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Site Specificity of Agonist and Second Messenger-Activated Kinases for Somatostatin Receptor Subtype 2A (Sst2A) Phosphorylation

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ABSTRACT

Somatostatin receptor subtype 2A (sst2A) mediates many of the endocrine and neuronal actions of somatostatin and is the target of somatostatin analogs in cancer therapy. As with many G-protein-coupled receptors, agonist stimulation causes sst2A receptor desensitization and internalization, events that require receptor phosphorylation. Furthermore, heterologous receptor activation of protein kinase C (PKC) also increases sst2A receptor phosphorylation and internalization. Here we analyzed a series of sst2A receptor mutants biochemically to identify residues in the receptor carboxyl terminus that were phosphorylated upon agonist stimulation, and we then generated four phosphorylation-sensitive antibodies to those residues. Once the selectivity of each antibody for its phosphorylated and nonphosphorylated target sequence was determined, the phospho-site-specific antibodies were used to demonstrate that somatostatin treatment of Chinese hamster ovary (CHO) cells expressing the wild type sst2A receptor increased phosphorylation on five residues in the receptor C terminus: Ser341, Ser343, Ser348, Thr353, and Thr354. Phorbol 12-myristate 13-acetate (PMA) increased receptor phosphorylation only on Ser343. Inhibition of PKC blocked PMA but not somatostatin stimulation, showing that different kinases catalyzed Ser343 phosphorylation. In contrast, somatostatin-stimulated sst2A receptor phosphorylation was inhibited by knockdown of G-protein coupled receptor kinase-2 with siRNA. Somatostatin increased sst2A receptor phosphorylation on the same five residues in GH4C1 pituitary cells as in CHO cells. However, PMA stimulated sst2A receptor phosphorylation on both Ser343 and Ser348 in GH4C1 cells. These results characterize the complex pattern of sst2A receptor phosphorylation by agonist and second messenger-activated kinases for the first time and indicate that cell type-specific regulation of sst2A receptor phosphorylation occurs.

G protein-coupled receptors (GPCRs), which constitute the largest family of membrane receptors, respond to ligand stimulation by activating heterotrimeric G proteins that regulate the activity of effector enzymes and ion channels, producing changes in the concentration of specific intracellular second messengers. GPCR action is regulated, in turn, by feedback inhibitory processes that reduce receptor responses to prolonged or repetitive ligand stimulation, a process called desensitization. The first step in GPCR desensitization involves receptor phosphorylation by two types of serine/threonine protein kinases. G protein-coupled receptor kinases (GRKs) specifically phosphorylate the agonist-occupied receptor and thereby trigger homologous receptor desensitization, whereas second messenger-activated kinases can phosphorylate GPCRs in either the ligand-bound or the unoccupied state (Reiter and Lefkowitz, 2006; Moore et al., 2007). Because GPCR phosphorylation by second messenger-activated kinases does not require receptor occupancy by agonist, these kinases can produce heterologous, as well as homologous, regulation of GPCRs. Once phosphorylated, agonist-occupied receptors bind molecules called arrestins, which sterically prevent further interaction between the receptor and G proteins, thus "arresting" receptor signaling (Gurevich and Gurevich, 2006; Moore et al., 2007). Arrestins

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; sst2, somatostatin receptor subtype 2A; PNGase F, peptide *N*-glycosidase F; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CT, carboxyl terminal; ELISA, enzyme-linked immunosorbent assay; SS14, the 14 amino acid form of somatostatin; WT, wild type; IC3, third intracellular loop; BSA, bovine serum albumin; WGA, wheat germ agglutinin; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; PPI, protease and phosphatase inhibitors; PAGE, polyacrylamide gel electrophoresis; HEK, human embryonic kidney; PKA cyclic AMP dependent protein kinase; PKC, protein kinase C; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride.

also facilitate the removal of cell surface GPCRs by acting as adaptors that promote receptor association with components of the endocytic machinery (Gurevich and Gurevich, 2006; Moore et al., 2007). Finally, in addition to damping G-protein-mediated signaling, arrestins act as scaffolds that recruit signaling kinases to their associated receptor and thus enable that receptor to initiate signaling events independently of G proteins (Dewire et al., 2007). Although the role of GRK-catalyzed receptor phosphorylation in arrestin-mediated GPCR regulation is well established, the mechanisms by which second messenger-activated kinases regulate GPCR signaling and trafficking is less well understood (Tobin, 2008).

The recognition that phosphorylation constitutes a critical event in GPCR regulation has generated great interest in understanding the specificity of the kinases that determine the phosphorylation state of this receptor family. However, chemical identification of the receptor residues phosphorylated in intact cells after receptor activation has proven difficult, and the phospho-acceptor sites involved have been identified in only a few instances (Maeda et al., 2003; Trester-Zedlitz et al., 2005). In fact, much of our understanding of the specificity of GRKs and second messenger-activated kinases for GPCR phosphorylation has been inferred from studies with receptor mutants. However, mutations can alter receptor phosphorylation both directly, by modifying phospho-acceptor sites, as well as indirectly, by changing receptor structure so as to affect the interactions between the mutated receptor and its kinase. To circumvent such problems with receptor mutants, complementary approaches have begun to be used. In particular, phospho-site-specific antibodies can identify individual phosphorylated residues in wild-type GPCRs and provide a sensitive assay for receptor phosphorylation in intact cells and tissues (Adams et al., 2003; McLaughlin et al., 2003; Pollok-Kopp et al., 2003; Tran et al., 2004).

In this study, we investigated the pattern of sst2A somatostatin receptor phosphorylation for the first time. This GPCR is an important inhibitor of neuroendocrine secretion in many tissues, including the pituitary and the endocrine pancreas, and also plays a critical role as a neuronal modulator in the central and peripheral nervous systems. Moreover, somatostatin analogs inhibit secretion from tumors that express the sst2A receptor and often inhibit tumor growth as well. As a result, the sst2A receptor is a therapeutic target in the clinical management of a variety of neuroendocrine cancers.

The sst2A receptor signals primarily by activating the $G_{i/o}$ family of G proteins, although pertussis-toxin-insensitive G proteins as well as G protein-independent signaling have been implicated in sst2A action (Liu and Wong, 2005; Florio, 2008). Like many GPCRs, the sst2A receptor rapidly desensitizes and internalizes upon agonist exposure (Hipkin et al., 1997; Elberg et al., 2002). Both the rate and the dose-response for receptor phosphorylation correlate closely with receptor internalization and desensitization (Hipkin et al., 1997; Elberg et al., 2002), and mutagenesis studies indicate that receptor phosphorylation is required for both desensitization and receptor endocytosis after somatostatin treatment (Liu et al., 2008).

Several kinases catalyze agonist-stimulated sst2A receptor phosphorylation. Using a yeast bioassay, Noble et al. (2003) showed that both GRK2 and GRK5 inhibit somatostatin-dependent growth in an sst2A receptor-expressing strain, whereas inactive forms of these kinases were without effect (Noble et al., 2003). Heterologous activation of protein kinase C, either by G_q-coupled receptors or by phorbol esters, produced a marked increase in sst2A receptor phosphorylation and stimulated receptor internalization (Hipkin et al., 2000; Elberg et al., 2002). Receptor phosphorylation is likely to be clinically as well as physiologically important, because the sst2A receptor is phosphorylated in vivo in human tumors continuously exposed to somatostatin (Liu et al., 2003). Yet the molecular details of sst2A receptor regulation are poorly understood and, moreover, are likely to be very different in targets such as neurons, which must respond to acute bursts of somatostatin release, compared with neuroendocrine tumors, which are subject to continuous somatostatin exposure during therapy.

In this study, we used complementary biochemical and immunological approaches, along with mutagenesis, to identify phosphorylation sites in the sst2A receptor and then generated phosphorylation specific antibodies to those sites. We subsequently used the phospho-site-specific antibodies to characterize the phosphorylation of the wild-type sst2A receptor in intact cells under basal conditions and after agonist or protein kinase C stimulation.

Materials and Methods

Materials. Cell culture medium and G418 were purchased from Invitrogen (Carlsbad, CA). Maleimide-activated bovine serum albumin was obtained from Pierce Biotechnology (Rockford, IL). SS14 was purchased from Bachem California (Torrance, CA). FuGENE 6 transfection reagent and 2,2-azino-di(3-ethylbenzthiozolinesulfonate-(6)) were purchased from Roche Diagnostics (Indianapolis, IN). The generation and specificity of the sst2A-receptor antiserum (R2-88) has been described previously (Gu and Schonbrunn, 1997). Leupeptin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, bacitracin, and Nonidet P-40 were obtained from Sigma (St. Louis, MO). *N*-Dodecyl β-D-maltoside was purchased from Calbiochem (La Jolla, CA). Peptide *N*-glycosidase F (PNGase F) and λ-protein phosphatase were purchased from New England BioLabs (Ipswich, MA). Bradford and electrophoresis reagents as well as the secondary antibodies, goat anti-rabbit IgG (heavy and light chains), and goat anti-mouse IgG (heavy and light chains) conjugated to horseradish peroxidase, were from Bio-Rad Laboratories (Hercules, CA). Mouse monoclonal anti-hemagglutinin (HA) epitope antibody (HA.11) was purchased from Covance (Berkeley, CA) and the GRK2 rabbit polyclonal antibody and GAPDH mouse monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GRK2 siRNA (ON-TARGETplus SMARTpool) and DharmaFECT 1 transfection reagent were purchased from Dharmacon, Inc (Lafayette, CO).

Plasmid Construction. Generation of a plasmid containing wild-type rat sst2A receptor containing three tandem repeats of the HA epitope (YPYDVPDYA) at the amino terminus has been described previously (Liu et al., 2005). Receptor mutants were produced by site-directed mutagenesis using either the Transformer kit (Clontech Laboratories, Inc., Mountain View, CA) or the QuikChange kit (Stratagene, La Jolla, CA) as reported previously (Liu et al., 2008). The vector used for all constructs was pcDNA3 (Invitrogen, Carlsbad, CA), and all plasmids were sequenced to ensure accuracy of the mutagenesis.

