The G protein-coupled sst2 somatostatin receptor is a critical negative regulator of cell proliferation. sstII prevents growth factor-induced cell proliferation through activation of the tyrosine phosphatase SHP-1 leading to induction of the cyclin-dependent kinase inhibitor p27\(^{kip}\). Here, we investigate the signaling molecules linking sst2 to p27\(^{kip}\). In Chinese hamster ovary-DG-44 cells stably expressing sst2 (CHO/sst2), the somatostatin analogue RC-160 transiently stimulates ERK2 activity and potentiates insulin-stimulated ERK2 activity. RC-160 also stimulates ERK2 activity in pancreatic acini isolated from normal mice, which endogenously express sst2, but has no effect in pancreatic acini derived from sst2 knock-out mice. RC-160-induced p27\(^{kip}\) up-regulation and inhibition of insulin-dependent cell proliferation are both prevented by pretreatment of CHO/sst2 cells with the MEK1/2 inhibitor PD98059. In addition, using dominant negative mutants, we show that sst2-mediated ERK2 stimulation is dependent on the pertussis toxin-sensitive G\(_\text{i/o}\) protein, the tyrosine kinase Src, both small G proteins Ras and Rap1, and the MEK kinase B-Raf but is independent of Raf-1. Phosphatidylinositol 3-kinase (PI3K) and both tyrosine phosphatases, SHP-1 and SHP-2, are required upstream of Ras and Rap1. Taken together, our results identify a novel mechanism whereby a G\(_{\text{ia}}\) protein-coupled receptor inhibits cell proliferation by stimulating ERK signaling via a SHP-1-SHP-2-PI3K/Ras-Raf/MEK pathway.

Somatostatin is a widely distributed peptide that plays an important inhibitory role in several biological processes, including neurotransmission, exocrine and endocrine secretions, and cell proliferation. Somatostatin acts via a family of five G protein-coupled receptors (GPCR)\(^1\) (sst1--sst5) that are variably expressed throughout numerous tissues ranging from the central nervous system to the endocrine and immune systems (1). Somatostatin receptors regulate diverse signal transduction pathways such as adenyl cyclase and guanylyl cyclase inhibition, phospholipase A\(_2\) stimulation, tyrosine phosphatase activation, or ionic conductance channels modulation (2). Somatostatin or its analogues promote growth inhibition of various normal and tumor cells both in vitro and in vivo. Somatostatin antiproliferative action results from inhibition of trophic or growth factors synthesis and/or secretion, or from inactivation of their related intracellular pathways (1, 3). Among the five somatostatin receptors, sst2 has been found to play a critical role in the negative control of normal and tumor cell growth and to act as a tumor suppressor gene for pancreatic cancer (4, 5). The signaling pathways coupling sst2 receptor to cell growth inhibition have not been fully elucidated. Using Chinese hamster ovary (CHO)-DG44 cells stably expressing sst2 (CHO/sst2), we have demonstrated that the somatostatin analogue, RC-160, promotes G\(_{\text{i}}\) cell cycle arrest of insulin-treated cells by a mechanism involving the stimulation of a tyrosine phosphatase we identified as SHP-1, and consequent up-regulation of the cyclin-dependent kinase inhibitor (CKI) p27\(^{kip}\) (6).

Activation of the extracellular regulated kinases (ERK1/2) mitogen-activated protein kinase (MAPK) pathway is generally related to growth promoting actions of growth factors, cytokines, and ligands for GPCR receptors (7). However, differentiation or growth inhibition may also be a consequence of ERK1/2 activation. For instance, nerve growth factor (NGF)- and hepatocyte growth factor-induced activation of ERK2 has been shown to result in NIH3T3 and HepG2 cell cycle arrest, respectively (8, 9). Activation of the cAMP pathway was reported to induce PC12 cell differentiation through sustained ERK1 activation (10). Transforming growth factor-\(\beta\) can block the proliferation of different cell lines through a rapid and transient activation of ERK1/2 (11). Thus, the biological outcome of ERK2 activation is dependent on cell types, extracellular factors, and their receptors. ERK1/2 are serine/threonine protein kinases activated upon dual phosphorylation by the MAPK kinases (MAPKK) MEK1/2, which are themselves activated following phosphorylation by the MAPKK kinases of the Raf family. Raf kinases are activated after their interaction with the GTP-bound and, hence, active form of the proto-oncogene Ras. Once associated with the active form of Ras, Raf kinases translocate from the cytosol to the membrane and are activated by a complex multistep process yet incompletely elucidated (12). Raf kinases

\[^1\] The abbreviations used are: GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; CKI, cyclin-dependent kinase inhibitor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPKK, MAPK kinase; MEK, MAPK kinase or ERK kinase; HA, hemagglutinin epitope; PI3K, phosphatidylinositol 3-kinase; MBP, myelin basic protein; PTK, Bovine endothelial cell kinase; GST, glutathione S-transferase; EGF, epidermal growth factor; NFG, nerve growth factor; MEM, minimum essential medium; DN, dominant negative; RBD, Ras or Rap binding domain; PMSF, phenylmethanesulfonyl fluoride; MBP, myelin basic protein; DTT, dithiothreitol; GEF, guanine nucleotide exchange factor.
have also been described as a point of convergence of other receptor-triggered signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K) or protein kinase C pathway (13, 14). Although Raf-1 is generally considered as the classic upstream activator of MEK1/2, a novel pathway has been recently described. This pathway involves the activation of B-Raf, another member of the Raf family kinases, by the Ras-like small GTPase Rap1 (15). Rap1 possesses an effector domain similar to that of Ras, indicating that both small GTPases might share common downstream effectors (16). Rap1 can antagonize Ras-dependent ERK1/2 signaling by interacting with Raf-1 cysteine-rich domain and preventing its activation (17). However, Rap1 activation can also lead to ERK1/2 phosphorylation via B-Raf. For instance, activation of the Rap1-B-Raf cascade has been found to be involved in Ras-independent activation of ERK1/2 by cAMP and to cooperate with Ras in inducing ERK1/2 activation in response to NGF in PC12 cells (18, 19).

