Prolonged Activation of Extracellular Signal-regulated Kinase by a Protein Kinase C-dependent and N17Ras-insensitive Mechanism Mediates the Proliferative Response of $G_{i/o}$-coupled Somatostatin sst$_4$ Receptors*

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The human sst$_4$ receptor, recombinantly expressed in Chinese hamster ovary cells, mediates proliferative activity of the peptide hormone somatostatin. This effect was shown to involve activation of pertussis toxin-sensitive G proteins and was inhibited by overexpression of the $\beta\gamma$-sequestrant, transducin. Somatostatin-induced proliferation was abolished by the MEK1 inhibitor, PD 98059, whereas the Src inhibitor, PP1, had no effect. A marked increase was observed in the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) 10 min after sst$_4$ receptor activation, which was blocked by pertussis toxin, decreased by PP1 and the $\beta\gamma$-sequestrant, but unaffected by PD 98059. In contrast, the somatostatin-induced phosphorylation of ERK obtained at 4 h, although sensitive to both pertussis toxin and transducin, was unaffected by PP1 but ablated by PD 98059. Protein kinase C inhibition also abolished this somatostatin-induced sustained phosphorylation of ERK, together with the associated increase in cell proliferation. Expression of dominant negative Ras (N17) failed to significantly reduce the proliferative effect mediated by the sst$_4$ receptor but markedly attenuated the acute phase of the somatostatin-induced phosphorylation of ERK obtained at 10 min. In contrast, the phosphorylation induced at 4 h was unaffected. We conclude that ERK activation by $G_{i/o}$-coupled sst$_4$ receptors involves a Src and Ras-dependent acute phase, but the proliferative response is dependent upon the prolonged ERK-induced activity, mediated by protein kinase C.

The peptide hormone somatostatin induces numerous biological actions, most of which are inhibitory, by interacting with cell membrane receptors of which five types, named sst$_1$–sst$_5$, have been heterologously expressed in different cells within the last few years (1). The growth inhibitory effects of somatostatin are well documented as it is considered to be the physiological regulator of growth hormone release (2). As well as reducing the circulatory levels of several other potential mitogenic hormones and growth factors (3), somatostatin has also been shown to have a direct action on cellular proliferation and tissue development, with therapeutic potential in retarding the growth of tumor (4) and vascular smooth muscle cells (5).

Numerous reports have demonstrated the expression of a high density of somatostatin receptors on a variety of human cancer cells, including most tumors of neuroendocrine origin (including gastroenteropancreatic tumors), small cell lung carcinomas, brain tumors (gliomas and meningiomas), lymphomas, and melanomas as well as colorectal, pituitary, kidney, and breast tumors (6, 7). The antiproliferative effect of either somatostatin or its analogue octreotide, however, does not correlate with this expression, having inhibitory actions on pancreatic (8) and breast tumors (9), although eliciting no effect on the growth of small cell lung (10) and colon tumors (11). Growth-promoting effects of somatostatin have also been described in vitro on human pancreatic carcinoid (12) and epidermoid carcinoma cells (13), whereas in both rat mesangial cells (14) and human pancreatic MIA-Pa-Ca-2 cells (15), somatostatin stimulates proliferation in the absence of serum but inhibits the growth of proliferating cells.

Little is known as to the identity of the receptor types mediating the proliferative or antiproliferative responses of somatostatin in tissues, and information has been restricted to studies involving partially selective receptor analogues (16). Activation of either mouse recombinant sst$_2$ or sst$_3$ receptors, however, has been shown to inhibit serum-induced proliferation (17), whereas stimulation of the human recombinant sst$_4$ receptor type induces proliferation in the absence of other mitogenic agents (18). Interestingly, the recently cloned rat sst$_{2b}$ receptor splice variant has also been shown to induce a proliferative response, in marked contrast to the antiproliferative property mediated by the rat sst$_{2a}$ receptor following recombinant expression in the same host cell line (19).