Cell Culture. The procedures used for transfection and clonal selection of CHO-K1 cell lines that stably express sst2A receptors have been published (Liu et al., 2005, 2008). The clonal cell lines that

stably express wild-type rat sst2A receptor without or with three tandem repeats of the HA epitope at the amino terminus have been described previously (Liu et al., 2003, 2005). Addition of this extracellular epitope tag affects none of the functional properties of the receptor, including ligand binding affinity, receptor internalization, or inhibition of cyclic AMP production (Liu et al., 2005). We also generated stable cell lines expressing three sst2A receptor mutants, all containing the triple HA epitope at the amino terminus (Liu et al., 2008): 1) *Ser−*, in which all serine residues in the third loop (Ser237, Ser238, Ser244, Ser245, and Ser250) and C terminus of sst2A (Ser316, Ser322, Ser333, Ser341, Ser343, Ser348, and Ser368) were mutated to alanine, 2) *Thr−*, in which all threonine residues were mutated to alanine (Thr353, Thr354, Thr356, Thr359, and Thr367), and 3) *Ser−/Thr−*, in which all the above serine and threonine residues were mutated to alanine. All three sst2A receptor mutants were expressed at the cell surface to similar levels, responded to nanomolar concentrations of agonist, and coupled normally to inhibition of adenylyl cyclase, demonstrating that the introduced mutations did not lead to general perturbations in receptor function (Liu et al., 2008). Stably transfected CHO-K1 cells were cultured in Ham's F12 medium containing 10% fetal bovine serum and 250 μg/ml G418 at 37°C and 5% CO_2. Experimental cultures were plated in 100-mm dishes in medium without G418 and used 2 to 3 days later with a medium change 18 to 24 h before use.

Other sst2A receptor mutants were expressed in CHO-K1 cells by transient transfection. with FuGENE 6 according to the manufacturer's instructions. Transfected cells were cultured for 48 h before experiments.

The GH-R2 pituitary cell line is a clonal cell line generated by transfecting wild-type sst2A receptors into GH4C1 cells that endogenously express both sst1 and sst2 receptors (Hipkin et al., 1997). GH-R2 cells were cultured as described previously (Hipkin et al., 1997).

Production of Phospho-Site-Specific Antibodies and Characterization by ELISA. Antigen peptides corresponding to different C-terminal regions of the sst2A receptor (Fig. 4) were synthesized with an amino-terminal cysteine and an amidated carboxyl terminus by Macromolecular Resources (Colorado State University, Fort Collins, CO). Peptides were purified to greater than 90% purity by high-pressure reversed-phase liquid chromatography, and their structure was confirmed by mass spectroscopy. Peptide antigens were conjugated to keyhole limpet hemocyanin through the N-terminal cysteine sulfhydryl and subsequently used to immunize either mice or rabbits. Monoclonal antibodies were generated by A&G Pharmaceutical, Inc. (Columbia, MD) and polyclonal antibodies were produced by Covance Research Products (Denver, PA). Standard protocols for immunization were followed and two to four animals were used for each antigen. Hybridoma clones were initially screened by differential ELISA reactivities toward phosphorylated and nonphosphorylated receptor peptides.

The sensitivity and specificity of each selected antibody was measured by competition ELISA essentially as described previously (Gu and Schonbrunn, 1997). Antigen peptides were conjugated to maleimide-activated BSA according to the instructions of the manufacturer (Pierce Biotechnology). BSA-conjugated peptides (0.2–2.5 ng/well) were adsorbed onto 96-well ProBind Assay Plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Nonspecific sites were then blocked by incubating with 1% gelatin in blocking buffer (50 mM Tris and 200 mM NaCl, pH 8.0) for 2 h. After washing, test antibody was added and incubated for 2 h in the presence of increasing concentrations of homologous or heterologous competing peptides. Plates were then incubated with goat anti-mouse or anti rabbit IgG (H+L)-conjugated horseradish peroxidase for 1 h and developed with 2,2-azino-di(3-ethylbenzthiozolinesulfonate-(6)) according to the manufacturer's instructions. Absorbance was measured at 450 nm. For simplicity, each phospho-site-specific antibody is named for the phosphorylated residue(s) that it recognized in these ELISA assays. The antibodies recognizing phosphoserines 341 plus 343 (αPSer341/3) or only phosphoserine 343 (αPSer343) are mouse monoclonal antibodies. The antibodies directed to phosphoserine 348 (αPSer348) and phosphothreonines 353 plus 354 (αPThr353/4) are polyclonal rabbit antibodies.

Receptor Purification, Deglycosylation, and Dephosphorylation. Receptors were purified by adsorption to wheat germ agglutinin (WGA) agarose (Vector Laboratories, Burlingame, CA) before immunoblotting, as described previously (Liu and Schonbrunn, 2001; Liu et al., 2003). In brief, CHO-K1 or GH4C1 cells expressing sst2A receptors were incubated in Ham's F12 medium with 5 mg/ml lactalbumin hydrolysate in the presence or absence of 100 nM SS14 or 200 nM PMA for 15 min at 37°C. The cells were then scraped into ice-cold HEPES-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, and 3 mM EGTA) containing protease and phosphatase inhibitors (PPI; 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 50 μg/ml bacitracin, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium vanadate, and 100 nM okadaic acid). After centrifugation, the cell pellet was solubilized in lysis buffer (HEPES-buffered saline containing 4 mg/ml dodecyl β-maltoside and PPI) for 60 min at 4°C. Detergent lysates were centrifuged at 100,000*g* for 30 min, and the protein content of the supernatants was assessed by the method of Bradford (1976). Sst2A receptors were then purified by lectin affinity chromatography followed by either deglycosylation or by both dephosphorylation and deglycosylation, as specified for each experiment. In brief, equal amounts of lysate protein (~ 0.5 mg/ml) were incubated at 4°C for at least 90 min with 100 μl (packed volume) of washed WGA agarose. After centrifugation, the agarose beads were washed vigorously with 1 ml of lysis buffer and subsequently incubated with 500 units of PNGase F at 37°C overnight in 50 μl of lysis buffer containing 0.1% SDS (v/v). In some experiments, WGA adsorbed proteins were treated with λ-phosphatase (300 units) at 37°C for 3 h in 50 mM HEPES, pH 7.5, containing 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij35, and 2 mM $MnCl_2$) before deglycosylation with PNGase F as described above. After enzyme treatments, 2× sample buffer [62.5 mM Tris-HCl, 2% SDS, 2% 2-mercaptoethanol (v/v), 6 M urea, and 20% glycerol, pH 6.8] was added, samples were incubated at 60°C for 15 min and then resolved on 12% SDS-polyacrylamide gels.

Immunoblotting. After polyacrylamide gel electrophoresis, resolved proteins were transferred to polyvinylidene difluoride membrane as described previously (Liu and Schonbrunn, 2001). Membranes were blocked for 2 h with bovine lacto-transfer optimizer (10 mM NaH_2PO_4, 10% nonfat dry milk, 10% glycerol, and 0.2% Tween 20) and incubated overnight at 4°C with the specified antibodies in bovine lacto-transfer optimizer. After repeated washing, immunoreactive proteins were detected with horseradish peroxidase-conjugated goat-anti-mouse/rabbit antibody and the ECL chemiluminescent detection system (Amersham, Chalfont St. Giles, Buckinghamshire, UK).

Total receptor protein was monitored in all samples to ensure similar receptor concentrations in every treatment group. When HA-tagged receptors were used, receptor loading was determined by immunoblotting with HA antibodies. Nontagged sst2A receptors were detected with the R2-88 antibody (Gu and Schonbrunn, 1997). Because R2-88 antibody binding is sensitive to the phosphorylation state of sst2A (Fig. 1), immunoblotting with R2-88 was carried out on samples that were dephosphorylated with λ-phosphatase before SDS-PAGE.

GRK2 Knockdown. HEK293 cells stably expressing the sst2A receptor were plated at 3.5×10^5 cells/well in a six-well plate and the following day were transfected with GRK2 siRNA (100 nM/35 mm well) in DharmaFECT 1 transfection reagent according to the manufacturer's instructions. To ensure high knockdown efficiency of GRK2, cells were transfected again with fresh siRNA 32 h after the initial treatment. After 72 h of transfection, cells were treated with or without 10 nM SS14 for 2 min and then harvested by solubilization in lysis buffer (HEPES-buffered saline containing 4 mg/ml do-

decyl β-maltoside and PPI) for 60 min at 4°C. Detergent lysates were centrifuged at 100,000*g* for 30 min and the protein content of the supernatants was assessed by the method of Bradford (1976). To determine GRK2 and GAPDH levels, aliquots of the protein lysates were diluted into 2× sample buffer (62.5 mM Tris-HCl, 2% SDS, 50 mM dithiothreitol, 6 M urea, and 20% glycerol, pH 6.8), heated to 95°C for 5 min, and then resolved on 10% SDS-polyacrylamide gels. Receptor was purified from the remainder of the detergent lysates by WGA chromatography and analyzed as described above.

Statistical Analysis and Curve Fitting. ELISA results are expressed as the mean ± S.E.M. of replicate determinations in a single experiment and are representative of at least two independent experiments. Values for the EC_{50} were calculated by least-squares nonlinear regression analysis of dose-response curves using Prism (version 4.0 for Macintosh; GraphPad Software, San Diego, CA). Each immunoblot shown is representative of at least two independent experiments. The intensity of receptor bands on immunoblots was quantitated using Scion Image (version 1.63; Scion Corporation, Frederick, MD) after scanning autoradiograms with a scanner (Expression 1680; Epson, Long Beach, CA). Band intensity with phospho-site-specific antibodies was normalized for total receptor levels in the same experiment and expressed as a percentage of the stimulation observed with SS14. Quantitated immunoblot data are expressed as mean ± S.E.M. or ± S.D. Differences between treatment groups were analyzed by two-way analysis of variance and Bonferroni post tests.