The ERK1/2 pathway is also elicited by somatostatin receptor activation, but its modulation and the biological outcome of its activation differ according to the receptor subtypes and the cellular environment. Indeed, when expressed in CHO-K1 cells, sst1 and sst4 activate the ERK1/2 pathway. However, induced expression of ERK1/2 by sst2 leads to Ras-independent cell proliferation, whereas sst1 inhibits cell growth through Ras- and Rap1-dependent transient activation of ERK1/2 (20, 21). Conversely, sst5-mediated cell growth inhibition requires ERK inhibition, which is mediated by guanylyl cyclase and consequent cGMP-dependent protein kinase inactivation (22). The role of the ERK1/2 pathway in sst2-mediated cell growth inhibition and the mechanisms involved are not well established. In human SY5Y neuroblastoma cells, sst2-mediated inhibition of platelet-derived growth factor (PDGF)-dependent ERK1/2 phosphorylation is associated with an inhibition of PDGF-induced cell proliferation, whereas in human U343 glioma cells, stimulation of sst2 only slightly affects epidermal growth factor (EGF)-promoted cell proliferation despite a strong inhibition of EGFr-dependent ERK1/2 hyperphosphorylation (23, 24). In CHO-K1 cells expressing sst2, although somatostatin mediates a sustained activation of ERK1/2, only p21Cip1/Waf1 induction was detected only following stimulation of both ERK1/2 and p38-MAPK (p38) phosphorylation, suggesting that ERK1/2 activation alone is necessary but not sufficient to mediate sst2 antiproliferative function (25).

In the present study, we investigated the role of ERK1/2 pathway in sst2-mediated inhibition of mitogenic insulin signaling in CHO/sst2 cells. We show that sst2-mediated cell growth inhibition is a consequence of a strong and transient stimulation of ERK2 activity. In addition, the intracellular events leading to sst2-mediated ERK2 activation were identified. sst2-mediated ERK2 activation is dependent on a Ras-Rap1/B-Raf/Raf1/MEK1/2 pathway. Furthermore, PI3K, SHP-1 and SHP-2 are required upstream of Ras and Rap1.

**EXPERIMENTAL PROCEDURES**

**Materials**—The somatostatin analogue RC-160 was a generous gift from A. V. Schally (Tulane University, New Orleans, LA). Insulin (100 IU/ml) was supplied by Eli Lilly and Co. Minimum essential medium alpha with ribonucleosides and deoxyribonucleosides ([α-32P]ATP (3000 Ci/mmol, 10 mCi/ml) was purchased from Amersham Biosciences. All other materials were obtained from Sigma unless otherwise stated.

**Cell Culture and Growth Assay**—Chinese hamster ovary DG-44 (CHO) cells stably expressing sst2 (CHO/sst2) (6), were routinely grown in αMEM supplemented with 5% fetal calf serum and Genetin (400 μg/ml) at 37 °C in a water-saturated 10% CO2 atmosphere. For treatment, cells were plated in 100-mm dishes at 5 × 105 cells/dish, grown for 24 h, and then serum-starved in αMEM for 18 h before inhibitor and/or peptide addition. All inhibitors were added 30 min prior to treatment except PTX (18 h). Control incubations were carried out to ensure that the vehicles (dimethyl sulfoxide or ethanol), concentrated at less than 0.1%, had no side effect. For growth assay, cells were plated in six-well dishes (11 × 104 cells/dish). After a 7-h attachment phase, cells were serum-starved for 18 h to induce quiescence and then cultured for 24 h in αMEM alone or containing 10 nM insulin in the presence or absence of 1 nM RC-160. Cell proliferation was measured by counting with a Coulter counter model Z1 (Coulter Electronics) as described previously (4).

**Actinomycin-sst2 knock-out C57Bl/129 mice (sst2 KO) were provided by S. M. Schaeffer (Merck Research Laboratories, Rahway, NJ) (26).** Corresponding wild-type mice (sst2 WT) were used as control. C57BL6-mem' mice (mem') were obtained by mating heterozygous C57BL6-mem' mice (Jackson Laboratories, Bar Harbor, ME) breeding pairs. Homozygous mem' were screened by reverse-transcription polymerase chain reaction as described previously (27). Mem' were identifiable by 10 days of age, owing to the moth-eaten appearance of the skin.

**Preparation of Mouse Pancreatic Acini**—sst2 KO or sst2 WT mice and mem' mice or their unaffected littermates were sacrificed at 2 weeks of age, and pancreases were removed. Pancreatic acini were prepared using an oxygenated Krebs-Ringer buffer containing 0.15 mg/ml collagenase, 0.01% soybean trypsin inhibitor, and 0.01% ascorbic acid and 0.01% dithiothreitol, as described previously (6). After washing in the oxygenated Krebs-Ringer buffer, acinar cells were incubated in the same buffer, in the presence or absence of inhibitors and/or RC-160 at 25 °C under gentle shaking, and then solubilized. Lysates were cleared by centrifugation, and supernatants were used for immunoblotting as described below.

**Plasmids and Transfection**—The plasmids encoding the dominant negative (DN) Ras mutant (pcDNA3/N17Ras), the activated Ras mutant (pcDNA3/V12Ras), the HA-tagged DN B-Raf mutant (pcDNA3/HA-NB-Raf) corresponding to the N-terminal region of quail B-raf (residues 1–443) or the pGEX plasmid for bacterial expression of a glutathione S-transferase (GST) fusion protein of RasGDS-Rap1 binding domain was generously provided by R. M. Oldberg (Developmental Biology and Cancer Research, Nice, France). The DN Rap1A mutant (pcPEA/N17Rap1) was generously provided by L. A. Quilliam (Indiana University School of Medicine, Indianapolis, IN). The expression vector pUSEamp that incorporating the kinase-inactive Src (K297R) mutant (pUSE/Src(K/R)) were from Upstate Biotechnology and were kindly donated by Dr. T. Florio (Advanced Biotechnology Center, Genova, Italy). The HA-tagged DN R-Raf-1 mutant (pcDNA3/HA-N-Raf-1), corresponding to the N-terminal region of human Raf-1 (residues 1–257) was obtained by subcloning the EcoRI-BamHI fragments of the HA-tagged wild-type Raf-1 into EcoRI-CfoI sites of pKS and inserting the EcoRI-ApoI fragments of pKS/N-Raf-1 back into EcoRI-ApoI sites of HA-pcDNA. The catalytically inactive murine SHP-1 (C4535) mutant (pcDNA3/SHP-1(C/S)) was provided by M. L. Thomas (Howard Hughes Medical Institute, Washington University, St. Louis, MO), and the catalytically inactive human SHP-2 (C4585) mutant (pcDNA3/SHP-2(C/S)) was provided by J. D. Roth (Cochin de Génétique Moléculaire, Paris, France). The c-Myc-tagged version of the SHP-2 (C4535) mutant (pcDNA3/c-Myc-SHP-2(C/S)) was obtained by inserting a double-stranded c-Myc tag oligonucleotide into XmnI-EcoRI sites at the C-terminal end.

