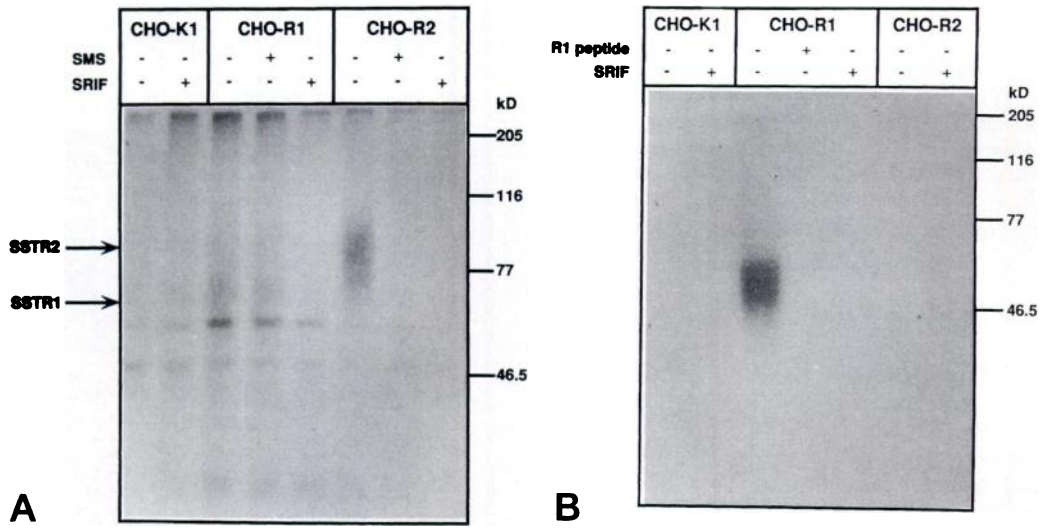

Fig. 3. Immunoprecipitation of photoaffinity-labeled receptor. *Left*, Membranes from CHO-K1 cells (125 μg/ml), CHO-R1 cells (63 μg/ml), or CHO-R2 cells (52 μg/ml) were incubated in the dark for 2 hr at 30° with [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF (334,780 cpm/ml or 0.092 nM) in the absence or presence of either 20 nM SMS 201–995 or 100 nM SRIF. After the binding incubation, the membranes were pelleted, resuspended in HEPES binding buffer, and irradiated under UV light for 10 min. Membranes were then solubilized in sample buffer and analyzed by SDS-PAGE and autoradiography. *Right*, Membranes from CHO-K1 cells (156 μg/ml), CHO-R1 cells (59 μg/ml), and CHO-R2 cells (174 μg/ml) were incubated with [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF (379,240 cpm/ml, 0.104 nM) in the presence or absence of 100 nM unlabeled SRIF for 2 hr. After receptor solubilization, photoaffinity labeling were carried out as described in Experimental Procedures. Affinity-labeled receptors were subsequently incubated with antiserum R1–201 (1:400 final dilution) in the presence or absence of 10 μM antigen peptide for 3 hr at 4°. After precipitation with Protein A-Sepharose, the pellets were dissolved in SDS sample buffer and analyzed by PAGE and autoradiography.

respectively. Unlabeled SRIF (100 nM) inhibited photoaffinity labeling of these bands, as expected for high affinity receptors. However, the SRIF analogue SMS 201–995 (10 nM) inhibited labeling only in CHO-R2 membranes, consistent with the known high affinity of this analogue for sst2 but not sst1 receptors (37, 38). No specific labeling was observed in the parental CHO-K1 cells. The molecular mass predicted for rat sst1 and sst2 receptors from their DNA sequence is 43 and 42 kDa, respectively. Both the higher apparent mass of the photoaffinity-labeled proteins and their diffuse migration in SDS polyacrylamide gels are consistent with the presence of multiple glycosylation sites in these two receptors. Thus, the proteins that were covalently labeled by [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF displayed the biochemical and binding properties expected for specific sst receptor subtypes.

To determine whether the 60-kDa protein from CHO-R1 cells could be specifically precipitated by the sst1 receptor antibody, membranes that had been preincubated with [^{125}I-Tyr^{11},ANB-Lys^{4}]SRIF in the presence or absence of 100 nM SRIF were solubilized and then irradiated to covalently label the receptors. The photoaffinity-labeled receptors were subjected to immunoprecipitation with antiserum R1–201 and then analyzed by SDS-PAGE and autoradiography (Fig. 3, *right*). No specifically labeled bands were observed in the immunoprecipitates from either CHO-K1 or CHO-R2 cells. However, a broad 60-kDa band was evident in the immunoprecipitate from CHO-R1 cells. Labeling of this band was prevented when the binding reaction was carried out in the presence of unlabeled SRIF. Furthermore, immunoprecipitation of this protein was competed by 10 μM antigen peptide. Therefore, the peptide antibody can be used to specifically precipitate the 60-kDa sst1 receptor protein.

