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High-Intensity p38 Kinase Activity Is Critical for $p21^{cip1}$ Induction and the Antiproliferative Function of G_i Protein-Coupled Receptors

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ABSTRACT

G protein-coupled receptors can stimulate the p38 kinase cascade, but the effect this has on cell growth remains poorly characterized. Here we show human somatostatin sst_2 and sst_4 receptors inhibit basic fibroblast growth factor (bFGF)-induced proliferation, via a mechanism that was blocked by the p38 inhibitor PD 169316. The sst_4 receptor could also induce a proliferative activity in the absence of bFGF, which was unaffected by PD 169316. In contrast, the sst_3 receptor had no effect on basal cell growth or on the proliferation evoked by bFGF. The extracellular signal-regulated kinase activity stimulated by the sst_3 receptor was transient in duration compared with a sustained activity induced by the sst_2 and sst_4 receptors and which was critical for the proliferative response of the latter receptor. In addition, activated sst_2 and sst_4 but not sst_3 receptors evoked a prolonged phosphorylation of p38 that was amplified by bFGF. The accumulation of the cell cycle inhibitor $p21^{cip1}$ was only apparent after sst_2 and sst_4 receptor activation in the presence of bFGF, which was sensitive to PD 169316 or pertussis toxin. Thus, the contrasting antiproliferative effects evoked by the human sst_2, sst_3, and sst_4 receptors can be accounted for by their differential abilities to activate p38. This activity is critical for $p21^{cip1}$ induction, blockade of entry into S phase, as indicated by the lack of retinoblastoma protein phosphorylation, and the associated antiproliferative activity of somatostatin. Furthermore, by changing the intracellular signaling threshold of p38 through cooperative effects of somatostatin and bFGF, the sst_4 receptor can mediate opposing effects on cell proliferation.

G protein-coupled receptors (GPCRs) stimulate mitogenesis, in part, via extracellular signal-regulated kinases (ERKs), which are members of the mitogen-activated protein (MAP) kinase family. The mechanism of activation of the different MAP kinase signaling pathways by GPCRs is poorly understood, although it is becoming evident that many of the same transduction intermediates as those activated by the receptor tyrosine kinases are often used. For example, ERK1 and ERK2 are regulated by GPCRs through a Ras-dependent pathway by stimulating the recruitment of the guanine nucleotide exchange factor mSos (van Biesen et al., 1995) into a plasma membrane-associated signaling complex, where it activates Ras by catalyzing GTP for GDP exchange. This recruitment is the consequence of receptor-induced stimulation of tyrosine protein kinases such as Src (Dikic et al., 1996; Luttrell et al., 1996; Daub et al., 1997), which phosphorylate adapter proteins, including Shc (Luttrell et al., 1996) and Gab1 (Daub et al., 1997), followed by the Grb-2-mediated docking of mSos to the plasma membrane.

Considerable attention has also been focused recently on the functional outcome induced by the alternative kinase cascades that culminate in the activation of the MAP kinase family members, the stress-activated protein kinases (SAPKs) and p38 (Kyriakis and Avruch, 1996; Fanger et al., 1997). In contrast to ERKs that are stimulated almost universally by mitogens, a number of findings indicate that the activation of SAPKs and p38 can play a decisive role in the control of cell death (Verheij et al., 1996; Yang et al., 1997). Furthermore, deprivation of neurotrophic factors in PC-12 cells not only activates SAPKs and p38 but also leads to a dramatic inhibition of the ERK pathway (Xia et al., 1995; Berra et al., 1997). It has thus been suggested that the proliferative outcome of a cell may be dictated by a critical balance between the signaling pathways involving the various MAP kinase family members (Canman and Kastan, 1996).

In the present study, we have examined the abilities of human G protein-coupled somatostatin receptors to activate the various MAP kinase pathways and correlated any differential stimulation with the distinct effects on cell proliferation mediated by the individual receptor types. Opposing effects of somatostatin on the proliferative activity of a num-

ABBREVIATIONS: GPCR, G protein-coupled receptor; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; SAPK, stress-activated protein kinase; sst, somatostatin; bFGF, basic fibroblast growth factor; Rb, retinoblastoma protein; CHO, Chinese hamster ovary; ATF-2, activating transcription factor 2; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20.

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ber of different cell types has been demonstrated, including inhibitory actions on prostatic (Brevini et al., 1993) and breast cell lines (Pagliacci et al., 1991), whereas growth-promoting effects of somatostatin have been described for human pancreatic carcinoid (Ishizuka et al., 1992), epidermoid carcinoma cells (Kamiya et al., 1993), and rat mesangial cells (Ruiz-Torres et al., 1993). However, little is known about the identity of the receptor types or the mechanisms involved in mediating the proliferative/antiproliferative functions of somatostatin in tissues.

We have previously shown that both activated human recombinant sst_2 and sst_5 receptors (Alderton et al., 1998) have no effect on basal proliferation, but can inhibit that induced by a submaximal concentration of basic fibroblast growth factor (bFGF). However, somatostatin can stimulate basal proliferation in cells expressing the human recombinant sst_4 receptor by a mechanism that is dependent on the sustained activation of ERK1 and ERK2, culminating in serine-phosphorylation of the transcription factor STAT3 (Sellers et al., 1999).

In addition to stimulating a survival/cell cycle progression pathway such as the ERK cascade, somatostatin may activate or inhibit apoptotic processes. Indeed, the well-documented antiproliferative effects of somatostatin may be the result of stimulating growth arrest rather than through a mechanism involving the direct inhibition of growth factor-activated transduction cascades. Although activation of p38 and SAPKs has been recently demonstrated for GPCRs (Yamauchi et al., 1997), the effect this activity has on regulating proliferative responses after stimulation by this receptor family is not well understood. Using a well-defined in vitro model (Sellers et al., 1999), we have examined the abilities of the human somatostatin sst_2, sst_3, and sst_4 receptor types to regulate cell proliferation by assessing changes in viable cell number, either in the presence or absence of bFGF. At intervals during the growth responses, the phosphorylation status of ERK1, ERK2, and p38 were determined to substantiate whether a correlation could be made with the resultant proliferative outcome and the activation of a particular kinase cascade. In addition, the induction of the cell cycle inhibitor $p21^{cip1}$ was assessed, as well as the involvement of the individual MAP kinase cascades in mediating its expression. Changes in the phosphorylation of the retinoblastoma protein (Rb) were also determined to substantiate if the levels of $p21^{cip1}$ were sufficient for cell cycle arrest.

Experimental Procedures

Materials. Chinese hamster ovary (CHO-K1) cells were obtained from The European Collection of Animal Cell Cultures (CAMR Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire, UK). Geneticin (G418 sulfate, specific activity 500 μg/ml) and reagents for culturing cells were obtained from Life Technologies, Inc. (Gaithersburg, MD) and plastic ware was from Costar (Cambridge, MA). Somatostatin and bFGF were obtained from Sigma (Poole, Dorset, UK). PD 98059 (2′-amino-3′-methoxyflavone), the pyridinyl imidazole compound PD 169316 [4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1*H*-imidazole], and *Bordetella pertussis* toxin were from Calbiochem (San Diego, CA). Antibodies to ERK1 (C-16) and ERK2 (C-14) were obtained from Santa Cruz Biochemicals (Santa Cruz, CA). Polyclonal antibodies specific for the dually phosphorylated and hence active forms of ERK1 and ERK2 (at Thr^{202} and Tyr^{204}) and phosphorylated p38 (Thr^{180} and Tyr^{182} of α, β, and δ isoforms) were obtained from New England Biolabs (Beverly, MA) together with an antibody to p38 with a specificity for the kinase independent of its phosphorylation state. Antibodies specific for the phosphorylated form of Rb and the transcription factor activating transcription factor 2 (ATF-2) were also obtained from New England Biolabs. A monoclonal antibody detecting $p21^{cip1}$ was supplied by Upstate Biotechnology (Lake Placid, NY). An antibody for STAT3 recognizing the phosphorylated form at a conserved tyrosine (Tyr^{705}), which allows dimerization of the transcription factor through reciprocal phosphotyrosine-SH2 domain interactions (Darnell et al., 1994), was obtained from New England Biolabs.