For transient transfections, CHO/sst2 cells were grown to 50–60% confluence in dishes of 100-mm diameter. Cells were washed with complete αMEM and transfected with 3–10 μg of the desired cDNAs using FuGENE 6 reagent as specified by the manufacturer. Transfected cells were then incubated for 24 h in complete medium before serum deprivation and treatment. Transient expression of proteins was confirmed 48 h post-transfection.
FIG. 1. Effect of RC-160 on ERK2, p38, and insulin-stimulated ERK2 activation in CHO/sst2 cells. A, serum-starved CHO/sst2 cells were treated for the indicated times with 1 nM RC-160. Soluble lysates were then subjected to ERK assay as described in “Experimental Procedures,” and radiolabeled MBP was analyzed using a PhosphorImager. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. B,
by immunoblot analysis as described below. Cells transfected with the appropriate empty vectors were used as control. Transfection efficiencies were determined by transfection of a plasmid encoding green fluorescent protein in CHO cells. Expression was detected by fluorescence microscopy (image-analysis system Visio-Lab 2000, Biocom) and was observed in ~34% of cells.

Immuno precipitation and Immunoblotting—Cells were rapidly washed with ice-cold phosphate-buffered saline and lysed on ice at 4 °C for 30 min in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM Na3P04, containing 1% Triton X-100, 20 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM/ml aprotinin, and 20 μM leupeptin). Soluble lysates were harvested by centrifugation at 13,000 rpm for 15 min at 4 °C. Protein content was measured using the Bradford method (Bio-Rad), and equal amounts of protein were either dissolved in 4× sample buffer (250 mM Tris-HCL, pH 6.8, 8% SDS, 20% β-mercaptoethanol, 50% glycerol, and bromphenol blue) and heated for 5 min at 95 °C for immunoblot analysis or submitted to immunoprecipitation.

Protein immunoprecipitation from cell lysates was performed using specific antibodies (1 μg/sample) pre-coupled to protein A-Sepharose beads. After incubation for 2 h at 4 °C, immunocomplexes were washed three times in HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, containing 0.2 mM sodium orthovanadate). The pellets were resuspended in HNTG buffer (in a total volume of 40 μl) and then either dissolved in 4× sample buffer for immunoblotting or utilized for kinase assays as described below. Immunocomplexes or total cell lysates were separated on SDS-PAGE followed by electrophoretic transfer onto nitrocellulose membranes (Pall Life Sciences). Membranes were then subjected to immunoblotting using horseradish peroxidase-conjugated secondary antibodies (Pierce) as described previously (6). Peroxidase activity was revealed using the enhanced chemiluminescence system (Pierce). Quantitative analyses were carried out by using a Biocom apparatus.

ERK and Raf Kinase Assays—Kinase activities were assayed using immunoprecipitated ERK2, Raf-1, or B-Raf, [γ-32P]ATP as a phosphate donor, and myelin basic protein (MBP) or a recombinant full-length MEK1 (Santa Cruz Biotechnology) as substrates. Kinase assays were performed at 25 °C under gentle shaking, by adding 5 μl of either 5× ERK buffer (50 mM magnesium acetate, 25 μM dithiothreitol (DTT), 25 μM ATP, 1 μCi of [γ-32P]ATP/sample and 7.5 μg of MBP/sample, in HNTG buffer) or 5× Raf kinase buffer (50 mM magnesium acetate, 500 μM DTT, 100 μM ATP, 5 μCi of [γ-32P]ATP/sample and 0.5 μg MEK1/sample, in HNTG buffer), to the 40 μl of immunoprecipitates. Reactions were stopped by the addition of 4× sample buffer and heating for 5 min at 95 °C. Radiolabeled MBP or MEK1 were separated from the immunocomplexes by 10% or 7.5% SDS-PAGE, transferred onto nitrocellulose membranes, and heated for 5 min at 95 °C for immunoblot analysis or submitted to immunoprecipitation.

For Ras and Rap1 Assays—Ras- and Rap1-dependent ERK2 activation was examined in CHO/sst2 cells (strain BL21(DE3)) and CHO cells (strain BL21(DE3)). raf1 and RalRD8-RBD GST fusion proteins were expressed in Escherichia coli (strain BL21(DE3)) by induction with 0.25 mM isopropyl-β-d-thiogalactopyranoside for 6 h at 30 °C. Expressed fusion proteins were affinity-purified on glutathione-agarose beads following bacterial lysis by sonication in TENGN buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 1% Nonidet-P-40, containing 1 μM DTT, 1 μg/ml aprotinin, and 1 mM PMSF). GST-Ras1/RBD-GST-RalGDS-RBD were then used to pull down activated Ras (Ras-GTP) or activated Rap1 (Rap1-GTP), respectively, using a protocol adapted from that of de Rooij and Bos (29). Briefly, cells were lysed in either Ras lysis buffer (50 mM Tris-HCl, pH 8.4, 150 mM MgCl2 containing 1% Triton X-100, 5 mM sodium deoxycholate, 2 μm sodium orthovanadate, 1 μm PMSF, 2 μg/ml aprotinin, and 20 μg/ml leupeptin) or Rap1 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM MgCl2, 5 mM EGTA, containing 1% Triton X-100, 0.25% sodium deoxycholate, 2 μm sodium orthovanadate, 1 μm PMSF, 2 μg/ml aprotinin, and 20 μg/ml leupeptin). Equal amounts of soluble lysates were mixed with 30 μg of GST fusion protein pre-coupled to agarose beads per sample. After incubation for 1 h at 4 °C, beads were pelleted, washed twice with Ras or Rap1 lysis buffer, resuspended in 4× sample buffer, and heated for 5 min at 95 °C. Precipitates were subjected to 12% SDS-PAGE followed by transfer onto nitrocellulose membranes. The presence of ERK2 and the specificity of immunoblot analysis using specific antibodies, as described above.

Statistical Analysis—Statistical analysis was performed using Student’s t test. p values < 0.05 indicate a statistically significant difference.

