Somatostatin Inhibits PC Cl3 Thyroid Cell Proliferation through the Modulation of Phosphotyrosine Phosphatase Activity

IMPAIRMENT OF THE SOMATOSTATINERGIC EFFECTS BY STABLE EXPRESSION OF E1A VIRAL ONCOGENE*

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In this study, we report the effects of somatostatin on the proliferation of PC Cl3 thyroid cell line and the intracellular mechanisms involved. We also evaluated the possible alterations, induced by E1A oncogene transformation on the intracellular pathways mediating somatostatin inhibition of cell proliferation. We showed that somatostatin was able to powerfully inhibit insulin and insulin + TSH-dependent cell proliferation by inducing a block in the G1/S progression in the cell cycle. These cytostatic effects were completely reverted by vanadate, suggesting that somatostatin may induce antiproliferative effects through the modulation of phosphotyrosine phosphatases. In the E1A-transformed cell line, somatostatin was completely ineffective. The lack of somatostatin inhibitory effects on cell proliferation were not due to alterations in the expression of somatostatin receptors, which were regularly expressed and coupled to adenyl cyclase activity, but were dependent on an alteration in their coupling with the phosphotyrosine phosphatase. In fact, although in PC Cl3 cells somatostatin increases by 100% phosphotyrosine phosphatase activity, it was completely ineffective in E1A-expressing cells. In conclusion we demonstrated that somatostatin activates phosphotyrosine phosphatases in PC Cl3 thyroid cells to inhibit cell proliferation and that the stable expression of E1A oncogene in these cells completely abolishes this antiproliferative effect.

Somatostatin is a powerful inhibitor of a wide range of biological activities including hormone secretion and cell proliferation (1, 2). Although somatostatin analogues have been used in the therapy of various human tumors (2, 3), the molecular mechanisms responsible for somatostatin antiproliferative activity are not yet completely clarified. The antiproliferative effects of somatostatin have been ascribed either to its inhibition of the release of growth promoting hormones (3) or to a direct antiproliferative activity. Although most of the information presently available is obtained in vitro on different cell lines (4–6), data from clinical trials also demonstrate that somatostatin is effective in the treatment of pituitary GH and prolactin-releasing adenomas, endocrine tumors of the gastroentero-pancreatic system, carcinoids, and breast and prostate cancers (2).

As far as the characterization of the intracellular mechanisms involved in the somatostatin antiproliferative activity is concerned, it has been recently emphasized that the modulation of phosphotyrosine phosphatase (PTPase) activity is one of the main intracellular pathways responsible for somatostatinergic inhibition of cell growth (7–10). It has been reported that the somatostatin-dependent increase of PTPase activity induces the dephosphorylation of the epidermal growth factor receptor, a tyrosine kinase activated by tyrosyl phosphorylation, and that this effect results in the inhibition of the proliferative activity of epidermal growth factor (8, 11). Moreover, somatostatin effect involved a single subset of PTPases rather than a nonspecific activation of all the components of this class of enzymes (12, 13).

Other neurohormones, such as dopamine (14) and luteinizing hormone-releasing hormone (15) have also been reported to induce growth arrest through the induction of PTPase activity, and in human breast cancer cells, the treatment with antiestrogens increases a membrane PTPase activity that is strictly correlated with their antiproliferative effects (16). Thus, hormonally regulated PTPases seem to play a key role in the control of cell proliferation, and somatostatin is suggested to be an important endogenous modulator of the activity of this class of enzymes.

