sst2 Somatostatin Receptor Mediates Cell Cycle Arrest and Induction of p27Kip1

EVIDENCE FOR THE ROLE OF SHP-1*

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Activation of the somatostatin receptor sst2 inhibits cell proliferation by a mechanism involving the stimulation of the protein-tyrosine phosphatase SHP-1. The cell cycle regulatory events leading to sst2-mediated growth arrest are not known. Here, we report that treatment of Chinese hamster ovary cells expressing sst2 with the somatostatin analogue, RC-160, led to G1 cell cycle arrest and inhibition of insulin-induced S-phase entry through induction of the cyclin-dependent kinase inhibitor p27Kip1. Consequently, a decrease of p27Kip1, cdk2 association, an inhibition of insulin-induced cyclin E-cdk2 kinase activity, and an accumulation of hypophosphorylated retinoblastoma gene product (Rb) were observed. However, RC-160 had no effect on the p21Waf1/Cip1. When sst2 was coexpressed with a catalytically inactive mutant SHP-1 in Chinese hamster ovary cells, mutant SHP-1 induced entry into cell cycle and down-regulation of p27Kip1 and prevented modulation by insulin and RC-160 of p27Kip1 expression, p27Kip1, cdk2 association, cyclin E-cdk2 kinase activity, and the phosphorylation state of Rb. In mouse pancreatic acini, RC-160 reverted down-regulation of p27Kip1 induced by a mitogen, and this effect did not occur in acini from viable motheaten (me+/me−) mice expressing a mutant SHP-1 with markedly deficient enzymes. These findings provide the first evidence that sst2 induces cell cycle arrest through the up-regulation of p27Kip1 and demonstrate that SHP-1 is required for maintaining high inhibitory levels of p27Kip1 and is a critical target of the insulin, and somatostatin signaling cascade, leading to the modulation of p27Kip1.

Somatostatin is a widely distributed inhibitory hormone that plays an important role in several biological processes including neurotransmission, inhibition of exocrine and endocrine secretions, and cell proliferation. The diverse biological effects of somatostatin are mediated through a family of five somatostatin receptors (sst1-sst5) that belong to the family of G-protein-coupled receptors and that regulate diverse signal transduction pathways including adenylate cyclase, phospholipase C-β, phospholipase A2, guanylate cyclase, ionic conductance channels, and tyrosine phosphatase (1, 2).

The ability of somatostatin and its stable analogues to promote inhibition of normal and tumor cell growth has been demonstrated in various cell types including mammary, prostatic, gastric, pancreatic, colorectal, and small cell lung cancer cells (3, 4). However, the mechanisms of cell growth arrest by somatostatin are still poorly understood. Somatostatin analogues induce a G1/G0 cell cycle arrest and thus prevent DNA synthesis in GH3 rat pituitary tumor cells, whereas they induce a transient G2/M blockade as well as apoptosis in MCF7 human mammary tumor cells (5, 6). These tumor cells express multiple somatostatin receptors and the question of whether different somatostatin receptor(s) may be involved in eliciting these effects still remains to be clarified. A specific role for sst3 in transducing apoptosis through an induction of p53 and Bax has been reported (7). Control of the cell cycle machinery by other receptors is an important problem that remains to be addressed.

Our studies on the expression of somatostatin receptor subtypes in heterologous systems led us to demonstrate that sst2 selectively mediates the antiproliferative effect of somatostatin analogues on serum- or insulin-induced cell growth through the stimulation of a protein-tyrosine phosphatase, which was recently identified as SHP-1 (9, 10). SHP-1, a protein-tyrosine phosphatase with two SH2 domains, plays a role in terminat-
sion and throughout S phase. One of the critical targets of cyclin-cdk complexes is the retinoblastoma gene product (Rb). Rb acts as a transcriptional repressor. In its hypophosphorylated form, it binds to the E2F family of cell cycle transcription factors during G1 phase and inhibits E2F activity. Rb is inactivated by cdk phosphorylation in mid to late G1 phase of the cell cycle and dissociates from E2F, leading to activation of genes containing E2F sites and a progression from G1 to S phase (reviewed in Ref. 12).