The molecular determinants that mediate the proliferative outcome of somatostatin receptors have not yet been fully clarified. All five human receptor types are functionally coupled to inhibition of adenylate cyclase via pertussis toxin-sensitive G proteins (20) and can mediate phospholipase C activation with subsequent calcium mobilization (21). Stimulation of sst$_1$ and sst$_3$ receptors has been shown to activate a protein-tyrosine phosphatase activity (22), and it has been suggested that such an activity may counteract the growth-promoting properties of receptors containing an intrinsic tyrosine kinase domain (23). The inhibition of basic fibroblast growth factor-stimulated proliferation by activation of human sst$_1$ receptors has also been proposed to be due to the induction of the cell cycle inhibitor, p21$^{Cip1/WAF1}$, shown in a recombinant system following the synergistic activation of extracellular signal-regulated kinase

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Thermaxon coverslips. Multiple parallel areas (400-μm wide) were denuded of cells, by dragging a Perspex comb across the surface of each coverslip according to the method described previously (18, 26). Coverslips were washed in phosphate-buffered saline and placed in a fresh well containing drug or vehicle in media without serum. Cells were allowed to undergo incubation for 24 h by washing the coverslip as above and adding 0.05% trypsin, 0.02% EDTA solution for 2-5 min. The digestion process was terminated by adding complete media and the single cell suspension was counted using a Coulter Counter™model Z1. Results were calculated from a minimum of three experiments with four replicates per test group and expressed as the arithmetic mean ± S.E. of the mean. Statistical analysis was by analysis of variance followed by Tukey's test (SigmaStat version 2).

**RESULTS**

**Effect of Somatostatin on the Proliferation of Chinese Hamster Ovary Cells Recombinantly Expressing Human sst_4 Receptors**—Following partial denudation of a confluent monolayer, the total number of CHO K1 cells recombinantly expressing human sst_4 receptors that remained on a single coverslip was 152 ± 3 × 10^3. After 24 h in the presence of incomplete media, this number had slightly increased to 166 ± 5 × 10^3, with less than 0.6% of the cells detaching from the coverslip over the time course examined. Application of somatostatin (100 nM) immediately following denudation in the absence of other exogenously added mitogenic factors caused a significant increase in cell number (Table I) that was comparable to that induced by

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1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; bFGF, basic fibroblast growth factor; CHO, Chinese hamster ovary; PDGF, platelet-derived growth factor; TBS, Tris-buffered saline; MEK, MAP kinase kinase.
Effect of various treatments on the proliferation induced by either somatostatin (100 nM) or bFGF (10 ng/ml) of CHO K1 cells expressing human recombinant sst4 receptors

| Treatment | Basal | Somatostatin bFGF |
|-----------|-------|-------------------|
| Pertussin, 100 ng/ml | 164 ± 2 | 167 ± 3 | 244 ± 4 |
| Genistein, 50 μM | 166 ± 4 | 166 ± 4 | 161 ± 4 |
| Lavendustin A, 11 nm | 162 ± 5 | 166 ± 6 | 160 ± 6 |
| Lavendustin B, 11 nm | 167 ± 6 | 241 ± 3 | 248 ± 5 |
| PD 98059, 2 μM | 167 ± 2 | 162 ± 6 | 201 ± 7 |
| PP1, 200 nm | 161 ± 8 | 239 ± 8 | 237 ± 9 |

* Treatment is significantly different (p < 0.001) from basal.

Effect of Pertussin Toxin Pretreatment—Pretreatment for 20 h with pertussis toxin (100 ng/ml) had no significant effect on either basal or bFGF-induced (10 ng/ml) proliferation (Table I). However, the increased proliferation induced by somatostatin (100 nM) was abolished following pretreatment with the toxin to values not significantly different from basal (Table I).

Effect of Protein-tyrosine Kinase Inhibitors—Neither tyrosine kinase inhibitor, genistein (50 μM) nor lavendustin A (11 nm), had any significant effect on basal proliferation (Table I) in the absence of exogenous growth factors. However, both inhibitors abolished the increase in proliferation induced by either somatostatin (100 nM) or bFGF (10 ng/ml) (Table I). Importantly, the inactive enantiomer of lavendustin A, lavendustin B (11 nm), had no effect on basal cell numbers or on the proliferation induced by either somatostatin or bFGF (Table I).

Effect of MEK1 or Src-family Inhibitors—The inhibitor of MEK1, PD 98059 (2 μM), had no significant effect on basal proliferation (Table I). The increase in proliferation elicited by somatostatin (100 nM) was abolished on co-incubation with PD 98059, whereas bFGF-induced (10 ng/ml) proliferation was only partially reduced (Table I). Pretreatment with PD 98059 for 1 h before partial denudation also completely inhibited somatostatin-induced increases in cell number (from 240 ± 4 × 10^3 to 164 ± 3 × 10^3) but again only partially inhibited bFGF-induced growth (from 244 ± 6 × 10^3 to 190 ± 4 × 10^3).