In this study we used the PC Cl3 clonal thyroid cell line to evaluate the role of somatostatin in the regulation of thyroid cell proliferation and the intracellular mechanisms involved. PC Cl3 cells retain in vitro most of the typical markers of
thyroid differentiation, such as dependence on TSH, thyroglobulin synthesis and secretion, and the ability to trap iodide from the medium (17). Although the role of somatostatin receptor (SSTR) activation in the control of thyroid cells proliferation has already been suggested in studies on the FRTL5 thyroid cell line (18), the aim of our study was the characterization of the intracellular mechanisms mediating this effect. By means of stable transfection of E1A viral oncogene in PC C13, we evaluated the possible alterations of the somatostatin inhibition of cell growth after oncogene transformation. Tumorigenesis is now considered a multi-step process, probably involving two or more genetic lesions and various still unknown epigenetic changes. The transfection of cellular and viral onco- genes in cells in culture is a valuable approach to examine the influence of a single genetic lesion on cell growth. E1A gene products are nuclear phosphoproteins acting as transactivator for the viral early genes and are reported to immortalize mammalian cells, block cell differentiation and induce cellular transformation in cooperation with other onco- genes (19). E1A proteins were reported to promote the entry in the G1 phase of the cell cycle bypassing the requirement for cellular components that are normally induced by external growth factors (20).

PC C13 subclone overexpressing E1A oncogene, named PC E1A, lost most of the differentiative features of normal thyroid cell and became partially independent on TSH for proliferation and functioning (21). Thus, it represents a good model to study the early modifications occurring during thyroid cell transformation and the alterations that this process may induce in the somatostatinergic network of cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—Somatostatin-14 was purchased from American Peptides. Vanadate was from Alomone Labs, and all other reagents were from Sigma unless otherwise specified.

Cell Culture—PC C13 and PC E1A cell lines were grown in Ham's F-12 medium, Coon's modification (Sigma) supplemented with 5% fetal calf serum (FCS) (ICN Flow), and six growth factors (10 nM TSH, 10 nM hydrocortisone; 100 nM insulin; 5 μM transferrin; 5 nM somatostatin; 20 μM glycolyl-histidyl-lysine) (6H), as previously reported (22). Because somatostatin reduced the proliferation rate of these cells, it was removed from the 6H mixture.

[3H]Thymidine Incorporation Assay—DNA synthesis activity was measured by means of the [3H]thymidine uptake assay as previously reported (14). Briefly, cells were plated at the density of 5 x 10^5 in 24-well plates. After 24 h cells were serum- and growth factor-starved for 24 h. Then, cells were treated with the test substances for 16 h and in the last 4 h cells were pulsed with 1 μCi/mL of [3H]thymidine (Amersham Corp.). At the end of the incubation time cells are trypsinized (15 min at 37°C), extracted in 10% trichloroacetic acid and 95% ethanol. The trichloroacetic acid-insoluble fraction was then counted in a scintillation counter.

Cell Cycle Analysis by Propidium Iodide Staining—Cells treated at different times were harvested by trypsinization, pelleted, washed twice with calcium- and magnesium-free phosphate-buffered saline, fixed and permeabilized with cold 70% ethanol, and then stored at 4°C. 1 ml of propidium iodide staining solution (50 μg/ml in phosphate-buffered saline, pH 7.4) containing 0.5 mg/ml DNase-free RNase was added to 2 x 10^6 cells (30 min at room temperature), and the DNA content of the cells was analyzed by a FACScan flow cytometry apparatus (Becton & Dickinson, Mountain View, CA). Cell cycle data analysis was performed on 20,000 events by CELL-FIT software (Becton & Dickinson), using the method of Hou. Pulse area versus pulse width gating was performed to exclude doublets from G2/M region.

Intracellular cAMP Assay—Cells were plated in 24-well dishes at the density of 500,000/well in culture medium containing 5% FCS and the 6H mixture without somatostatin. Before starting the experiments, the medium in the wells was replaced with FCS- and 6H-free medium supplemented with 50 μM isobutylmethylxanthine and the test substances. The reaction was stopped after 1 h by rapid aspiration of the medium that was replaced by a mixture of acidic ethanol (1 ml of 1 n HCl/100 ml of ethanol) to extract the intracellular cAMP. Samples were kept at 4°C overnight, neutralized with 1 n NaOH, dried, resuspended in 50 μl of a solution of 0.05 n Tris, pH 7.5/4 μl of EDTA, and stored at -80°C. The amount of cAMP produced was determined using the [3H]cAMP assay system (Amersham Corp.).

