Somatostatin regulates multiple biological functions by acting through a family of five G protein-coupled receptors, somatostatin receptors (SSTRs) 1–5. Although all five receptor subtypes inhibit adenylate cyclase activity and decrease intracellular cAMP levels, specific receptor subtypes also couple to additional signaling pathways. In CCL39 fibroblasts expressing either human SSTR1 or SSTR2, we demonstrate that activation of SSTR1 (but not SSTR2) attenuated both thrombin- and integrin-stimulated Rho-GTP complex formation. The reduction in Rho-GTP formation in the presence of somatostatin was associated with decreased translocation of Rho and LIM kinase to the plasma membrane and fewer focal contacts. Activation of Rho resulted in the formation of intracellular actin stress fibers and cell migration. In CCL39-R1 cells, somatostatin treatment prevented actin stress fiber assembly and attenuated thrombin-stimulated cell migration through Transwell membranes to basal levels. To show that native SSTR1 shares the ability to inhibit Rho activation, we demonstrated that somatostatin treatment of human umbilical vein endothelial cells attenuated thrombin-stimulated Rho-GTP accumulation. These data show for the first time that a G protein-coupled receptor, SSTR1, inhibits the activation of Rho, the assembly of focal adhesions and actin stress fibers, and cell migration.

The low molecular mass GTPase Rho plays a central role in regulating organization of the actin-based cytoskeleton in mammalian cells. Activated, GTP-bound Rho promotes the formation of contractile actin filaments into stress fibers and the assembly of cell adhesion complexes (1, 2). Through its coordinate regulation of actin filaments, contractility, and cell adhesion, Rho also plays a critical role in cell migration (3, 4) and in tumor invasion (5, 6). Rho is activated by transmembrane receptors, including the integrin family of adhesion receptors (7) and a subset of heptahelical G protein-coupled receptors (GPCRs) (8). Although the signaling pathway linking integrin receptors to Rho has not been determined, GPCRs, including those for lysophosphatidic acid (9–11) and thrombin (11–13), activate Rho through the heterotrimeric GTPases G12 and G13. However, GPCRs linked to the inhibition of Rho and downstream cytoskeletal reorganization have not been identified.

We now report that somatostatin (SST), acting at the GPCR subtype SSTR1, inhibits Rho activity, attenuates the assembly of actin stress fibers and focal adhesions, and inhibits cell migration. Five distinct SSTR subtypes that are activated by SST have been identified, and these receptors generally have potent inhibitory effects on diverse cell functions such as hormone secretion, neurotransmitter release, smooth muscle contractility, and cell proliferation (14, 15). Effector pathways regulated by SSTRs, including inhibition of adenylate cyclase and Ca2+ channel activity and stimulation of K+ channel and phosphatase activity, are mediated by pertussis toxin (PTX)-sensitive mechanisms, most likely involving GTPases of the Go family (15–17).

The rationale for studying SST effects on Rho and the cytoskeleton was based on our previous studies with the Na+/H+ exchanger NHE1. NHE1 acts downstream of Rho to play a critical role in regulating cytoskeletal organization (18). NHE1 is phosphorylated directly by the Rho-associated kinase ROCK (19), and activation of NHE1 by GPCRs, such as those for lysophosphatidic acid and thrombin, and by integrin receptors is mediated by Rho and ROCK (19, 20). Moreover, through its direct association with the ERM (ezrin/radixin/mo esiin) family of actin-binding proteins, NHE1 acts as a plasma membrane anchor for actin filaments to control the assembly of cortical stress fibers and focal adhesions (21). In contrast to lysophosphatidic acid and thrombin, NHE1 activation is regulated by SSTR1 (but not SSTR2) subtype, inhibits NHE1 activity (16). We reasoned that inhibition of NHE1 by SSTR1 might be associated with an inhibition of Rho.

Consistent with this rationale, the findings from this study indicate that SSTR1 (but not SSTR2) stably expressed in fibroblasts inhibits Rho activation, cytoskeletal reorganization, and cell migration by thrombin. We previously reported that in human umbilical vein endothelial cells (HUVECs), which express endogenous SSTR1, but not SSTR2, SST attenuates stress fiber assembly by serum (22), and we have now determined that this attenuation is associated with a decrease in activated, GTP-bound Rho. Moreover, in contrast to most effects of SSTRs that are abolished by PTX, SSTR1 inhibition of NHE1 (16) and, as we now report, Rho activity is PTX-insensitive.