The Src-family inhibitor, PP1 (200 nm), had no significant effect on basal proliferation (Table I). The increased cell counts induced by either somatostatin or bFGF were also unaffected by incubation with PP1 (Table I). Pretreatment of the cells with PP1 for 1 h before partial denudation, again elicited no significant effect on proliferation induced by somatostatin (237 ± 13 × 10^3 and 242 ± 5 × 10^3 in the presence and absence of PP1, respectively) or bFGF (240 ± 10 × 10^3 and 247 ± 4 × 10^3). As a positive control for PP1, proliferation induced by platelet-derived growth factor (PDGF-BB) was examined. The PDGF-evoked increase in cell number (using 5 ng/ml) was significantly reduced by PP1 from 268 ± 7 × 10^3 to 185 ± 10 × 10^3.

Changes to the Phosphorylation Status of ERK1 and ERK2—Activation of MEK1 in mediating the proliferative action of somatostatin was substantiated by assaying change to the phosphorylation status of ERK1 and ERK2 using an antibody that recognizes only the dually phosphorylated, active form of ERK. To distinguish between effects on either the acute or prolonged phases of MAP kinase activation, the phosphorylation status of ERK1 and ERK2 was examined at 10 min and 4 h following partial denudation. For all treatment groups there was no detectable change in the expression of ERK1 or ERK2 protein at the time points investigated (Fig. 1A). An increase in the phosphorylation status over basal was observed for both ERK1 and ERK2 following somatostatin treatment (100 nM), but that induced at 10 min was considerably greater than that observed at 4 h post-denudation (Fig. 1, B and C), consistent with previous observations (18). The enhanced phosphorylation of ERK1 and ERK2 induced by somatostatin at both time points was abolished by pertussis toxin pretreatment (20 h at 100 ng/ml) and unaffected by genistein (50 μM) (Fig. 1, B and C). Neither pertussis toxin nor genistein had any detectable effect on the basal level of phosphorylation of ERK1 or ERK2 at either time point (Fig. 1, B and C).

Differential effects of PD 98059 (2 μM) and PP1 (200 nm) treatments, however, were observed on the somatostatin-induced increase in ERK phosphorylation at the times examined. PD 98059 had no detectable effect on basal or somatostatin-induced phosphorylation obtained 10 min post-denudation (Fig. 1B). In contrast, the somatostatin-mediated increase was reduced following PP1 treatment, with the Src inhibitor showing no detectable effect on basal phosphorylation levels (Fig. 1B). After 4 h of regenerative processes, the somatostatin-induced phosphorylation of ERK1 and ERK2 was unaffected by PP1 but abolished by PD 98059, with neither inhibitor having any observable effect on the basal level of phosphorylation (Fig. 1C).

Effect of a Dominant Negative Mutant of Ras on Cell Proliferation and ERK Phosphorylation—To evaluate the involvement of Ras in mediating the activation of ERK by somatostatin sst4 receptors, transient expression of the dominant negative mutant of Ras (N17) was performed. This Ras mutant, in which amino acid 17 (serine) is changed to asparagine, is thought to function by inhibiting guanine nucleotide exchange factors (27). Transient transfection with the empty vector had no significant effect on the proliferation induced in CHO K1 cells expressing the sst4 receptor by either somatostatin (100 nM) or bFGF (10 ng/ml), as determined by counting the number of cells forming the regenerating monolayers 24 h after partial denudation (Fig. 2A). Transient expression of N17Ras also failed to significantly effect the increase in cell number evoked by somatostatin treatment, but in contrast that induced by bFGF was markedly attenuated (Fig. 2A). Transfection with either the empty vector or that incorporating N17Ras cDNA failed to significantly effect basal cell counts (data not shown). The increase in N17Ras levels after transfection was evaluated by immunoblotting cell extracts immediately before partial denudation with a polyclonal antibody to Ras. The inset in Fig. 2A shows that in mock-transfected cells, the immunoreactivity with the anti-Ras antibody was almost undetectable compared with the intense reactivity obtained from the same number of cells transfected with pUSEamp(+) plasmids containing dominant negative Ha-ras.