RESULTS

RC-160 Induces a Rapid and Transient Activation of ERK2 in CHO/sst2 Cells—We first examined the action of RC-160, a stable analogue of somatostatin, on ERK2 activity in CHO cells stably expressing sst2. These cells have been widely characterized and used as model systems for the study of somatostatin signaling via the sst2 receptor (4, 6). ERK2 activity was assessed by using an in vitro kinase assay, with MBP serving as a substrate, after immunoprecipitation of endogenous ERK2 from treated cells lysates. Treatment of CHO/sst2 cells with 1 nM RC-160 induced a time-dependent increase in ERK2 activity (Fig. 1A). An increment in ERK2 activity became apparent 3 min after RC-160 treatment, reached a maximum after 5 min (667 ± 119%), persisted up to 10 min, and rapidly decreased to basal levels thereafter. In contrast, RC-160 had no effect on ERK2 activity in wild-type CHO cells (data not shown). Previous reports demonstrated that sst2 and sst4 can also trigger a strong and sustained activation of p38 (30). To determine whether p38 activation was also affected by somatostatin in our cellular model, RC-160-treated CHO/sst2 cell lysates were subjected to immunoblot analysis using a polyclonal antibody specific to the phosphorylated, and hence activated, form of p38. As shown in Fig. 1B (left panel), RC-160 had no effect on p38 activation throughout the entire time course. The anti-phospho-p38 antibodies specificity was confirmed on total cell extracts from C-6 glioma cells treated or not with p38 activator anisomycin (Fig. 1B, right panel). These results support a model in which RC-160 induces sst2-mediated rapid and transient increase in ERK2 activity but does not influence p38 activation.

RC-160 Potentiates Insulin-stimulated ERK2 Activation in CHO/sst2 Cells—It is well known that insulin can induce ERK1/2 activation (31). Exposure of CHO/sst2 cells, which express endogenous insulin receptors, to 10 nM insulin actually stimulates ERK2 activity (data not shown). Nevertheless, although RC-160-mediated ERK2 activity was monophasic (Fig. 1A), insulin-induced ERK2 activity exhibited a biphasic pattern. A transient increase in ERK2 activity peaking at 5 min was observed (588 ± 54% of control, data not shown), but at later time points, ERK2 activity was still found to be significantly elevated after 4 h of insulin treatment (352 ± 69% of control, data not shown). We next examined the action of RC-160 on insulin-stimulated ERK2 activity. Incubation of CHO/sst2 cells with RC-160 or insulin alone for 5 min similarly

serum-starved CHO/sst2 cells were treated for the indicated times with 1 nM RC-160. Soluble lysates were then subjected to SDS-PAGE and analyzed by immunoblotting with anti-phospho-p38 (P-p38) antibodies. Control cell extracts from C-6 glioma cells treated (lane 2) or not (lane 1) by anisomycin were used to assess anti-phospho-p38 antibodies efficiency. Immunoblotting confirming equivalent protein loading was performed using anti-α-tubulin antibodies (28). Western blots were performed using antibodies to activated ERK1 and ERK2 (1:10,000). The specificity of the anti-phospho-ERK1/2 antibodies was confirmed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting.
increased ERK2 activity (5-fold) (Fig. 1C). Interestingly, the level of ERK2 activity induced by RC-160 in the presence of insulin was greater than the sum of that obtained with each peptide alone (13-fold). However, RC-160 did not affect insulin-mediated increase in ERK2 activity after a 3-h co-treatment (data not shown). Similar results were obtained using a monoclonal antibody specific to active phosphorylated ERK1 and ERK2 isoforms (Fig. 1D), thus validating the use of this antibody to monitor ERK2 activity. Furthermore, a pretreatment of CHO/sst2 cells with the MEK1/2-specific inhibitor PD98059 (50 μM) completely abolished RC-160-induced ERK2 activation (Fig. 1E) as well as potentiation of insulin-stimulated ERK2 activation (data not shown), indicating that RC-160 effects are MEK1/2-dependent.

**RC-160 Activates ERK1/2 via sst2 in Mouse Pancreatic Acini**—To further support the physiological role of sst2 in RC-160-induced ERK1/2 activation, experiments were performed using mouse pancreatic acini, which express a high level of endogenous sst2 receptors (32). As shown in Fig. 2A, incubation of pancreatic acini with 1 nM RC-160 induced a rapid and transient ERK1/2 phosphorylation, reaching maximum levels between 3 and 10 min, and then gradually diminished. As expected, RC-160-mediated ERK1/2 phosphorylation was completely inhibited upon preincubation with the MEK1/2 inhibitor PD98059 (Fig. 2B). To confirm that RC-160-mediated ERK1/2 activation observed in this model was sst2-dependent, pancreatic acini were prepared from sst2 KO mice and treated with RC-160 for 3 min. As observed in Fig. 2C, RC-160 had no effect on ERK1/2 activity in pancreatic acini derived from sst2 KO mice, thus demonstrating that RC-160-mediated ERK1/2 phosphorylation observed in pancreatic acini isolated from sst2 WT mice is specifically triggered by sst2. Moreover, these results indicate that sst2-mediated ERK1/2 phosphorylation is physiologically relevant.

**RC-160-mediated CHO/sst2 Cell Growth Inhibition and p27Kip1 Up-regulation Depend on ERK1/2 Activation**—We previously reported that RC-160 inhibited the mitogenic effect of insulin in CHO/sst2 cells (4, 33). To investigate whether RC-160-mediated ERK1/2 activation is implicated in this effect, CHO/sst2 cells were pretreated or not with PD98059 (20 μM) before treatment with 10 nM insulin in the presence or absence of 1 nM RC-160 for 24 h. Exposure of cells to the MEK1/2 inhibitor abrogated RC-160 inhibitory effect on insulin-mediated cell proliferation, whereas exposure to vehicle did not, indicating that suppression of the insulin mitogenic effect by RC-160 involves ERK1/2 activation. In addition, PD98059 affected neither basal cell proliferation nor mitogenic effect of insulin (Fig. 3A).

We previously demonstrated that sst2 activation blocks insulin-induced S-phase entry through p27Kip1 induction (6). To
investigate the potential involvement of ERK1/2 activation in sst2-mediated up-regulation of p27Kip1, CHO/sst2 cells were pretreated or not with PD98059 (50 \textmu M) prior to a 3-h treatment with 10 nM insulin in the presence or absence of 1 nM RC-160. p27Kip1 protein expression was measured by immunoblotting using specific antibodies. In agreement with our previous results, RC-160 increased the level of p27Kip1, an effect completely reversed when cells were preincubated with PD98059 (Fig. 3B). This result indicates that ERK1/2 activation contributes to sst2-mediated G1 cell cycle arrest. Taken together, these results clearly demonstrate that ERK1/2 activation plays a critical role in the negative growth signal promoted by sst2 activation.