Stable Expression of Human Somatostatin sst_2, sst_3, and sst_4 Receptors in Chinese Hamster Ovary Cells. The cDNA encoding each of the human receptors was subcloned into the mammalian expression vector pAlphaCA12 harboring a neomycin-resistant gene as a selection marker. CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) containing 10% (v/v) fetal calf serum and 1 mM Glutamax I and transfected in the absence of serum using a cationic liposome formulation-mediated transfer (LipofectAMINE; Life Technologies). Selection was performed in the presence of complete medium containing 1 mg/ml G418 sulfate and clonal cell lines expressing the cDNA were isolated by single cell cloning. Receptor expression was assessed by binding of ^{125}I-Tyr^{11}-somatostatin and the estimated B_{max} values for the clonal cell lines used were similar: 3.7 ± 0.5, 4.2 ± 1.2, and 3.3 ± 0.7 pmol/mg membrane protein for the sst_2 ($CHOsst_2$), sst_3 ($CHOsst_3$) and sst_4 ($CHOsst_4$) receptor-expressing cell lines, respectively ($n = 3$ for all data sets). No specific binding was detected in untransfected CHO-K1 cells. All cultures were routinely maintained in their appropriate growth medium at 37°C in humidified air containing 5% (v/v) carbon dioxide and passaged when 95% confluence was reached.

Partial Denudation of Confluent Cell Monolayers and Assessment of Change in Cell Number. To assess the effect of various treatments on cell number, the clonal cell lines were grown to confluence in complete media on Thermanox coverslips (Nunc, Naperville, CT). Multiple denuded areas (400 μm wide) were created by dragging a Perspex comb across the surface of the coverslip, according to the method described previously (Sellers, 1999). The Perspex comb was designed so that 50% of the confluent monolayer was removed by the partial denudation process, leaving parallel strips of cells. Repopulation of the denuded areas was investigated by placing the coverslip into a fresh well containing drug or vehicle in media without serum. Cells were harvested after incubation for 24 h by washing the coverslip in phosphate-buffered saline and adding 0.05% (w/v) trypsin/0.02% (w/v) EDTA solution for 2 to 5 min. The digestion process was terminated by adding complete media and the single cell suspension counted using a Coulter counter model Z1. Results were calculated from a minimum of three experiments with three replicates per test group and expressed as the arithmetic mean of the number of cells harvested from a single coverslip ± S.E.M. Statistical analysis was by Student's t test taking $P < 0.05$ as the level of significance.

Determination of the Phosphorylation Status of ERK1, ERK2, p38, STAT3, and ATF-2 or the Induction of $p21^{cip1}$. To analyze changes in the phosphorylation status of the MAP kinase family members or the transcription factors STAT3 and ATF-2 at various stages during the repopulation process, whole cell protein extract was combined from four coverslips for each treatment group. Immediately before partial denudation, cells forming the confluent monolayers will be in either G_0 or early G_1 of the cell cycle. Producing multiple denuded areas on a single coverslip dramatically increases the number of cells recruited into the growth responses and amplifies the resultant biochemical signals. Subsequent analysis of changes induced in the phosphorylation state of effector kinases involved in basal or growth factor-stimulated processes will thus reflect those of a large, synchronized cell population as well as from a small, contact-inhibited subpopulation localized to the central regions of the confluent strips.

Termination of the phosphorylation events was achieved by washing the clonal CHO-K1 cell monolayers in ice-cold phosphate-buff-

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ered saline before applying SDS-polyacrylamide gel electrophoresis sample buffer (50 μl of 3× strength) to each test well [1× sample buffer: 4% (w/v) sodium dodecyl sulfate, 5% (v/v) glycerol, 60 mM Tris, and 0.01% (w/v) bromophenol blue; pH 6.8] under reducing conditions (50 mM 2-mercaptoethanol). After solubilization of cellular protein by rapid mixing, the well contents were transferred to a separate tube and combined with two further washings of the well with deionized water (50 μl). Samples were vortexed, centrifuged at 10,000*g* for 2 min, and heated at 95°C for 5 min. Equivalent amounts of protein per sample were electrophoretically resolved on 10% polyacrylamide gels or 15% for the subsequent detection of $p21^{cip1}$.

After electrophoretic transfer onto nitrocellulose (0.22 μm), the membrane was washed briefly in Tris-buffered saline (TBS: 50 mM Tris and 250 mM NaCl; pH 7.5) and saturated overnight in TBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) dried milk. A 1:1000 dilution of the anti-phosphospecific antibodies was used and antibodies recognizing the kinases or transcription factors independent of their phosphorylation state were at a 1:2000 dilution. Anti-$p21^{cip1}$ antibody was at a 1:500 dilution. All primary incubations were for 1 h at 22°C in TBS containing 0.1% (v/v) Tween 20 (TBST) followed by washing five times for 10 min each in TBST. Membranes were incubated for 1 h at 22°C with a 1:3,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% (w/v) dried milk. Excess antibody was removed by washing as described above and immunocomplexes were visualized using enhanced chemiluminescence detection, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). The Western blots shown are representative of at least three separate experiments and each panel is taken from a single immunoblot.

Cell Cycle Analysis. The extent of Rb phosphorylation was measured directly on Western blots from 7.5% gels using specific antibodies supplied by New England Biolabs (at a 1:500 dilution).

Results

Effect of Somatostatin on Proliferation of Chinese Hamster Ovary Cells Recombinantly Expressing Human sst_2, sst_3, or sst_4 Receptors. The total number of CHO-K1 cells that remained on a single coverslip immediately after partial denudation of a confluent monolayer was $131 \pm 4 \times 10^3$, $140 \pm 5 \times 10^3$, and $138 \pm 7 \times 10^3$ for sst_2 ($CHOsst_2$), sst_3 ($CHOsst_3$), and sst_4 ($CHOsst_4$) receptor-expressing cell lines, respectively. After 24 h in the presence of incomplete media, this number had slightly increased for all recombinant lines (Fig. 1), with less than 0.7% of the cells detaching from the coverslip over the time course examined. Application of somatostatin (100 nM), immediately after denudation in the absence of other exogenously administered mitogenic factors, had no significant effect on the number of $CHOsst_2$ (Fig. 1A) or $CHOsst_3$ (Fig. 1B) cells counted 24 h later, compared with basal values. In contrast, somatostatin (100 nM) caused a significant increase in $CHOsst_4$ cell number (Fig. 1C), in agreement with previous observations (Sellers et al., 1999) and which was comparable with that induced by bFGF, using a concentration (10 ng/ml) that produced 80% of its maximal response.

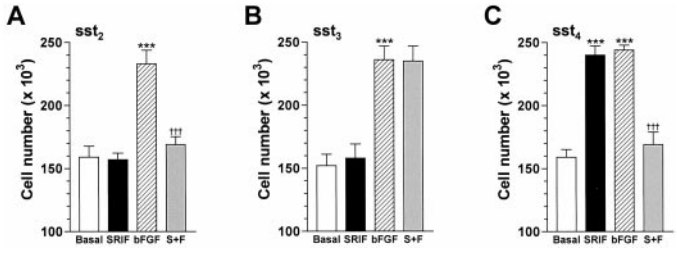

Fig. 1. Effect of somatostatin on cell proliferation in the presence and absence of bFGF in CHO-K1 cells recombinantly expressing human sst_2, sst_3, or sst_4 receptors. The mean number of $CHOsst_2$ (A), $CHOsst_3$ (B), and $CHOsst_4$ (C) cells harvested from a single coverslip after incubation with incomplete media (basal; □), somatostatin (100 nM; SRIF; ■), bFGF (10 ng/ml; ▨), or somatostatin and bFGF in combination (S+F; ▤) determined 24 h after application to partially denuded cell monolayers ($n = 3$). ***, significantly different from basal ($P < 0.001$); †††, significantly different from that incubated in the presence of bFGF alone ($P < 0.001$).

Effect of Somatostatin on bFGF-Induced Proliferation. In $CHOsst_2$ cells, the bFGF-induced (10 ng/ml) proliferative effect (Fig. 1A) was abolished by coapplication with somatostatin (100 nM) to values not significantly different from basal. However, somatostatin (100 nM) at the sst_3 receptor type had no significant effect on the increase in cell number induced by the growth factor (Fig. 1B). Paradoxically, activation of the sst_4 receptor abolished the bFGF-mediated proliferative effect using the same concentration of somatostatin (100 nM) that induced an increase in cell number in the absence of other mitogenic agents (Fig. 1C).

Effect of Pertussis Toxin Pretreatment on Proliferative Responses. Pretreatment with pertussis toxin (18 h at 100 ng/ml) had no significant effect on basal cell numbers determined 24 h after partial denudation of confluent monolayers of the recombinant cell lines (Table 1). $CHOsst_2$ and $CHOsst_3$ cell numbers after incubation with somatostatin (100 nM) were similarly unaffected by pertussis toxin, whereas the increased cell count mediated by sst_4 receptors was abolished by the toxin (Table 1). Pertussis toxin pretreatment had no effect on the proliferation induced by bFGF in all recombinant lines (Table 1) and stimulation of the sst_3 receptor in pertussis toxin-treated cells failed to significantly affect the bFGF-induced increase in cell number. However, the inhibition of bFGF-induced proliferation, mediated by somatostatin at the sst_2 and sst_4 receptor types, was abolished by pertussis toxin pretreatment (Table 1) to values not significantly different from those obtained after treatment with the growth factor alone.