sst receptor expression in secretory cells. High affinity sst receptors have been identified in both GH_4C_1 rat pituitary cells and AR4–2J rat pancreatic acinar cells (4, 32). To determine whether these cells express sst1 receptor protein, GH_4C_1 and AR4–2J cell membranes were incubated with [^{125}I-Tyr^{11}]SRIF, and the ligand/receptor complex was solubilized and immunoprecipitated with the sst1 receptor antibody (Fig. 4, *top*). The antiserum precipitated 44% of the total [^{125}I-Tyr^{11}]SRIF/sst receptor complex from GH_4C_1 cells but <1% of the complex from AR4–2J cells. Therefore, functional sst1 receptor protein was expressed only by GH_4C_1 cells.

We next analyzed the cell-specific expression of sst receptor mRNAs (Fig. 4, *bottom*). Total RNA was isolated from CHO-K1, GH_4C_1, and AR4–2J cells and used for reverse transcription-polymerase chain reaction analysis to identify mRNA encoding sst1 and sst2 receptor subtypes. Our PCR strategy made use of primers from conserved regions of sst receptors located in the first and seventh transmembrane domains and Southern blotting with sst receptor subtype-specific oligonucleotides located in the third extracellular loop and adjacent membrane spanning regions. This procedure ensured both high sensitivity and specificity; PCR amplification of rat brain RNA without subsequent Southern blotting produced multiple products that presumably represented homologous receptors (data not shown). However, sst receptor subtype specificity was absolute with the inclusion of the hybridization step. When the PCR products were probed with the sst1 receptor-specific oligonucleotide (Fig. 4, *bottom*), the 747-bp sst1 receptor-specific PCR product was detected in GH_4C_1 cells but not in CHO-K1 or AR4–2J cells. Quantification with a Betascope analyzer showed that there was no significant difference between the reactions with and without RT for either AR4–2J or CHO-K1 RNA. However, the 747-bp band from the reaction with GH_4C_1 cell RNA contained 1900 cpm above background. Because the primer sequences used for PCR were highly conserved between sst1 and sst2 receptors, the blot was stripped of the sst1 receptor