RC-160-mediated ERK2 Activation in CHO/sst2 Cells Requires B-Raf—To further investigate the signaling pathway by which RC-160 stimulates ERK2 activity, we determined
FIG. 4. Involvement of Raf-1 and B-Raf in RC-160-mediated ERK2 activation in CHO/sst2 cells. A and B, serum-starved CHO/sst2 cells were treated for the indicated times with 1 nM RC-160. Soluble lysates were then submitted to either Raf-1 (A) or B-Raf (B) immunoprecipitation. Raf-1 and B-Raf kinase assays were performed using MEK1 as a substrate as described under “Experimental Procedures” and radiolabeled MEK1 was analyzed using a PhosphorImager. Equal loading of immunoprecipitated Raf-1 or B-Raf was confirmed by immunoblotting. The diagrams represent densitometric quantifications of the bands, and results are expressed as percentage of control values obtained from untreated cells. Values are mean ± S.E. of three separate experiments. (Statistical comparison between RC-160-treated and untreated cells; *, p < 0.05.) C, CHO/sst2 cells were transfected with empty vector (10 μg; vector), co-transfected with empty vector (2 μg) and HA-NRaf-1 (8 μg), co-transfected with V12Ras (2 μg) and empty vector (8 μg) or co-transfected with V12Ras (2 μg) and HA-NRaf-1 (8 μg), serum-starved, and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. Immunoblots of the same lysates were carried.
whether Raf-1 and/or B-Raf was responsible for ERK2 phosphorylation in response to RC-160. CHO/sst2 cells were treated with 1 nM RC-160 for periods ranging from 1 to 5 min. Raf-1 and B-Raf kinase activities were then measured by immune complex kinase assays, using MEK1 as a substrate. Raf-1 activity was not affected by RC-160 (Fig. 4A). In contrast, RC-160 induced a rapid and transient increase in B-Raf activity, which reached its maximum (2-fold) at 2–3 min (Fig. 4B). Western blot analysis of cell homogenates indicated that both Raf-1 and B-Raf are expressed in CHO/sst2 cells. To further confirm that RC-160-stimulated ERK2 activation was independent of Raf-1 activation, the capacity of Raf-1 (NRaf-1) and B-Raf (NB-Raf) dominant negative mutants to modulate ERK2 activation was examined. HA-tagged versions of NRaf-1 or NB-Raf were transiently transfected, and ERK2 phosphorylation was evaluated after exposure of CHO/sst2 cells to RC-160 for 5 min. Exogenous expression of NRaf-1 had no effect on RC-160-mediated ERK2 activation (Fig. 4C). However, expression of NB-Raf strongly inhibited RC-160-induced ERK2 phosphorylation (86 ± 12%) (Fig. 4, D and E). To control NRaf-1 efficiency, CHO/sst2 cells were cotransfected with NRaf-1 and a constitutively activated Ras mutant (V12Ras). As shown in Fig. 4C, expression of V12Ras could induce ERK2 activation in the absence of RC-160 and expression of NRaf-1 inhibited V12Ras-induced ERK2 phosphorylation, confirming that the NRaf-1 construct effectively behaves as a dominant negative mutant of Raf-1. Together, these results indicate that in CHO/sst2 cells, RC-160-induced ERK2 activation involves B-Raf but not Raf-1.

RC-160-mediated ERK2 Activation in CHO/sst2 Cells Requires Both Ras and Rap1—Raf family kinases are activated by members of the Ras-like superfamily of small monomeric GTP-binding proteins (12). Among them, although Ras has been shown to activate Raf-1 and B-Raf equally in many systems, Rap1 exhibits a more specific action toward B-Raf (17, 34, 35). We thus investigated whether Ras and/or Rap1 could be required in the signal triggered by sst2. We therefore measured Ras and Rap1 activation in response to RC-160 using pull-down assays based on the capacity of active GTP-loaded Ras or Rap1 to selectively interact with a GST-Raf-1-RBD or a GST-Ral-GDS-RBD fusion protein, respectively. Treatment of CHO/sst2 cells with 1 nM RC-160 resulted in a time-dependent increase in the amount of both Ras- and Rap1-GTP precipitated (Fig. 5, A and B). The activation of both GTPases was detected after 2 min of treatment, peaked at 5 min, and rapidly decreased to basal levels thereafter. In both cases, RC-160 stimulation resulted in a 5- to 6-fold increase over basal activity. These results suggest that both Ras and Rap1 may be involved in RC-160-mediated ERK2 activation. We further investigated this hypothesis by analyzing whether Ras and Rap1 could regulate ERK2 activation in CHO/sst2 cells. We investigated this hypothesis by analyzing whether transient transfection of dominant negative mutants of Ras (N17Ras) or Rap1 (N17Rap1) could modulate ERK2 phosphorylation in CHO/sst2 cells treated with RC-160 for 5 min. Expression of either N17Ras or N17Rap1 alone resulted in a partial inhibition of RC-160-mediated ERK2 activation (Fig. 5, C and D). However, when cotransfected, both mutants severely blocked ERK2 phosphorylation (Fig. 5E). Indeed, as shown in Fig. 5F, N17Ras or N17Rap1 inhibited RC-160-induced ERK2 phosphorylation by 59.9 ± 9.5% and 60.8 ± 8.8%, respectively, whereas a 93.1 ± 3.5% inhibition was observed following cotransfection. Altogether, these results demonstrate that sst2-mediated ERK2 activation is dependent on both Ras and Rap1 activation and that Ras and Rap1 cooperate to fully activate ERK2.