Changes in the Phosphorylation Status of ERK1 and ERK2. Activation of MAP kinase family members during the initial repopulation events after partial denudation of confluent monolayers of the recombinant cell lines was assessed by monitoring changes in the kinase phosphorylation status using Western analysis. A time course of the immunoreactivity detected in whole cell extracts of the clonal lines with the anti-phosphospecific ERK1 and ERK2 antibody over the initial 4 h of basal repopulation and that in the presence of either bFGF (10 ng/ml) or somatostatin (100 nM) is shown in Fig. 2. During this period and irrespective of drug treatment, there was no detectable change in the expression of the ERK kinases examined for any of the recombinant lines, and the immunoreactivity obtained under basal conditions using phosphorylation state-independent pan antibodies is provided for the $CHOsst_2$ cells (Fig. 2A).

Before and immediately after partial denudation, phosphorylated forms of ERK1 and ERK2 were undetectable in all recombinant lines (Fig. 2). Under basal repopulation conditions, ERK1 and ERK2 were similarly activated in a weak and transient manner for all transfected CHO-K1 cells, reaching a maxima at 20 min and falling to undetectable levels by 60 min after denudation (Fig. 2B). In the presence of bFGF (10 ng/ml), the recombinant repopulating cell lines

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showed a marked increase in the phosphorylation status of ERK1 and ERK2, and the time profile of ERK activation for $CHOsst_2$ cells is shown in Fig. 2C. The kinetics of ERK1 and ERK2 phosphorylation in all cell types by the growth factor was similar and seemed biphasic. A transient increase in ERK activation occurred between 10 and 20 min with a second peak of activity after 4 h denudation, although a persistent increase in phosphorylation over basal levels was observed throughout the entire time course for all cell types. Application of somatostatin (100 nM) immediately after partial denudation to $CHOsst_2$, $CHOsst_3$, or $CHOsst_4$ cells also evoked a marked increase in the phosphorylation of both ERK1 and ERK2. However, in contrast to that induced by bFGF treatment, the time profile of ERK activation was monophasic and showed kinetic differences after activation by the various somatostatin receptor types. In $CHOsst_2$ cells, maximal ERK phosphorylation was observed at 10 min, and although this level subsequently declined, that observed at 4 h after denudation was substantially increased over basal (Fig. 2D). Although the onset of ERK1 and ERK2 phosphorylation by activated sst_3 receptors was rapid, the peak of the response was delayed compared with that of $CHOsst_2$ cells and was not observed until 30 min after denudation (Fig. 2E). Again in contrast to that exhibited by both $CHOsst_2$ and $CHOsst_4$ cells, the phosphorylation of ERK1 and ERK2 by sst_3 receptors was transient and had declined to undetectable levels by 2 h after denudation (Fig. 2E). The time profile of ERK activation by sst_4 receptors (Fig. 2F) was similar to that of the sst_2 receptor.

Changes to the Phosphorylation Status of ERK1, ERK2, p38, and ATF-2 in the Presence of bFGF. Because ERK1 and ERK2 can be activated by either somatostatin or bFGF in all recombinant lines, we examined the effect on MAP kinase phosphorylation of the combined drug treatments, mimicking the conditions when somatostatin can exhibit an antiproliferative activity. Whole cell extracts from the recombinant cell lines were analyzed by Western blotting at both 20 and 120 min after denudation to determine the phosphorylation status during the transient and sustained phases of the kinase activity profiles. The expression of p38 was unaffected by any of the treatments in all recombinant cell lines and unchanged between the time points investigated and that detected immediately after denudation (data not shown). Phosphorylated p38 was only just detectable in confluent CHO-K1 cell monolayers and remained consistent in intensity after partial denudation and at all time points investigated throughout the basal repopulation processes (Fig. 3).

Application of both somatostatin (100 nM) and bFGF (10 ng/ml) to partially denuded $CHOsst_2$, $CHOsst_3$, or $CHOsst_4$ cells induced a strong phosphorylation of ERK1 and ERK2 at 20 min after denudation (Fig. 3A), although the intensity of the immunoreactivity detected with the anti-phosphospecific antibody was comparable with that obtained in the presence of somatostatin alone. However, phosphorylation of ERK1 and ERK2 was greater for $CHOsst_2$ and $CHOsst_4$ cells after 120 min of repopulation processes in the presence of somatostatin (100 nM) with bFGF (10 ng/ml) than for cells treated with either agent alone (Fig. 3B). This enhancement of the ERK activity status by the addition of bFGF to somatostatin-treated samples was not evident for $CHOsst_3$ cells at 120 min after denudation (Fig. 3B). Figure 3 also shows that in addition to the distinct transient nature of ERK phosphorylation after sst_3 receptor stimulation, the intensity of ERK activation obtained at 20 min after denudation was lower than that detected for either $CHOsst_2$ and $CHOsst_4$ cells at equivalent protein loading.

Application of bFGF (10 ng/ml) to each of the repopulating cell monolayers failed to induce an increase in the phosphorylation of p38 at either 20 or 120 min after denudation (Fig. 3, A and B). In contrast, phosphorylation of p38 by somatostatin treatment was apparent in both $CHOsst_2$ and $CHOsst_4$ cell lines, but that detected at 20 min after denudation (Fig. 3A) was greater than that observed at 120 min (Fig. 3B). However, the phosphorylation of p38 by somatostatin alone in $CHOsst_2$ or $CHOsst_4$ cells was also enhanced by the presence of the growth factor, particularly during the sustained phase of its activation (Fig. 3B). Activation of sst_3 receptors failed to induce phosphorylation of p38 either in the presence or absence of bFGF for 20 or 120 min (Fig. 3, A and B). In addition, further analysis showed the sst_3 receptor to have no effect on the phosphorylation status of p38 at any time point examined over a 4-h exposure period to somatostatin, in contrast to the sustained activity profile obtained for the sst_2 receptor type (Fig. 3C).

To determine that the time-related phospho-p38 immunoreactivity changes observed in $CHOsst_2$ and $CHOsst_4$ cells could be correlated with an increase in the activity status of this kinase, we examined the effect of somatostatin on the phosphorylation levels of ATF-2, a known substrate for p38 kinase. Activation of this transcription factor requires dual phosphorylation at Thr^{69} and Thr^{71}, enabling subsequent

TABLE 1

Effect of pertussis toxin and the p38 kinase inhibitor on the proliferative response induced by somatostatin and in combination with bFGF in CHO-K1 cells expressing human recombinant sst_2, sst_3, or sst_4 receptors

Immediately after denudation, cell monolayers were incubated in incomplete media (basal), somatostatin (100 nM; SRIF), bFGF (10 ng/ml), or somatostatin and bFGF (SRIF+bFGF) either in the presence and absence of PD 169316 (10 μM) or after an initial pretreatment of confluent monolayers with pertussis toxin (18 h at 100 ng/ml). Values are expressed as the mean cell number ($\times 10^3 \pm$ S.E.M.) harvested from a single coverslip 24 h after partial denudation ($n = 3$)

	Basal	SRIF	bFGF	SRIF+bFGF
$CHOsst_2$	162 ± 6	169 ± 13	248 ± 3[a]	179 ± 11[b]
+Pertussis	157 ± 6	175 ± 10	249 ± 4	**245 ± 2**
+PD 169316	148 ± 3	157 ± 11	234 ± 4	**238 ± 3**
$CHOsst_3$	154 ± 4	152 ± 9	239 ± 5[a]	249 ± 10[a]
+Pertussis	160 ± 1	155 ± 10	237 ± 1	244 ± 5
+PD 169316	145 ± 2	152 ± 8	236 ± 4	237 ± 6
$CHOsst_4$	152 ± 4	238 ± 7[a]	236 ± 16[a]	158 ± 2[b]
+Pertussis	157 ± 10	**160 ± 4**	252 ± 3	**234 ± 7**
+PD 169316	146 ± 5	236 ± 5	236 ± 6	**230 ± 1**

[a] Treatment is significantly different ($P < 0.01$) from basal.
[b] Treatment is significantly different from that in the presence of bFGF alone ($P < 0.01$).
Values highlighted in bold indicate the treatment is significantly different ($P < 0.01$) from that in the absence of pertussis toxin and PD 169316.