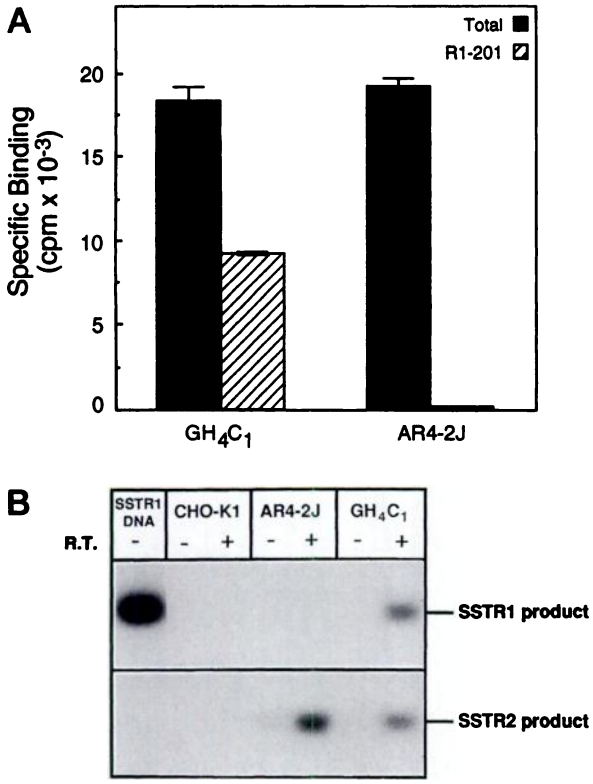

Fig. 4. Expression of sst1 receptor protein and mRNA in GH_4C_1 and AR4–2J cells. *Top*, Membranes from either GH_4C_1 cells (318 μg/ml) or AR4–2J cells (182 μg/ml) were incubated with [^{125}I-Tyr^{11}]SRIF for 2 hr at 30° in the absence or presence of 100 nM SRIF. After detergent solubilization with 4 μg/ml DβM and 0.8 mg/ml CHS, the amount of soluble [^{125}I-Tyr^{11}]SRIF/sst receptor complex was quantified by PEG precipitation as described in Experimental Procedures. Antiserum R1–201 was then added to the solubilized receptors from either GH_4C_1 cells (477 μg membrane protein/300 μl) or AR4–2J cells (137 mg membrane protein/150 μl) to a final dilution of 1:800 and incubated for 3 hr at 4°. After precipitation with Protein A-Sepharose, specific binding was measured in the pellets as described in Experimental Procedures. *Top*, mean ± standard error for the specific binding in either the PEG precipitates (Total) or the immunoprecipitates (R1-201). *Bottom*, Total RNA (10 μg) prepared from CHO-K1, GH_4C_1, or AR4–2J cells was reverse transcribed and PCR amplified as described in Experimental Procedures. Each reaction was carried out both without (*−RT*) or with (*+RT*) reverse transcriptase to control for DNA contamination. The PCR products were electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled sst receptor subtype-specific oligonucleotides. Autoradiography was carried out for 15 min. The plasmid pBS-BR2 (100 pg) encoding the rat sst1 receptor (18) was used as a positive control. *Top strip* shows hybridization to an sst1 receptor-specific oligonucleotide. After removal of the sst1 receptor probe, the membrane was rehybridized with an sst2 receptor-specific oligonucleotide (*bottom strip*).

probe and rehybridized with an sst2 receptor-specific oligonucleotide. The sst1 receptor-specific probe was completely removed as shown by the elimination of the 747-bp band in the lane containing 100 pg of pBS-BR2 plasmid encoding the sst1 receptor (Fig. 4, *bottom*). However, the 753-bp sst2 receptor-specific PCR product was detected in both GH_4C_1 and AR4–2J cells. RNA from GH_4C_1 and AR4–2J cells resulted in the hybridization of 400 and 660 cpm above background, respectively. Therefore, the reverse transcription-polymerase chain reaction analysis demonstrates that sst2 receptor mRNA is expressed in both GH_4C_1 and AR4–2J cells, whereas only GH_4C_1 cells express sst1 receptor mRNA. Therefore, these results corroborate the finding that functional sst1 receptor protein is expressed only in GH_4C_1 cells.

We also determined whether AtT20 mouse pituitary cells and RINm5F insulinoma cells expressed sst1 receptor protein. Both of these cell lines have been reported to contain high affinity sst receptors (31, 39) as well as sst1 receptor mRNA (22, 40). However, antiserum R1–201 immunoprecipitated only 0.1% and 6.2% of the soluble [^{125}I-Tyr^{11}]SRIF/receptor complex from AtT20 and RINm5F cells, respectively (data not shown). Therefore, AtT20 cells appear not to make any sst1 receptor protein, and this receptor constitutes only a very small proportion of the receptors in RINm5F cells.

In summary, of four cell lines widely used to study sst receptor function, only GH_4C_1 pituitary cells express high levels of sst1 receptor protein. Immunoprecipitation with R1–201 allows the purification of this receptor subtype from the other sst receptors present in these cells.

Coprecipitation of coupled G proteins with sst1 receptors. One of the characteristic features of G protein-coupled receptors is that their binding affinity for agonists is reduced in the presence of GTP or nonhydrolyzable GTP analogues (5). Therefore, the addition of GTP to a preformed agonist/receptor/G protein complex causes rapid dissociation of the ligand by converting the receptors from a high affinity, G protein-coupled state to a low affinity, uncoupled state (5). The effect of GTP analogues on sst1 receptor binding was initially determined by incubating CHO-R1 membranes with [^{125}I-Tyr^{11}]SRIF in the absence or presence of 10 μM GTPγS (Fig. 5, *top*). The equilibrium binding of [^{125}I-Tyr^{11}]SRIF to CHO-R1 membranes was inhibited 52% by the addition of 10 μM GTPγS to the incubation buffer, indicating that the sst1 receptor is coupled to G proteins. Increasing the concentration of GTPγS to 100 μM had no greater effect (data not shown). We next determined whether the G proteins coupled to sst1 receptors were sensitive to pertussis toxin. Membranes from toxin-treated and -untreated CHO-R1 cells were incubated with [^{125}I-Tyr^{11}]SRIF in the absence or presence of GTPγS. The results in Fig. 5 (*top*) show that hormone binding was reduced by 75% in pertussis toxin-treated membranes and, furthermore, that pertussis toxin pretreatment abolished the GTPγS effect on binding. Thus, in CHO cells, the sst1 receptor is coupled to pertussis toxin-sensitive G proteins and does not appear to be coupled to toxin-insensitive G proteins.