RC-160-mediated ERK2 Activation in CHO/sst2 Cells Involves a PTX-sensitive G Protein and Src—We and others have previously demonstrated that in CHO/sst2 cells, sst2 is coupled to Gαq and Gα12, PTX-sensitive G proteins (36, 37). To determine whether a Gαq protein is involved in RC-160-induced ERK2 activation, CHO/sst2 cells were pretreated or not with PTX (100 ng/ml) for 18 h and then treated with 1 nM RC-160 for 5 min. ADP-ribosylation of Gαq by PTX partially inhibited RC-160-induced ERK2 phosphorylation (35.4 ± 6.9%) (Fig. 6, A and D). These results argue in favor of a role for Gαq protein in RC-160-mediated ERK2 activation. Because other Gαq protein-coupled receptors have been shown to activate ERK2 through Src-like kinases (38), we therefore investigated the involvement of Src family kinases in RC-160-induced ERK2 signaling. Preincubation of CHO/sst2 cells with the Src-like kinases inhibitor PP2 (10 μM) for 30 min partially inhibited RC-160-induced ERK2 phosphorylation (Fig. 6B). Among the Src family kinases, several studies have documented the requirement of Src in Gαq protein-coupled receptor-mediated activation of ERK1/2 (38). To establish whether Src is actually implicated in RC-160-mediated ERK2 activation, CHO/sst2 cells were transiently transfected with a dominant negative mutant (Src(K/R)) before treatment with RC-160 for 5 min. As shown in Fig. 6C, expression of Src(K/R) resulted in a partial inhibition of RC-160-induced ERK2 phosphorylation. This inhibition was similar to that induced by PP2 pretreatment (61.8 ± 10.3% and 64.5 ± 7%, respectively; Fig. 6D), strongly arguing in favor of a role for Src in mediating sst2-dependent ERK2 activation. This result is consistent with recent studies from our group demonstrating that, in CHO/sst2 cells, Src is the unique member of the Src family that communoprecipitates with sst2 (39).

RC-160-mediated ERK2, Ras, and Rap1 Activation Involves PI3K in CHO/sst2 Cells—Many studies have shown a requirement of PI3K activity for ERK2 activation by GPCRs (40). To investigate whether PI3K is an upstream component of the sst2 pathway, we analyzed the effect of two different PI3K-specific inhibitors, LY294002 and wortmannin, on RC-160-induced ERK2 activation. Pretreatment of CHO/sst2 cells with LY294002 (25 μM) or wortmannin (100 nM) for 30 min partially decreased RC-160-mediated ERK2 phosphorylation (Fig. 7A). Both compounds similarly inhibited ERK2 activation (61 ± 11.7% and 59.1 ± 5.4%, respectively). These data suggest that PI3K is involved in RC-160-mediated ERK2 activation. It has been reported that PI3K interacts with GTP-bound Ras, which can act both as an effector and as a regulator of PI3K. Furthermore, other studies have suggested that Rap1 activation requires PI3K (40, 41). This prompted us to investigate the potential role of PI3K in RC-160-induced Ras and Rap1 activation in CHO/sst2 cells. Inhibition of PI3K activity by LY294002 completely blocked RC-160-mediated Ras activation (Fig. 7B). In addition, the PI3K inhibitor blocked the ability of RC-160 to activate Rap1 (Fig. 7C). These results indicate that in CHO/
Involvement of Ras and Rap1 in RC-160-mediated ERK2 activation in CHO/sst2 cells. A and B, serum-starved CHO/sst2 cells were treated for the indicated times with 1 nM RC-160. Equal amounts of soluble lysates were then submitted to either Ras (A) or Rap1 (B) activation assay using GST fusion proteins as described under “Experimental Procedures.” Activated Ras (Ras-GTP) or Rap1 (Rap1-GTP) were detected by immunoblotting using anti-Ras or anti-Rap1 antibodies. Equal fractions of the same lysates were subjected to direct immunoblotting using anti-Ras or anti-Rap1 antibodies to visualize total Ras or Rap1 level.

C and D, CHO/sst2 cells were transfected with empty vector (vector) or N17Ras (5 μg) (C) and with empty vector (vector) or N17Rap1 (5 μg) (D), serum-starved, and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. Immunoblots of the same lysates were carried out using anti-Ras or anti-Rap1 antibodies to confirm the expression of transfected proteins.

E, CHO/sst2 cells were transfected with empty vector (6 μg; vector), co-transfected with empty vector (3 μg) and N17Ras (3 μg) or N17Rap1 (3 μg), or co-transfected with N17Ras and N17Rap1, serum-starved and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies.

F, quantitative analysis of ERK2 phosphorylation. The percentage of ERK2 phosphorylation compared to control (vector) was measured by densitometric analysis of the immunoblots. Statistical analysis was performed using a one-way ANOVA followed by a Bonferroni post hoc test. *p < 0.05 compared to control (vector).
sšt2 cells, both RC-160-mediated Ras and Rap1 activation require upstream PI3K activity.

**RC-160-mediated ERK2 Activation in CHO/sšt2 Cells Involves Both SHP-1 and SHP-2 Activities**—In various mammalian cell lines, including CHO/sšt2 cells, sšt2 mediates inhibitory effect on cell growth through a rapid stimulation of the SH2-containing tyrosine phosphatase SHP-1 (5, 37). Therefore, to address whether RC-160 mediates ERK2 activation through SHP-1, CHO/sšt2 cells were transiently transfected with a catalytically inactive mutant of SHP-1 (SHP-1(C/S)), and ERK2 phosphorylation was monitored after 5 min of RC-160 treatment. Expression of SHP-1(C/S) resulted in a 47.7 ± 14.8% inhibition of RC-160-mediated ERK2 activation (Fig. 8, A and D). To further validate the involvement of SHP-1, we next compared the effect of RC-160 on ERK1/2 activation in pancreatic acini isolated from normal or mev mice, which express a nearly inactive SHP-1 mutant (27). Both normal and mev mouse pancreatic acini express endogenous sšt2 at similar levels (32). In mev pancreatic acini incubated with 1 nM RC-160 for 3 min, ERK1/2 phosphorylation was, however, decreased by 50% as compared with normal acini (Fig. 8B). Altogether, these results suggest that SHP-1 is a component of RC-160-mediated ERK2 activation in both CHO/sšt2 cells and mouse pancreatic acini.

SHP-2, another member of the SH2-containing tyrosine phosphatase family, is also known to play a critical role in ERK1/2 stimulation following growth factor receptor or GPCR activation (43, 44). To determine whether SHP-2 contributes to sšt2-dependent signaling to ERK2, CHO/sšt2 cells were transiently transfected with a c-Myc-tagged, catalytically inactive mutant of SHP-2 (SHP-2(C/S)), and ERK2 activation was evaluated after exposure to RC-160 for 0.5 min. Expression of SHP-2(C/S) strongly inhibited (88.4 ± 3.1%) RC-160-mediated ERK2 activation (Fig. 8, C and D). Thus, it appears that in CHO/sšt2 cells, RC-160-mediated ERK2 activation is also dependent on SHP-2 activity. This result is consistent with our recent data demonstrating that, in CHO/sšt2 cells, RC-160 stimulates SHP-2 phosphatase activity, this effect being involved in RC-160-mediated cell growth inhibition (39).