EXPERIMENTAL PROCEDURES

Cell Culture—CCL39 hamster lung fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum (FBS). Human SSTR1 and SSTR2 were stably expressed in CCL39 cells using the inducible mammalian expression vector pCMV (16). Cells were cotransfected with pRSV-neo using calcium phosphate precipitation, and G418-resistant clones were selected and examined for their
ability to bind [125I-Tyr11]SST-14 and to mediate SST inhibition of cAMP accumulation, as previously described (16). To determine sensitivity to PTX, cells were pretreated with the toxin (List Biologicals) at 100 ng/ml for 18 h. Primary HUVECs (Clonetics, Walkersville, MD) were cultured in endothelial cell basal medium supplemented with 5% FBS, 1% antibiotic-antimycotic, 20 ng/ml basic fibroblast growth factor, 10 mM hoptanamin/aminopterin B, and 0.4% bovine brain extract (Clonetics).

Affinity Precipitation of Rho-GTP and Rac-GTP—The abundance of activated, GTP-bound Rho was determined by a modification of previously described methods (7, 9) using affinity adsorption with a GST fusion protein of the Rho-binding domain of the Rho-associated kinase ROCK (a gift from Dr. S. Naressed). The BL21 bacterial strain was transformed with this construct, and expression of the fusion protein was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. Bacteria were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM diethiothreitol, and 1% Triton X-100. Bacterial lysates were sonicated with four 15-s pulses and then cleared by centrifugation at 14,000 rpm for 10 min. A 40-μl aliquot of the supernatant was collected for determination of total cellular Rho. The remaining supernatant was added to 75 μg of GST fusion protein-coated beads and incubated for 1 h at 4°C; the beads were washed four times with wash buffer (Tris containing 0.5% FBS, 100 mM NaCl, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 mM orthovanadate). The lysates from three plates were pooled, aspirated three times through a 26-gauge needle, and then cleared by centrifugation at 13,000 × g for 3 min at 4°C. A 40-μl aliquot of the supernatant was collected for determination of total cellular Rac. The remaining supernatant was added to 75 μg of GST fusion protein-coated beads and incubated for 1 h at 4°C; the beads were washed four times with buffer (Tris with 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and proteins were separated by 12% SDS-PAGE. GTP-bound Rho and total cellular Rac were detected by Western blotting using a monoclonal antibody to Rac (Transduction Laboratories) and quantified as described for determining the percentage of GTP-bound Rho.

Immunocytochemistry—Cells grown on 22-mm glass coverslips were maintained for 24 h in 0.5% FBS and then treated with 30 nM thrombin in the absence or presence of 100 nM SST for 20 min. Cells were washed briefly with phosphate buffer and fixed in 2% paraformaldehyde at room temperature for 1 min. After thorough washing with PBS to remove excess fixative, cells were permeabilized with 0.1% Triton X-100; immersed in 5% normal calf serum; and incubated with primary antibodies, including those against paxillin (1:200 for 1 h at 23°C; Zymed Laboratories Inc., RhôA (1:250 for 4 h at 4°C; Transduction Laboratories), and LIMK (1:200 for 18 h at 4°C; Transduction Laboratories). The bound antibodies were detected using the relevant fluorescent isothiocyanate-conjugated secondary antibody (either goat anti-mouse or donkey anti-rabbit IgG, Jackson Immunoresearch Laboratories, Inc.) at a dilution of 1:500 for 1 h at room temperature. The coverslips were mounted on glass slides with Vectashield anti-fade mounting medium (Vector Labs, Inc.) and screened using a Zeiss Axiosph microscope (magnification ×1000). Representative images were collected using a Spot2 camera (Diagnostic Instruments, Inc.) and imported into Adobe Photoshop.