Another level of regulation of cdk activity results from the action of cdk inhibitors that bind cyclin-cdk complexes and either inhibit their kinase activities or prevent their activation by cdk-activating kinase (reviewed in Refs. 13 and 14). In mammalian cells, cdk inhibitors comprise two classes of proteins, the Ink4 family including p16 Ink4a, Ink4b, p18 Ink4c, and p19 Ink4d, which specifically inhibit cyclin D-dependent kinases, cdk4 and cdk6 (12), and the p21 family including p21, p27 Kip1, and p57 Kip2 (15–19), which can interact with different cyclin-cdk complexes. Among them, p27 Kip1 is a widely distributed cdk inhibitor that has an important role regulating entry into and exit from the cell cycle. p27 Kip1 is abundantly expressed in normal quiescent cells and is down-regulated by mitogens. The decrease in p27 Kip1 expression occurs through protein degradation via the ubiquitin-proteasome pathway after p27 Kip1 phosphorylation by cyclin E-cdk2 complexes (20–22). Increased levels of p27 Kip1 induced by transforming growth factor β, contact inhibition, serum deprivation, rapamycin, or staurosporine have been associated with a G1 arrest (18, 23, 24). In contrast, an overexpression of p27 Kip1 antisense cDNA results in mitogen-independent G1 progression, demonstrating the importance of p27 Kip1 in controlling cell cycle exit (25, 26). The involvement of p27 Kip1 in the negative regulation of cell proliferation is related to its binding and subsequent inhibition of the kinase activity of cdk2-cyclin complexes (17, 18, 23, 27).

In this study, we investigated the potential effects of sst2 on cell cycle progression and expression of cell cycle regulatory proteins in CHO cells expressing sst2 (CHO/sst2). Activation of sst2 caused a G1 cell cycle arrest in-phase accompanied by an increased expression of cdk inhibitor p27 Kip1, which resulted in an increase of its association with cdk2 and a decrease in cdk2 activity and led to dephosphorylation of protein Rb. The role of SHP-1 in sst2-mediated regulatory mechanisms was investigated in CHO cells coexpressing sst2 and a negative SHP-1 (C453S) mutant as described previously (18). SHP-1 (C453S) mutant (a gift of Dr. C. Nahmias, ICGM, Paris) was stably co-transfected in CHO cells using Lipofectin reagent with pSV2 neo vector for somatostatin binding and the presence of SHP-1 as described (9).

**Materials—**CHO-DG44 stably expressing sst2 (CHO/sst2) or sst2 and CHO-C453S (CHO/sst2-CHO-C453S) were cultured in αMEM containing 10% fetal calf serum and G418 (200 μg/ml) as described previously (8). After an overnight attachment phase, cells were serum-starved in αMEM for 18 h without geneticin before peptide addition.

**Flow Cytometry Analysis—**Cells were harvested by trypsin (0.5 mg/ml) and EDTA (0.02 mg/ml), washed twice with phosphate-buffered saline and then with buffer B. Cells were then incubated to 2.0 × 10⁶ cells for 30 min at 25 °C for indicated times. Cells were then pelleted by centrifugation at 900 × g for 10 min at 4 °C and used for immunoprecipitation or immunoblotting.

**Immunoprecipitation and Immunoblotting—**Cells were washed with buffer A, acinar cells were incubated in the same buffer in the presence or absence of peptides at 25 °C for indicated times. Acini were then pelleted by centrifugation at 900 × g for 10 min at 4 °C and used for immunoprecipitation or immunoblotting.

**Kinase Assay—**Immunoprecipitated proteins with anti-cdk2 or anti-tyrosine kinase antibodies, followed by respectant antibody and peroxidase-labeled antibody. Since the reactions were conducted on the same membrane, measurement of the autoradiographic density was a convenient way of monitoring the activity of the various kinases.

**Experimental Procedures**

**Materials—**Monoclonal anti-p27 Kip1 and anti-Rb antibodies, known to react with murine and human proteins, were purchased from Transduction Laboratories and Pharmingen, respectively. Polyclonal anti-human p27 Kip1 antibodies were from Transduction Laboratories. Polyclonal anti-mouse p27 Kip1 antibodies and anti-cdk2 antibodies that react with human and murine proteins were from Santa Cruz Biotechnology. Monoclonal antibovine Rb antibodies react with human and murine proteins and were from Calbiochem. RC-160 was synthesized as described previously (28). [γ-32P]ATP (3,000 Ci/mmol) was purchased from Isotrade (France). Histone H1 was from Sigma, and the enhanced chemiluminescence (ECL) immunodetection system was from Amersham Pharmacia Biotech.