**RC-160-mediated Ras and Rap1 Activation in CHO/sšt2 Cells Requires Both SHP-1 and SHP-2 Activities**—We next examined the potential role of SHP-1 and/or SHP-2 in RC-160-induced Ras and Rap1 activation. Ras and Rap1 activation was monitored in CHO/sšt2 cells transiently transfected with SHP-1 or SHP-2 mutants. Expression of either SHP-1(C/S) or SHP-2(C/S) mutant prevented the effect of RC-160 on Ras activation (Fig. 8E). Likewise, RC-160-induced Rap1 activation was abrogated following expression of SHP-1(C/S) as well as SHP-2(C/S) (Fig. 8F). These results indicate that in CHO/sšt2 cells activation of both SHP-1 and SHP-2 is required for RC-160-mediated Ras and Rap1 activation and may constitute an early event upstream of both GTPases and ERK2 activation.

**DISCUSSION**

This study shows that, in CHO/sšt2 cells, GPCR sšt2 somatostatin receptor can inhibit insulin-mediated cell proliferation by a mechanism dependent on ERK1/2 activation, which leads to CKI p27Kip1 induction. The somatostatin analogue, RC-160, rapidly and transiently activates ERK2 through the coordinated stimulation of several signaling molecules. We show that sšt2-dependent activation of ERK2 requires PTX-sensitive Gij protein, Src, and the transient activation of the two small GTPases Rap1 and Ras, and that of the MAPK kinase B-Raf, but not Rap-1. Furthermore, sšt2 mediates Ras- and Rap1-GTP loading through a mechanism dependent on PI3K and both SHP-1 and SHP-2 tyrosine phosphatases (Fig. 9).

The present data indicate that activation of sšt2 transiently stimulates basal ERK2 activity and potentiates insulin-stimulated ERK2 activity, leading to accumulation of p27Kip1 and inhibition of insulin-induced cell proliferation. Support for the role of a marked ERK2 activation in sšt2-mediated p27Kip1 induction and inhibition of insulin-mediated cell proliferation comes from the observation that both RC-160 effects are inhibited by the MEK1/2 inhibitor PD98059. Interestingly, blocking the MEK1/2-ERK1/2 pathway with PD98059 prior to insulin treatment did not inhibit CHO/sšt2 cell proliferation, suggesting that, in contrast to that observed with other growth factors, e.g. basic fibroblast growth factor (25), insulin-induced CHO cell proliferation requires activation of other signals than ERK1/2 pathway. An ERK1/2-independent mitogenic effect of insulin has also been observed in rat hepatic stellate cells and in MBA-15.4 mouse and MG-63 human preosteoblasts (45, 46). Because RC-160-mediated p27Kip1 induction and antiproliferative effects occurred only when cells where co-treated with both insulin and RC-160, we suggest that the cellular decision to induce p27Kip1 and consequent G1 cell cycle arrest is dictated by the cumulative effect of both agents on ERK1/2 signal. We also detect a RC-160-mediated transient MEK1/2-dependent ERK1/2 activation, in normal mouse pancreatic acini, a more integrated model in which we have previously observed that RC-160 induces p27Kip1 accumulation (6). Mouse pancreatic acini express endogenous sšt2, and the lack of RC-160 effect on ERK1/2 activity in pancreatic acini derived from sšt2 KO mice argues in favor of the specific role of sšt2 in ERK1/2 activation. This further strengthens the physiological relevance of our results obtained in CHO/sšt2 cells. Such a peptide-induced stimulation of ERK1/2 correlated with inhibition of growth factor- or serum-mediated cell proliferation has been observed for other GPCRs, including sst1 somatostatin, α1B adrenergic, and B1 bradykinin receptors (47–49). As for sšt2, activation of these receptors enhances or potentiates growth factor-mediated ERK1/2 stimulation and increases the expression of p27Kip1 CKIs, suggesting that the strength of ERK1/2 activation can determine their effect on CKI expression and cell proliferation. A role for ERK1/2 activation in p27Kip1 induction has been observed for other agents inducing cell growth arrest, including phorbol 12-myristate 13-acetate, oncostatin, and transforming growth factor-β (50–52). However, the molecular mechanisms involved are poorly understood. p27Kip1 is regulated at the level of transcription, translation, sequestration, nuclear localization, and proteolysis (53–55). Our preliminary results do not argue in favor of a role for sšt2 at the level of p27Kip1 mRNA. It is possible that RC-160-mediated p27Kip1 induction results, at least in part, from ERK1/2-mediated inhibition of p27Kip1 degradation as observed in hepatoma cells activated by oncostatin (51). However, other post-transcriptional mechanisms cannot be excluded.

Besides the ERK1/2 pathway, activation of p38 has been reported to participate in cell growth arrest or differentiation (30, 56). However, we show that p38 is not phosphorylated following sšt2 activation whatever the time of treatment, pre-

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C. Bousquet, personal communication.
FIG. 6. Involvement of G_{ia} protein and Src in RC-160-mediated ERK2 activation in CHO/sst2 cells. A and B, serum-starved CHO/sst2 cells were pretreated for 18 h with vehicle alone or with 100 ng/ml PTX (A), or pretreated for 30 min with vehicle alone or with 10 μM PP2 (B) and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. C, CHO/sst2 cells were transfected with empty vector (vector) or Src(K/R) (5 μg), serum-starved and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. Immunoblot of the same lysates was carried out using anti-Src antibodies to confirm the expression of transfected proteins. D, P-ERK2 immunoblots from A, B, and C were densitometrically quantified and results are expressed as percentage of control values obtained from RC-160-treated cells pretreated with vehicle alone or transfected with empty vector, which were taken as 100% (open bar). Values are mean ± S.E. of three separate experiments. (Statistical comparison between RC-160-treated cells pretreated with PTX or with PP2 or transfected with Src(K/R), and cells pretreated with vehicle alone or transfected with empty vector; *, p < 0.02; **, p < 0.01.)
including the implication of this pathway in sst2-mediated p27^Kip1 induction and cell growth inhibition in CHO/sst2 cells.