Cell Migration Assay—Migration of CCL39-R1 and CCL39-R2 cells was assessed using Transwell membranes (8-μm pore size and 6.5-mm diameter) in 24-well plates (Corning Costar Corp.). The cells were detached from the culture plates, and 200 μl of cell suspension at a density of 1 × 10⁵/ml in Dulbecco’s modified Eagle’s medium containing 1% FBS were placed in the top chamber of the filter. Thrombin (30 nM final concentration) was added to the top chamber in the absence or presence of 100 nM SST, and migration of cells through the Transwell membrane was compared with untreated cells. The plates were incubated at 37°C for 8 h, with agonists reapplied at 4 h. After 8 h, 2% paraformaldehyde was added to the top and bottom wells, and cells were fixed overnight. After washing with PBS, the cells in the upper well were removed by aspiration, and the nuclei of cells that had migrated through to the bottom surface of the filter were stained with 1 μg/ml 4,6-diamidino-2-phenylindole for 15 min at room temperature. To count migrated cells, the filter was cut out of the holder and placed bottom side up on a glass slide. Five fields at magnification ×40 were captured using a Spot2 camera, and the number of migrating cells was counted using NIH Image. The percentage of cells that had migrated through to the bottom surface of the filter was calculated using a formula: (number of cells in the bottom well/number of cells in the top well) × 100. The amount of migration was expressed as mean ± S.E., and statistical significance was assessed by Student’s unpaired t test, with p < 0.05 considered significant. After removal of the Transwell, the individual wells were checked to determine whether cells that had migrated through the filter, detached, and subsequently attached to the base of the 24-well plate.

Regulation of Tyrosine-phosphorylated p190RhoGAP and c-Src—Tyrosine phosphorylation of p190RhoGAP was determined in cell preparations similar to those described for detecting Rho-GTP. Following fibronectin plating or addition of secretagogues to adherent cells, cells were washed with PBS containing 1% orthovanadate, lysed with a 500 μl of lysis buffer (50 mM Tris (pH 7.4), 500 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM orthovanadate). The lysates from three plates were pooled, aspirated three times through a 26-gauge needle, and cleared by centrifugation at 13,000 × g for 3 min at 4°C. A 40-μl aliquot of the supernatant was collected for determination of total cellular Rho. The remaining supernatant was added to 75 μg of GST fusion protein-coated beads and incubated for 1 h at 4°C. The supernatant was collected using 100 μl of wash buffer (Tris containing 1% Nonidet P-40, 100 mM NaCl, 10 mM MgCl₂, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and proteins were separated by 12% SDS-PAGE. GTP-bound Rho and total cellular Rac were detected by Western blotting using a monoclonal antibody to Rac (Transduction Laboratories) and quantified as described for determining the percentage of GTP-bound Rho.

LIMK Translocation—The subcellular localization of Rho and LIMK was determined by immunostaining and immunoblotting. For immunoblotting, cells plated on 100-mm plates were maintained for 24 h in 0.5% FBS and then treated with thrombin (30 nM) in the absence or presence of SST (100 nM) for 20 min. Cells were washed twice with PBS; collected by scraping in containing 50 mM Heps, 135 mM NaCl, 3 mM KCl, and 3 mM EDTA (pH 7.5); and lysed by sonication. Immunoblotting was performed on NaRSSR1 Inhibits Rho
RESULTS

SSTR1 (but Not SSTR2) Inhibits Actin Stress Fiber Assembly and Rho Activation by Thrombin—Our initial studies investigated whether SST regulates stress fiber assembly in CCL39 fibroblasts stably expressing human SSTR1 (CCL39-R1) and human SSTR2 (CCL39-R2). Data are expressed as a percentage of forskolin (10 μM) stimulation. Also included are data of cAMP accumulation by forskolin (10 μM) plus SST (100 nM) with and without pretreatment with PTX (100 μg/ml) for 18 h. Forskolin-induced increases in cAMP accumulation were not significantly different in the absence or presence of PTX (data not shown). Expression of SSTR1 and SSTR2 was determined by radioligand binding of cell membranes with [125I]-Tyr11[SST-14 and are expressed as fmol/mg of protein. Data represent the means ± S.E. of three separate cell preparations for cAMP accumulation and the means of four separate membrane preparations for radioligand binding. B, shown are the results from phallolidin labeling of actin filaments in quiescent cells (Control) and in cells treated with thrombin in the absence and presence of SST. Images are representative of >80% of the total population of cells in four separate preparations.

In the presence of SST (100 nM), thrombin-induced stress fiber formation was unchanged in wild-type CCL39 cells and in CCL39 cells expressing SSTR2 (CCL39-R2), but was strikingly inhibited in CCL39-R1 cells expressing SSTR1 (CCL39-R1) (Fig. 1A). Actin stress fibers were absent in the cell body, and their abundance and size were markedly decreased in the cortex.