The ability of the receptor antibody to coprecipitate coupled G proteins with sst1 receptors was tested in the experiment in Fig. 5 (*bottom*). Control and pertussis toxin-treated CHO-R1 membranes, prelabeled with [^{125}I-Tyr^{11}]SRIF in the presence and absence of GTPγS, were solubilized and then immunoprecipitated with anti-sst1 receptor antiserum. We have previously shown that preincubation of the membrane receptor with ligand stabilizes the sst receptor/G protein complex and that the integrity of this complex is maintained during solubilization with DβM (32). Comparison of the top with the bottom in Fig. 5 shows that [^{125}I-Tyr^{11}]SRIF binding in the immunoprecipitates paralleled that in membranes. Thus, when the binding incubation was carried out in the presence of GTPγS, the amount of immunoprecipitated [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex was reduced by 73%. Hormone binding was also reduced in the immunoprecipitates from pertussis toxin-treated membranes, and GTPγS no longer had an effect on binding. These results indicate that

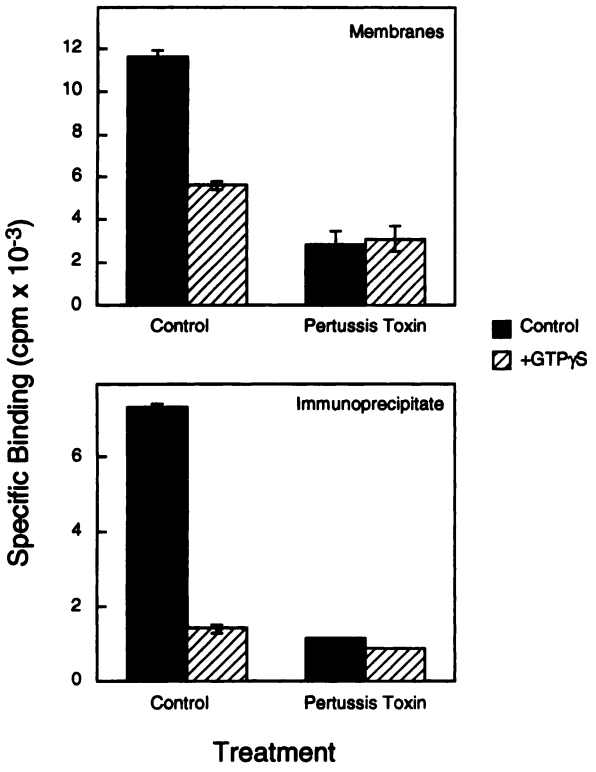

Fig. 5. Effect of GTPγS and pertussis toxin in sst1 receptor binding and immunoprecipitation. *Top*, Membranes were prepared from CHO-R1 cells pretreated for 16 hr either without (control) or with 50 ng/ml pertussis toxin and subsequently incubated (≈80 μg/ml) with [^{125}I-Tyr^{11}]SRIF (190,000 cpm/ml, 0.052 nM) for 2 hr at 30°. Binding reactions were carried out in the absence or presence of 10 μM GTPγS as indicated. Specific binding was measured as described in Experimental Procedures and is expressed per 50 μg of membrane protein. *Bottom*, Membranes from the experiment represent in the *top* were solubilized with DβM/CHS at a detergent/protein ratio of 5:1 and then immunoprecipitated with antiserum R1–201 at a final dilution of 1:400. The amount of specifically bound radioactivity in the immunoprecipitate was determined as described in Experimental Procedures and is expressed per 50 μg of starting membrane protein. In both panels, data represent the mean ± standard error of triplicate samples.

the antibody immunoprecipitated the high affinity, G protein-coupled form of the receptor.

To directly test whether the receptor antibody binds the intact receptor/G protein complex, immunoprecipitated [^{125}I-Tyr^{11}]SRIF-receptor was resuspended in buffer either without or with 100 μM GTPγS and incubated at 30° to allow some of the bound ligand to dissociate (Fig. 6). In the absence of guanine nucleotide, 53% of the specifically bound [^{125}I-Tyr^{11}]SRIF dissociated from the precipitated receptor complex after the 10-min incubation at 30°. The addition of GTPγS increased [^{125}I-Tyr^{11}]SRIF dissociation so that 78% of the initially bound hormone was released (Fig. 6). The ability of GTPγS to stimulate ligand dissociation from the immunoprecipitated receptor complex demonstrates that functional G proteins remain bound to the receptor. Together, our studies show that the sst1 receptor is coupled to pertussis toxin-sensitive G proteins when expressed in CHO-K1 cells, that the immunoprecipitated receptor remains G protein coupled, and, therefore, that the epitope recognized by the antibody must not be essential for receptor/G protein interactions.