PTPase Assay—Cells, plated at 50% confluency in 10-cm Petri dishes, were preincubated with the test substances for 2 h in FCS-free medium at 37°C in a CO2 incubator. Then the cells were washed with phosphate-buffered saline and mechanically scraped in a buffer containing 0.32 M sucrose, 10 μM Tris, pH 7.5, 5 μM EGTA, and 1 μM EDTA, and the membranes were isolated as previously reported (24). Nuclei were removed by centrifugation at 2,000 x g at 4°C for 10 min. Membrane fraction was sedimented by a further centrifugation at 15,000 x g at 4°C for 10 min, resuspended in a buffer containing 250 μM HEPES, pH 7.2, 140 mM NaCl, 1% Nonidet P-40, and phenylmethylsulfonyl fluoride and leupeptin as protease inhibitors, and assayed for protein content using the method of Bradford (25) with bovine serum albumin as standard and the Bio-Rad reagent. 20 μg of control or treated membranes were used in the PTPase assay. PTPase assay was performed using the synthetic substrate para-nitrophenylphosphate (p-NPP) in a spectrophotometric assay. p-NPP is a general phosphatase substrate that in presence of inhibitors of Ser/Thr phosphatases is specific for PTPases (8, 14). Membranes were preincubated for 5 min at 30°C in 80 μl of volume containing 20 μl of a 5 x reaction buffer (250 μM HEPES, pH 7.2, 50 mM dithiothreitol, 25 mM EDTA, 500 μM microcystin-leucine-arginine (Alomone Labs.) as a Ser/Thr phosphatases inhibitor, and 50 μM p-NPP). The reaction was started by adding 20 μl of 50 mM p-NPP, carried out for 30 min at 30°C and stopped by adding 900 μl of 0.2 n NaOH. The absorbance of the sample, directly proportional to the amount of dephosphorylated substrate, was measured at 410 nm (26). The extinction coefficient for p-NPP, at this wavelength is 1.78 x 10^5 M^-1 cm^-1 (26).

Phosphorysine Immunoblot—Immunolabeling of PC C13 total proteins, size fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred on polyvinylidene difluoride membranes (Bio-Rad), was performed, as previously reported (27), using anti-phosphotyrosine antibody (1:500) (Amersham Corp.). The detection of the immunocomplexes was performed using the ECL chemiluminescence kit (Amersham Corp.).

RNA Isolation and Reverse Transcription (RT)-PCR—Total RNA was isolated using the acidic phenol technique (28). RT-PCR was performed by adding 2.5 μl of 20 μl of buffer A (0.2 mM Tris, pH 8.3, and 1.0 mM KCl) 2.5 μl of 20 μl buffer B (30 mM MgCl2, 15 mM MnSO4, 0.1 μg of up- and down-stream primers, 0.2 mM dCTP, dGTP, and dTTP, 0.02 μM dATP, 5 μl of [32P]dATP, and 5 units of Retrotroph RT (Epicentre Technology) 5 μl containing 0.5 μg of total RNA. The samples, in a final volume of 50 μl, were incubated for 5 min at 50°C and then 10 min at 70°C for the first strand synthesis. Second strand was synthesized by 1 cycle of 1 min at 94°C, 1 min at 55°C, and 5 min at 72°C. After a brief RNase treatment, samples were run in 6% polyacrylamide-7 M urea gel in 1 x TBE buffer and analyzed by autoradiography.

Primers used for RT-PCR were a forward primer targeting SSTR1, 5'-sense primer corresponded to the amino acids 86–91 and 3'-antisense primer corresponded to the amino acids 211–217 of SSTR1 sequence; SSTR2, 5'-sense primer corresponded to the amino acids 86–91 and 3'-antisense primer corresponded to the amino acids 210–215 of SSTR2 sequence; SSTR4, 5'-sense primer corresponded to the amino acids 283–290 and 3'-antisense primer corresponded to the amino acids 367–374 of SSTR4 sequence; SSTR5, 5'-sense primer corresponded to the amino acids 271–276 and 3'-antisense primer corresponded to the amino acids 364–369 of SSTR5 sequence. Expected lengths for the amplified products were the following: SSTR1, 393 bp; SSTR2, 387 bp; SSTR4, 273 bp; SSTR5, 294 bp.