To determine whether SSTR1 attenuation of stress fiber assembly is associated with an inhibition of Rho activity, we measured the abundance of GTP-bound Rho by thrombin with and without pretreatment with PTX. In the absence of PTX, thrombin increased the abundance of GTP-bound Rho, with maximal stimulation occurring at 10–20 min. Activation decreased between 30 and 60 min, but remained higher than in control cells (data not shown). In the presence of SST (100 nM), maximal Rho activation by thrombin at 20 min was inhibited by 75% in CCL39-R1 cells, but was unchanged in wild-type CCL39 cells (Fig. 2A). In the presence of SST (100 nM), thrombin-induced stress fiber formation was unchanged in wild-type CCL39 cells and in CCL39-R2 cells, but was strikingly inhibited in CCL39-R1 cells expressing SSTR1 (CCL39-R1) (Fig. 1B). Actin stress fibers were absent in the cell body, and their abundance and size were markedly decreased in the cortex.

FIG. 2. SST inhibits activation of Rho by thrombin. The abundance of GTP-bound Rho complexed with the Rho-binding domain of ROCK and total Rho in post-nuclear supernatants was determined by immunoblotting, and the abundance of Rho-GTP is expressed as a percentage of total Rho immunoreactivity. A, the abundance of Rho-GTP and immunoblots of Rho-GTP and total Rho obtained from wild-type CCL39 cells and CCL39-R1 and CCL39-R2 cells. Data were obtained from quiescent cells (Control) and cells treated with thrombin (30 nM) for 20 min in the absence and presence of SST (100 nM) and are representative of five separate cell preparations. B, the abundance of Rho-GTP and immunoblots of Rho-GTP and total Rho obtained from control and ligand-treated CCL39-R1 cells pretreated with PTX (100 ng/ml) for 18 h. Data are representative of three separate cell preparations. C, the abundance of Rho-GTP and immunoblots of Rho-GTP and total Rho acquired from quiescent (Control) HUVECs and HUVECs treated with thrombin (30 nM) for 20 min in the absence or presence of SST (100 nM). Data are representative of three separate cell preparations.

FIG. 1. SST inhibits cAMP accumulation and actin stress fiber assembly. A, cAMP accumulation was determined in wild-type CCL39 cells and in CCL39-R1 and CCL39-R2. Data are expressed as a percentage of forskolin (10 μM) stimulation. Also included are data of cAMP accumulation by forskolin (10 μM) plus SST (100 nM) with and without pretreatment with PTX (100 ng/ml) for 18 h. Forskolin-induced increases in cAMP accumulation were not significantly different in the absence or presence of PTX (data not shown). Expression of SSTR1 and SSTR2 was determined by radioligand binding of cell membranes with [125I]-Tyr11[SST-14 and are expressed as fmol/mg of protein. Data represent the means ± S.E. of three separate cell preparations for cAMP accumulation, and the means of four separate membrane preparations for radioligand binding. B, shown are the results from phallolidin labeling of actin filaments in quiescent cells (Control) and in cells treated with thrombin in the absence and presence of SST. Images are representative of >80% of the total population of cells in four separate preparations.
absence of thrombin, SST had no effect on the abundance of Rho-GTP in all three cell types (data not shown), indicating that SSTR1 inhibits stimulated (but not basal) Rho activity.

Although most effector actions of SST are abolished by PTX, SST inhibition of NHE1 is PTX-insensitive (16, 24). Consistent with this finding, the inhibition of Rho activity and stress fiber assembly by SST was not blocked by preincubation with PTX (Fig. 2B), although the inhibition of cAMP accumulation was completely reversed (Fig. 1A). In PTX-treated cells, however, basal and thrombin-stimulated Rho-GTP levels were consistently less than in untreated cells, suggesting that PTX attenuates or blunts the activation of Rho.

To confirm that SSTR1 inhibition of Rho activity is not an artifact of receptor overexpression, we examined this response in HUVECs, which express endogenous SSTR1, but not SSTR2. In HUVECs, the activation of Rho by thrombin (30 nM) for 20 min was inhibited by 80% in the presence of SST (Fig. 2C).