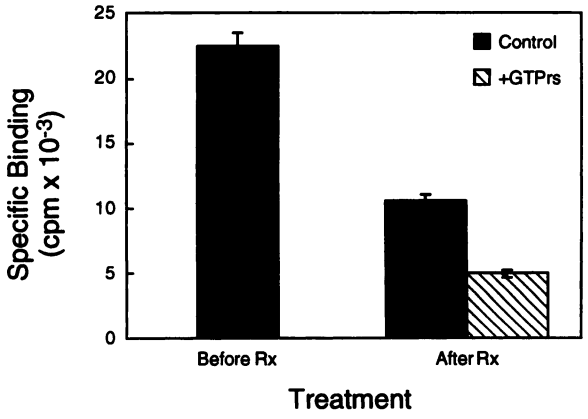

Fig. 6. Effect of GTPγS on ligand binding to immunoprecipitated sst1 receptors. CHO-R1 cell membranes (180 μg/ml) were incubated with [^{125}I-Tyr^{11}]SRIF (561,230 cpm/ml, 0.155 nM) in the absence or presence of 100 nM SRIF for 2 hr at 30°. After solubilization of the ligand/receptor complex, receptor was immunoprecipitated with antiserum R1–201 (final dilution 1:200) as described in Experimental Procedures. The immunoprecipitate was then resuspended in cold HEPES binding buffer containing 0.25 μg/ml DβM. Aliquots (60 μg membrane protein) were either counted immediately (*Before Rx*) or incubated without or with 100 μM GTPγS for 10 min at 30° followed by recentrifugation (*After Rx*). Specific binding was measured as described in Experimental Procedures.

Characterization of sst1 receptor/G protein interactions in GH_4C_1 cells. We next examined the nature of the G proteins coupled to sst1 receptors endogenously expressed in GH_4C_1 cells (Fig. 7). GH_4C_1 membranes from both control

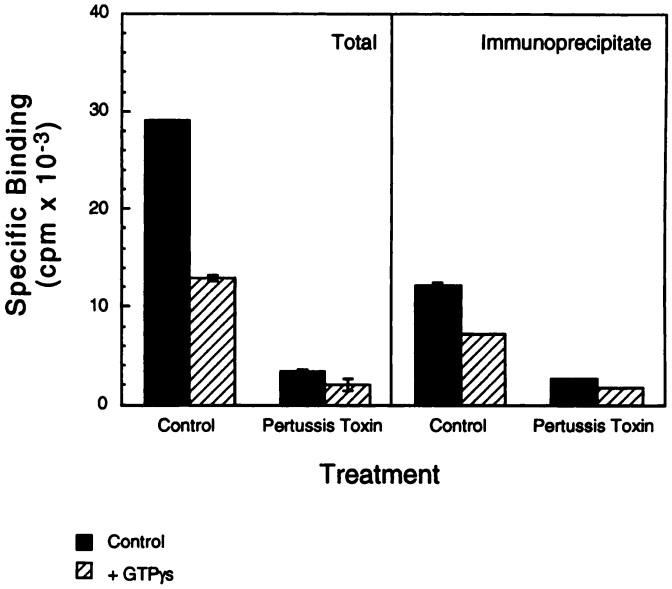

Fig. 7. G protein coupling of the sst1 receptor in GH_4C_1 cells. *Left*, Membranes were prepared from GH_4C_1 cells pretreated for 24 hr either without (*control*) or with 50 ng/ml pertussis toxin and subsequently incubated with [^{125}I-Tyr^{11}]SRIF (266,000 cpm/ml, 0.07 nM) for 2 hr at 30°. Binding reactions were carried out in the absence or presence of 1 μM GTPγS as indicated. After solubilization with 1 mg/ml DβM and 0.2 mg/ml CHS, the amount of [^{125}I-Tyr^{11}]SRIF specifically bound to solubilized receptors was measured by PEG precipitation and is expressed per 60 μg of soluble protein. *Right*, Antiserum was added to the solubilized receptor to a final dilution of 1:100, and the amount of specifically bound radioactivity in the immunoprecipitate was subsequently determined as described in Experimental Procedures. In both panels, data represent the mean ± standard error of the specific binding in triplicate samples per 60 μg of soluble protein added.

and pertussis toxin-pretreated cells were prelabeled with [^{125}I-Tyr^{11}]SRIF in the presence and absence of GTPγS. Under these conditions, [^{125}I-Tyr^{11}]SRIF binds to both sst1 and sst2 receptors (41). Receptors were then solubilized with DβM/CHS and either precipitated with PEG to quantify the total hormone/receptor complex or precipitated with antiserum to measure binding only to sst1 receptors. Fig. 7 (*left*) shows that both guanine nucleotides and pertussis toxin inhibited hormone binding to solubilized receptors, as previously shown for the membrane receptors in these cells (6, 7). Fig. 7 (*right*) shows that 42% of the [^{125}I-Tyr^{11}]SRIF/receptor complex was precipitated by the receptor antiserum. Moreover, GTPγS and pertussis toxin decreased hormone binding in the immunoprecipitate by 41.5% and 78.8%, respectively. In three independent experiments, [^{125}I-Tyr^{11}]SRIF binding in the immunoprecipitate was reduced 34 ± 5% by GTPγS and by 55 ± 12% by pertussis toxin. Therefore, although both sst1 and sst2 receptors are expressed in GH_4C_1 cells, specific immunoprecipitation showed that the sst1 receptor couples primarily to pertussis toxin-sensitive G proteins in this pituitary cell line.