Results

Identification of sst2A Receptor Phosphorylation Sites. Treatment of intact cells with the native agonist SS14 leads to the rapid phosphorylation of the sst2A receptor on Ser and Thr residues in two regions: the third intracellular loop (IC3) and the carboxyl terminus (CT) (Hipkin et al., 1997; Hipkin et al., 2000; Elberg et al., 2002; Liu et al., 2008). The IC3 and CT domains of the sst2A receptor contain 17 potential phosphorylation sites (Fig. 1). To identify specific Ser and Thr residues phosphorylated in the sst2A receptor in intact cells, we investigated two changes that we had observed in the behavior of this receptor after SS14 treatment. First, we noticed that the binding of an sst2A receptor antibody (Gu and Schonbrunn, 1997) was reduced after SS14 incubation (Fig. 2). Second, we observed that SS14 produced a phosphorylation-dependent shift in the migration of the sst2A receptor on SDS-PAGE gels (Fig. 3). These effects were specific to SS14 and either did not occur or were much reduced with phorbol ester treatment. In the following series of experiments, we identified the phosphorylation events responsible for these SS14-induced changes in the sst2A receptor.

We had previously generated an antibody to a peptide from the sst2A receptor (residues 339–359; Fig. 4) that contained 7 of the 12 potential phosphorylation sites in the C terminus (Gu and Schonbrunn, 1997). However, the precise antibody recognition sequence within the antigen peptide had not been identified. We first determined the sensitivity of this antibody to mutation or phosphorylation of Ser or Thr residues within this receptor region. Figure 2A shows the reactivity of the R2-88 antibody with the wild-type sst2A receptor and with three receptor mutants: 1) *Ser−*, in which all 12 serine residues in the third loop (Ser237, Ser238, Ser244, Ser245, and Ser250) and CT of sst2A (Ser316, Ser322, Ser333, Ser341, Ser343, Ser348, and Ser368) were mutated to alanine, 2) *Thr−*, in which all five threonine residues in the CT of the receptor (Thr353, Thr354, Thr356, Thr359, and Thr367) were mutated to alanine, and 3) *Ser−/Thr−*, which contained both sets of mutations. Immunoblotting with an HA antibody served as a control for receptor loading because this antibody recognizes a sequence tag on the extracellular amino terminus of the receptor. Whereas mutation of all the Ser residues in the third intracellular loop and CT domains of the receptor did not significantly affect R2-88 antibody reactivity, mutation of the Thr residues abolished antibody binding (Fig. 2A). These results indicate that the R2-88 antibody reacts with the hydroxyl groups of one or more Thr residues in the CT region of the sst2A receptor.

We next determined whether phosphorylation of Thr would reduce R2-88 antibody affinity as did Thr mutation to Ala. Figure 2B shows the ability of several nonphospho and phospho peptides to compete for binding to the R2-88 antibody in an ELISA. The sequences of the peptides tested are

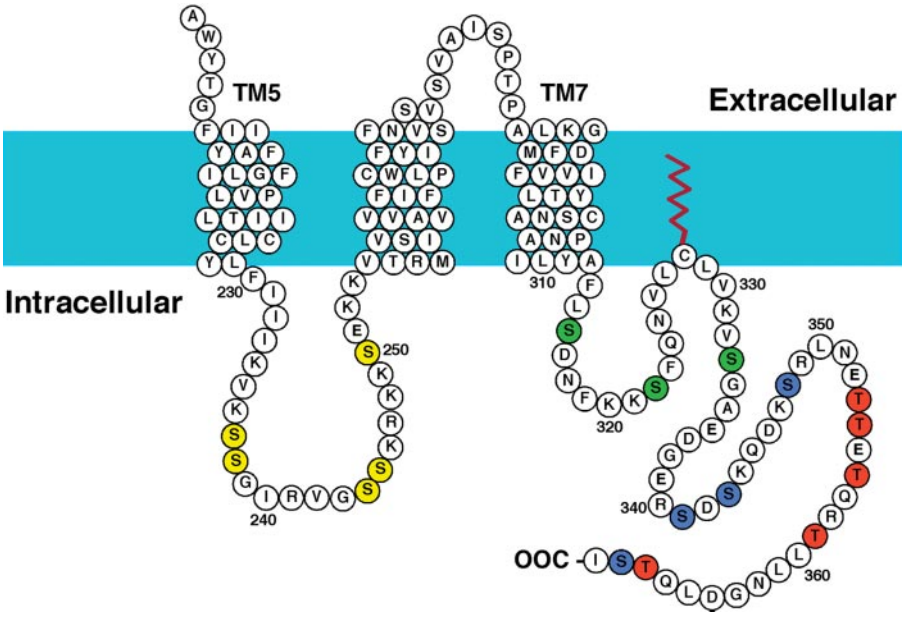

Fig. 1. Diagram of the potential phosphorylation sites in the sst2A receptor. The structure depicts the carboxyl-terminal portion of the rat sst2A receptor with the potential phosphorylation sites in the third intracellular loop and C terminus of the receptor highlighted. The serine and threonine residues mutated to alanine as a group in the constructs used to identify phosphorylated residues are colored identically (see *Results*). The numbers indicate the amino acid positions in the receptor protein.

shown in Fig. 4 and Tables 1 and 2 summarizes the results of from two to five independent ELISA experiments. The peptide CT335–347, which lacks the N-terminal sequence containing the Thr residues in the immunizing peptide, was unable to compete for R2-88 binding even at a concentration of 100 μM (Fig. 2B, Table 2). In contrast, the C-terminal peptide CT344–357 competed for antibody binding with an EC_{50} of 1 μM. However, when either Thr353 or Thr354 or both residues (peptide pThr353/354 in Table 2) were phos-

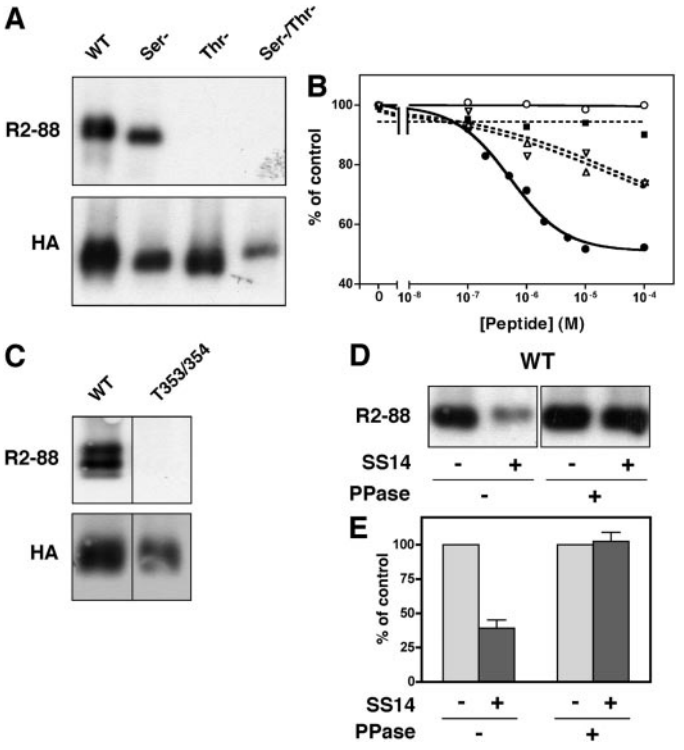

Fig. 2. Identification of Thr phosphorylation sites in the sst2A receptor. A, CHO cells stably expressing wild-type, *Ser−*,*Thr−*, and *Ser−/Thr−* sst2A receptors were solubilized in lysis buffer and adsorbed to WGA agarose. The adsorbed proteins were deglycosylated by PNGase F, subjected to SDS-PAGE, and immunoblotted with both R2-88 and HA antibodies. The results demonstrate that the R2-88 antibody does not recognize the sst2A receptor with C-tail Thr→Ala mutations. B, the C-terminal peptide CT339–362 (5 ng) conjugated to BSA was adsorbed onto wells of a 96-well plate and incubated with R2-88 antibody (1:10,000) and increasing concentrations of five different competing peptides. The two nonphosphorylated peptides are shown in solid lines [CT344–357 (●), CT335–347 (○)] and the three phosphorylated peptides are shown in dashed lines [pThr353 (△), pThr354 (▽), and pThr353/4 (■)]. Peptide sequences are given in Fig. 4. ELISAs were carried out as described under *Materials and Methods*. Nonlinear regression analysis was performed using GraphPad Prism (version 4). Data show the mean values from two to five independent experiments, each carried out with triplicate samples. The equilibrium binding constants are summarized in Tables 1 and 2. The results show that high-affinity R2-88 antibody binding requires both Thr353 and Thr354 in the unphosphorylated form. C, CHO cells transiently transfected with either wild-type sst2A or the T353/354A receptor mutant were solubilized in lysis buffer and processed as described in A. Replicate samples were subjected to SDS-PAGE and immunoblotted with R2-88 and HA antibodies. The results demonstrate that R2-88 antibody does not recognize the T353/354A sst2A mutant receptor. D, CHO cells stably expressing the wild-type sst2A receptor were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization, equal protein samples from control and SS14 treated cells were adsorbed to WGA agarose and deglycosylated with PNGase F in the absence (left) or presence (right) of λ-phosphatase. Samples were then subjected to SDS-PAGE and immunoblotted with the R2-88 antibody. The intensity of each receptor band was then quantitated as described under *Materials and Methods* and normalized to the receptor level in unstimulated samples. The data in E show the mean values from four independent experiments. SS14 treatment reduced R2-88 reactivity with the receptor by over 60%, and this decrease was reversed by phosphatase treatment.