Because activation of Rho is associated with its translocation from the cytoplasm to the plasma membrane, we determined whether changes in Rho translocation are associated with the SSTR1-mediated decrease in Rho-GTP. Immunoblotting for Rho indicated that thrombin increased the abundance of Rho in the P100 fraction by 2-fold in CCL39-R1 cells compared with quiescent (control) cells (Fig. 3A). In the presence of SST, this increase was inhibited by 92%. The serine/threonine kinase LIMK is activated downstream of Rho and ROCK, and this activation promotes the translocation of LIMK from the cytosol to the plasma membrane. Consistent with this redistribution by activated Rho, we found that thrombin and SST regulated the abundance of membrane-associated LIMK. Compared with quiescent CCL39-R1 cells, thrombin induced a 1.5-fold increase in the abundance of LIMK in the P100 fraction (Fig. 3B). In the presence of SST, the abundance of LIMK in the P100 fraction was 60% less than that of quiescent cells and 90% less that that in the presence of the thrombin. Because SST did not reduce the level of Rho-GTP below that observed in quiescent cells, additional Rho-independent signaling mechanisms likely contribute to SST inhibition of membrane-associated LIMK in control cells.

The caveat of using subcellular fractionation to determine protein localization is that the P100 fraction does not reveal which membranes are involved. We therefore used immunostaining to determine whether changes in the abundance of Rho and LIMK in the P100 fraction reflected changes in their localization at the plasma membrane. In thrombin-treated CCL39 and CCL39-R1 cells, the distribution of Rho immunoreactivity shifted from a predominant intracellular localization seen in quiescent cells to more marked staining at the cell membrane (Fig. 3C). Pretreatment with SST blocked this redistribution of Rho immunoreactivity in CCL39-R1 cells, but not in wild-type CCL39 cells, indicating that SSTR1 inhibition of Rho-GTP is also associated with an inhibition of its translocation to the plasma membrane. The distribution of LIMK immunoreactivity in quiescent CCL39-R1 cells revealed a perinuclear and diffuse cytoplasmic localization (Fig. 3D). In the presence of thrombin, there was no detectable perinuclear immunoreactivity, but instead diffuse cytoplasmic staining and punctate accumulations in plasma membrane protrusions. These findings suggest that LIMK immunoreactivity in the P100 fraction of immunoblots from quiescent cells primarily reflects association with intracellular membranes, but that in thrombin-treated cells, it reflects, in part, association with the plasma membrane. In the presence of SST, LIMK staining was predominantly perinuclear, with no detectable immunoreactivity at the plasma membrane.

**Fig. 3.** SST inhibits the membrane-associated abundance of Rho and LIMK immunoreactivity in response to thrombin. A and B, immunoblots of Rho and LIMK abundance, respectively, in post-nuclear supernatants (pNs) and in S100 and P100 fractions obtained from control CCL39-R1 cells and CCL39-R1 cells treated with thrombin (30 nM) in the absence and presence of SST (100 nM). Data represent one-tenth of the total post-nuclear supernatants, one-fourth of the S100 fraction, and one-half of the P100 fraction and were similar in three separate cell preparations. C and D, immunostaining for Rho and LIMK, respectively, in control CCL39-R1 cells and CCL39-R1 cells treated with thrombin (30 nM) in the absence and presence of SST (100 nM). Images are representative of >80% of the cells in three separate cell preparations.

**SSTR1 Inhibits Rho Activity and Focal Adhesion Assembly by Integrins**—In addition to its central role in mediating GPCR regulation of cytoskeletal reorganization, Rho is also activated by integrin receptors, and it regulates integrin-induced assembly of focal adhesions. CCL39 cells express α5β1 integrins, which can be activated by platelet the cells on fibronectin to promote the assembly of actin stress fibers and paxillin-rich focal adhesions (20). To determine whether SSTR1 also inhibits activation of Rho by integrins, we determined the effects of SST in CCL39-R1 and CCL39-R2 cells plated on fibronectin. Compared with control cells plated on poly-l-lysine, plating on fibronectin for 60 min induced an increase in Rho activity in both cell types. Although preincubating cells with SST for 5 min prior to plating had no effect on cell attachment (data not shown), it inhibited Rho activation by fibronectin in CCL39-R1 cells, but not in CCL39-R2 cells (Fig. 4A). Moreover, SST treatment had a dramatic effect on the assembly of focal adhesions and stress fibers by fibronectin in CCL39-R1 cells. In the absence of SST, immunostaining showed that the focal adhesion-associated protein paxillin was localized in densely packed bundles within peripheral focal adhesions, and phalloidin staining revealed densely packed actin filaments predomi-
nantly at the cortex (Fig. 4B). With SST treatment, however, paxillin immunostaining revealed smaller, punctate focal complexes, indicating impaired assembly of focal adhesions (Fig. 4B). Additionally, in the presence of SST, fibronectin-induced stress fiber formation was dramatically inhibited in both the cell body and cortex (Fig. 4B). Hence, SSTR1 inhibits the activation of Rho and cytoskeletal reorganization in response to both GPCR- and integrin-mediated signals.