**DNA Transfection—**The 1.2-kilobase XbaI fragment of mouse sst2A cDNA subcloned into pCMV6c vector was stably co-transfected in CHO (DG44 variant) cells using Lipofectin reagent and pSV2neo as described (kindly donated by Dr. G. I. Bell, Howard Hughes Medical Institute, University of Chicago and Dr. T. Reisine, University of Pennsylvania, School of Medicine, Philadelphia) (8). Stable transfectants were selected in αMEM (minimal essential medium) containing geneticin at 600 μg/ml. Geneticin-resistant clones expressing sst2 (CHO/sst2) were screened for somatostatin binding using [125I-Tyr11]somatostatin as tracer as described (8). The 2.1-kilobase HindIII/NotI fragment of human SHP-1 cDNA (a gift of Dr. M. L. Thomas, Howard Hughes Medical Institute, Washington University, St. Louis, MO) was subcloned into the expression vector pcDNA I neo vector (Invitrogen). The SHP-1 (C453S) mutant (a gift of Dr. C. Nahmias, ICGM, Paris) was constructed as described (10). The mouse sst2 gene in the pCMV6c vector was stably co-transfected in CHO cells using Lipofectin reagent with the SHP-1 (C453S) mutant in pcDNA I neo. Stable colonies obtained by selection with G418 (100 μg/ml) were screened for somatostatin binding and the presence of SHP-1 as described (9).

**Cell Culture—**CHO-DG44 stably expressing sst2 (CHO/sst2 or sst2 and CHO-C453S (CHO/sst2-CHO-C453S) were cultured in αMEM containing 10% fetal calf serum and G418 (200 μg/ml) as described previously (8). After an overnight attachment phase, cells were serum-starved in αMEM for 18 h without geneticin before peptide addition.

**Pancreatic Acini from Fasted Mice—**Acini from fasted mice were prepared as described (40). After washing with buffer A, acinar cells were incubated in the same buffer in the presence or absence of peptides at 25 °C for indicated times. Acini were then pelleted by centrifugation at 900 × g for 10 min at 4 °C and used for immunoprecipitation or immunoblotting.

**Flow Cytometry Analysis—**Cells were harvested by trypsin (0.5 mg/ml) and EDTA (0.02 mg/ml), washed twice with phosphate-buffered saline and then with buffer B. Cells were then incubated to 2.0 × 10⁶ cells for 30 min at 25 °C for indicated times. Cells were then pelleted by centrifugation at 900 × g for 10 min at 4 °C and used for immunoprecipitation or immunoblotting.

**Immunoprecipitation and Immunoblotting—**Cells were washed with phosphate-buffered saline and then with buffer B. Cells were lysed in 500 μl of buffer B containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin. After a 15-min incubation at 4 °C, the lysate was collected and centrifuged at 13,000 × g for 10 min at 4 °C and used for immunoprecipitation or immunoblotting.

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RESULTS

Somatostatin Promotes G1 Cell Cycle Arrest and Blocks Induction of the S Phase—We previously reported that in CHO cells expressing sst2, the addition of the somatostatin analogue, RC-160, for 24 h to the culture medium led to inhibition of the mitogenic effect of insulin (10). To analyze whether RC-160-mediated inhibition of cell proliferation reflects a stage-specific arrest of the cell cycle, cells were rendered quiescent by serum deprivation and incubated with 100 nM insulin in the presence or absence of 1 nM RC-160 and then analyzed by flow cytometry. Cells grown in the absence of fetal calf serum were taken as control values. The treatment of cells with insulin increased the percentage of cells in the S phase, which reached 27% at 6 h and increased up to 50% at 24 h of treatment (data not shown). The simultaneous treatment of cells with insulin and RC-160 for 6 h prevented cells from entering into the S phase, RC-160 causing a decrease in the percentage of cells in the S phase (43%) and an accumulation of cells in the G1 phase, which increased from 57% in the absence of RC-160 to 72% (Fig. 1). For longer treatment, RC-160 had no significant effect on the G1/S transition (data not shown). We concluded that activation of sst2 by ligand induces a G1 cell cycle arrest in CHO/sst2 cells. Furthermore, treatment of cells with 1 μM orthovanadate suppressed the RC-160-induced decrease of number of cells in the S phase as well as the increase of cells in the G1 phase, indicating that a tyrosine phosphatase was required in the RC-160 effects (data not shown).