The level of phosphorylation of ERK1 and ERK2 induced by somatostatin (100 nM) at either time point examined (10 min and 4 h) was unchanged by transfection with the empty plasmid (Fig. 2B). After transient expression of N17Ras, the somatostatin-induced phosphorylation observed 10 min after partial denudation was substantially decreased, whereas that obtained at 4 h was unaffected (Fig. 2B). Neither transfection with pUSEamp or pUSEamp(N17ras) showed any effect on basal levels of ERK phosphorylation observed at 10 min or 4 h (Fig. 2B). To show consistency in protein loading, detection of ERK1 and ERK2 using phosphorylation state-independent pan antibodies was also made (Fig. 2C). Electrophoretic mobility shifts for both ERK1 and ERK2 could be observed in those treatment groups where a marked change in the phosphorylation status of these proteins had occurred.
It has previously been shown using the same model system as employed in this study that bFGF (10 ng/ml) induces a sustained phosphorylation of both ERK1 and ERK2 (18). However, in contrast to that evoked by somatostatin, the time profile for the growth factor-stimulated phosphorylation was biphasic, producing peaks at 10 min and again at 4 h post-denudation. In this study, ERK phosphorylation induced by bFGF (10 ng/ml) at 10 min and 4 h was unaffected by transfection with pUSEamp (Fig. 2D). However, transient expression of N17Ras inhibited the growth factor-induced phosphorylation at both time points examined (Fig. 2D). Neither transfection with pUSEamp or pUSEamp(N17ras) showed any effect on basal levels of ERK phosphorylation observed at 10 min or 4 h (Fig. 2D), and the level of ERK protein expression remained unchanged across treatment groups (data not shown).

Effect of Protein Kinase C Inhibition on Cell Proliferation and ERK Phosphorylation—The protein kinase C inhibitor, Ro 31-8220 (50 nM), had no significant effect on the number of CHO K1 cells (expressing the human recombinant sst4 receptor) maintained in the absence of serum for 24 h after partial denudation (Fig. 3A). However, Ro 31-8220 abolished the increase in cell number induced by either somatostatin (100 nM) or bFGF (10 ng/ml) (Fig. 3A). Down-regulation of protein kinase C following pretreatment of cells with the phorbol ester phorbol 12-myristate 13-acetate (100 ng/ml for 24 h) also reduced the somatostatin-induced increase in cell counts from $244 \pm 6 \times 10^3$ to $172 \pm 11 \times 10^3$. Incubation with Ro 31-8220 abolished the induced phosphorylation of ERK1 and ERK2 observed following somatostatin treatment for 4 h (Fig. 3B). However, there was no detectable change in the level of somatostatin-induced immunoreactivity detected with the anti-phosphospecific antibody obtained 10 min after partial denudation (Fig. 3B). Similarly, bFGF-induced ERK phosphorylation at 10 min was unaffected by Ro 31-8220, whereas that obtained at 4 h was markedly attenuated (Fig. 3C). Ro 31-8220 had no apparent effect on the basal level of ERK phosphorylation observed at either time point (Fig. 3, B and C) or on the expression levels of the kinases (data not shown).

Effect of Transient Transfection with Transducin on Cell Proliferation and ERK Phosphorylation—Neither mock transfection with pCDNA3 nor pCDNA3 incorporating transducin cDNA had any significant effect on basal proliferation of CHO K1 cells recombinantly expressing the sst4 receptor, determined 24 h following partial denudation in the absence of exogenous growth factors (data not shown). The increased cell number induced by the submaximal concentration of bFGF (10 ng/ml) was also unaffected following transfection with either plasmid (Fig. 4A). However, the proliferation induced by somatostatin (100 nM) was significantly reduced following transient expression of transducin, whereas that following mock transfection was unaffected (Fig. 4A). The inset in Fig. 4A shows a representative immunoblot of protein extract from cells transfected with either empty vector or that incorporating transducin cDNA showed marked immunoreactivity as detected by an anti-transducin antibody compared with those transfected with the empty vector.