To further characterize the interactions between sst1 receptors and G proteins, the [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex immunoprecipitated from GH_4C_1 cells was resuspended in buffer with or without 20 μM GTPγS, a concentration that maximally inhibits agonist binding to the membrane receptors. The rate of dissociation of the prebound ligand was then monitored at 25° as an indicator of changes in the affinity state of the receptors (Fig. 8). In the absence of guanine nucleotide, hormone dissociation followed first order kinetics with a half-time of 43 min (Fig. 8, *top*). In the presence of 20 μM GTPγS, a biphasic dissociation was observed (Fig. 8). Approximately 60% of the ligand dissociated 10 times more rapidly ($t_{1/2}$ = 4.2 min) in the presence of guanine nucleotide than in its absence. The remaining 40% dissociated at the same rate under both conditions ($t_{1/2}$ = 43 min). In a second experiment with a different preparation of GH_4C_1 cell membranes, 80% of the immunoprecipitated [^{125}I-Tyr^{11}]SRIF/receptor complex was sensitive to GTPγS ($t_{1/2}$ = 3.1 min) and 20% was insensitive ($t_{1/2}$ = 65 min). These results indicate that there are two high affinity states of the sst1 receptor. The majority of the receptors are coupled to G proteins that bind GTPγS with high affinity. The remainder (20–40%) are much less sensitive to this guanine nucleotide and continue to exhibit high affinity hormone binding in its presence.

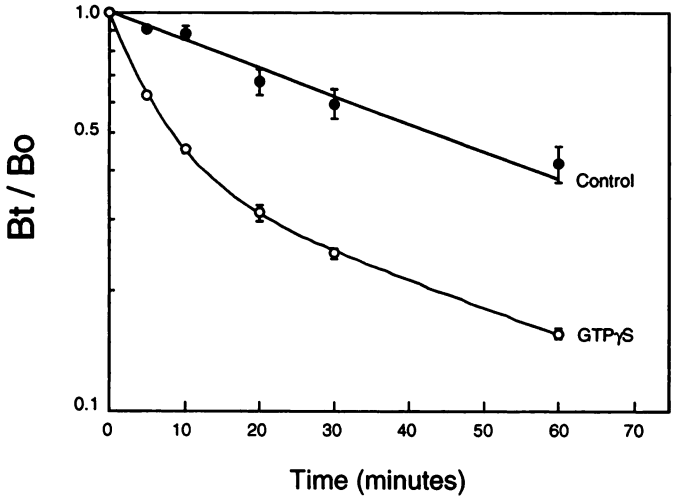

Fig. 8. Effect of GTPγS on ligand dissociation from sst1 receptors. GH_4C_1 cell membranes (101 μg/ml) were incubated with [^{125}I-Tyr^{11}]SRIF (309,400 cpm/ml, 0.085 nM) in HEPES binding buffer in the absence and presence of 100 nM SRIF for 2 hr at 30°. After solubilization and immunoprecipitation with antiserum R1–201, the immunoprecipitate was resuspended in buffer containing 10 nM SRIF with or without 20 μM GTPγS. Incubation was performed at 25°. At the times shown, aliquots were chilled and centrifuged, and the specifically bound ligand present in the immunoprecipitate was quantified. The graph shows the ratio of the specific binding at each time point (*B*) to the specific binding in the immunoprecipitate before the initiation of the dissociation reaction (*Bo*). The data obtained in the absence of GTPγS were fitted to a single exponential equation ($B/Bo = Ae^{-kt}$) with k = 0.016 min^{-1}. The binding data in the presence of GTPγS were fitted to the equation $B/Bo = Ae^{-kt} + (1 - A)e^{-k't}$, with fitted values of A = 0.606, k = 0.167 min^{-1}, and k' = 0.016 min^{-1}.