G1 progression depends on an orderly and coordinated expression of cyclins that bind to and activate cdks, the activity of which is negatively regulated by their association with a family of cdk inhibitory proteins. Therefore we investigated whether sst2-mediated cell cycle arrest is associated with a change in the expression of the cdk inhibitors.

Somatostatin Analogue Induces a Rapid Accumulation of p27\textsuperscript{kip1}—We first examined the expression of the Kip/Cip family cdk inhibitor p27\textsuperscript{kip1}, which has been demonstrated to be involved in the regulation of cell cycle progression induced by various antiproliferative stimuli that cause G1-phase arrest (23–26). CHO/sst2 cells were treated for various times with insulin in the presence or not of RC-160, and the level of p27\textsuperscript{kip1} was investigated by Western blot analysis. As observed in Fig. 2, p27\textsuperscript{kip1} was expressed at high level in growth-arrested control cells, and after 3 h of insulin treatment, its expression decreased by 45% (p < 0.05), consistent with previous results reported for mitogenic signals (26, 31, 32). The decrease of p27\textsuperscript{kip1} level was transient, the level of this cdk inhibitor being not significantly different from that in control cells by 24 h. The addition of RC-160 resulted in a 4-fold increase (p < 0.02) in the level of p27\textsuperscript{kip1} during the first 3 h. Elevated levels of p27\textsuperscript{kip1} were found to return to control levels at 24 h of treatment with RC-160. Treatment of cells with 1 μM orthovanadate for 3 h suppressed the RC-160-induced increase of p27\textsuperscript{kip1}, indicating that this effect is dependent on a tyrosine phosphatase (Fig. 2).

The expression of the other member of the Kip/Cip family cdk inhibitors, p21\textsuperscript{Waf1/Cip1}, was also examined in CHO/sst2 cells. In contrast to p27\textsuperscript{kip1}, p21\textsuperscript{Waf1/Cip1} was found to be barely detectable in control cells (Fig. 3), as observed by others in quiescent cells (33). As reported for mitogens in other cell
systems (32, 34), insulin induced an increase of its expression up to 24 h, suggesting that elevated p21^{Waf1/Cip1} is not related to insulin-mediated G1/S transition. However, the addition of RC-160 did not significantly modify the insulin-induced expression of p21^{Waf1/Cip1} irrespective of the time of treatment, suggesting that this inhibitor is not involved in the somatostatin-mediated growth arrest.

**Somatostatin Analogue Induces Inhibition of cdk2 Kinase Activity**—It has been shown in many cell types that among the G1 cyclin-cdk complexes negatively regulated by p27^{Kip1}, the up-regulation of p27^{Kip1} in response to growth inhibitory factors favors its association with cyclin E-cdk2, resulting in kinase inhibition and contributing to cell growth arrest (34). Therefore, we first tested whether somatostatin analogue-mediated increases in the level of p27^{Kip1} should be reflected in a change of the kinase activity of cdk2-associated complexes, as measured by an *in vitro* assay on cdk2 immunoprecipitates using histone H1 protein as a substrate. In comparison with the control activity detected in resting CHO/sst2 cells, cdk2 kinase activity was increased by about 2-fold after 3 h of treatment with insulin, as revealed by the heavily phosphorylated histone H1 level. When RC-160 was added to the culture, the cdk2-dependent kinase activity was inhibited by 80% during the first 3 h of culture (Fig. 4A). Similarly, RC-160 induced a decrease of about 80% insulin-induced increase of cyclin E-cdk2 associated kinase activity (Fig. 4B). In addition, the amount of p27^{Kip1} associated with cdk2 was decreased by 70% after treatment of cells for 3 h with insulin, whereas the addition of RC-160 increased the level of the complexes by 87% (Fig. 4C). These results indicate that insulin and RC-160 could contrarily inhibit S-phase Entry