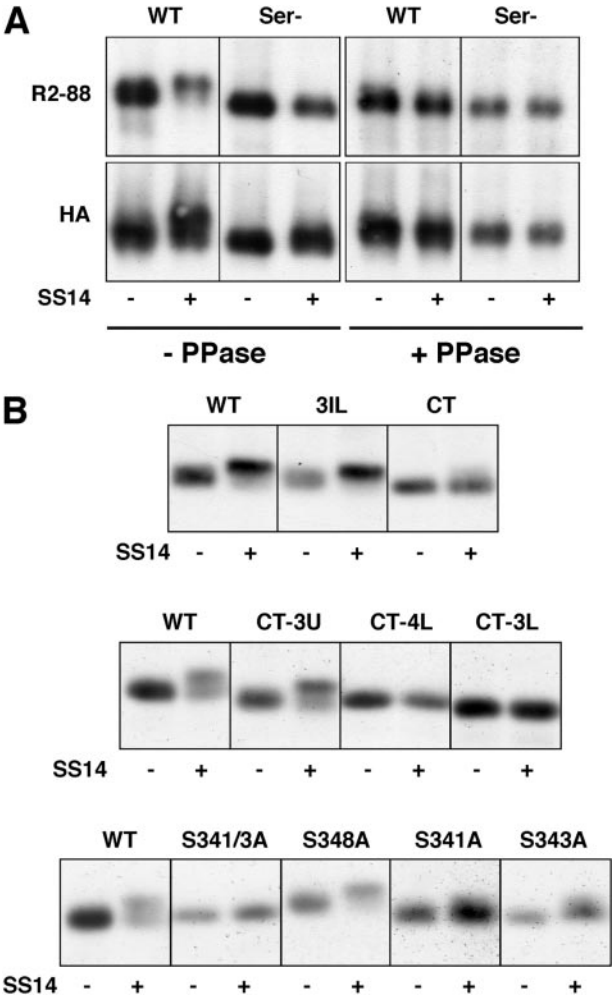

Fig. 3. Identification of serine phosphorylation sites in the sst2A receptor. A, CHO cells stably expressing HA-tagged wild-type or *Ser−* receptors were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization, equal protein samples from control and SS14-treated cells were adsorbed to WGA agarose and deglycosylated with PNGase F in the absence (left) or presence (right) of λ-phosphatase. Samples were solubilized in sample buffer, subjected to SDS-PAGE, and immunoblotted with both R2-88 (top row) and HA (bottom row) antibodies. The results shown are representative of three independent experiments. SS14 treatment produced a shift in the migration of the wild-type receptor and λ-phosphatase reversed this band shift. In contrast, SS14 did not affect the migration of the *Ser−* mutant. Thus, altered mobility of the sst2A receptor results from SS14 stimulated phosphorylation on Ser residues. B, CHO cells transiently transfected with either wild-type or mutant sst2A receptors were incubated without or with 100 nM SS14 for 15 min. After detergent solubilization, protein samples from control and SS14-treated cells were adsorbed to WGA agarose. The adsorbed proteins were deglycosylated by PNGase F, subjected to SDS-PAGE, and immunoblotted with R2-88 antibody. In the IC3 and CT constructs, Ala was substituted for either the five Ser residues in IC3 (Fig. 1, yellow) or the seven Ser residues in the C-terminal tail (Fig. 1, green and blue), respectively. The residues mutated in the CT-3U and CT-4L constructs represent the upper or lower Ser clusters, respectively, in the C-tail of the receptor and are shown in green or blue, respectively in Fig. 1. In the CT-3L plasmid, only residues Ser341, Ser343, and Ser348 were mutated to Ala. The results are representative of four to nine independent experiments with each mutant. They demonstrate that SS14 treatment does not change the mobility of sst2A receptors in any construct containing the S341/343A double mutation but is able to induce a band-shift when other Ser residues in the CT and IC3 regions of the receptor are mutated.

phorylated in CT344–357, peptide affinity was reduced at least 50-fold even though Thr356 remained unphosphorylated. These results show that the R2-88 antibody recognizes the receptor sequence containing Thr353 and Thr354 in a nonphosphorylated state.

To test whether the specificity of the R2-88 antibody observed in ELISA corresponded with its recognition site in the intact receptor, we compared antibody binding to WT sst2A and the T353/354A receptor mutant. Figure 2C shows that mutation of just the two Thr residues, T353A and T354A, prevented R2-88 binding to the sst2A receptor. Together, these results indicate that the R2-88 antibody requires the hydroxyl groups on Thr353 and Thr354 for high-affinity binding: modifying the structure of these Thr either by mutation to Ala (Fig. 2C) or by phosphorylation (Fig. 2B) eliminated or reduced antibody recognition.

Because the R2-88 antibody was sensitive to Thr353/Thr354 phosphorylation (Fig. 2B), we next determined the effect of SS14 treatment on antibody binding to the receptor. Exposure of cells to 100 nM SS14 for 15 min markedly reduced the receptor signal produced by the R2-88 antibody in immunoblots (Fig. 2, D and E). Furthermore, this decrease was reversed by phosphatase treatment showing that Thr353 and/or Thr354 in the sst2A receptor are phosphorylated upon stimulation of cells with agonist.

In addition to decreasing the reactivity of the wild-type sst2A receptor with the R2-88 antibody, SS14 treatment also altered the mobility of the receptor in SDS gels, as we previously reported (Liu et al., 2003). The observation that phosphatase digestion reversed the SS14-induced mobility shift indicated that the change in receptor migration was produced by receptor phosphorylation. Therefore, to identify the residues required for this change in receptor mobility, we determined the effect of SS14 treatment in a series of receptor mutants using large gels to sensitively detect the change in receptor migration upon phosphorylation (Fig. 3).

Incubation of cells with 100 nM SS14 for 15 min reduced the electrophoretic mobility of the WT receptor but not that of the *Ser−* mutant (Fig. 3A). As in our previous study (Liu et al., 2003), the change in receptor mobility was abolished by phosphatase treatment. Therefore, the SS14-induced shift in sst2A receptor migration must result from receptor phosphorylation on Ser residues.

To identify the specific Ser(s) phosphorylated upon agonist stimulation, we determined the effect of SS14 on the electrophoretic mobility of a series of sst2A receptor mutants with Ser→Ala substitutions at different sites (Fig. 3B). Cells transiently transfected with sst2A receptor plasmids were treated with 100 nM SS14 for 15 min and, after solubilization and deglycosylation, the receptors were analyzed by SDS-PAGE and immunoblotting. Figure 3B, top, shows the effect of SS14 treatment on the mobility of the WT receptor and two receptor mutants in which either all five Ser residues in the IC3 or all seven Ser residues in the CT were mutated to Ala. SS14 treatment did not produce a mobility shift in the CT mutant, whereas it reduced the electrophoretic migration of both the WT and the IC3 receptors. Thus, the band shift must result from somatostatin-stimulated phosphorylation of one of the C-terminal Ser residues. To determine which C-terminal Ser residues were involved, we next tested sst2A receptors with either the three upper (Fig. 1, green) or the four lower (Fig. 1, blue) C-tail Ser residues mutated to Ala. The results in Fig. 3B, middle, show that SS14 treatment produced a mobility shift in the CT-3U receptor but not in the CT-4L receptor. Furthermore, mutating only three of the four lower Ser residues (i.e., S341A, S343A, and S348A) also abrogated the SS14-induced mobility shift. In the final series, we tested the effect of SS14 on sst2A receptors with Ser341, Ser343, or Ser348 mutated to Ala individually or in combination (Fig. 3B, bottom). Whereas SS14 produced a mobility shift in the S348A receptor mutant, the shift was markedly reduced in the individual S341A and S343A mutants and completely eliminated in the S341A/S343A double mutant. Together, these results indicate that phosphorylation on Ser341 and Ser343 is responsible for the reduced electrophoretic mobility of the sst2A receptor after SS14 treatment.

In summary, our biochemical studies suggest that four residues (i.e., Ser341, Ser 343, Thr353, and Thr354) are rapidly phosphorylated upon stimulation of cells with SS14.

Development of Phospho-Site-Specific Antibodies to the sst2A Receptor. To study receptor phosphorylation in

Peptide	Sequence	Antibody
	330 340 350 360	
Sst2A C-tail	VKVSGAEDGERSDSKQDKSRLNETTETQRTLLNGDLQTSI	
CT339-362	CERSDSKQDKSRLNETTETQRTLLN	
CT339-359	CERSDSKQDKSRLNETTETQRT	R2-88
CT335-347	CAEDGERSDSKQDK	
CT344-357	CKQDKSRLNETTETQ	
pS341/343	CAEDGERS(P)DS(P)KQDK	αpS341/3, αpS343
pS348	CDGERSDSKQDKS(P)RLN	αpS348
pT353/354	CKQDKSRLNET(P)T(P)ETQ	αpT353/4

Fig. 4. Peptides used to produce and characterize phospho-site-specific antibodies. The peptides used to generate and characterize sst2A receptor antibodies are shown aligned to the C-tail of the receptor. All peptides were synthesized with a carboxyl-terminal cysteine to allow coupling to carrier proteins. The antibodies produced in response to each peptide are shown in the right column. CT339–362, CT339–359, CT335–347, and CT344–357 are nonphosphorylated peptides homologous to different regions of the sst2A receptor C terminus. pSer341/3, pSer348, and pThr353/4 are phosphopeptides containing the specified phosphorylated residues. The singly phosphorylated peptides named pSer341, pSer343, pThr353, and pThr354 are not shown for simplicity: they differ from their diphosphorylated homologs only by the site of phosphorylation. R2-88 is a previously described antibody raised to CT339–359 (Gu and Schonbrunn, 1997). αpSer341/3 and αpSer343 are mouse monoclonal antibodies and αpSer348 and αpThr353/4 are rabbit polyclonal antibodies produced in response to the indicated phospho-peptide antigen and named according their observed specificity in ELISA competition assays (see Fig. 5 and Tables 1 and 2).

detail, we generated antibodies to the phosphorylated residues that we had identified in Figs. 2 and 3. In addition, because sst2A receptor phosphorylation is increased by activation of PKC but not PKA (Hipkin et al., 2000; Elberg et al., 2002), we raised antibodies to phosphorylated Ser348, which is predicted to be a phosphorylation target for PKC (KinasePhos 2.0) (Wong et al., 2007) or PKA (NetPhosK) (Blom et al., 2004) by phosphorylation site prediction programs.