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binding to both activator protein-1 and cAMP response element DNA binding domains (Gupta et al., 1995). ATF-2 phosphorylation was only just detectable under basal conditions at 60 min after denudation and was unaffected by the presence of bFGF in all recombinant cell lines (Fig. 3D). However, a slight increase in ATF-2 phosphorylation was evoked by somatostatin at 60 min in $CHOsst_2$ and $CHOsst_4$ cells, but not in $CHOsst_3$ cells (Fig. 3D). The phospho-ATF-2 immunoreactivity obtained with somatostatin was also amplified by the presence of bFGF (Fig. 3D), consistent with the enhanced phosphorylation of p38 in the presence of the growth factor in both $CHOsst_2$ and $CHOsst_4$ cells. In addition, ATF-2 phosphorylation was abolished by the p38 inhibitor PD 169316 (10 μM) in $CHOsst_2$ and $CHOsst_4$ cells (Fig. 3D) but unaffected by the MEK1 inhibitor PD 98059 (1-h pretreatment at 40 μM). Basal levels of ATF-2 phosphorylation (data not shown) and that obtained in the presence of somatostatin in $CHOsst_3$ cells were unaffected by application of either inhibitor (Fig. 3D). The expression levels of ATF-2 were also unaffected by the treatments at the time point investigated and for all the recombinant lines (data not shown).

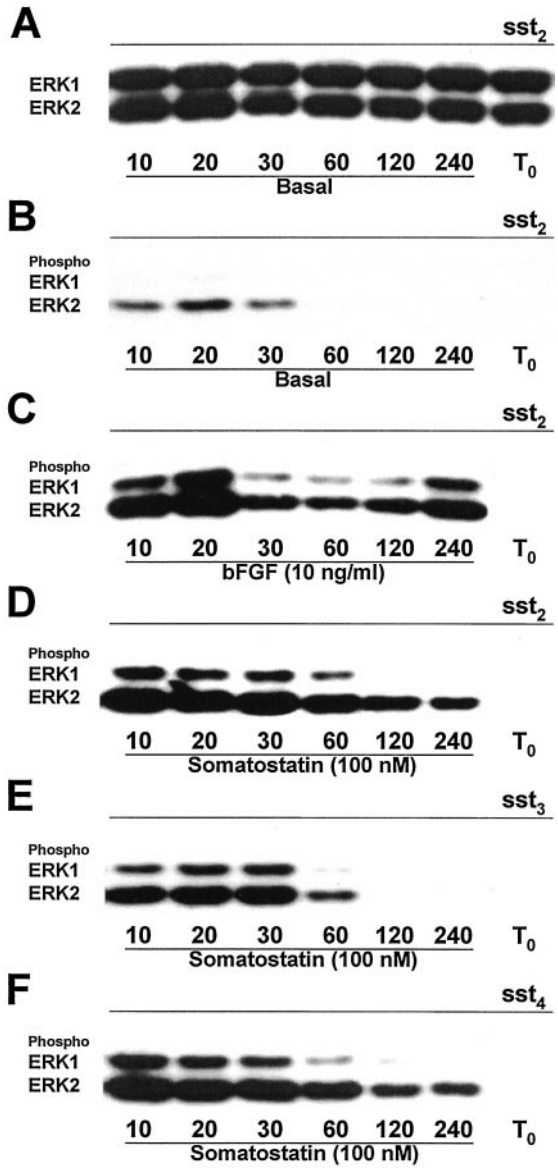

Fig. 2. Changes induced in the phosphorylation status of ERK1 and ERK2 during initial processes in the repopulation of partially denuded monolayers of the recombinant cell lines, as determined by Western analysis. Whole cell extracts were prepared from confluent monolayers immediately after denudation (T_0) and after incubation with incomplete media (basal), bFGF (10 ng/ml), or somatostatin (100 nM) for the times shown in minutes. Consistency of protein loading was substantiated by determining the immunoreactivity of samples with phosphorylation state-independent anti-ERK antibodies as shown in A for $CHOsst_2$ cells. Phosphorylation changes were demonstrated by detection with an antibody to ERK1 and ERK2 that recognizes only the dually phosphorylated and active forms (at Thr^{202} and Tyr^{204}) in $CHOsst_2$ (B–D), $CHOsst_3$ (E), and $CHOsst_4$ (F) cells.

Effect of MEK1 or p38 Inhibition and Pertussis Toxin on the Induced Phosphorylation of ERK1, ERK2, and p38. The effect of pretreatment with the selective MEK1 inhibitor PD 98059 or pertussis toxin on somatostatin-induced phosphorylation of ERK1 and ERK2 either in the presence or absence of bFGF was examined. At 20 min after denudation, ERK activation in response to either somatostatin (100 nM) or bFGF (10 ng/ml) was partially reduced by PD 98059 (1-h pretreatment at 40 μM) in each of the recombinant cell lines (data not shown). By 60 min after denudation, the induced phosphorylation of ERK1 and ERK2 by either drug alone or in combination was abolished by PD 98059 pretreatment in all three cell lines (Fig. 4). The sustained activity of ERK detectable in $CHOsst_2$ and $CHOsst_4$ cell lines after 120 min in the presence of somatostatin or bFGF was also abolished by the MEK1 inhibitor, as was the enhanced phosphorylation observed after treatment with the drugs in combination (data not shown). Pretreatment with pertussis toxin (18 h at 100 ng/ml) had no effect on bFGF-induced phosphorylation of ERK1 and ERK2 at 20, 60 (Fig. 4), or 120 min after denudation in any of the recombinant cell lines. In contrast, ERK activation induced by somatostatin (100 nM) at all three receptor types was abolished by pretreatment with the toxin at the equivalent time points (Fig. 4). In addition, the additive effect of somatostatin and bFGF on the sustained ERK phosphorylation induced in $CHOsst_2$ (Fig. 4A) and $CHOsst_4$ (Fig. 4C) cells was also abolished by pertussis toxin, resulting in levels comparable with those obtained in the presence of bFGF alone. In contrast, the p38 inhibitor PD 169316 (10 μM) had no significant effect on either basal levels of ERK phosphorylation or on that induced by somatostatin for 60 min in any of the recombinant cell lines (Fig. 4). Treatment with the kinase inhibitors or pertussis toxin had no effect on the expression levels of ERK1 and ERK2 at the time points investigated (data not shown). Pertussis toxin pretreatment abolished the induced phosphorylation of p38 after incubation for 60 min with somatostatin in $CHOsst_2$ and $CHOsst_4$ cells (Fig. 4D). Pertussis toxin had no effect on the low levels of basal p38 phosphorylation (Fig. 4D) or on the expression level of the kinase in any of the recombinant lines (data not shown).

Induction of $p21^{cip1}$ by Somatostatin and the Effect of the MEK1 Inhibitor or Pertussis Toxin. To determine whether activation of the individual somatostatin receptor types could regulate the accumulation of the cell cycle inhibitor $p21^{cip1}$, whole cell protein extract from each of the recombinant cell lines was analyzed, 24 h after partial denudation of confluent monolayers. Western analysis of equivalent protein loadings showed incubation in the combined presence of somatostatin (100 nM) and bFGF (10 ng/ml) to induce the accumulation of $p21^{cip1}$ in $CHOsst_2$ (Fig.

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5A) and CHOsst$_4$ (Fig. 5C) cells but not in CHOsst$_3$ (Fig. 5B) cells. Treatment with somatostatin or bFGF alone had no effect on the basal expression level of p21^{cip1} in any of the cell types at the time point investigated.

Pretreatment with pertussis toxin (18 h at 100 ng/ml) or PD 98059 (1 h at 40 μM) immediately before partial denudation of confluent monolayers had no apparent effect on the basal level of p21^{cip1} expression, detected 24 h later either in the presence or absence of somatostatin or bFGF in each of the repopulating recombinant cell lines (Fig. 5). However, the increased accumulation of the inhibitor protein by somatostatin and bFGF in combination in both CHOsst$_2$ (Fig. 5A) and CHOsst$_4$ (Fig. 5C) cells was reduced by either pertussis toxin or PD 98059 to levels similar to those obtained under basal conditions. Pretreatment with either pertussis toxin or PD 98059 had no effect on p21^{cip1} accumulation in CHOsst$_3$ cells incubated with both somatostatin and bFGF (Fig. 5B).