There was no detectable change in the basal level or somatostatin-induced phosphorylation of ERK1 or ERK2 in samples allowed to regenerate for either 10 min or 4 h after mock transfection (data not shown). However, overexpression of transducin reduced the somatostatin-induced phosphorylation of ERK1 and ERK2 observed at both 10 min and 4 h after denudation compared with mock-transfected cells (Fig. 4B). Transducin overexpression had no apparent effect on basal phosphorylation levels at either time point examined (Fig. 4B).
DISCUSSION

Activation of the MAP kinase cascade, which in most systems requires Ras and Raf, is a universal downstream response to the stimulation of most receptor protein-tyrosine kinases and has been demonstrated following activation of the G protein-coupled somatostatin sst4 receptor (18, 28). Using a well

FIG. 3. Effect of the protein kinase C inhibitor, Ro 31-8220, on somatostatin- and bFGF-induced cell proliferation and ERK phosphorylation in CHO K1 cells recombinantly expressing human sst4 receptors. Panel A shows the mean number of cells harvested from a single coverslip after application of incomplete media (Basal; open histograms), somatostatin (100 nM; SRIF; closed histograms), or bFGF (10 ng/ml; hatched histograms) in the presence and absence of Ro 31-8220 (50 nM; Ro). Values are expressed as the mean cell number ± S.E., obtained 24 h after partial denudation of a previously confluent monolayer (n = 3, 4 replicates). Groups labeled with * are significantly different from basal (p < 0.001), and those labeled with # are significantly different from values in the presence of either somatostatin or bFGF but in the absence of Ro 31-8220 (p < 0.001). The effect of Ro 31-8220 on the phosphorylation of ERK1 and ERK2 induced by either somatostatin (100 nM; SRIF) or bFGF (10 ng/ml; FGF) at 10 min and 4 h immediately after partial denudation is shown in panels B and C, respectively. Control samples incubated in incomplete media (CON) with or without Ro 31-8220 (Ro) at both time points are also shown. Detection was made by Western analysis using the phosphospecific ERK antibody. Each panel has been taken from a single immunoblot and is a representative of three separate experiments.

FIG. 2. Effect of dominant negative Ras on somatostatin- and bFGF-induced cell proliferation and ERK phosphorylation in CHO K1 cells recombinantly expressing human sst4 receptors. The effect of transient expression of N17Ras (-Ras) on the increased cell number induced by somatostatin (100 nM; SRIF; closed histograms) or bFGF (10 ng/ml; hatched histograms) 24 h after application to partially denuded cell monolayers is shown in panel A. The number of cells obtained after incubation in the presence of incomplete media is shown by the open histogram (Basal), and the effect of transfection with the empty plasmid, pUSEamp, on somatostatin- and bFGF-stimulated proliferation is represented by the histograms labeled Mock. Values are expressed as the mean cell number harvested from a single coverslip (n = 3, 4 replicates). The vertical bars represent the S.E., and those groups labeled with * are significantly different from basal (p < 0.001). The treatment group labeled with # is significantly different from that incubated in the presence of bFGF without transfection (p < 0.01). The inset in panel A shows an immunoblot of protein from cell samples extracted immediately before partial denudation that had been transfected 48 h previously with either pUSEamp (Mock) or that incorporating dominant negative Ha-ras (-Ras). After separation by 15% polyacrylamide gel electrophoresis and transfer onto nitrocellulose, detection was made with an anti-β-actin antibody to demonstrate consistency of protein loading as well as with an antibody to Ras. The effect of transient transfection with N17Ras (-Ras) on the somatostatin-induced (100 nM; SRIF) phosphorylation of ERK1 and ERK2, as detected by the phosphospecific antibody using Western analysis, is shown in panel B. Samples from cells transfected with the empty plasmid 48 h prior to partial denudation are labeled Mock, and a comparison is shown of the phosphorylation status of ERK1 and ERK2 determined at both 10 min and 4 h post-denudation. Samples labeled CON were incubated for the appropriate times in the presence of incomplete media. Consistency of protein loading was substantiated by the evenness of the immunoreactivity obtained following detection of the samples shown in panel B with the anti-ERK antibodies (panel C). Panel D shows the effect of transient expression of N17Ras on bFGF-induced (10 ng/ml; FGF) phosphorylation of ERK1 and ERK2. Western blots shown are a representative from at least three separate experiments, and each panel has been taken from a single immunoblot.
from a single immunoblot. from at least three separate experiments, and the phosphorylation status of ERK1 and ERK2 determined at both 10 min and 4 h post-denudation. The Western blot shown is a representative panel B