The peptides used as antigens and the antibodies induced by each peptide are shown in Fig. 4. For each antigen, the antibody giving the strongest, phosphorylation-specific signal with the sst2A receptor in immunoblots was chosen for detailed characterization. For simplicity, the selected antibodies are named according to their specificity as determined in peptide competition experiments using ELISA (Fig. 5). The binding of each antibody to the phosphorylated peptide immunogen was competed with varying concentrations of the antigen itself or with partially phosphorylated as well as nonphosphorylated homologs (Fig. 4). Antibodies were used at low concentrations, ranging from 1:30,000 to 1:80:000 dilution, so that these competition experiments provide a good measure of the relative affinity of each antibody for different phosphorylated peptides. Figure 5, A and B show the specificity of two different monoclonal antibodies generated in response to the diphosphorylated pSer341/343 peptide. The results in Fig. 5A show that the peptides pSer341/343 and pSer343 both competed for antibody binding at nanomolar concentrations, whereas pSer341 and the nonphosphorylated peptide CT339–362 did not compete. These results demonstrate that the αpSer343 antibody requires a phosphorylated Ser at the 343 position and that its affinity is not greatly affected by phosphorylation on Ser341. For our second antibody (B), the peptide pSer341/343 competed for antigen binding much more effectively than either monophosphorylated peptide pSer341 or pSer343. The nonphosphorylated peptide CT339–362 again did not compete. Therefore, the αpSer341/343 antibody exhibits a 10-fold selectivity for the diphosphorylated pSer341/343 peptide over either of the singly phosphorylated peptides pSer341 or pSer343. Figure 5C shows that the rabbit antibody αpSer348 demonstrates a 10,000-fold preference for the peptide containing phosphorylated Ser348 compared with the nonphosphorylated peptide. Finally, the rabbit antibody αpThr353/354 is highly selective for the peptide phosphorylated on both Thr353 and Thr354 compared with either of the monophosphorylated peptides

TABLE 1

Site specificity of phospho-serine-sensitive antibodies

The affinity of each antibody for different phosphorylated and nonphosphorylated peptides was determined in competition ELISA experiments such as those shown in Fig 5. The EC_{50} values were determined by nonlinear regression curve fitting and values represent results from two to five independent experiments.

Competing Peptide	EC_{50}		
	αpSer341/3	αpSer343	αpSer348
	μM	*nM*	*nM*
CT339–362	N.C.*	N.C.	N.C.
pSer341	11.5 ± 3.1	N.C.	
pSer343	12.7 ± 1.6	2.83 ± 0.09	
pSer341/3	1.27 ± 0.66	1.49 ± 0.09	
pSer348			0.53 ± 0.09

N.C., no competition at 1 μM; N.C.*, no competition at 100 μM.

TABLE 2

Site specificity of phospho-threonine-sensitive antibodies

The affinity of each antibody for different phosphorylated and nonphosphorylated peptides was determined in competition ELISA experiments such as those shown in Fig 5. The EC_{50} values were determined by nonlinear regression curve fitting and values represent results from two to five independent experiments.

Competing Peptide	EC_{50}	
	R2–88	αpT353/4
	μM	*nM*
CT339–362		N.C.
CT335–347	N.C.*	
CT344–357	0.52 ± 0.11	
pThr353	41 ± 16	N.C.
pThr354	60 ± 26	N.C.
pThr353/354	N.C.*	0.27 ± 0.02

N.C., no competition at 1 μM; N.C.*, no competition at 100 μM.

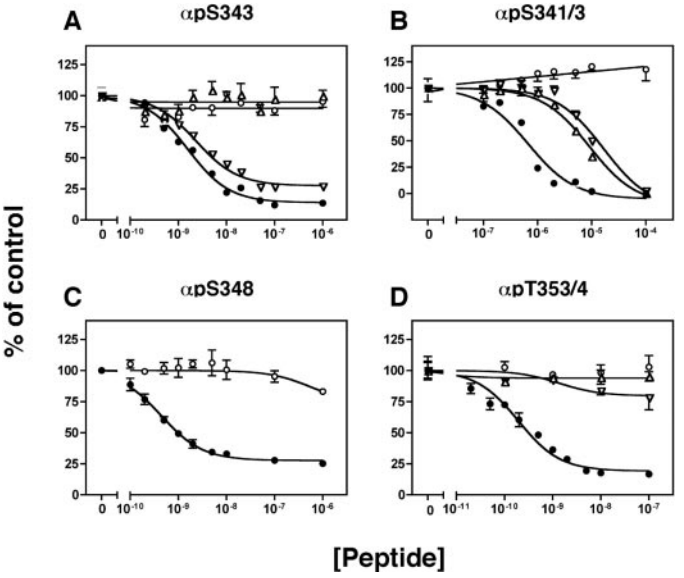

Fig. 5. Specificity of phospho-peptide induced antibodies. Antigen phosphopeptides were absorbed onto wells of a 96-well plate and then incubated with the complementary antibody and increasing concentrations of the phosphorylated or nonphosphorylated competing peptides specified. ELISAs were performed as described under *Materials and Methods*, and each point shows the mean ± S.E.M. of triplicate samples in a representative experiment. Nonlinear regression curve fitting was carried out using GraphPad Prism (version 4). Data from a single experiment is shown and is representative of at least two independent experiments. Results from multiple experiments are summarized in Tables 1 and 2. A, wells were coated with pSer341/343 (2.5 ng) and incubated with the monoclonal mouse αpSer343 antibody and increasing concentrations of either the antigen diphosphopeptide pSer341/343 (●), the nonphosphorylated peptide CT339–362 (○), or the monophosphorylated peptide pSer341 (△) or pSer343 (▽). The data show that antibody recognition requires a phosphorylated Ser at position 343. B, wells were coated with pSer341/343 (2.5 ng) and incubated with the monoclonal mouse αpSer341/3 antibody and increasing concentrations of either the antigen diphosphopeptide pSer341/343 (●) or with CT339–362 (○), pSer341 (△), or pSer343 (▽). The data show that this antibody binds with a 10-fold higher affinity to the doubly phosphorylated peptide pSer341/343 than to either of the singly phosphorylated peptides pSer341 or pSer343. It does not recognize the nonphosphorylated peptide. C, wells were coated with pSer348 (0.2 ng) and incubated with the polyclonal rabbit αpSer348 antibody and increasing concentrations of either the antigen phosphopeptide pSer348 (●) or the nonphosphopeptide CT339–362 (○). The antibody shows more than 1000-fold selectivity for the phosphorylated peptide over the nonphosphorylated peptide. D, wells were coated with pThr353/4 (0.2 ng) and incubated with the polyclonal rabbit αpThr353/354 antibody and various concentrations of either the antigen phosphopeptide pThr353/4 (○), the nonphosphorylated peptide CT339–362 (●), or the monophosphorylated peptides pThr353 (△) and pThr354 (▽). The data demonstrate that the antibody binds the diphosphorylated peptide with a much higher affinity than either the singly phosphorylated peptides or the nonphosphorylated peptide.

and does not recognize the nonphosphorylated sequence at all (Fig. 5D). Tables 1 and 2 summarize the results from multiple independent experiments and provide a quantitative measure of the selectivity of each phospho-specific antibody.

Identification of the Sites of sst2A Receptor Phosphorylation with Phospho-Site-Directed Antibodies. We next determined whether our new antibodies recognized the wild-type sst2A receptor and whether they did so in a phosphorylation-specific manner (Fig. 6). Cells were incubated without or with 100 nM SS14 for 15 min, and the sst2A receptor was isolated as described under *Materials and Methods*. Each sample was then split in two and incubated with or without phosphatase. Replicate aliquots were subjected to SDS PAGE and analyzed by immunoblotting either with HA antibody, as a measure of total receptor concentration, or one of the phospho-site directed antibodies (Fig. 6). Three of the antibodies (αpSer341/343, αpSer348, and αpThr353/4) showed no reactivity with receptor from control cells but gave a strong signal after SS14 treatment. In contrast, αpSer343 showed some reactivity with receptor from untreated cells although SS14 markedly stimulated the signal produced by this antibody as well. Phosphatase treatment eliminated the reactivity of all four phospho-site-directed antibodies with receptor (Fig. 6). Finally, none of the antibodies produced a signal in preparations from nontransfected CHO-K1 cells that do not express the sst2A receptor, either with or without SS14 treatment (data not shown). These results demonstrate that our antibody panel recognizes the sst2A receptor only in a phosphorylated state. To confirm that each antibody recognized the same phosphorylated residue(s) in the intact sst2A receptor as it did in peptide ELISAs, we next examined antibody reactivity with receptors in which the target phospho-Ser or phospho-Thr residues had been mutated to prevent phosphorylation. Figure 7, A and B, shows the reaction of the three phospho-Ser–directed antibodies with sst2A receptor mutants in which Ser341, Ser343, Ser348, or Ser341/343 were mutated to Ala. Figure 7C shows the reactivity of the phospho-Thr–directed antibody with receptors in which Thr353, Thr354, or both residues were mutated. Antibodies were tested with receptors isolated from both control and SS14-treated cells, and the level of receptor expression was determined in each experiment with a control antibody. As expected from the peptide ELISA, the αpSer341/343 antibody did not recognize sst2A receptors containing either the S341A or S343A mutation, demonstrating that it requires both these phosphorylated Ser residues for reactivity (Fig. 7A). Also as expected, the αpSer343 antibody did not recog-