Discussion

The specificity of G protein coupling by heptahelical receptors is known to depend both on the types of G proteins expressed in a host cell and on the level of receptor expression, with low affinity receptor/G protein interactions becoming of functional significance at high receptor densities (25, 26). Therefore, it is not surprising that the use of different cell lines expressing high levels of transfected sst1 receptors have led to conflicting conclusions regarding the specificity of sst1 receptor/G protein coupling. In some cells, the sst1 receptor appeared unable to couple to G proteins or inhibit adenylyl cyclase (15–17). In others, it inhibited adenylyl cyclase in a pertussis toxin-sensitive manner (18–22) or produced both toxin-sensitive and -insensitive effects (23). Nevertheless, cell-specific differences cannot account for all of the disparate observations reported as conflicting results have been observed regarding sst1 receptor/G protein coupling in the same cell line by different investigators (15, 23, 42). However, the coupling of endogenously expressed sst1 receptors has not been examined in any cell type, so it is not known which of the established expression systems mimics the normal behavior of this receptor.

Several practical difficulties have prevented examination of sst1 receptor/G protein coupling in normal cells. First, a cell or tissue that expresses only the sst1 receptor subtype has not been identified. Target tissues such as the brain, pituitary, and gastrointestinal tract all express mRNAs for multiple sst receptors (21, 43–45), as do clonal cell lines such as GH_3, GH_4C_1, and AtT20 pituitary cells (22, 27, 40, present report). Second, no specific agonists have been identified for the sst1 receptor, so that it is not yet possible to study the function of this receptor in the presence of other sst receptor subtypes (46). No SRIF receptor antagonists are known. Third, although two groups have developed sst2 receptor antibodies (40, 47), it has not been possible to physically isolate the sst1 receptor from other sst receptor subtypes. To overcome these limitations, we prepared a polyclonal antibody to a unique peptide located close to the COOH terminus of the sst1 receptor. Although this region varies greatly among sst receptors, it is entirely conserved between the mouse, human, and rat sst1 receptor. The peptide antibody immunoprecipitated the sst1 receptor with high efficiency: 70% of the ligand/receptor complex was routinely recovered. Immunoprecipitation of the sst1 receptor was completely pre-

vented by the antigenic peptide, and other sst receptor subtypes were not precipitated, showing that the antibody was receptor specific. Moreover, immunoprecipitation of the photoaffinity-labeled sst1 receptor showed that it migrated as a broad 60-kDa band on SDS gels. To our knowledge, we describe the first antibody generated to the sst1 receptor, providing a unique and powerful new tool for studies of this receptor subtype.

Because of the confusion regarding signal transduction by the sst1 receptor, we determined whether the peptide antibody could coprecipitate receptor-associated G proteins. For our initial studies, we chose a cell line (CHO-K1 cells stably transfected with the sst1 receptor) in which SRIF inhibits both adenylyl cyclase and protein tyrosine phosphates via pertussis toxin-sensitive G proteins (18, 48). Although Hershberger *et al.* demonstrated an effect of GTP analogues on adenylyl cyclase inhibition in these cells, they did not observe any effect of guanine nucleotide on hormone binding (18). In our experiments, we reproducibly found that GTPγS reduced [^{125}I-Tyr^{11}]SRIF binding to CHO-R1 membranes (Fig. 5). We have been unable to identify the reason for the difference in GTP regulation in the two studies; perhaps subtle variations in the protocol used for membrane preparation were responsible. Nevertheless, both we (Fig. 5) and Hershberger *et al.* (18) found that pertussis toxin pretreatment reduced hormone binding to CHO-R1 membranes, indicating that the sst1 receptor was coupled to toxin-sensitive G proteins in this cell line.

Two types of experiments showed that the anti-receptor antibody recognized the G protein-coupled form of the sst1 receptor. First, GTPγS directly stimulated the dissociation of prebound hormone from the immunoprecipitated receptor. Second, hormone binding to the immunoprecipitated receptor was reduced in pertussis toxin-treated membranes. Although the third cytoplasmic loop is critical for the interaction of many heptahelical receptors with G proteins, regions within the carboxyl terminus also play a role in several instances. For example, alternative splicing of the carboxyl-terminal tail of prostaglandin E receptor subtype EP3 generates multiple splice variants that differ in their capacity to transduce a signal as well as to couple to specific G proteins (49, 50). Similarly, in the sst receptor family, carboxyl-terminal splice variants of the sst2 receptor have been reported to differ in their efficiency to inhibit adenylyl cyclase (51, 52). Our observation that the sst1 receptor can bind simultaneously to G proteins and the R1–201 antibody indicates that the region of the carboxyl terminus that forms the antibody epitope is not essential for the interaction of this receptor with G proteins.