**SSTR1 Inhibits Rho Activity Independently of Rac and Src Activity**—In addition to the stimulation of Rho and accumulation of stress fibers, activation of thrombin receptors is known to stimulate the migration of fibroblasts. To investigate the possibility that SST treatment inhibits thrombin-stimulated cell migration, a single-cell suspension of CCL39 and CCL39-R1 cells was added to the upper well of a Transwell chamber. Addition of thrombin (30 nM) to the upper well resulted in a significant increase in the number of both cell types migrating through the pores of the Transwell membranes (Fig. 5). Although the migration of control cells was less in CCL39 cells compared with CCL39-R1 cells, the percent increase with thrombin in both cell types was similar (50% increase in CCL39 cells and 46% increase in CCL39-R1 cells). Co-treating the cells with SST (100 nM) and thrombin had no effect on the movement of wild-type CCL39 cells through to the lower surface of the membrane, but it abolished thrombin-stimulated migration of CCL39-R1 cells ($p < 0.05; n = 4$) (Fig. 5).

**SSTR1 Inhibits Rho**

**FIG. 4.** SST inhibits activation of Rho and the assembly of focal adhesion in response to integrin activation. **A,** the abundance of GTP-bound Rho complexed with the Rho-binding domain of ROCK and total Rho in CCL39-R1 and CCL39-R2 cells was determined by immunoblotting, and the abundance of Rho-GTP is expressed as a percentage of total Rho immunoreactivity. Data were obtained from cells plated for 60 min on poly-L-lysine (PLL) (control) or fibronectin (FN) in the absence or presence of SST and are representative of three separate cell preparations. **B,** the abundance of actin filaments, determined by phalloidin labeling, and the abundance of focal adhesions, determined by paxillin staining, are shown for CCL39-R1 cells plated for 60 min on fibronectin in the absence and presence of SST. Images are representative of $>85\%$ of the cells observed in four separate preparations.

**FIG. 5.** SST inhibits cell migration in response to thrombin. In wild-type CCL39 cells and CCL39-R1 cells, thrombin (30 nM) added to the upper well stimulated migration of cells through to the lower surface of the Transwell membrane. Co-addition of SST (100 nM) to the upper well failed to affect the thrombin-induced migration of CCL39 cells, but reversed the migratory effect and reduced the number of cells on the lower membrane surface to below basal levels. Data represent the means $\pm$ S.E. of three separate cell preparations.
Phosphatase-2 (25 ng/ml) for 10 min in the absence and presence of protein growth factor-BB (25 ng/ml) for the indicated times and are representative of duplicate cell preparations. B, and C, the abundance of tyrosine-phosphorylated p190RhoGAP was determined in CCL39-R1 cells plated for the indicated times on poly-l-lysine (PL) or fibronectin (FN) in the absence or presence of SST or plated for 48 h and treated with thrombin (T; 30 ng/ml) for 20 min in the absence or presence of SST (S). p190RhoGAP was immunoprecipitated, and immune complexes were separated by 5% SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine antibody (4G10) and p190RhoGAP antibody (total p190RhoGAP). Data are representative of three separate cell preparations. D, GTP-bound Rho complexed with the Rho-binding domain of ROCK was determined in CCL39-R1 cells in the absence or presence of the c-Src inhibitor protein phosphatase-2 (PP2; 25 μg/ml; 10-min pretreatment). Data are shown for quiescent cells and cells treated with thrombin (30 ng/ml) for 20 min in the absence or presence of SST (100 ng/ml). Data are representative of duplicate cell preparations. E, total cell lysates were prepared from quiescent CCL39-R1 cells and CCL39-R1 cells treated with FBS (10%) for 10 min in the absence and presence of protein phosphatase-2 (25 μg/ml). Tyrosine-phosphorylated c-Src (PY-Src) and total c-Src were determined by immunoblotting.