Fig. 4. Effect of the βγ sequestrant, transducin, on somatostatin-induced cell proliferation and ERK phosphorylation in CHO K1 cells recombinantly expressing human sst4 receptors. The effect of transient expression of transducin (Trans) on the increased cell number induced by somatostatin (100 nM; SRIF; closed histograms) or bFGF (10 ng/ml; hatched histograms) 24 h after application to a partially denuded cell monolayer is shown in panel A. The number of cells obtained after incubation in the presence of incomplete media is shown by the open histogram (Basal), and the effect of transfection with the empty plasmid, pCDNA3, on somatostatin- and bFGF-stimulated proliferation is represented by the histograms labeled Mock. Values are expressed as the mean cell number harvested from a single coverslip (n = 3, 4 replicates). The vertical bars represent the S.E., and those groups labeled * are significantly different from basal (p < 0.001). The treatment group labeled # is significantly different from that incubated in the presence of somatostatin without transfection (p < 0.01). The inset in panel A shows an immunoblot of protein from cell samples extracted immediately before partial denudation that had been transfected 48 h previously with either pCDNA3 (Mock) or that incorporating transducin cDNA (Trans). Western detection was made with an anti-β actin antibody to demonstrate consistency of protein loading as well as by the anti-transducin antibody (Trans). The effect of transient expression of transducin (Trans) on the phosphorylation of ERK1 and ERK2 induced by somatostatin (100 nM; SRIF) as well as that obtained after incubation with incomplete media (CON), as detected by the phosphospecific antibody using Western analysis, is shown in panel B. Samples from cells transfected with the empty plasmid 48 h before partial denudation are labeled Mock, and a comparison is shown of the phosphorylation status of ERK1 and ERK2 determined at both 10 min and 4 h post-denudation. The Western blot shown is a representative from at least three separate experiments, and the panel has been taken from a single immunoblot.

characterized model to determine the re-population of denuded areas in an otherwise confluent monolayer, we have previously demonstrated that the somatostatin-induced proliferative response of this receptor type depends on the sustained activation of ERK1 and ERK2 and independent of a robust transient phase (18). One of the advantages of this model is that cells are synchronized in G0 or early G1 of the cell cycle at the onset of the investigative period, and to substantiate that effects are on a proliferative rather than a motogenic process, the total number of cells forming the regenerating monolayer after 24 h in the presence of test agents was determined in the current study. The aim of this investigation was to attempt to identify the transduction effectors involved in mediating the sustained activation of ERK1 and ERK2 by somatostatin sst4 receptors and, hence, those responsible for inducing the proliferative response.

The protein-tyrosine kinase inhibitors, genistein and laven-dustin A, had no effect on basal cell numbers at concentrations that abolished the increased proliferation induced by bFGF. The signaling cascades activated by bFGF receptors would thus seem dependent on this type of phosphate transfer process for transduction of the proliferative function and is compatible with the well characterized mechanism through which this family of receptors mediate their mitogenic effects (29). It would also appear that transmission of the growth-promoting activity of sst4 receptors is similarly dependent on a protein-tyrosine kinase activity. However, in contrast to the growth factor receptor, which contains an intrinsic tyrosine kinase domain within the COOH terminus of each subunit forming the active dimer, the site of intervention of these kinase blockers in the transduction process for sst4 receptor-mediated growth must be localized to secondary effectors. The lack of effect of genistein on the somatostatin-induced ERK phosphorylation determined at time points representative of the acute and sustained phases of MAP kinase activation suggests that either a parallel cooperative pathway utilizing a tyrosine kinase is essential for growth or, alternatively, that the kinase lies exclusively downstream from MAP kinase. Evidence for a pertussis toxin-sensitive pathway mediating tyrosine phosphorylation of the transcription factor STAT3 has recently been provided for the sst4 receptor (18), and it may be that the genistein-sensitive effector is situated within this particular cascade (see Fig. 5). Interestingly, it has also been shown in this previous study that only after the additional phosphorylation on serine residues of this same transcription factor as a consequence of prolonged MAP kinase activation, could a proliferative response be induced by somatostatin.

The growth-promoting effect of somatostatin is additionally dependent on a pertussis toxin-sensitive pathway that distinguishes its proliferative mechanism from that of bFGF. Other G protein-coupled receptors have been shown to mediate rapid tyrosine phosphorylation of several proteins that participate in mitogenic signal transduction such as the adapter protein Sbc (30), which is a major substrate for Src kinase. The mechanism whereby these receptors stimulate tyrosine phosphorylation is poorly understood, although activation of the Src-family kinases by several G protein-coupled receptors has been reported (31). In addition, activation of Src seems to account for the Gq-mediated tyrosine phosphorylation events that direct recruitment of the Shc and Grb2 adaptor proteins to the membrane (32), thus providing a route into the Ras-ERK cascade (Fig. 5).