To identify appropriate model systems in which the coupling of endogenously expressed sst1 receptors could be examined, we screened several cell lines for the presence of the sst1 receptor protein. Expression of sst1 receptor mRNA has been reported in RINm5F insulinoma cells (22), AR4–2J pancreatic acinar cells (16), and AtT20 pituitary cells (40). We found that only 6% of the occupied receptors from RINm5F cells could be precipitated by the sst1 receptor antibody, and we did not detect any functional sst1 receptor protein in either the AR4–2J or AtT20 cell lines. Moreover, when we tested our AR4–2J cells for the presence of sst1 receptor mRNA, we did not find any. Therefore, sst1 receptor expression can vary in the same cell line among different laboratories. Moreover, mRNA expression does not provide a quantitative indication of the level of sst receptor protein.

The GH_4C_1 pituitary cell line has been one of the most widely used model systems for studies of SRIF action (53, 54). We found that these cells contain mRNA for both sst1 and sst2 receptors and, using the R1–201 antibody, showed that they express functional sst1 receptor protein. The sst2 receptor protein has also been identified in these cells (55). The presence of multiple sst receptor subtypes raises interesting questions about the specific role that each receptor plays in mediating the different actions of SRIF. Somatostatin inhibits hormone secretion by GH_4C_1 cells in a pertussis toxin-sensitive manner, and this inhibition results from a decrease in both cAMP and intracellular calcium (7, 30, 56, 57). The latter effect is at least in part due to K^+ channel activation involving SRIF stimulation of serine/threonine phosphatase activity (9). In addition, recent studies show that SRIF stimulates protein tyrosine phosphatases in these cells (48). Using antisense plasmids to reduce the expression of $G_{\alpha o}$ and $G_{\alpha i\text{-}2}$ in GH_4C_1 cells, Liu *et al.* concluded that SRIF inhibition of calcium influx through voltage-dependent calcium channels occurred via $G_{\alpha o}$, whereas $G_{\alpha i\text{-}2}$ mediated SRIF inhibition of adenylyl cyclase (58). However, affinity purification of sst receptors from GH_4C_1 cells indicated that $G_{\alpha i\text{-}2}$ and $G_{\alpha i\text{-}3}$ were predominantly associated with these receptors, whereas the association of $G_{\alpha i\text{-}1}$ and $G_{\alpha o}$ was negligible (59). The explanation for the apparent inconsistencies between these two studies is unclear, and the sst receptor subtypes that couple to the different G protein subunits cannot be determined from the data available.

Using specific immunoprecipitation to separate sst1 from sst2 receptors, we show here that both GTPγS and pertussis toxin pretreatment reduced hormone binding to the sst1 receptor from GH_4C_1 cells. This demonstrates, for the first time, that an endogenously expressed sst1 receptor couples to pertussis toxin-sensitive G proteins. To further characterize sst1 receptor/G protein interactions, we examined the effect of GTPγS on the immunoprecipitated [^{125}I-Tyr^{11}]SRIF/sst1 receptor complex in detail. The guanine nucleotide stimulated the dissociation of prebound hormone 10-fold, consistent with an uncoupling of the receptor/G protein complex in the immunoprecipitate. However, even in the presence of maximal concentrations of GTPγS, ~30% of the receptors were insensitive to the nucleotide such that the original slow rate of ligand dissociation for this fraction was unaffected. The nucleotide-insensitive form of the receptor was very stable as it survived the solubilization and immunoprecipitation procedures. This stability suggests that the GTPγS-insensitive form of the receptor results either from stable associations with other proteins or from covalent modification, such as phosphorylation. Interestingly, partial sensitivity to guanine nucleotides has been recognized for numerous G protein-coupled receptors in membranes (60–63), although the biochemical nature of this GTP-insensitive, high affinity receptor state has not been elucidated. The classic ternary complex model of hormone-receptor/G protein interactions defines the high affinity receptor as a complex between receptor and G protein (64). Binding of guanine nucleotides to the G protein in this complex dissociates it from the receptor and converts the latter to a state with low affinity for agonists. According to this model, the GTP-insensitive agonist binding to sst1 receptors is best explained by the existence of

a G protein/receptor complex that is not sensitive to guanine nucleotide under the conditions used. However, a new model containing a high-affinity, G protein-uncoupled receptor state has been postulated recently to explain the binding properties of a constitutively active mutant of the β_2-adrenergic receptor (65). Our results are also consistent with the existence of a high-affinity, G-protein-uncoupled form of the sst1 receptor. The immunoprecipitating receptor antibodies we describe should be helpful in distinguishing between these possibilities and elucidating the biochemical nature of the GTP-insensitive form of the sst1 receptor.

In summary, we have generated a subtype-specific antibody to the sst1 receptor and used it to isolate this receptor in association with G proteins. The ability to purify the sst1 receptor from both normal cellular targets and from transfected cells will allow its biochemical properties to be analyzed and will allow the proteins stably associated with this receptor to be identified.

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