**SHP1 Is Required for Somatostatin Analogue-mediated Inhibition of S-phase Entry**—SHP-1 has been previously demonstrated to play a role in the negative feedback of growth factor signaling and to be required for early events in somatostatin-activated sst2 signaling (9–11). However the role of SHP-1 in the regulation of cell cycle machinery has not been delineated. To investigate whether SHP-1 is critical for cell cycle arrest and p27^{Kip1} regulation, a mutated SHP-1 cDNA in which the active site of SHP-1 was mutated to a catalytically inactive enzyme. We transfected the cDNA coding for the SHP-1 mutant and sst2 in CHO cells and selected the clones (CHO/sst2-SHP1(C453S)) that expressed sst2 receptors at a level similar with that observed in CHO/sst2 cells (10). These clones overexpressed the SHP-1 mutant protein approximately 4-fold as observed by Western blotting (not shown).

Analysis of CHO/sst2-SHP1(C453S) cells by flow cytometry revealed that in the absence of serum, cells did not undergo G1 arrest as observed with CHO/sst2 cells, and 22% of cells remained in S phase. Furthermore, insulin and RC-160 did not modify the S phase, indicating that expression of SHP-1 mutant promoted G1 progression of cells and nullified the modulatory effect of insulin and RC-160 on cell cycle progression

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**Fig. 3.** Effect of insulin and RC-160 on p21^{Waf1/Cip1} expression in CHO/sst2 cells. A, Serum-starved CHO/sst2 cells were incubated at 37°C for indicated times with 0.1 μM insulin and with (Ins + RC) or without (Ins) 1 nM RC-160 or were not treated (control (Cont)) and solubilized as described under "Experimental Procedures." A, soluble proteins were subjected to SDS-PAGE and immunoblotted (Blot) with anti-p21^{Waf1/Cip1} antibodies. The arrow indicates the position of p21^{Waf1/Cip1}. B, immunoblots were analyzed densitometrically, and the data were plotted as the percentage of control values obtained from cells incubated in serum-free MEM at time 3, 6, and 24 h. Data from three separate experiments are presented as mean ± S.E.
SHP-1 Involved in sst2-mediated Cell Cycle Arrest

**Fig. 4.** Effect of insulin and RC-160 on cyclin E- and cdk2-associated kinase activity, the amount of p27<sup>Kip1</sup> associated with cdk2, and the expression of cyclin E and cdk2 protein in CHO/sst2 cells. Serum-starved CHO/sst2 cells were incubated at 37°C for 3 h with 0.1 μM insulin with or without 1 nM RC-160 or were not treated and then solubilized. A and B, soluble proteins were subjected to immunoprecipitation (Ip) with anti-cdk2 (A) or anticyclin E (B) antibodies. cdk2 kinase activity was assayed in cdk2 or cyclin E immunoprecipitates using histone H1 as substrate, followed by SDS-PAGE and autoradiography. C, to detect the amount of p27<sup>Kip1</sup> associated with cdk2, soluble proteins were subjected to immunoprecipitation with anti-p27<sup>Kip1</sup> antibodies. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting (Blot) with anti-cdk2 antibodies. The arrow indicate the position of cdk2. D, soluble proteins were resolved by SDS-PAGE and analyzed by immunoblotting (Blot) with anti-cdk2 or anticyclin E antibodies. Arrows indicate the position of cdk2 and cyclin E.

**Fig. 5.** Effect of insulin and RC-160 on pRb phosphorylation in CHO/sst2 cells. CHO/sst2 cells were incubated for indicated times with 0.1 μM insulin with or without 1 nM RC-160 or were not treated and then solubilized. Soluble proteins were subjected to SDS-PAGE and immunoblotted with anti-pRb antibodies. The upper band represents the hyperphosphorylated (pRb<sup>hyp</sup>), whereas the lower band (pRb) represents the hypophosphorylated form of protein pRb.

(Fig. 6). These results are in agreement with the previously observed increase in basal proliferation and abrogation of regulatory effect of insulin as well as RC-160 on cell proliferation in CHO/sst2-SHP-1C453S cells (10) and argue in favor of a role for SHP-1 in maintenance of cell quiescence.