To determine whether activation of the MAP kinase cascade was a prerequisite for processing the growth effects induced by somatostatin, the selective MEK1 inhibitor, PD 98059 (33), was used in the proliferation model. It is well documented that the dual-specific kinase MEK stimulates ERK by phosphorylation on threonine (Thr-183) and tyrosine (Tyr-185) residues, which following subsequent translocation into the nucleus, activates transcription factors, resulting in enhanced cell growth (34). In this study, the proliferative effect of somatostatin was abolished by PD 98059, confirming that the MAP kinase cascade is critical for the growth-promoting effect of somatostatin by the sst4 receptor. In contrast, bFGF-stimulated proliferation in the
same host cell was only partially inhibited on co-application with PD 98059, which is consistent with the ability of this receptor type to recruit a multitude of secondary effectors and initiate a number of distinct, yet parallel signaling pathways. An involvement of MEK1 in the sst4 receptor-mediated proliferative response was further supported by the demonstration of increased phosphorylation of ERK1 and ERK2 following somatostatin treatment. However, although both the acute and sustained phases of MAP kinase activation were abolished by pertussis toxin, a differential effect on the temporally distinct activities was observed following MEK1 inhibition. Abolition of the sustained phase with PD 98059, although having no observable effect on the transient activity, is supportive evidence for the requirement of the prolonged activation of MAP kinase in mediating cell growth. The lack of effect of the MEK1 inhibitor on the marked transient phosphorylation of ERK is possibly due to the ineffectiveness of the concentration of PD 98059 used. However, genistein is a nonselective tyrosine kinase inhibitor, and it may be that in this system, Src activity is unaffected by the concentration of genistein used.

Several Gi-coupled receptors have been shown to mediate MAP kinase activation through the \( \beta \gamma \)-component of the G protein possibly through the activation of the Src-family of tyrosine kinases (35). The proliferative response induced by somatostatin was inhibited following overexpression of the \( \beta \gamma \)-sequestering protein, transducin, in contrast to the lack of effect on bFGF-induced growth. However, the Src-family inhibitor, PP1 (36), failed to reduce the proliferative effect induced by either mitogen in this cell line. The bFGF results were somewhat unexpected since it is well known that Src is a co-transducer of mitogenic signals arising from a number of tyrosine kinase growth factor receptors, such as platelet-derived growth factor or epidermal growth factor receptors (37). However, the association of Src with bFGF receptors appears to be cell-specific (38), and in this respect, bFGF-induced proliferation in vascular smooth muscle cells has been shown to be partially inhibited by the Src inhibitor PP1, as determined by the same model system employed in this current study. PP1 in CHO K1 cells transfected with the somatostatin sst4 receptor was shown to reduce platelet-derived growth factor-stimulated growth.

The inability of PP1 to inhibit somatostatin-induced proliferation suggests Src is not involved in this response mechanism, and therefore the process through which MEK is activated appears to be very different to that employed by other Gi protein-coupled receptors (35). However, further examination of the somatostatin-induced ERK phosphorylation showed that the transient phase was sensitive to the Src inhibitor, in contrast to the PP1-independent prolonged phosphorylation. The attenuation of the transient phosphorylation of ERK by the Src inhibitor without any resultant effect on the proliferative response again suggests that it is the sustained activation of MAP kinase that is critical for proliferation. In addition, the sensitivity of the transient and not the prolonged phase of ERK phosphorylation to Src-inhibition provides evidence that different transduction events are involved in mediating the temporally distinct MAP kinase activities. The involvement of Src in mediating the acute phase of ERK phosphorylation and the insensitivity of this component to genistein seem incompatible. However, genistein is a nonselective tyrosine kinase inhibitor, and it may be that in this system, Src activity is unaffected by the concentration of genistein used.

The mechanisms by which Gi and Gs-coupled receptors typically activate MAP kinase are through Ras-dependent or protein kinase C-dependent pathways, respectively. However, a

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2 L. A. Sellers, unpublished data.
few exceptions to this rule have been recently reported for Gα-coupled receptors in that MAP kinase can be activated through a pertussis toxin-insensitive but protein kinase C-independent pathway (39). In this study, we have demonstrated that the transient phosphorylation of ERK by sst4 receptors is sensitive to both transducin and dominant negative Ras (N17) but unaffected following protein kinase C inhibition. These results are also in accord with the βγ-mediated Src stimulation utilized by other Gα-coupled receptors to activate MAP kinase through a Ras-dependent mechanism (Fig. 5). In addition, these data are also consistent with the acute phase of MAP kinase activity not being involved in mediating a growth response, as expression of N17Ras had no effect on somatostatin-induced proliferation or the prolonged activation of MAP kinase.