Effect of the p38 Inhibitor on p21^{cip1} Induction, Rb Phosphorylation, and Antiproliferative Activity of Somatostatin Receptors. The specific inhibitor of p38 kinase, PD 169316 (10 μM), had no significant effect on basal cell numbers obtained 24 h after partial denudation of CHOsst$_2$ or CHOsst$_3$ cells either in the presence or absence of somatostatin (100 nM) (Table 1). In CHOsst$_4$ cells, the increase in

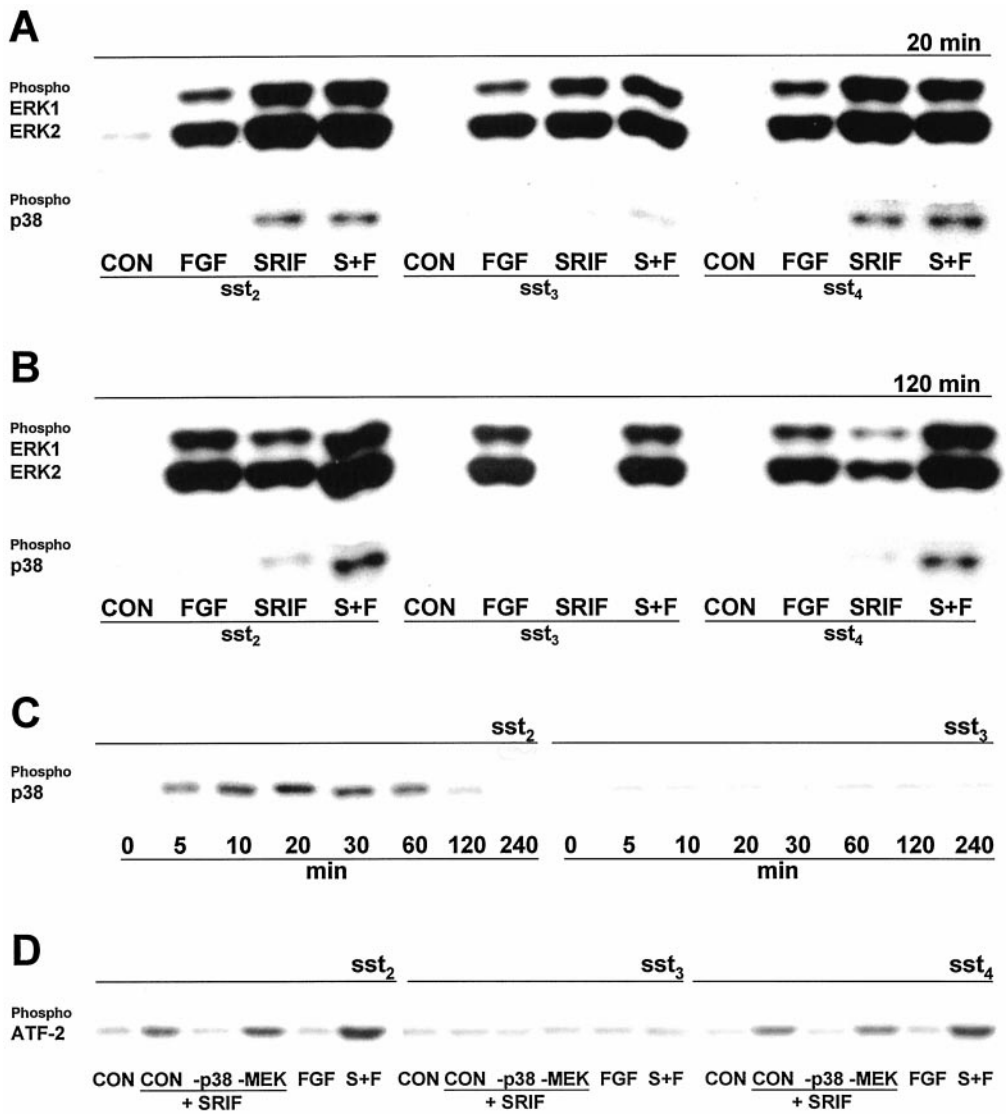

Fig. 3. Comparison of the changes induced by somatostatin and bFGF in the phosphorylation status of ERK1, ERK2, p38, and ATF-2 at various intervals during initial processes in the repopulation of partially denuded monolayers of CHOsst$_2$, CHOsst$_3$, and CHOsst$_4$ cells. Analysis at 20 (A) and 120 (B) min after partial denudation of confluent monolayers was determined by Western detection using antibodies recognizing the active forms of ERK1, ERK2, and p38. Activation of p38 was assessed using an antibody specific for the doubly phosphorylated form at residues Thr^{180} and Tyr^{182} within the TGY sequence. Whole cell protein extracts were prepared from partially denuded monolayers incubated in the presence of incomplete media (CON), bFGF (10 ng/ml; FGF), somatostatin (100 nM; SRIF), or somatostatin in the presence of bFGF (S+F). Equivalent amounts of protein were analyzed for the three cell types and each panel has been taken from a single immunoblot. C, kinetic analysis of the change in p38 phosphorylation over a 4-h time course immediately after partial denudation (shown in minutes) in the presence of somatostatin (100 nM) for both CHOsst$_2$ and CHOsst$_3$ cells. D, changes in the phosphorylation of the transcription factor ATF-2 in CHOsst$_2$, CHOsst$_3$, and CHOsst$_4$ cells, incubated for 60 min after partial denudation in the presence of incomplete media (CON), bFGF (10 ng/ml; FGF), somatostatin (100 nM; SRIF), somatostatin in the presence of bFGF (S+F), somatostatin in the presence of the p38 inhibitor PD 169316 (10 μM; -p38), or somatostatin in the presence of the MEK1 inhibitor PD 98059 (1-h preincubation at 40 μM; -MEK). Immunoreactivity was detected by Western analysis using the phosphospecific ATF-2 antibody, and each panel has been taken from a single immunoblot.

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proliferation elicited by somatostatin (100 nM) was unaffected by coincubation with PD 169316 and basal values were similarly unaffected (Table 1). The p38 inhibitor also had no significant effect on the increase in cell number induced by bFGF (10 ng/ml) in any of the recombinant cell lines (Table 1). However, both the sst_2 and the sst_4 receptor-mediated inhibition of the bFGF-induced proliferative effect was abolished by the p38 inhibitor (Table 1). Cell numbers determined after incubation of $CHOsst_3$ cells in the presence of bFGF and somatostatin with PD 169316 were not significantly different from values obtained for treatment with bFGF alone (Table 1).

The induction of $p21^{cip1}$ by the combined effect of somatostatin (100 nM) and bFGF (10 ng/ml) in $CHOsst_2$ and $CHOsst_4$ cells, 24 h after application to partially denuded monolayers, was abolished by treatment with the p38 inhibitor (10 μM) (Fig. 6A). However, the basal level of $p21^{cip1}$ expression as detected by Western analysis in cells allowed to repopulate in the presence of incomplete media, somatostatin, or bFGF was also slightly reduced by the presence of PD 169316 in all cell lines (Fig. 6A).

Cell cycle arrest at G_1/S has been attributed to increased levels of $p27^{Kip1}$ and $p21^{cip1}$, which inhibit the activity of the cyclin-dependent kinases (cdks), thus preventing cell cycle progression (Sherr and Roberts, 1995). The cell cycle gate in late G_1 marks the end of a requirement for external growth factor stimulation and correlates with the phosphorylation of Rb by cdks. We thus determined whether the up-regulation of $p21^{cip1}$ by somatostatin in the presence of bFGF was sufficient for cycle arrest in late G_1, by monitoring changes in the phosphorylation status of Rb in the sst_2, sst_3, and sst_4 receptor-expressing cell lines. There was a marked increase over basal levels of Rb phosphorylation after 24 h in the presence of bFGF (10 ng/ml) in all three recombinant cell lines (Fig. 6B). Somatostatin (100 nM) also induced Rb phosphorylation in $CHOsst_4$ cells, but was without effect in $CHOsst_2$ and $CHOsst_3$ cells. Application of somatostatin and bFGF in combination inhibited the bFGF-mediated phosphorylation of Rb in $CHOsst_2$ and $CHOsst_4$ cells but not in $CHOsst_3$ cells. In $CHOsst_4$ cells treated with PD 169316 (10 μM), Rb phosphorylation induced by somatostatin or bFGF was unaffected, whereas the reduced level of phosphorylated Rb observed after treatment with these drugs in combination was antagonized by the p38 inhibitor (Fig. 6B). A similar effect of PD 169316 on Rb phosphorylation was observed in $CHOsst_2$ cells treated with somatostatin in the presence of bFGF (data not shown).

Effect of Somatostatin on Tyrosine Phosphorylation of STAT3 in the Recombinant Cell Lines. Tyrosine phosphorylation of STAT3 is required for dimerization of the transcription factor and subsequent binding to DNA (Darnell et al., 1994). Western analysis of $CHOsst_2$, $CHOsst_3$, and $CHOsst_4$ cells incubated for 10 min after partial denudation in the presence of incomplete media or somatostatin (100 nM) showed similar levels of STAT3 protein expression (Fig. 7A). Regeneration in the presence of somatostatin resulted in a marked increase in the tyrosine phosphorylation of STAT3 compared with basal levels for $CHOsst_4$ cells but had no effect in $CHOsst_2$ or $CHOsst_3$ cells (Fig. 7B). Multiple products with discrete electrophoretic mobilities were detected by the anti-phosphospecific antibody, although the increased immunoreactivity observed after sst_4 receptor activation was primarily of the band with the greatest mobility.