**FIG. 6.** *SSTR1 does not regulate GTP binding to Rac or tyrosine phosphorylation of p190RhoGAP.* A, the abundance of GTP-bound Rac complexed with the GTPase-binding domain of PAK1 and total Rac in post-nuclear supernatants was determined in CCL39-R1 cells by immunoblotting. Data were obtained from quiescent cells (control (C)) and cells treated with SST (S; 100 ng/ml) and SSTR2, and SSTR5 or platelet-derived growth factor-BB (P; 25 ng/ml) for the indicated times and are representative of duplicate cell preparations. B, and C, the abundance of tyrosine-phosphorylated p190RhoGAP was determined in CCL39-R1 cells plated for the indicated times on poly-l-lysine (PL) or fibronectin (FN) in the absence or presence of SST or plated for 48 h and treated with thrombin (T; 30 ng/ml) for 20 min in the absence or presence of SST (S). p190RhoGAP was immunoprecipitated, and immune complexes were separated by 5% SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine antibody (4G10) and p190RhoGAP antibody (total p190RhoGAP). Data are representative of three separate cell preparations. D, GTP-bound Rho complexed with the Rho-binding domain of ROCK was determined in CCL39-R1 cells in the absence or presence of the c-Src inhibitor protein phosphatase-2 (PP2; 25 μg/ml; 10-min pretreatment). Data are shown for quiescent cells and cells treated with thrombin (30 ng/ml) for 20 min in the absence or presence of SST (100 ng/ml). Data are representative of duplicate cell preparations. E, total cell lysates were prepared from quiescent CCL39-R1 cells and CCL39-R1 cells treated with FBS (10%) for 10 min in the absence and presence of protein phosphatase-2 (25 μg/ml). Tyrosine-phosphorylated c-Src (PY-Src) and total c-Src were determined by immunoblotting.

fibronectin at both 20 and 60 min was not different (Fig. 6B). We also found no change in tyrosine phosphorylation of p190RhoGAP in 48-h adherent CCL39-R1 cells treated with thrombin in the absence and presence of SST for 20 min (Fig. 1C). At earlier time points of 5 and 10 min, there was also no effect of thrombin or SST on tyrosine phosphorylation of p190RhoGAP (data not shown). In neutrophils, however, activation of p190RhoGAP by β2 integrin has been shown to occur through an increased association of p190RhoGAP and p125RasGAP, independent of changes in tyrosine phosphorylation (28). Considering that SSTR1 might regulate p190RhoGAP activity independently of phosphorylation, we investigated upstream regulation by determining the effect of Src activity. In CCL39 cells, however, SST attenuation of Rho-GTP levels with thrombin was not different in the absence (68% decrease) or presence (72% decrease) of the Src inhibitor protein phosphatase-2 (Fig. 6D). To confirm that protein phosphatase-2 was effectively inhibiting Src activity, we determined that it completely abolished the increase in tyrosine phosphorylation of Src in response to serum (10%, 10 min) (Fig. 6E). Together, these data suggest that coupling of SSTR1 to the inhibition of Rho activity is not mediated by the activation of Rac or Src or by increased tyrosine phosphorylation of p190RhoGAP.

**DISCUSSION**

This study demonstrates for the first time that a GPCR, viz. SSTR1, inhibits activation of Rho and that this effect is correlated with a decrease in actin stress fiber assembly and cell migration. This effect was not shared by SSTR2, which is a member of a second subgroup of SSTRs. SSTRs have been subdivided based on sequence homology into two subgroups: SSTR1 and SSTR4; and SSTR2, SSTR3, and SSTR5. The major functions of the latter group are well known because they have higher affinities for most commonly available SST analogs, including octreotide, which is widely used in a number of clinical settings (29, 30). The functions of SSTR1 and SSTR4 remain unclear because, until recently (31–33), there were no receptor-specific analogs for members of this subgroup.

The SSTR family has widespread and overlapping cellular expression patterns, with many cell lineages expressing two or more subtypes. Most cells express SSTR2 and/or SSTR5 as well as SSTR1 and/or SSTR4, making it difficult to discriminate precisely which receptor is responsible for a given biological function without the aid of receptor-specific analogs. The consequence of multiple receptor subtype expression becomes particularly problematical when the formation of heterodimers between different SSTRs is taken into consideration (34, 35). To circumvent this problem we investigated receptor-specific signaling using CCL39 hamster lung fibroblasts stably expressing either human SSTR1 or SSTR2.