Statistical Analysis—Experiments were performed in quadruplicate and repeated at least three times. Statistical analysis was performed by means of one-way ANOVA. A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

The growth characteristics of PC C13 and PC E1A cell lines have been studied by means of both flow cytometry and [3H]thymidine incorporation analysis.

Fig. 1 depicts the cell cycle distribution of the two clonal cell lines (PC C13, Fig. 1, A and B; PC E1A, Fig. 1, C and D) grown in medium supplemented with FCS and 6H mixture (Fig. 1, A and C) and after 48 h of growth factors removal (Fig. 1, B and D). Although the PC E1A clone shows a higher proliferative
rate compared with the normal cell line, both cell lines are
clearly blocked in G0/G1 phases after FCS and 6H mixture
withdrawal (more than 90% of the cell populations analyzed
are found in these phases of the cell cycle). Among the growth
factors studied, insulin and TSH showed the more relevant
proliferative activity in both cell lines. In fact, in G0/G1 syn-
chronized cell population the readdition of insulin (100 nM)
significantly increased the synthesis of DNA in both cell lines
(Table I), and TSH (100 nM), although
perse ineffective, greatly
potentiated insulin effects (Table I). The insulin and insulin
1 TSH stimulation of DNA synthesis was comparable with that
induced by 5H (6H growth factor mixture without TSH) and 6H
mixtures, respectively, showing that the proliferative effects
of these growth factors are mainly dependent on the presence
of these two hormones (data not shown). The treatment of both
cell lines with forskolin (100 nM) mimicked the effects of TSH
(data not shown), confirming that TSH effects are dependent
on the activation of the adenylyl cyclase enzyme.

On the contrary, somatostatin exerted a negative role in the
control of proliferation of PC Cl3 cells. Although somatostatin
was present in a very small amount (5 nM) in the 6H growth
mixture, its withdrawal from the mixture resulted in a 30 or
40% increase in [3H]thymidine incorporation in G0/G1 synchro-
nized cells, in both the presence and the absence of FCS,
respectively (data not shown). Thus, somatostatin was removed
from the 6H mixture.

Then we analyzed the effects of somatostatin on both insulin
or insulin + TSH stimulation of PC Cl3 and PC E1A cell
proliferation. In both cell lines, somatostatin was ineffective in
G0/G1-synchronized cells (Fig. 2). On the contrary, in PC Cl3
Table I

|    | Basal | TSH | Insulin | Insulin + TSH |
|----|-------|-----|---------|---------------|
| PC Cl3 | 100 ± 3 | 105 ± 4 | 1266 ± 33a | 2548 ± 16a |
| PC E1A | 100 ± 5 | 107 ± 3 | 1544 ± 15a | 3302 ± 21a |

*a p < 0.01 versus respective basal values.
values comparable with those of the cells grown in presence of FCS and growth factors (11.5 and 16% for PCCl3 and PCE1A, respectively). Longer treatments did not further increase these percentages (Tables II and III).

A similar increase was observed also for the number of cells in G2/M phases, but in this case the peak of activity was reached after 32 h of treatment for the normal cells and after 24 h for the PCE1A cells (data not shown). More striking results were obtained by the combined treatment with insulin and TSH. In these experimental conditions the percentage of cells in the S and G2/M phases was higher than the percentage of cells in the same phases of the cell cycle, when they were kept in regular culture medium containing FCS and the 6H mixture. The number of cells in the S phase, in both cell lines, remained elevated over the values of the nonsynchronized cells, up to 32 h of treatment (Tables II and III), whereas after 40 h of treatment the percentage of cells in G2/M phases was still higher than in nonsynchronized cells (data not shown).