Discussion

Considerable attention is currently being focused on the role played by MAP kinase cascades in the control of cell

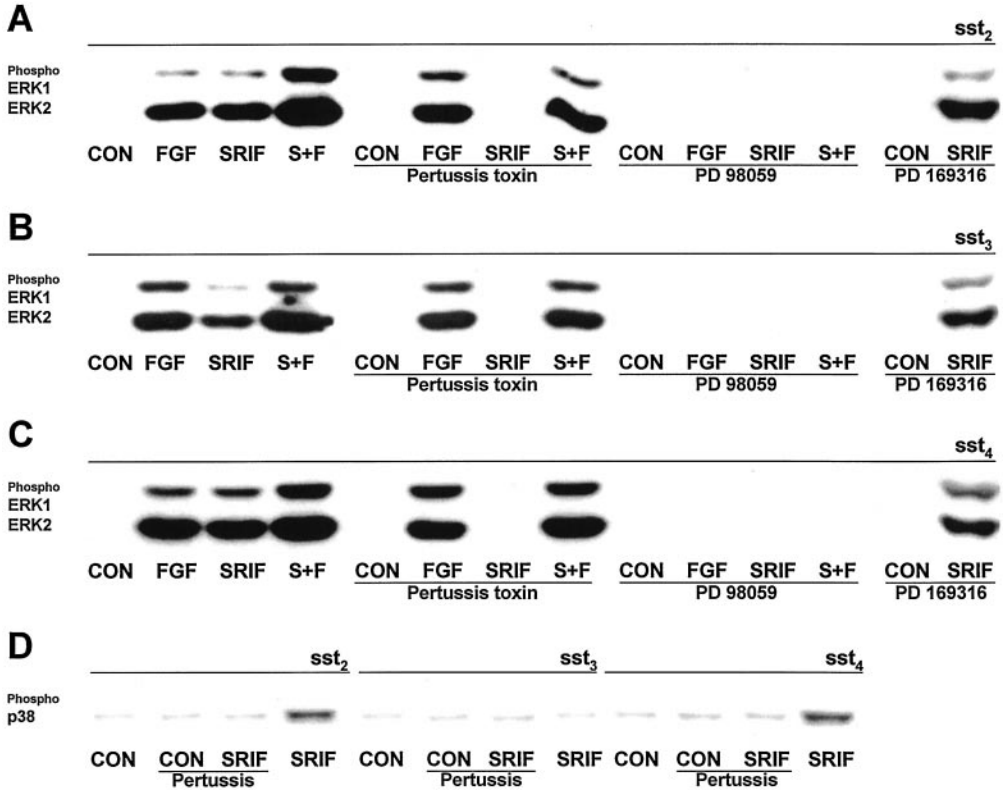

Fig. 4. Effect of pertussis toxin, PD 98059, and PD 169316 on the phosphorylation status of ERK1, ERK2, and p38 induced by somatostatin. Confluent monolayers of $CHOsst_2$ (A), $CHOsst_3$ (B), and $CHOsst_4$ (C) cells were treated with pertussis toxin (18 h at 100 ng/ml), PD 98059 (1 h at 40 μM), or PD 169316 (10 μM) immediately before partial denudation and in the presence of incomplete media (CON), bFGF (10 ng/ml; FGF), somatostatin (100 nM; SRIF), or somatostatin in the presence of bFGF (S+F) for 60 min after partial denudation. Detection was made by Western analysis of whole cell extract using phosphospecific ERK1 and ERK2 antibodies (A–C) or p38 antibodies (D).

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survival or programmed death mechanisms. Several G protein-coupled receptors, including the human somatostatin sst_1 (Florio et al., 1999) and sst_4 (Sellers, 1999) receptor types have been shown to stimulate the ERK pathway, and in this report we demonstrate that the human sst_2 and sst_3 receptors similarly activate this cascade through a pertussis toxin-sensitive mechanism. However, no correlation could be made with the activation of the ERK pathway by the somatostatin receptor types and the proliferative outcome, a finding that is in accord with the role of ERK in the signaling pathways of both mitogenic and antimitogenic agents (Cospedal et al., 1999; Wang et al., 2000). ERK phosphorylation of similar intensity and with identical kinetics was obtained after sst_2 and sst_4 receptor activation, although only the latter receptor induced a proliferative response. In contrast, the sst_2 and sst_3 receptors both lacked mitogenic activity, but showed differences in both the maxima as well as the duration of the induced ERK phosphorylation.

One aspect that is fundamental to the resultant cellular effect induced by the activation of the ERK cascade will be which transcription factors are present and whether they are restricted in localization to the nucleus. In every case examined thus far, sustained ERK activation is associated with translocation of the kinases to the nucleus (Dikic et al., 1994; Traverse et al., 1994). Transient activation, which does not induce cytoplasmic-nuclear migration, will therefore have very different consequences for gene expression compared with that of sustained ERK activity. However, because both sst_2 and sst_4 receptor types induce prolonged activation of ERK, it is unlikely that differential activation of nuclear-located transcription factors can account for their contrasting effects on cell proliferation. Stimulation of sst_4 receptors has been shown to phosphorylate the transcription factor STAT3, which is present in a latent form in the cytoplasm and becomes phosphorylated on a single tyrosine, obligatory for STAT activation (Darnell et al., 1994). Phosphorylation of STAT3 and prolonged activation of ERK have both been shown to be critical for the proliferative activity of sst_4 receptors (Sellers et al., 1999). In this study, the sst_2 receptor had no effect on the tyrosine phosphorylation status of STAT3, which may account for the lack of proliferative activity exhibited by this receptor type despite its ability to induce sustained ERK phosphorylation. In addition, the sst_3 receptor, which also has no proliferative activity, similarly failed to phosphorylate STAT3.

Whereas the pathway linking cell surface receptors to ERKs has been partially elucidated (Widmann et al., 1999), the mechanism of activation of p38 and SAPKs is poorly understood. This is particularly so for members of the G protein-coupled receptor family, which have only recently been shown to use these alternative MAP kinase cascades for transduction purposes. Activation of p38 (Yamauchi et al., 1997) and SAPKs (Coso et al., 1996) has been demonstrated after stimulation of the G_q/G_{11}-coupled m_1 and G_i-coupled m_2 muscarinic acetylcholine receptors and a role for p38 in directly controlling cell growth has recently been demonstrated for the rat somatostatin $sst_{2(a)}$ receptor splice variant (Sellers et al., 2000). In the current study we show the antiproliferative effect of both the human sst_2 and sst_4 receptors as well as the induction of the cell cycle inhibitor $p21^{cip1}$ to be depen-

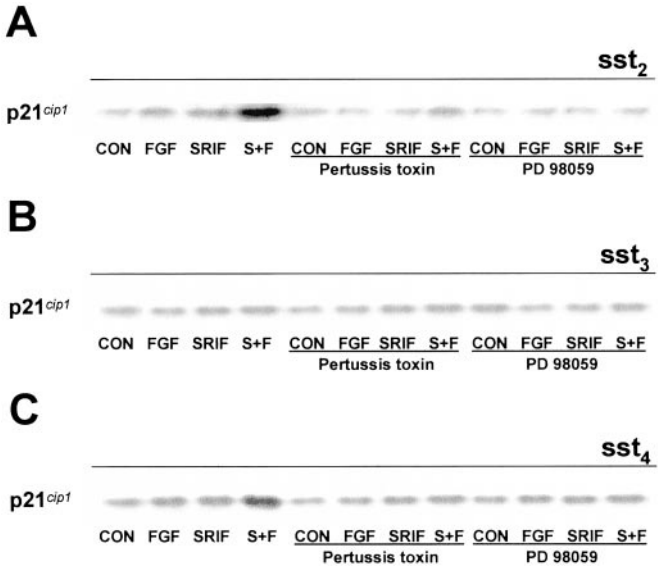

Fig. 5. Induction of the cell cycle inhibitor $p21^{cip1}$ after activation of human recombinant sst_2 (A), sst_3 (B), and sst_4 (C) receptors and the effect of pertussis toxin and PD 98059 pretreatments. Immediately after denudation, cell monolayers were incubated in the presence of incomplete media (CON), bFGF (10 ng/ml; FGF), somatostatin (100 nM; SRIF), or somatostatin and bFGF (S+F) with and without an initial pretreatment of confluent monolayers with pertussis toxin (18 h at 100 ng/ml) or PD 98059 (1 h at 40 μM). Whole cell protein extracts were prepared 24 h after partial denudation and analyzed by an anti-$p21^{cip1}$ antibody. Western detection was also made with an anti-β actin antibody to demonstrate consistency of protein loading (data not shown).