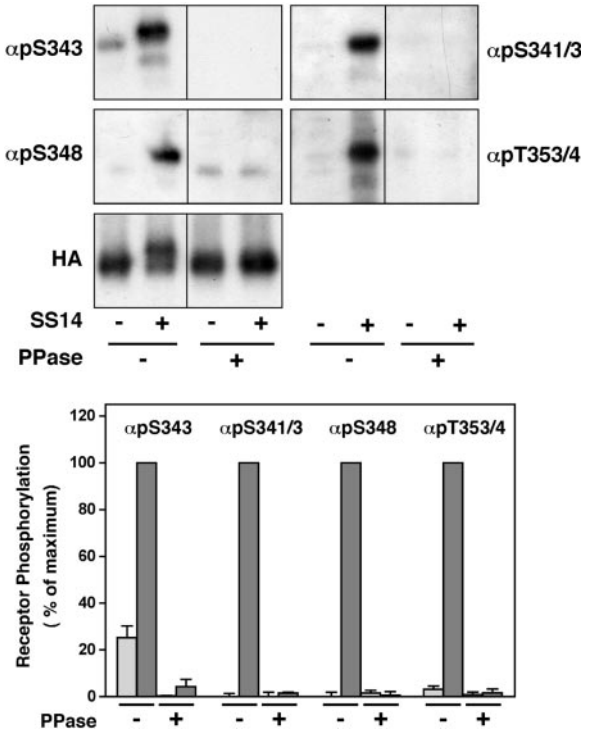

Fig. 6. Reactivity of phospho-site-specific antibodies with the sst2A receptor. CHO cells expressing HA tagged, wild-type sst2A receptor were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization, equal protein samples from control and SS14-treated cells were adsorbed to WGA agarose and subjected to deglycosylation by PNGase F in the absence or presence of λ-phosphatase. Top, aliquots from each reaction were subjected to SDS-PAGE and immunoblotted with the antibodies specified. The HA antibody shows the amount of receptor in each sample. Bottom, intensity of each receptor band was quantitated as described under *Materials and Methods* and normalized for both total receptor and the maximum stimulation in each experiment. The data show the mean ± S.E.M. for each treatment group in five independent experiments. Light gray bars, control samples; dark gray bars, SS14-treated samples. All the phospho-site-specific antibodies showed preferential reactivity with receptor from SS14-treated cells, and in each case, antibody reactivity was abolished by phosphatase treatment.

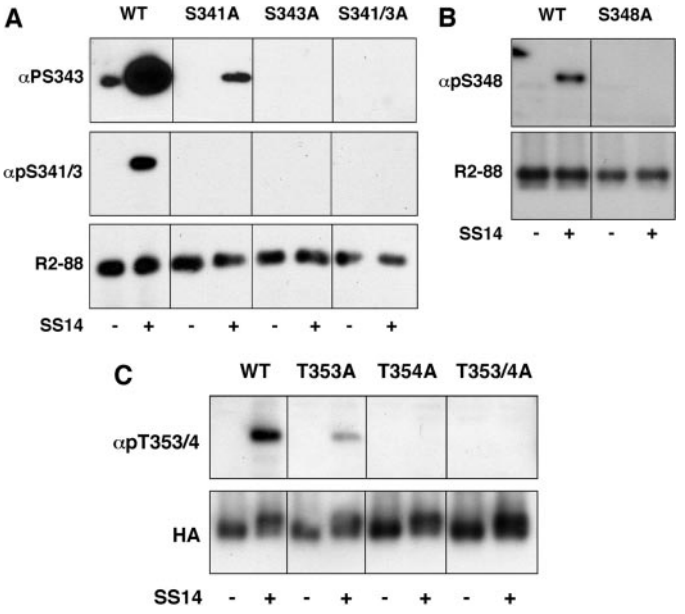

Fig. 7. Identification of receptor recognition sites for the phospho-site-specific antibodies. A and B, CHO cells transiently transfected with wild-type, S341A, S343A, S341/3A, or S348A mutant sst2A receptors were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization, equal protein samples from control and SS14 treated cells were adsorbed to WGA agarose, deglycosylated, and then analyzed by SDS-PAGE and immunoblotting with the phosphospecific antibodies shown. To determine receptor loading, samples were treated with phosphatase before electrophoresis and immunoblotted with the R2-88 antibody. C, CHO cells transiently transfected with HA-tagged wild-type, T353A, T354A, and T353/4A mutant sst2A receptors were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization, receptor samples were prepared as described above and analyzed by SDS-PAGE and immunoblotting with αPThr353/4 and HA antibodies. The results shown are representative of three to five independent experiments.

nize the S343A mutant receptor but did react with receptors containing the S341A mutation. However, the SS14-stimulated signal with the S341A mutant was much less intense than with the WT receptor (12.5 ± 3.4%, $n = 5$) (Fig. 7A), although the similar affinity of the αpSer343 antibody for the pSer341/343 and pSer343 peptides in ELISA would predict that this antibody should bind phosphorylated Ser343 similarly in both the WT and the S341A mutant. These results therefore suggest that the low signal observed with the αpSer343 antibody with the S341A receptor compared with the WT receptor (Fig. 7A) results from decreased SS14-stimulated phosphorylation of Ser343 in the mutant. The αpSer348 antibody gave no signal with the S348A mutant receptor, entirely consistent with the peptide ELISA results, (Fig. 7B). Finally, the phospho-Thr−directed antibody gave a much weaker signal with the T353A mutant (29 ± 3.5% of wild-type, $n = 3$) than with the wild-type receptor and did not recognize receptors containing the T354A mutation (Fig. 7C). Because this antibody bound the monophosphorylated peptides much more poorly than the diphosphorylated peptide in ELISA (Fig. 5), the receptor data indicate that the wild-type receptor is phosphorylated on both Thr353 and Thr354. The low level of phosphorylation detected in the T353A mutant is explained by the weak reactivity of the phospho-Thr-directed antibody with the monophosphorylated pThr354 peptide and the relatively high antibody concentration (1:1000 dilution) used for immunoblotting. Together, these results indicate that the phospho-site-directed antibodies exhibit the same specificity in their reaction with the intact sst2A receptor as in peptide competition ELISA and they identify five residues—Ser341, Ser343, Ser348, Thr353, and Thr354—as sites of sst2A receptor phosphorylation upon stimulation of intact cells with SS14.

Role of GRK and Second Messenger-Activated Kinases in sst2a Receptor Phosphorylation. Stimulation of protein kinase C with either PMA or by activation of G_q-coupled receptors rapidly increases sst2A receptor phosphorylation, whereas PKA stimulation has no detectable effect (Hipkin et al., 2000; Elberg et al., 2002). To determine whether any of the residues phosphorylated upon agonist stimulation were also substrates for second messenger-activated kinases, we determined the effect of PKC or PKA stimulation on sst2A receptor phosphorylation using our antibody panel (Fig. 8). Cells were treated with 100 nM SS14, 200 nM PMA, or 10 μM forskolin for 15 min, receptors were isolated as described previously, and then immunoblotted with each of the phospho-site-specific antibodies (Fig. 8). Reactivity with HA antibody was used to determine receptor loading. Whereas SS14 increased receptor phosphorylation at all residues tested, as in our previous experiments, PMA stimulated sst2A phosphorylation only on Ser343. Forskolin did not affect receptor phosphorylation at any site.

Many G_i-coupled receptors, including sst receptors, activate phospholipase C-β3 via the βγ subunits of pertussis-toxin-sensitive G proteins (Murthy et al., 1996). As a result, they increase the formation of diacylglycerol and thereby stimulate protein kinase C. Because SS14 and PMA both increased sst2A receptor phosphorylation at Ser343, we next determined whether the effect of SS14 involved either receptor signaling via pertussis-toxin-sensitive G proteins or activation of protein kinase C. The results in Fig. 9 show that the PKC inhibitor GF109203X specifically blocked stimulation of Ser343 phosphorylation by PMA but not by SS14. Moreover, pretreatment of cells with pertussis toxin did not prevent Ser343 phosphorylation upon either SS14 or PMA treatment. These results demonstrate that stimulation of Ser343 phosphorylation by PMA involves PKC activation. However, the effect of SS14 requires neither pertussis-toxin-sensitive G proteins nor protein kinase C. Thus, Ser343 is phosphorylated by two different kinases after SS14 and PMA treatment.

To directly assess whether G protein-coupled receptor kinases were involved in agonist stimulated sst2A receptor phosphorylation, we next determined the effect of GRK knockdown on receptor phosphorylation in the human HEK293 cell line. Although a yeast bioassay system was previously used to show that SS14 stimulation of sst2A receptor signaling can be inhibited by coexpression of GRK2 (Noble et al., 2003), the role of endogenous GRKs in sst2A receptor phosphorylation has not been investigated. GRK2 is highly expressed in HEK293 cells, and we found that SS14 treatment of HEK293 cells stably expressing the sst2A receptor produced the same pattern of receptor phosphorylation as that observed in CHO cells (data not shown). Transfection of HEK293 cells with human GRK2 siRNA reduced GRK2 protein levels by more than 95% while decreasing

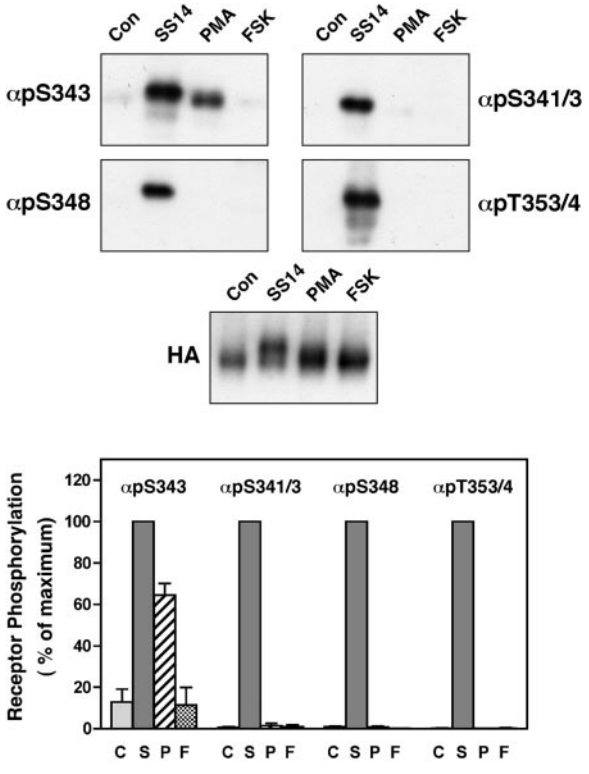

Fig. 8. Effect of second messenger-activated kinases on sst2A receptor phosphorylation. CHO cells stably expressing the wild-type sst2A receptor were incubated in the absence or presence of 100 nM SS14, 200 nM PMA, or 10 μM forskolin (FSK) for 15 min. Top, after detergent solubilization, equal protein samples from each group were adsorbed to WGA agarose, deglycosylated, subjected to SDS-PAGE, and then immunoblotted with HA and the phosphospecific antibodies shown. Bottom, the intensity of each receptor band was quantitated as described under *Materials and Methods* and normalized for both total receptor and the maximum stimulation in each experiment. The data show the mean values in two independent experiments. PMA specifically increased Ser343 phosphorylation but forskolin did not stimulate sst2A receptor phosphorylation at any site.