The simultaneous treatment with insulin and somatostatin for 16 h prevented PCCl3 cells from entering in the S phase, keeping the percentage of PC13 cell cycle population in S phase superimposable to that of cells not treated with growth factors (Table I). For longer treatments the effect of somatostatin disappeared, and there was no difference between the cells treated with insulin alone or insulin + somatostatin (Table I). Similar results were obtained on the insulin-dependent increase in the percentage of cells in the G2/M phases (data not shown). In the normal cell line, the somatostatinergic inhibition of the G2/S transition after the treatment with insulin + TSH was much more pronounced (Table II), and the inhibition of entering the G2/M phases lasted more than 24 h (data not shown). Conversely, in the PC13 cell line, somatostatin treatment was completely ineffective in inhibiting either insulin or insulin + TSH proliferative stimuli (Table III), causing only a slight reduction in the percentage of cells in G2/M phases after 24 h of treatment (data not shown).

The presence of SSTRs in both cell lines was demonstrated by RT-PCR using primers specific for SSTR1, SSTR2, SSTR4, and SSTR5. In both cell lines SSTR4 subtype was the only SSTR mRNA to be detected (Fig. 4).

To study the intracellular mechanisms mediating the somatostatinergic inhibition of cell proliferation, we focused our attention on two of the main intracellular pathways modulated by insulin, TSH, and, in the opposite direction, somatostatin: the tyrosine phosphorylation cascade and the modulation of the adenyl cyclase activity. It is well known that although the insulin receptors have intrinsic tyrosine kinase activity (29) and the stimulation of the TSH receptors activates the adenyl cyclase enzyme (30), both the activation of PTPases and the inhibition of the production of cAMP represent two of the transducing mechanisms modulated by SSTRs activation (24, 12). Thus, somatostatin may control thyroid cell proliferation directly counteracting the intracellular signals activated by both insulin and TSH and an alteration in the modulation of these pathways could be responsible for the loss of responsivity in the PC E1A cell line.
stimulated progression from G1 to the S phase of the cell cycle and treatment.

reduced the stimulatory effects of the insulin (14). Somatostatin, which powerfully inhibited insulin- and ade of PTPases, resulted in an activation of DNA synthesis in the normal and transformed cell lines, confirming treatment with vanadate increased basal and stimulated DNA synthesis in the presence or the absence of the PTPase activity was only slightly lower in PC E1A than in PC Cl3 cells, was completely ineffective in preventing insulin-stimu-

lation of PTPases, we tested the inhibitory effects of somatostatin (1 μM).

PC Cl3 cells were deprived of FCS and 6H mixture for 48 h before the experiment and then treated for the indicated times with the test hormones. Control cells received medium without FSC and 6H mixture during all the time of the experiment. The data represent the means of the percentage of the cell in S phase ± S.E. in three independent experiments. 20,000 events were analyzed for each experimental point.

| Hours of treatment | 8   | 16  | 24  | 32  | 40  |
|--------------------|-----|-----|-----|-----|-----|
| Control            | 4.9 ± 0.1 | 4.4 ± 0.11 | 2.3 ± 0.04 | 1.7 ± 0.07 | 4.4 ± 0.2 |
| Insulin            | 4.7 ± 0.3 | 11.5 ± 1.2* | 10.5 ± 0.4* | 9.4 ± 0.14* | 11.4 ± 1.7* |
| Insulin + somatostatin | 3.0 ± 0.4 | 6.3 ± 0.5* | 10.5 ± 0.6 | 8.3 ± 0.4 | 8.7 ± 0.17 |
| Insulin + TSH      | 5.1 ± 0.08 | 27.0 ± 0.6* | 17.6 ± 0.9* | 16.2 ± 0.4* | 13.4 ± 0.5* |
| Insulin + TSH + somatostatin | 4.1 ± 0.2 | 8.2 ± 0.4* | 14.2 ± 0.6 | 16.1 ± 0.6 | 11.1 ± 0.4 |

* p < 0.01 versus respective control value.