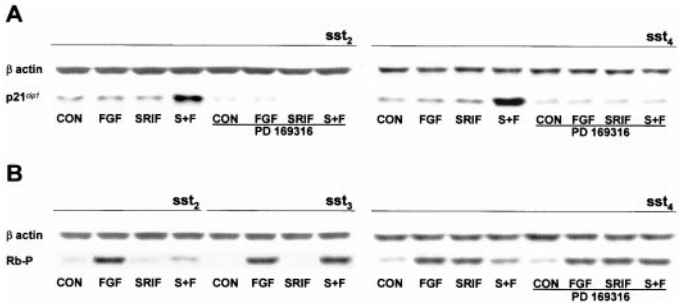

Fig. 6. Effect of the p38 kinase inhibitor on the induction of the cell cycle inhibitor $p21^{cip1}$, and phosphorylation of Rb. Immediately after denudation, cell monolayers were incubated with incomplete media (CON), bFGF (10 ng/ml; FGF), somatostatin (100 nM; SRIF), or somatostatin and bFGF (S+F) either in the presence or absence of PD 169316 (10 μM). Whole cell protein extracts were prepared 24 h later from human recombinant sst_2 and sst_4 receptors and analyzed by Western blotting using an anti-$p21^{cip1}$ antibody (A) or from all three somatostatin receptor-expressing cells analyzed by phosphospecific Rb antibodies (B). Detection was also made with an anti-β actin antibody to demonstrate consistency of protein loading.

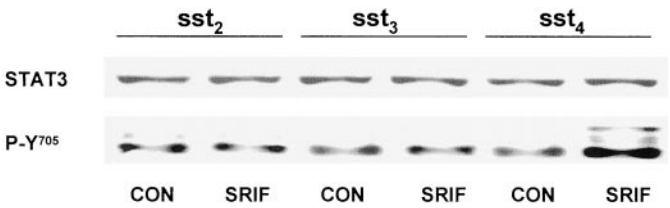

Fig. 7. Changes in the phosphorylation status at tyrosine-705 of STAT3 during initial events in the repopulation of $CHOsst_2$, $CHOsst_3$, or $CHOsst_4$ cells. Whole cell extracts were prepared from partially denuded confluent monolayers allowed to recover for 10 min in the presence of incomplete media (CON) or somatostatin (SRIF; 100 nM). Top, Western analysis using the anti-STAT3 antibody; bottom, the immunoreactivity obtained with the antibody recognizing the phosphorylated form ($P\text{-}Y^{705}$).

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dent on p38 activation, which was blocked by pertussis toxin. ERK activation of G_i-coupled somatostatin receptors is dependent on $\beta\gamma$-release (Sellers, 1999) and it may be that the p38 kinase cascade is similarly activated, consistent with the demonstration that overexpression of $G\beta\gamma$ can stimulate p38 activity in human embryonic kidney 293 cells (Yamauchi et al., 1997) and expression of transducin can inhibit the p38-dependent antiproliferative activity of the rat $sst_{2(a)}$ receptor (Sellers et al., 2000). The pertussis toxin sensitivity of p38 phosphorylation in the current study is also in agreement with the known coupling of G_i proteins to the rat sst_2 receptor homolog (Sellers et al., 2000).

The proliferative effect of both sst_4 and bFGF receptors was unaffected after inhibition of p38 MAP kinase, suggesting that this kinase cascade is not involved in the mitogenic effects observed in this study. There are few reports demonstrating p38 activation through bFGF receptors, although it has been implicated in bFGF-mediated tube formation by endothelial cells (Tanaka et al., 1999) and in bFGF-induced interleukin-6 synthesis in osteoblasts (Kozawa et al., 1999). Here we demonstrate that bFGF, despite having no effect on p38 phosphorylation in CHO-K1 cells, can enhance the phosphorylation of p38 and its substrate ATF-2 after stimulation of either the sst_2 or the sst_4 receptor types. The abolition of the somatostatin-induced ATF-2 phosphorylation by PD 169316 confirms that p38 activity is increased after sst_2 and sst_4 receptor stimulation. In addition, the antiproliferative function of both somatostatin receptors against bFGF-induced growth was shown to be critically dependent on this kinase activity. The inability of the sst_3 receptor to phosphorylate p38 either in the presence or absence of bFGF, may account for its lack of antiproliferative activity.

As well as prolonging and amplifying the activity of p38 by the concomitant effect of somatostatin and the growth factor in $CHOsst_2$ and $CHOsst_4$ cells, an enhanced phosphorylation of ERK during the sustained phase of its activity profile was also observed. In contrast, the transient ERK activation by sst_3 receptor types was unaffected by the presence of the growth factor. Because bFGF, sst_2, and sst_4 receptors have the capacity to stimulate a prolonged activation of ERK1 and ERK2, the amplification of this signal as observed in the presence of somatostatin and the growth factor was perhaps expected. However, the mechanism by which bFGF increases the intensity of somatostatin-induced p38 phosphorylation is unclear. It is possible that bFGF in addition to activating ERK1 and ERK2 may additionally inhibit members of the dual-specificity phosphatase family, which reverse MAP kinase activities, enabling high-intensity signals to be observed for both p38 and ERK in the presence of somatostatin. Taken together, these data demonstrate that a complex interplay exists in the transduction cascades activated by distinct receptor types.

A further example of the influence of stimulating two receptor types on the net activity of a particular signaling pathway was also demonstrated in this study for the induction of the cell cycle inhibitor $p21^{cip1}$. The increased accumulation of $p21^{cip1}$ required a sustained activation of both p38 and ERK with a critical signal strength that was provided in this system by the cooperative effects of both the growth factor and sst_2 or sst_4 receptor activities. The importance of p38 activity in mediating the induction of $p21^{cip1}$ was further supported by the lack of effect on the expression of this protein by activated sst_3 receptors. The role of ATF-2 in regulating the accumulation of $p21^{cip1}$ remains to be determined, although it should be noted that in contrast to the abolition of the induced accumulation of the cell cycle inhibitor by PD 98059, ATF-2 phosphorylation was unaffected. In addition to the sustained activity of p38, ERK is also necessary for the increased accumulation of $p21^{cip1}$ by sst_2 and sst_4 receptors. Although PD 98059 blocked the induction of $p21^{cip1}$ by bFGF and somatostatin in $CHOsst_2$ and $CHOsst_4$ cells, it only partially inhibited the transient phase of ERK phosphorylation and abolished the sustained phase. This is in accord with previous reports showing that amplification of the ERK cascade is necessary for increased accumulation of this cell cycle inhibitor and growth arrest (Sewing et al., 1997) and consistent with observations from this study that the duration of ERK activation is also critical for $p21^{cip1}$ induction.

Further support for the involvement of the p38 cascade in mediating the antiproliferative effect of somatostatin was demonstrated by determining changes in the phosphorylation of the retinoblastoma tumor suppressor protein Rb. Phosphorylation of Rb by cyclin–cyclin-dependent kinase complexes prevents binding to various regulatory target proteins such as the E2F family of transcription factors and regulates cell proliferation by controlling progression through the restriction point within the G_1 phase of the cell cycle (Sherr and Roberts, 1995). The ability of bFGF to induce Rb phosphorylation is in agreement with other studies demonstrating that various mitogens that can sustain ERK1 and ERK2 activation can also inactivate Rb (Lavoie et al., 1996). Activation of sst_2 or sst_4 receptor types, however, inhibited Rb phosphorylation by the growth factor, which was blocked by the p38 kinase inhibitor. The p38 kinase cascade has also been shown to inhibit the expression of cyclin D1, required for cdk4 activity and progression through the restriction point (Lavoie et al., 1996). Therefore, in the present study, it is possible that cells are arrested in late G_1 before Rb phosphorylation by a combined effect of the reduction of cyclin D1 proteins, which could lead to a redistribution of sequestered $p21^{cip1}$ to cdk2 complexes, together with the increased total levels of $p21^{cip1}$. The expression of $p21^{cip1}$ is transcriptionally regulated by p53 and its function is critical for p53-dependent G_1 growth arrest (Kachnic et al., 1999). The p53 gene is mutated in approximately half of all human cancers (Ullrich et al., 1993) and it is possible that activation of somatostatin receptors in certain tumors may not result in the induction of this potent antiproliferative activity. This could perhaps explain the poor effects of somatostatin analogs observed in the clinical setting, to effectively treat the growth of some cancers cells (Macaulay et al., 1991). However, the loss of an antiproliferative activity in certain cancers such as colorectal and pancreatic adenocarcinomas has been show to correlate with the decreased expression of sst_2 receptor types (Benali et al., 2000).

There is an increasing number of examples in the literature where the functional outcome in response to a mitogenic agent is not only determined by the strength but also the duration of the stimulus and small differences in signal input can generate large differences in transcriptional response. Such quantitative variations can control physiological decisions and has been demonstrated in this study by the switch from a proliferative to an antiproliferative activity, as observed for the sst_4 receptor. This seems to be caused by coupling to transduction cascades that culminate in the ac-

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tivation of STAT3, a sustained activation of ERK1 and ERK2, and a weak activation of p38. However, with an additional signal from bFGF that amplifies and prolongs the MAP kinase cascades, induction of $p21^{cip1}$ is increased and an antiproliferative activity results, demonstrating the importance of defining the functional outcome in the context of the activity status of the cell.