**SHP-1 Is Required for Somatostatin Analogue-mediated Induction of p27<sup>Kip1</sup>, Inhibition of cdk2 Activity, and pRb Hypophosphorylation.—** We then examined whether SHP-1 mutant affected p27<sup>Kip1</sup> protein levels. Western blotting analysis demonstrated that in cells expressing mutant SHP-1, the basal level of p27<sup>Kip1</sup> decreased significantly as compared with control CHO/sst2 cells (57 ± 5.6% of control) (Fig. 7A). Furthermore, the dominant negative mutant SHP-1 was found to prevent insulin-mediated down-regulation as well as RC-160-induced up-regulation of p27<sup>Kip1</sup> (Fig. 7B). These results strongly suggest that SHP-1 might dephosphorylate some key substrate(s) in the insulin- and sst2-mediated signaling pathway in order for p27<sup>Kip1</sup> regulation to occur. We previously demonstrated that the phosphotyrosine insulin receptor is an early substrate of somatostatin-activated SHP-1 (11), suggesting that SHP-1 may exert at least part of its effects on p27<sup>Kip1</sup> expression by dephosphorylating the insulin receptor.

These results prompted us to examine the effect of mutant SHP-1 on cdk2 and cyclin E-associated kinase activities. Expression of mutant SHP-1 prevented the effect of insulin and RC-160 on cdk2 as well as cyclin E-associated kinase activity (Fig. 8A). In addition, the decrease of p27<sup>Kip1</sup> basal level observed in cells expressing mutant SHP-1 was paralleled by a decrease in the association of p27<sup>Kip1</sup> with cdk2 (Fig. 8B). The amount of p27<sup>Kip1</sup> associated with cdk2 was decreased by 45% under basal conditions, and insulin and RC-160 no longer had an effect on the association p27<sup>Kip1</sup>-cdk2. These results provide evidence that SHP-1 is involved in the retargeting of p27<sup>Kip1</sup> to cyclin E-cdk2 complexes and, in turn, in inhibition of the associated kinase activity.

The effect of mutant SHP-1 on p27<sup>Kip1</sup> and cdk2-associated kinase activity suggests that mutant SHP-1 may affect the extent of pRb phosphorylation. Immunoblotting of pRb showed that only the hyperphosphorylated inactive form of pRb was detected in CHO/sst2-SHP-1C453S grown in serum-starved conditions and that mutant SHP-1 prevented the mobility shift from hypo- to hyperphosphorylated pRb as well as from hyper- to hypophosphorylated after growth in the presence of insulin and RC-160, respectively (Fig. 8C).

To further examine the role of SHP-1 in p27<sup>Kip1</sup> regulation, experiments were performed using isolated pancreatic acini from viable motheaten (me<sup>−</sup>) mice. These animals express negligible SHP-1 catalytic activity consequent to loss-of-function mutation in the gene encoding SHP-1 (35). Pancreatic acini from normal as well as me<sup>−</sup> mice expressed a high level of sst2 receptors as revealed by anti-sst2 immunoblotting. However, the p27<sup>Kip1</sup> protein level was decreased by about 50% in the me<sup>−</sup> pancreatic acinar cells as compared with normal acini (Fig. 9). We observed that incubation of normal acini for 3 h with 1 nM epidermal growth factor (EGF) at 25°C induced a down-regulation of p27<sup>Kip1</sup>. This effect was reversed by the addition of 1 nM RC-160, in agreement with the mitogenic effect of EGF and the antiproliferative effect of somatostatin on pancreatic acini (36). In contrast, when acini were isolated from me<sup>−</sup> mice, EGF down-regulated p27<sup>Kip1</sup> as observed in control acini, but RC-160 had no more significant effect on the level of p27<sup>Kip1</sup> (Fig. 9).

**DISCUSSION**

Despite evidence for the role of somatostatin in negative growth control in various cellular types, our understanding of the mechanisms involved remains limited. Somatostatin analog, octreotide, inhibits cell proliferation as a result of a blockade in G<sub>S</sub> to G<sub>0</sub>/M in GH<sub>3</sub> rat pituitary tumor cell, in contrast to a transient accumulation of cells in G<sub>2</sub>/M and apoptosis in MCF-7