In CCL39 fibroblasts stimulated by thrombin, parallel arrays of actin stress fibers were seen throughout the cytoplasm, as noted previously in fibroblasts (11, 36) and endothelial cells (37, 38) and consistent with the activation of Rho (1). Although thrombin-stimulated endothelial cells undergo rounding (38), no change in the overall shape of the CCL39 cells was noted in the present experiments. Treatment of thrombin-stimulated cells with SST attenuated stress fiber formation in CCL39-R1 fibroblasts, but not in wild-type CCL39 or CCL39-R2 fibroblasts. This is consistent with our previous finding that SST decreases the abundance and size of actin stress fibers in HUVECs expressing endogenous SSTR1 (22).

The signaling mechanism whereby SSTR1 inhibits Rho activity remains to be determined. Down-regulation of Rho activity has been shown to occur in response to activation of Rac (25); however, in CCL39-R1 cells, Rac activity was not regulated by SST, although it was markedly increased by platelet-derived growth factor (Fig. 6A). SSTR1 could attenuate Rho activity by stimulating a Rho-dependent GAP or by inhibiting a Rho-specific guanine nucleotide exchange factor (GEF). In fibroblasts, a Src-dependent activation of p190RhoGAP transiently inhibits Rho activity by integrin engagement (26). Although we confirmed that a transient increase in tyrosine phosphorylation of p190RhoGAP occurs in response to integrin activation, we found no effect of SSTR1 activation. Moreover, although the Src inhibitor protein phosphatase-2 effectively blocked tyrosine phosphorylation of Src in response to serum, it had no effect on SST attenuation of Rho-GTP activity. Hence, if SSTR1 couples to the inhibition of Rho by activating a GAP, our findings suggest that this is independent of p190RhoGAP and Src.

An alternative mechanism mediating SSTR1 attenuation of Rho-GTP could be inhibition of a Rho-specific GEF. Rho can be activated by p115RhoGEF, which acts downstream of trimeric G proteins G11 and G13. Although p115RhoGEF binds to both G12 and G13, its activation of Rho is stimulated by G13, but not G12; and activation of G12 inhibits G13 stimulation of p115RhoGEF (39). Hence, one possible mechanism whereby SSTR1 inhibits...
SSTR1 Inhibits Rho

Rho is through G_{12} blocking the G_{12}P11\textsuperscript{RhoGTP}-Rho signal. Consistent with this possibility, we previously determined that G_{12} is the only trimeric G protein α-subunit shown to inhibit NHE1, which is regulated downstream of Rho (40). SSTR1 inhibition of Rho is likely not mediated by members of the G_{i} family of trimeric G proteins because PTX failed to prevent this effect or by members of the G_{q} family, which couple to the activation of Rho.

SSTR1 might also regulate a Rho guanine nucleotide dissociation inhibitor to decrease GTP binding and plasma membrane translocation of Rho. Two CAAX domains in Rho are thought to mediate lipid anchoring; and in the GDP-bound conformation, these domains may be masked, preventing the association of Rho with the plasma membrane (41). Immuno-cytochemical studies in resting and SST-treated CCL39-R1 cells indicated that, although some Rho immunoreactivity is present as a diffuse stain in the cytoplasm, a proportion is associated with small vesicular structures scattered throughout the cell, with little evidence of plasma membrane, Golgi, or nuclear staining. Overexpression of green fluorescent protein-tagged RhoA and RhoB in COS-1 and Madin-Darby canine kidney cells demonstrated that in quiescent cells, RhoA is predominantly present as a diffuse cytoplasmic fluorescence with no discernible plasma membrane staining, whereas RhoB is localized to the plasma membrane and Golgi stack (41). The staining we obtained with CCL39-R1 cells is consistent with the localization of RhoA in the Madin-Darby canine kidney and COS-1 cells.

In addition to its central role in mediating GPCR regulation of cytoskeletal reorganization, Rho is stimulated by plating cells on a fibronectin substrate and activation of integrin receptors (7). In the present study, the ability of SSTR1 to inhibit Rho activation, strongly implicating this receptor in the anti-angiogenic effects of SST.

In summary, our data show for the first time that activation of a GPCR, SSTR1, inhibits the GTP binding of Rho and its translocation to the plasma membrane. The decrease in activated Rho correlates with a decreased assembly of actin stress fibers and focal adhesions and impaired cell migration.

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