We show in this study that the duration of the p38 MAP kinase cascade, as has been well documented for ERK activation (Marshall, 1995), is also critical for dictating functional responses. The contribution of several input signals, such as that from bFGF and somatostatin receptors, can generate large differences in the duration of the p38 MAP kinase activity and the subsequent regulation of transcriptional events such as ATF-2 phosphorylation and protein expression. The induction of $p21^{cip1}$, for example, requires activation of both the p38 and ERK cascades mediated by the interplay of bFGF and sst_2 or sst_4 receptors. The dependence on p38 for $p21^{cip1}$ expression also suggests that p38 activity may have a dual role in not only mediating apoptotic processes but also inhibiting cell proliferation. This is analogous to that of ERK activation, which can promote mitogenesis and provide protection against apoptosis (Berra et al., 1997).

References

Alderton F, Lauder H, Feniuk W, Fan T-P and Humphrey PPA (1998) Differential effects of somatostatin and angiopeptin on cell proliferation. *Br J Pharmacol* **124:**323–330.

Benali N, Ferjoux G, Puente E, Buscail L and Susini C (2000) Somatostatin receptors. *Digestion* **62(Suppl 1):**27–32.

Berra E, Municio MM, Sanz L, Frutos S, Diaz-Meco MT and Moscat J (1997) Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. *Mol Cell Biol* **17:**4346–4354.

Brevini TAL, Bianchi R and Motta M (1993) Direct inhibitory effect of somatostatin on the growth of the human prostatic cancer cell line LNCaP: possible mechanism of action. *J Clin Endocrinol Metab* **77:**626–631.

Canman CE and Kastan MB (1996) Signal transduction. Three paths to stress relief. *Nature (Lond)* **384:**213–214.

Coso OA, Teramoto H, Simonds WF and Gutkind JS (1996) Signaling from G protein-coupled receptors to c-Jun kinase involves $\beta\gamma$ subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J Biol Chem* **271:**3963–3966.

Cospedal R, Lobo M and Zachary I (1999) Differential regulation of extracellular signal-regulated protein kinases (ERKs) 1 and 2 by cAMP and dissociation of ERK inhibition from anti-mitogenic effects in rabbit vascular smooth muscle cells. *Biochem J* **342:**407–414.

Darnell JE Jr, Kerr IM and Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (Wash DC)* **264:**1415–1421.

Daub H, Wallasch C, Lankenau A, Herrlich A and Ullrich A (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J* **16:**7032–7044.

Dikic I, Schlessinger J and Lax I (1994) PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. *Curr Biol* **4:**702–708.

Dikic I, Tokiwa G, Lev S, Courtneidge SA and Schlessinger J (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature (Lond)* **383:**547–550.

Fanger GR, Gerwins P, Widmann C, Jarpe MB and Johnson GL (1997) MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev* **7:**67–74.

Florio T, Yao H, Carey KD, Dillon TJ and Stork PJS (1999) Somatostatin activation of mitogen-activated protein kinase via somatostatin receptor 1 (SSTR1). *Mol Endocrinol* **13:**24–37.

Gupta S, Campbell D, Derijard B and Davis RJ (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science (Wash DC)* **267:**389–393.

Ishizuka J, Beauchamp RD, Evers BM, Townsend CM Jr and Thompson JC (1992) Unexpected growth-stimulatory effect of somatostatin analogue on cultured human pancreatic carcinoid cells. *Biochem Biophys Res Commun* **185:**577–581.

Kachnic LA, Wu B, Wunsch H, Mekeel KL, DeFrank JS, Tang W and Powell SN (1999) The ability of p53 to activate downstream genes p21(WAF1/cip1) and MDM2, and cell cycle arrest following DNA damage is delayed and attenuated in scid cells deficient in the DNA-dependent protein kinase. *J Biol Chem* **274:**13111–13117.

Kamiya Y, Ohmura E, Arai M, Fujii T, Hayakawa F, Ito J, Kawaguchi M, Tsushima T and Sakuma N (1993) Effect of somatostatin and its analogue on proliferation of human epidermoid carcinoma cells in vitro. *Biochem Biophys Res Commun* **191:**302–307.

Kozawa O, Tokuda H, Matsuno H and Uematsu T (1999) Involvement of p38 mitogen-activated protein kinase in basic fibroblast growth factor-induced interleukin-6 synthesis in osteoblasts. *J Cell Biochem* **74:**479–485.

Kyriakis JM and Avruch J (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J Biol Chem* **271:**24313–24316.

Lavoie JN, L'Allemain G, Brunet A, Müller R and Pouysségur J (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271:**20608–20616.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ and Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor- and $G\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271:**19443–19450.

Macaulay VM, Smith IE, Everard MJ, Teale JD, Reubi JC and Millar JL (1991) Experimental and clinical studies with somatostatin analogue octreotide in small cell lung cancer. *Br J Cancer* **64:**451–456.

Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80:**179–185.

Pagliacci MC, Tognellini R, Grignani F and Nicoletti I (1991) Inhibition of human breast cancer cell (MCF-7) growth *in vitro* by the somatostatin analog SMS 201–995: effects on cell cycle parameters and apoptotic cell death. *Endocrinology* **129:**2555–2562.

Ruiz-Torres P, Lucio FJ, González-Rubio M, Rodríguez-Puyol M and Rodríguez-Puyol D (1993) A dual effect of somatostatin on the proliferation of cultured rat mesangial cells. *Biochem Biophys Res Commun* **195:**1057–1062.

Sellers LA (1999) 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. *J Biol Chem* **274:**24280–24288.

Sellers LA, Alderton F, Carruthers AM, Schindler M and Humphrey PPA (2000) Receptor isoforms mediate opposing proliferative effects through $G\beta\gamma$-activated p38 or Akt pathways. *Mol Cell Biol* **20:**5974–5985.

Sellers LA, Feniuk W, Humphrey PPA and Lauder H (1999) Activated G protein-coupled receptor induces tyrosine phosphorylation of STAT3 and agonist-selective serine phosphorylation via sustained stimulation of mitogen-activated protein kinase. Resultant effects on cell proliferation. *J Biol Chem* **274:**16423–16430.

Sewing A, Wiseman B, Lloyd AC and Land H (1997) High-intensity Raf signal causes cell cycle arrest mediated by $p21^{Cip1}$. *Mol Cell Biol* **17:**5588–5597.

Sherr CJ and Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* **9:**1149–1163.

Tanaka K, Abe M and Sato Y (1999) Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis. *Jpn J Cancer Res* **90:**647–654.

Traverse S, Seedorf K, Paterson H, Marshall CJ, Cohen P and Ullrich A (1994) EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr Biol* **4:**694–701.

Ullrich SJ, Sakaguchi K, Lees-Miller SP, Fiscella M, Mercer WE, Anderson CW and Appella E (1993) Phosphorylation at Ser-15 and Ser-392 in mutant p53 molecules from human tumors is altered compared to wild-type p53. *Proc Natl Acad Sci USA* **90:**5954–5958.

van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM and Lefkowitz RJ (1995) Receptor-tyrosine-kinase- and $G\beta\gamma$-mediated MAP kinase activation by a common signaling pathway. *Nature (Lond)* **376:**781–784.

Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, et al. (1996) Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. *Nature (Lond)* **380:**75–79.

Wang QM, Studzinski GP, Chen F, Coffman FD and Harrison LE (2000) $p53/56^{lyn}$ antisense shifts the 1,25-dihydroxyvitamin D3-induced G1/S block in HL60 cells to S phase. *J Cell Physiol* **183:**238–246.

Widmann C, Gibson S, Jarpe MB and Johnson GL (1999) Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* **79:**143–180.

Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science (Wash DC)* **270:**1326–1331.

Yamauchi J, Nagao M, Kaziro Y and Itoh H (1997) Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of $G\beta\gamma$ and $G_{\alpha q/11}$ subunits. *J Biol Chem* **272:**27771–27777.

Yang X, Khosravi-Far R, Chang HY and Baltimore D (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell* **89:**1067–1076.

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Correction to "High-intensity p38 kinase activity is critical for p21^{cip1} induction and the antiproliferative function of G_i protein-coupled receptors"

In the above article [Alderton F, Humphrey PPA and Sellers LA (2001) *Mol Pharmacol* **59:**1119–1128], the received and accepted dates were reversed. The correct dates are:

Received September 7, 2000; Accepted January 22, 2001.

We regret this error and apologize for any confusion or inconvenience it may have caused.

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