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<sup>2</sup> F. Lopez and G. Ferjoux, personal communication.
breast cancer cells and AtT-20 mouse pituitary tumor cells (5, 37). All these cell types express multiple somatostatin receptor subtypes, and the involvement of each receptor subtype in somatostatin response remains to be clarified. We have previously demonstrated the role of sst2 in the somatostatin-mediated inhibition of cell growth and the involvement of SHP-1 in the transduction of the inhibitory growth signal (8–10, 38). The present investigation was undertaken to further delineate the basis of sst2-mediated control of cell cycle machinery. We have demonstrated that in CHO cells expressing sst2, the inhibition of proliferation in response to somatostatin analogue results from the suppression of cell cycle progression and the arrest of cells in the G0/G1 phase, which correlates with an increase in expression of p27\textsuperscript{Kip1} but not p21\textsuperscript{Waf1/Cip1}. This is accompanied by an increase of association of p27\textsuperscript{Kip1} with cdk2, a concomitant inhibition of cyclin E- cdk2 activity and a consequent decrease in the phosphorylation of pRb that precedes the inhibition of entry into S phase. On the other hand, the data presented provide strong support for the involvement of the tyrosine phosphatase SHP-1 in maintaining cell quiescence as well as sst2-induced cell cycle arrest. The expression of dominant negative SHP-1 is sufficient to induce G1/S transition, allowing mitogen-independent cell proliferation and abrogating the inhibitory effect of somatostatin. Consistent with its effect on cell cycle progression, expression of dominant negative SHP-1 down-regulates p27\textsuperscript{Kip1}, which results in a decrease of association of p27\textsuperscript{Kip1}, an increase of cyclin E/cdk2 kinase activity, and a subsequent inactivation of the growth-suppressive function of pRb protein, thus linking SHP-1 to control of p27\textsuperscript{Kip1} expression.

The pivotal role of p27\textsuperscript{Kip1} in controlling cdk function and, thus, cell cycle progression is well established. p27\textsuperscript{Kip1} mediates cell cycle arrest in response to various antimitogenic signals, including transforming growth factor β, rapamycin, cAMP, cell-cell contact, and anti-epidermal growth factor antibody (18, 39–41). Overexpression of p27\textsuperscript{Kip1} leads to cell cycle arrest (17) and inhibition of both normal and transformed human mammary epithelial cell growth (42). Antisense inhibition of p27\textsuperscript{Kip1} expression can prevent quiescence upon withdrawal of growth factor (25, 26). In this study, insulin treatment of CHO/sst2 cells reduces the level of p27\textsuperscript{Kip1}, as expected for mitogenic signaling pathways. In the presence of soma-
sst2-SHP1 (C453S) cells were incubated at 37 °C for 3 h with 0.1% serum-free medium and plotted as percentages of control values obtained from cells incubated in serum-free medium. The decrease of the amount of p27Kip1 is associated with cyclin E-cdk2 complexes, inactivates cyclin E-cdk2 complexes, and induces the disappearance of the hyperphosphorylated form of pRb protein. It has been reported that p27Kip1 inactivates cyclin E-cdk2-associated kinases (23), and conversely, inactivation of cyclin E-cdk2-associated kinases can lead to accumulation of p27Kip1, because cyclin E-cdk2-associated kinases phosphorylate p27Kip1 and induce its destruction by the ubiquitin pathway (21). Somatostatin also decreases cyclin E protein level. However, cyclin E overexpression does not prevent cell cycle exit (44), and there is no significant relationship between cyclin E expression and cyclin E-cdk2 activity (45). It is likely that the negative regulation by somatostatin of the cdk2 activity could be one of the consequences of the rise of p27Kip1, leading to an increased interaction of p27Kip1 with cyclin E-cdk2 complexes and, thus, resulting in an inhibition of their activity.