SS14 stimulated sst2A receptor phosphorylation by 50% (Fig. 10). These results indicate that although SS14-stimulated sst2A receptor phosphorylation is catalyzed by endogenous GRK2 in HEK293 cells, other GRK subtypes are also likely to be involved.

Cell Specificity for sst2A Receptor Phosphorylation. CHO cells are readily transfectable and thus provided an experimentally useful system for our studies requiring transient expression of a large number of receptor mutants. However, they do not express somatostatin receptors endogenously. Therefore, we determined whether the pattern of sst2A receptor phosphorylation observed in CHO cells was reflected in pituitary cells, which do express this receptor (Gu and Schonbrunn, 1997) and have provided an outstanding model for elucidating the mechanism of somatostatin action and regulation in electrically excitable endocrine cells (Schonbrunn et al., 1996; Hipkin et al., 2000). A clonal GH4C1 cell line expressing the sst2A receptor (GH-R2 cells) was incubated with either SS14 or PMA for 15 min, and the pattern of sst2A receptor phosphorylation was then examined in immunoblots (Fig. 11). In contrast to CHO cells, we did not observe basal phosphorylation on Ser343. However, as in CHO cells, SS14 treatment increased sst2A receptor phosphorylation in GH-R2 cells at all sites recognized by our antibody panel. PMA also increased Ser343 phosphorylation in GH-R2 cells, as it had in CHO cells. It is noteworthy, however, that PMA treatment also stimulated phosphorylation on Ser348, an effect not previously observed in CHO cells. Thus, although agonist produced the same pattern of sst2A receptor phosphorylation in GH-R2 and CHO cells, PKC activation increased sst2A receptor phosphorylation on both Ser343 and Ser348 in GH-R2 cells but only on Ser343 in CHO cells. Furthermore, basal phosphorylation on Ser343 occurred only in CHO cells.

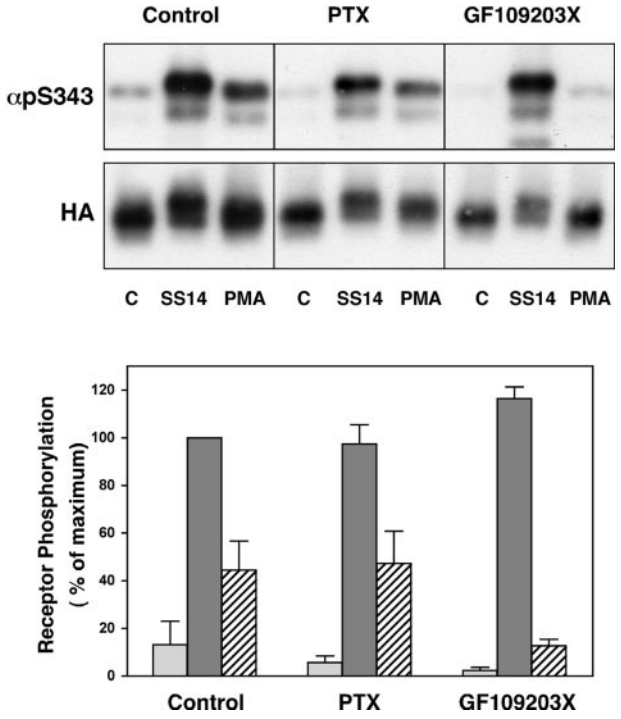

Fig. 9. Effect of pertussis toxin or PKC inhibition on SS14 and PMA-stimulated phosphorylation at Ser343. CHO cells stably expressing wild-type sst2A receptor were incubated at 37°C in the absence or presence of either pertussis toxin (PTX; 100 ng/ml) overnight or the protein kinase C inhibitor GF109203X (4 μM) for 15 min. Subsequently, the cells were treated without or with 100 nM SS14 or 200 nM PMA for 15 min in the continued absence or presence of inhibitors. Top, after detergent solubilization, equal protein samples from control and SS14- and PMA-treated cells were adsorbed to WGA agarose, deglycosylated, subjected to SDS-PAGE and then immunoblotted with HA and αPSer343 antibodies. Bottom, the intensity of each receptor band was quantitated as described under *Materials and Methods* and normalized for both total receptor and the maximum stimulation in each experiment. The data show the mean values from two independent experiments. Light gray bars, control samples; dark gray bars, SS14-treated samples; hatched bars, PMA-treated samples. Two-way analysis of variance showed that PTX pretreatment did not significantly affect either SS or PMA stimulation ($p > 0.05$), whereas GF109203X specifically inhibited the PMA effect ($p < 0.05$).

Discussion

The sst2A receptor undergoes desensitization and internalization within minutes of agonist exposure (Hipkin et al., 1997; Elberg et al., 2002). Although receptor phosphorylation is required for both these regulatory events, different residues are involved such that phosphorylation at particular Ser/Thr sites has distinct functional consequences (Liu et al., 2008). However, the specific residues at which the sst2A receptor is phosphorylated had not been identified, and little is known about the molecular mechanisms by which this receptor is regulated either by agonists or by heterologous hormones. In this study, we identify for the first time five residues in the C-tail of the sst2A receptor that are phosphorylated upon SS14 stimulation in both CHO cells and GH4C1 pituitary cells (Ser341, Ser343, Ser348, Thr353, and Thr354).

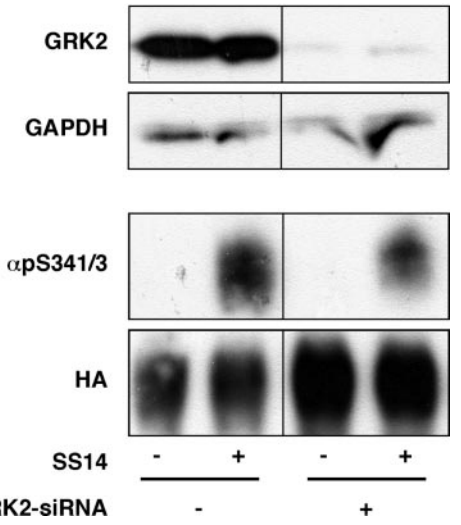

Fig. 10. Effect of GRK2 knockdown on sst2A receptor phosphorylation. HEK293 cells stably expressing the wild-type sst2A receptor were transfected with human GRK2 siRNA for 72 h and subsequently incubated in the absence or presence of 10 nM SS14 for 2 min. After solubilization in lysis buffer and protein determination, an equal amount of protein lysate was directly added to SDS sample buffer for GRK2 and GAPDH quantitation by immunoblotting. The rest of the lysates were adsorbed to WGA agarose and subsequently eluted with SDS sample buffer. Samples were subjected to SDS-PAGE and then immunoblotted with GRK2, GAPDH, HA, or αpSer341/3 antibodies as shown. Staining with the GAPDH and HA antibodies provided a measure of the amount of cell lysate and receptor loaded in each lane, respectively, and was used to standardize the intensity of the signals obtained with the GRK2 and αPSer341/3 antibodies. GRK2 levels were reduced by 97% in the siRNA-treated samples compared with untreated controls, and SS14-stimulated sst2A receptor phosphorylation at Ser341/3 was reduced by 49% by the siRNA treatment. In two independent experiments, GRK2 knockdown reduced sst2 receptor phosphorylation by 40 ± 9% (mean ± range).

Of these, protein kinase C activation specifically increases only Ser343 phosphorylation in CHO cells but stimulates both Ser343 and Ser348 phosphorylation in GH4C1 pituitary cells. In addition, in CHO cells, a small and variable amount of basal receptor phosphorylation is detected only on Ser343, indicating that the sst2A receptor is subject to heterologous regulation under normal culture conditions. These studies elucidate the complex pattern of sst2A receptor phosphorylation and show how cell-type specific regulation of the sst2A receptor may be achieved by differential phosphorylation at particular phosphorylation sites.

In developing a combined biochemical and mutagenesis approach to identify receptor phosphorylation sites, we were sensitive to the fact that mutation of particular Ser/Thr residues could inhibit receptor phosphorylation in two ways: either because the mutated residue was itself phosphorylated or because the mutation changed receptor structure in such a way as to inhibit phosphorylation at a distal site. Therefore, although we initially identified putative phosphorylation sites using site-directed mutagenesis, we subsequently confirmed phosphorylation at each site in the wild-type receptor using phospho-site-specific antibodies the specificity of which was independently determined in peptide competition experiments. These ELISA assays, which used low antibody concentrations, provided a quantitative measure of the specificity and selectivity of each antibody for the phosphorylated peptide used as the immunogen, as well as for partially phosphorylated and nonphosphorylated homologs. Subsequently, we determined the reactivity of each antibody with wild-type receptors and receptors in which selected Ser or Thr residues were mutated to Ala. Because immunoblotting of receptors is carried out using an antibody excess to increase sensitivity, this method is more sensitive than ELISA to low-affinity interactions of the antibodies with partially phosphorylated forms of the receptor. Nonetheless, with three of our four antibodies, namely αpSer341/343, αpSer348, and αpThr353/354 the reactivity observed in peptide ELISA matched antibody reactivity with mutant receptors in immunoblots. However, this was not the case for the αpSer343 antibody, where a discrepancy between peptide competition and receptor mutagenesis was observed. ELISA experiments showed that αpSer343 bound the monophosphorylated pSer343 peptide and the diphosphorylated pSer341/343 peptide with similar affinities, indicating that it recognized pSer343 independently of the phosphorylation state of Ser341. Thus, the observation that this antibody gave only a weak signal after SS14 treatment of the S341A receptor mutant (12.5 ± 3.4% of WT) cannot be explained by antibody specificity, and indicates that phosphorylation of Ser343 is markedly inhibited by mutating the neighboring Ser341 to Ala. It is not known how frequently distal effects occur during the phosphorylation of other mutated GPCRs but our data now provide a precedent for such action.