PC E1A cells were deprived of FCS and 6H mixture for 48 h before the experiment and then treated for the indicated times with the test hormones. Control cells received medium without FSC and 6H mixture during all the time of the experiment. The data represent the means of the percentage of the cell in S phase ± S.E. in three independent experiments. 20,000 events were analyzed for each experimental point.

| Hours of treatment | 8   | 16  | 24  | 32  | 40  |
|--------------------|-----|-----|-----|-----|-----|
| Control            | 6.1 ± 0.1 | 5.2 ± 0.11 | 3.6 ± 0.04 | 4.3 ± 0.07 | 4.2 ± 0.2 |
| Insulin            | 4.7 ± 0.3 | 16.2 ± 1.2* | 11.4 ± 0.4* | 7.2 ± 0.14* | 6.7 ± 1.7 |
| Insulin + somatostatin | 4.4 ± 0.4 | 16.1 ± 0.5 | 10.5 ± 0.6 | 6.9 ± 0.4 | 7.5 ± 0.17 |
| Insulin + TSH      | 5.1 ± 0.08 | 30.6 ± 2.1* | 25.0 ± 1.9* | 18.1 ± 0.4* | 15.6 ± 0.5* |
| Insulin + TSH + somatostatin | 6.3 ± 0.2 | 31.4 ± 1.4 | 23.9 ± 0.8 | 16.3 ± 0.6 | 14.9 ± 0.4 |

* p < 0.01 versus respective control value.

To directly evaluate the second messenger systems involved in the somatostatin antiproliferative activity, we tested the effects of SSTRs activation in PC Cl3 and PC E1A cell lines on cAMP production and PTPase activity. In PC Cl3 cells somatostatin, although ineffective in basal conditions, induced a significant inhibition of TSH-dependent stimulation of cAMP accumulation. TSH (100 nM) increased by 475% the intracellular cAMP content and somatostatin treatment (1 μM) significantly reduced this effect (−49% versus TSH stimulation) (Table IV). In PC E1A cells, although basal values were slightly lower than in normal cells (−20%), TSH increased cAMP accumulation by 547% and somatostatin, although did not affect basal cAMP levels, largely reduced TSH stimulatory effects (−62%) (Table IV).

The effects of somatostatin on PTPase activity were measured by both a spectrophotometric assay of the enzyme activity in membrane preparations and immunoblot of total cell proteins labeled with anti-phosphotyrosine antibody.

In the PTPase activity assay, the incubation with the test substances was performed in serum-free culture medium and lasted for 2 h, because it was previously shown that 2 h was the time of maximal activation of PTPases by somatostatin (12). PTPase activity was only slightly lower in PC E1A than in PC Cl3 control cells and was not modified by incubation with insulin or insulin + TSH (Fig. 7). On the contrary, in PC Cl3 cell lines, both in control and insulin- or insulin + TSH-stimulated conditions somatostatin (1 μM) greatly increased PTPase activity, although it was completely ineffective in E1A-transformed cells (Fig. 7). In both cell lines, vanadate (50 μM) completely abolished p-Npp hydrolysis even in presence of somatostatin (Fig. 7).

The specificity of this effect was demonstrated by anti-phosphotyrosine immunoblot in PC Cl3 cells. In these experiments pretreatment (data not shown). In PC E1A cells, somatostatin was ineffective both in control and in vanadate-treated cells, although vanadate increased basal and stimulated DNA synthesis (Fig. 6).