The mechanism by which the ERK1/2 pathway can lead to cell cycle arrest is not completely understood. In many normal and tumor cells, expression of activated Ras or its downstream effector Raf-1 can elicit ERK1/2 activation and growth arrest (57–59). More recently, another Ras family member, Rap1, and its downstream effector B-Raf, have also been shown to be involved in ERK1/2 activation and consequent cell growth arrest and/or differentiation through a Ras-independent mechanism (18, 60). However, in other cell lines, B-Raf is the only Raf kinase involved in the Ras-mediated activation of ERK1/2 (28, 61). Ras and Rap1 can also act in concert to modulate ERK1/2 signal duration and to induce cell differentiation, transient Ras, and sustained Rap1 activation eliciting transient and sustained activation of ERK1/2, respectively (19, 34, 41, 62).
FIG. 8. Involvement of SHP-1 and SHP-2 in sst2-mediated ERK2 activation. A and C, CHO/sst2 cells were transfected with empty vector (vector) or SHP-1(C/S) (5 μg) (A), or transfected with empty vector (vector), or c-Myc-SHP-2(C/S) (5 μg) (C), serum-starved and treated or not for 5 min with 1 nM RC-160. Soluble lysates were then submitted to ERK2 immunoprecipitation followed by immunoblotting with anti-phospho-ERK1/2 (P-ERK2) antibodies. Equal loading of immunoprecipitated ERK2 was confirmed by immunoblotting. Immunoblot of the same lysates was carried out using anti-SHP-1 or anti-c-Myc antibodies to confirm the expression of transfected proteins. B, pancreatic acini were isolated from normal mice (normal) or from mev/mev mice (mev) as indicated under “Experimental Procedures” and incubated in oxygenated Krebs-Ringer buffer in the presence or not of 1 nM RC-160 for 3 min. Acini were solubilized, and extracts were subjected to SDS-PAGE and analyzed by immunoblotting with anti-phospho-ERK1/2 (P-ERK1/2) antibodies. Immunoblotting confirming equivalent protein loading was performed using anti-ERK2 antibodies. D, P-ERK2 immunoblots from A and C were densitometrically quantified, and results are expressed as percentage of control values obtained from RC-160-treated cells transfected with empty vector, which were taken as 100% (open bar). Values are mean ± S.E. of five separate experiments. (Statistical comparison between RC-160-treated cells transfected with SHP-1(C/S) or c-Myc-SHP-2(C/S) and empty vector; *, p < 0.01; **, p < 0.001.) E and F, CHO/sst2 cells were transfected with empty vector (vector), SHP-1(C/S) (5 μg), or c-Myc-SHP-2(C/S) (5 μg), serum-starved and treated or not for 5 min with 1 nM RC-160. Equal amounts of soluble lysates were then submitted to either Ras (E) or Rap1 (F) activation assay.
Using dominant negative mutants, we have identified B-Raf as the only Raf kinase activated by sst2 and involved in ERK1/2 activation. Furthermore, the striking resemblance in the kinetics of Ras, Rap1, and B-Raf activation suggests that both small GTP-binding proteins act together upstream of B-Raf to activate ERK2. Experiments showing that RC-160-mediated ERK2 activation is only partially prevented by either dominant negative mutants of Ras or Rap1, but is totally precluded when both mutants are expressed, further support this hypothesis.

Our results highlight the concept that coordinated transient Rap1 and Ras activation is essential and sufficient for B-Raf-dependent transient activation of ERK2 in response to activation of sst2. The ability of $G_{i\alpha}$-coupled receptors to activate ERK1/2 pathway is well documented, ERK1 being activated by $G_{i\alpha}$ and $G_{b\gamma}$-dependent pathways (63, 64). Here, the involvement of $G_{i\alpha}$ protein in sst2-mediated ERK2 activation is based on the sensitivity of RC-160-mediated ERK2 activation to PTX. This is in agreement with the PTX-induced abrogation of sst2-mediated signaling we previously observed in CHO/sst2 cells (37). In addition, we present evidence that Src kinase is involved in sst2-mediated ERK2 activation. Activation of Src family kinases is known to play a role in GPCR-mediated Ras- or Rap1-dependent activation of ERK1/2 pathway (65, 66). Several mechanisms have been shown to mediate Src activation by GPCRs such as a direct interaction with $G_{b\gamma}$ subunits and subsequent activation of Src via the binding of $\beta$-arrestin to its SH3 and catalytic domains (67). $\beta$-Arrestin has been shown to be recruited by activated sst2 in CHO cells stably expressing sst2 (68). Furthermore, we recently demonstrated that, in CHO/sst2 cells, Src is associated with sst2 in resting cells and that activated sst2 stimulates Src activity by a $G_{b\gamma}$-dependent mechanism (39). Thus, one possibility is that sst2-induced $G_{b\gamma}$-dependent Src activation mediates ERK2 activation.

Besides tyrosine kinases, PI3K has been shown to act as an early intermediate of ERK1/2 activation in response to GPCRs, PI3K being positioned upstream or downstream of Ras (40). CI class I PI3Ks can contribute to GPCR-mediated ERK1/2 ac-
activation upstream from Ras by a mechanism involving Gi/o-coupled receptors, have been identified, but C3G and CalDAG-GEF, which presumably mediate activation of GTPase-activating proteins (GAP). Many GEF, including those activated by a molecular switch between GDP- to GTP loading as well as ERK2 activation. The role of SHP-1 in sst2-mediated ERK2 activation is strengthened by results obtained in pancreatic acini derived from m\textsuperscript{o} mice, which express an inactive SHP-1, where RC-160-mediated ERK1/2 activation is highly attenuated. We recently reported that sst2 activates SHP-1 and SHP-2 and that both tyrosine phosphatases control sst2-mediated growth inhibition (37, 39). SHP-2 is known to be required for ERK1/2 activation by GPCR but the mechanisms involved are not completely elucidated (21, 69, 72, 73). An SHP2-dependent pathway involving Gab1 and PI3K was recently identified as essential for Ras activation under lysophosphatidic acid stimulation (44, 69). There is little evidence for a participation of SHP-2 in Ras activation under lysophosphatidic acid stimulation (44, 69). There is little evidence for a participation of SHP-2 in Rap1 activation. To our knowledge, a link between SHP-2 and Rap1 has never been reported for SHP-2, SHP-1 acts mainly as a negative regulator of Ras activation upstream from Ras by a mechanism involving G

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