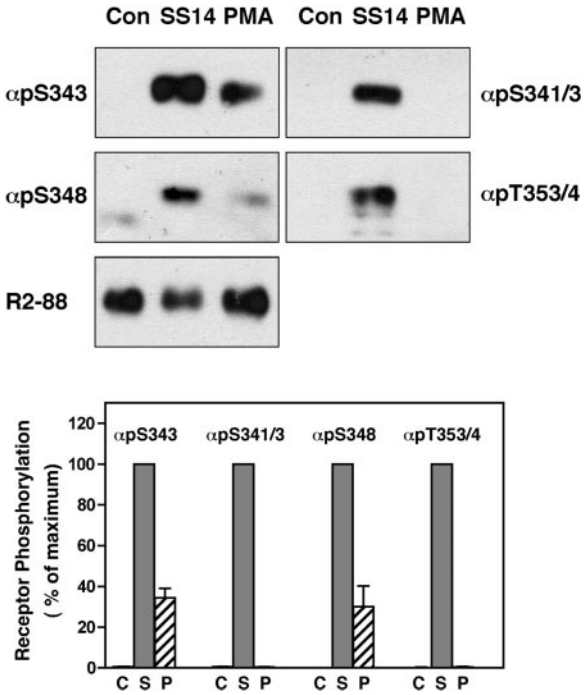

Fig. 11. Phosphorylation of the sst2A receptor in pituitary cells. GH4C1 pituitary cells expressing wild-type sst2A receptor were incubated in the absence or presence of 100 nM SS14 or 200 nM PMA for 15 min. Top, after detergent solubilization, equal protein samples from each group were adsorbed to WGA agarose, deglycosylated, and analyzed by SDS-PAGE and immunoblotting with the phospho-site-specific antibodies shown. To determine receptor loading, samples were treated with phosphatase before SDS-PAGE and then detected with the R2-88 antibody. Bottom, the intensity of each receptor band was quantitated as described under *Materials and Methods* and normalized for both total receptor and the maximum stimulation in each experiment. The data show the mean values from two independent experiments.

The phosphorylation sites identified in this study, as well as the surrounding sequence between residues 340 and 355, are completely conserved in all mammalian sst2A receptors characterized to date, as well as in the chicken receptor (Horn et al., 2003). Although the Ser in position 348 is mutated to Ala in the homologous receptor from *Xenopus laevis*, the remainder of this C-tail sequence is also identical to the rat receptor. Even in fish (*Fugu rubripes*, *Haplochromis burtoni*, *Carassius auratus*), four of the five identified phosphorylation sites in the C-tail are conserved: only Thr in position 353 is altered. It is noteworthy, however, that there is little sequence similarity in this region with other somatostatin receptor subtypes, consistent with their distinct modes of regulation (Hukovic et al., 1996; Hipkin et al., 1997; Liu and Schonbrunn, 2001; Tulipano et al., 2004).The high degree of sequence identity in this region of the sst2A receptor indicates that substantial evolutionary pressure was present to maintain C-tail phosphorylation sites and argues for their functional importance.

In human tissues, the only form of the sst2 receptor identified to date is the sst2A variant. However, in rodents sst2 receptor mRNA undergoes alternative splicing in a tissue-specific manner to produce two receptor forms, sst2A and sst2B, that differ in the sequence and length of their carboxyl termini (Cole and Schindler, 2000). Because the sst2B receptor ends after residue 332, it is missing all five of the phosphorylation sites identified in this study. Consistent with this observation, a recent report showed that phosphorylation of the sst2B receptor was not detectable after agonist stimulation of colonic adenocarcinoma cells, whereas the sst2A receptor was rapidly phosphorylated under the same conditions (Holliday et al., 2007). In these adenocarcinoma cells, SS14 caused rapid desensitization of the sst2A but not the sst2B receptor. However, this difference in desensitization has not been consistently observed by other investigators (Cole and Schindler, 2000). Indeed, the sst2A receptor is

known to be phosphorylated in the third intracellular loop as well as the C-tail (Hipkin et al., 2000; Elberg et al., 2002), and of course this sequence is shared by the two sst2 receptor variants. Thus the functional consequences of different phosphorylation patterns for the sst2A and sst2B receptors remain to be elucidated. Nonetheless, such differences in receptor phosphorylation are likely to be particularly important in the brain and the gastrointestinal tract, where different amounts of the two sst2 splice variants are found.

Our results demonstrate that at least two classes of kinases are involved in sst2A receptor phosphorylation and that these phosphorylate the receptor in a distinct pattern. Agonist binding increases receptor phosphorylation on Ser341, Ser343, Ser348, Thr353, and Thr354: five clustered residues in the receptor C terminus. In preliminary studies, we have observed that overexpression of each of the widely expressed GRK isoforms, namely GRK2, GRK3, GRK5, or GRK6 in HEK cells increased sst2A receptor phosphorylation (Q. Liu and A. Schonbrunn, unpublished observations) indicating that multiple GRKs are able to phosphorylate this receptor. Here we show that extensive knockdown of GRK2 expression reduced receptor phosphorylation at Ser341/3 but did not block it completely, supporting the conclusion that multiple GRKs catalyze agonist induced sst2A receptor phosphorylation. In contrast to agonist stimulated receptor phosphorylation, the phorbol ester PMA increased sst2A phosphorylation only on Ser343 in CHO cells and on both Ser343 and Ser348 in GH4C1 pituitary cells. Forskolin did not affect sst2A receptor phosphorylation. The PKC inhibitor GF109203X blocked PMA but not agonist-stimulated phosphorylation of Ser343, demonstrating that only the former is catalyzed by protein kinase C. Thus, all five identified residues seem to be GRK substrates, but only Ser343 is phosphorylated upon PKC activation in CHO cells. It is noteworthy that under the conditions of our experiments, the PMA-induced phosphorylation is consistently less than that produced by agonist. The different pattern of sst2A receptor phosphorylation by GRKs and PKCs is striking because both increase receptor internalization (Hipkin et al., 2000; Elberg et al., 2002) and indicates that distinct mechanisms are likely to be involved.

In ^{32}P incorporation experiments with intact CHO cells, we previously observed that the sst2A receptor was phosphorylated to a small extent even in the absence of agonist stimulation (Liu et al., 2008). In this study, we found that the sst2A receptor was specifically phosphorylated on Ser343 in the absence of agonist although the magnitude of this basal phosphorylation varied between experiments. Ben-Shlomo et al. (2007) suggested that the sst2 receptor has constitutive activity based on the observation that reducing receptor mRNA expression increased baseline intracellular cAMP levels. If the sst2A receptor has inherent constitutive activity, the constitutively active conformation of the receptor should be recognized by GRKs and phosphorylated in the same manner, if not to the same extent, in the absence of agonist as when stimulated by SS14. However, in the absence of hormone, the sst2A receptor in CHO cells was phosphorylated only on Ser343, and it was not phosphorylated on any residue in GH4C1 pituitary cells. Although low levels of constitutive receptor phosphorylation could have fallen below the sensitivity of our phospho-site-specific antibodies, the absence of detectable basal phosphorylation at any of the GRK-specific sites indicates that no more than a small fraction of the sst2A receptor exists in a constitutively active form.

The observation that basal phosphorylation of sst2A occurs specifically on Ser343 suggested that PKC might be involved. However, inhibition of basal phosphorylation by GF109203X did not reach statistical significance. Thus, further studies will be required to determine whether PKC or another kinase is responsible for basal sst2A receptor phosphorylation.

To characterize our panel of phospho-site-specific antibodies with sst2A receptor mutants, we needed a cell line that was easily transfected and grew rapidly. We chose CHO cells for these studies because they are a widely studied model for GPCR regulation. However, previous reports have shown that GPCR phosphorylation can vary between cell types (Ozcelebi et al., 1996; Tobin, 2008) and CHO cells do not express sst2A receptors endogenously. Therefore, we also determined the pattern of sst2A receptor phosphorylation in GH4C1 pituitary cells that represent a commonly used model for mechanistic studies of both somatostatin receptor signaling and regulation and retain most aspects of somatostatin action observed in normal pituitary cells (Schonbrunn and Koch, 1987). In fact, the first demonstration that receptor phosphorylation was involved in the homologous and heterologous regulation of the sst2A receptor was carried out in this model cell line (Hipkin et al., 1997, 2000). Our results show that the general pattern of agonist-stimulated sst2A receptor phosphorylation is the same in CHO and GH4C1 pituitary cells: the same five residues are phosphorylated in the receptor C terminus. Furthermore, PKC activation also results in Ser343 phosphorylation in both cell lines. However, PMA increased sst2A receptor phosphorylation on Ser348 in GH4C1 cells, but not in CHO cells. Although the biological significance of this difference remains to be determined, the demonstration that phosphorylation at a particular receptor residue by a second messenger-activated kinase occurs in a cell-specific manner clearly shows that cell-type differences in sst2A receptor function may occur as a consequence of distinct patterns of receptor phosphorylation.

In conclusion, we have identified specific phosphorylated residues in a somatostatin receptor for the first time and characterized the pattern of receptor phosphorylation under basal conditions, upon agonist stimulation, and upon heterologous activation of protein kinase C. We show that receptor phosphorylation under basal conditions is inconsistent with constitutive receptor activity and instead indicates that the unoccupied receptor is subject to heterologous regulation in a cell specific manner. These studies provide a major step toward understanding the biochemical mechanisms by which the trafficking and signaling of the sst2A receptor is regulated.

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