A critical finding of the present study is that unlike CHO cells expressing sst2, which accumulate in G0/G1, CHO cells coexpressing sst2 and a dominant negative mutant SHP-1 remain distributed through the cell cycle after serum withdrawal, with a high portion of cells being in the S phase. These cells express a constitutively low level of p27Kip1; this in turn leads to an activation of cyclin E-cdk2 complexes and accumulation of hyperphosphorylated pRb. Furthermore, the expression of dominant negative SHP-1 circumvents the requirement for insulin in G1 cell cycle progression and is associated with a resistance to the antiproliferative effect of somatostatin. Ours results provide strong support for the hypothesis that SHP-1 is required to revert mitogen-induced down-regulation of p27Kip1 and clearly demonstrate the importance of SHP-1 in sst2 signaling for blockade of p27Kip1 down-regulation. This hypothesis is strengthened by our results, obtained with acinar cells isolated from mev mice that express a high level of sst2 and a defective SHP-1 (35). As observed in CHO/sst2 cells expressing a mutant SHP-1, acini from mev mice express a low level of p27Kip1 that can be no longer up-regulated by somatostatin, demonstrating the importance of SHP-1 in the retention of high levels of p27Kip1 and its central role as the downstream target of the sst2 signaling pathway leading to up-regulation of p27Kip1. The functional role of SHP-1 in pancreatic cells is not fully understood, but it may play a critical role in the regulation of cell cycle progression and proliferation. Further studies are needed to elucidate the mechanisms by which SHP-1 regulates the expression and activity of cyclin E-cdk2 complexes and their interaction with pRb.
known, but the demonstration that SHP-1 is necessary for regulation of $p27^{kip1}$ suggests that SHP-1 may be important for the pancreatic cell development. It is notable that EGF down-regulates $p27^{kip1}$ in acini from me$^x$ mice, suggesting that SHP-1 is not the only negative regulatory protein-tyrosine phosphatase in growth factor signaling or that another protein-tyrosine phosphatase can substitute for SHP-1 when it is not functional. Most of the previous studies have focused on the role of SHP-1 in response of quiescent cells to mitogenic stimulation. We and others identified SHP-1 as a critical negative regulator of cytokine as well as growth factor signaling; the recruitment of this enzyme to activated membrane receptors causes dephosphorylation of the receptors or of downstream signaling molecules (10, 46). The results presented here extend these observations by demonstrating that SHP-1 is necessary for negative regulation of cell cycle progression in the G1 phase and is a key mediator of sst2 signaling pathway in controlling high inhibitory levels of $p27^{kip1}$.

Regulation of $p27^{kip1}$ occurs by different mechanisms, including transcriptional, post-transcriptional, or post-translational mechanisms. Indeed, post-transcriptional mechanisms have been implicated in the up-regulation of $p27^{kip1}$ protein induced by antiestrogen-transforming growth factor-$b$ (47) and interferon $\gamma$ (48), whereas post-translational mechanisms involving dephosphorylation of $p27^{kip1}$ as a consequence of down-regulation of the N-myc gene (49) or degradation of the protein (50) have been proposed for the retinoic acid- and lovastatin-induced increases in $p27^{kip1}$, respectively. It has been reported that the mitogen-induced decrease in $p27^{kip1}$ expression occurs through posttranscriptionally regulated protein degradation via the ubiquitin-proteasome pathway (20). Recent data demonstrated the key role for Ras signaling pathway in the down-regulation of $p27^{kip1}$ and the involvement of RhoA in ubiquitin-mediated $p27^{kip1}$ degradation (51). However, a regulation at a transcriptional level can also occur, as shown in v-Src oncoproteins-transformed cells, v-Src reducing the level of $p27^{kip1}$ mRNA and preventing cellular quiescence (52). The mechanisms involved in the SHP-1-induced up-regulation of $p27^{kip1}$ remain to be elucidated. We previously demonstrated that SHP-1 is associated with activated insulin receptor and is involved in down-regulation of insulin signaling. In addition, upon sst2 stimulation, somatostatin negatively regulates insulin signal transduction by controlling first the recruitment of SHP-1 to insulin receptor and its activation and then causing a dephosphorylation and an inactivation of insulin receptor and its substrates, thus leading to an inhibition of the insulin downstream signaling (12). In agreement with these results, activated SHP-1 may regulate the level of $p27^{kip1}$ as a consequence of SHP-1-induced dephosphorylation of insulin receptors and blockade of the insulin-induced catalytic cascade leading to down-regulation of $p27^{kip1}$. However, other downstream effectors of somatostatin-activated SHP-1, different from growth factor receptors and not yet identified, could also be involved in the SHP-1-induced $p27^{kip1}$ regulation.

In conclusion, this investigation shows that activation of sst2 promotes cell growth arrest through the ability of somatostatin to maintain high levels of $p27^{kip1}$ and inactivate cyclin E-cdk2 complexes, thus leading to hypophosphorylation of $pRb$. Our findings provide evidence that SHP-1 may be required for accumulation of $p27^{kip1}$ and inhibition of cell cycle progression and indicate that SHP-1 is a key mediator of sst2-induced $p27^{kip1}$ up-regulation and subsequent cell cycle arrest.

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