Since it appears that the sustained activation of MAP kinase, required for the somatostatin-induced proliferative effect, utilizes a distinct but convergent pathway to that mediating the transient Ras-dependent ERK phosphorylation, we examined the involvement of protein kinase C, which can activate the Ras-ERK cascade at the point of Raf (40) (Fig. 5). Both the proliferation and sustained phosphorylation of ERK1 and ERK2 induced by somatostatin were abolished following protein kinase C blockade. This suggests that protein kinase C involvement is critical for the growth response and is placed upstream to ERK activation, consistent with other reports investigating MAP kinase stimulation through Gα-coupled receptors (39). Receptor tyrosine kinase-mediated activation of Raf-1 is coupled to Ras, and bFGF-induced proliferation of CHO K1 cells used in this study was N17Ras-sensitive together with both the acute and sustained phases of ERK phosphorylation. By contrast, protein kinase C-mediated activation of Raf-1 is thought to be Ras-independent and is in keeping with the lack of effect on the prolonged MAP kinase phosphorylation and the induced proliferative response observed in this study following application of somatostatin to cells overexpressing N17Ras. Activation of Raf-1 by protein kinase C has been shown to be insensitive to dominant negative Ras (41), indicating that protein kinase C activates Raf by a mechanism distinct from that initiated by activation of receptor tyrosine kinases. Although in this study the acute phase of ERK phosphorylation induced by bFGF was unaffected by a protein kinase C inhibitor, the sustained component was reduced, and the proliferative effect was abolished. It thus appears that it is the sustained ERK activity that is also critical for bFGF to induce cell proliferation. However, in contrast to that following sst4 receptor activation, the sustained phase of ERK phosphorylation induced by bFGF appears to involve both Ras- and protein kinase C-dependent mechanisms, and both seem to be required for the proliferative effect.

The protein kinase C family has at least 11 members, 6 of which (δ, ε, γ, η, μ, θ) have been shown to be expressed in the CHO K1 cells used in this study. Both the typical and atypical protein kinase C isoforms are activated by diacylglycerol, which is produced by the metabolism of phosphatidylinositol. Although sst4 receptors have been shown to mediate inositol 1,4,5-trisphosphate production (21), it has not been determined here if through βγ release, the subsequent stimulation of phospholipase C-β is mandatory for the sustained ERK phosphorylation induced by somatostatin. Activation of MAP kinase after sst4 receptor stimulation has been shown to be dependent on phosphoinositide 3-OH kinase (42). Signaling targets of the lipid products of this kinase activity include the calcium-independent protein kinase C isoforms (43), raising the possibility that ERK activation through protein kinase C in this study could be via a calcium-independent pathway. Involvement of phosphoinositide 3-OH kinase in the prolonged activation of ERK would also be in keeping with the Gβγ dependence of both this and the proliferative events observed after somatostatin application, as a phosphoinositide 3-OH kinase responsive to Gβγ subunits has recently been cloned (44).

Very little evidence is currently available as to the identity of the molecular determinants responsible for the sustained activation of MAP kinase. Recently it has been shown that in PC12 cells, Ras must be activated for the initial phase of ERK activation following stimulation of the nerve growth factor receptor (TrkA), but the sustained phase involves another small GTPase, Rap1 (45). In this study we have demonstrated that sst4 receptor stimulation can stimulate cellular proliferation through transduction mechanisms with a critical requirement for a sustained, protein kinase C-dependent activation of MAP kinase. Stimulation of MAP kinase has been shown to regulate a diverse range of functional responses, sometimes with opposing effects. For example, although we have shown a critical requirement for ERK activity in the sst4 receptor-mediated proliferative effect, this activity also appears to be necessary for the growth inhibitory response of sst1 receptor types (24). These apparent conflicting functional processes following activation of the MAP kinase cascade will be better explained once the kinetics of ERK activation as well as the strength of the stimulus for a given receptor type have been fully evaluated and elements of the transduction machinery required for the temporally distinct activities have been identified.

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