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**TABLE III**

Time course of the percentage of PC E1A cell population entering the S phase of the cell cycle assayed by flow cytometry after treatment with insulin (100 nM) or insulin + TSH (100 nM) with or without somatostatin (1 μM).

| Hours of treatment | 8   | 16  | 24  | 32  | 40  |
|--------------------|-----|-----|-----|-----|-----|
| Control            | 7.8 ± 0.2 | 11.4 ± 0.04 | 17.6 ± 0.14 | 21.0 ± 0.6 | 23.9 ± 0.4 |
| Insulin            | 7.5 ± 0.04 | 18.2 ± 0.34 | 25.0 ± 0.64 | 30.6 ± 0.84 | 36.2 ± 1.0 |
| Insulin + somatostatin | 5.1 ± 0.08 | 12.6 ± 0.24 | 17.6 ± 0.34 | 22.0 ± 0.44 | 26.6 ± 0.6 |
| Insulin + TSH      | 6.3 ± 0.14 | 21.0 ± 0.44 | 26.0 ± 0.64 | 31.6 ± 0.84 | 38.2 ± 1.0 |
| Insulin + TSH + somatostatin | 5.1 ± 0.08 | 12.6 ± 0.24 | 17.6 ± 0.34 | 22.0 ± 0.44 | 26.6 ± 0.6 |

**Fig. 4. SSTRs subtypes mRNA expression in PC Cl3 and PC E1A cell line assayed by means of RT-PCR techniques.** Only a 273-bp cDNA fragment, corresponding to the SSTR4 mRNA, was amplified in both cell lines. The NULL lanes represent the incubation of a mixture of all the primers without mRNA in the RT-PCR reaction.

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To evaluate the role of the somatostatin-dependent activation of PTPases, we tested the inhibitory effects of somatostatin on DNA synthesis in the presence or the absence of the PTPases inhibitor, vanadate (31). As shown in Fig. 5, the treatment with vanadate increased basal and stimulated DNA synthesis in the normal and transformed cell lines, confirming that increased tyrosine phosphorylation, induced by the blockade of PTPases, resulted in an activation of DNA synthesis (14). Somatostatin, which powerfully inhibited insulin- and insulin + TSH-stimulated DNA synthesis in control PC Cl3 cells, was completely ineffective in preventing insulin-stimulated [3H]thymidine incorporation after pretreatment with 25 μM vanadate and in these experimental conditions only slightly reduced the stimulatory effects of the insulin + TSH treatment.

Similar results were obtained by flow cytometry cell cycle analysis. Indeed, the blockade of insulin- or insulin + TSH-stimulated progression from G0 to the S phase of the cell cycle induced by somatostatin was completely reverted by vanadate treatment (data not shown). In PC E1A cells, somatostatin was ineffective both in control and in vanadate-treated cells, although vanadate increased basal and stimulated DNA synthesis (Fig. 6).
total proteins of PC Cl3 cells kept in the 6H mixture containing insulin and TSH, without somatostatin, or total proteins of PC Cl3 cells treated with the 6H mixture plus 1 mM somatostatin, were size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred on polyvinylidene difluoride membranes, and probed with anti-phosphotyrosine antibody. Somatostatin induction of PTPase activity caused a selective dephosphorylation of some protein bands that were highly phosphorylated in the cells not treated with somatostatin (Fig. 8). This effect was specific because the phosphorylation state of all the other proteins was not modified by somatostatin treatment (Fig. 8).

**DISCUSSION**

In the present study we have characterized the effect of somatostatin on the proliferation rate of PC Cl3 clonal thyroid cells and the modifications that occurred in the somatostatin control of cell growth after stable expression of the E1A oncogene. PC Cl3 cells are dependent on insulin and TSH for their proliferation. Interestingly, although in FRT L5 thyroid cell line the increase of cAMP levels induced by TSH or other adenylyl cyclase-stimulating agents is sufficient to induce cell proliferation (30,18), in PC Cl3 cells neither TSH nor forskolin alone induced cell proliferation, but both agents highly synergized with insulin. This observation is in line with previous studies where the cooperation between the cAMP/PKA system and the tyrosine kinases-activated pathway was demonstrated to be involved in the regulation of thyroid cell proliferation (32).

The expression of the E1A oncogene, although it induced a higher proliferation rate in normally cultured cells, did not significantly change the responsivity to insulin and TSH in G0/G1-synchronized cells, confirming that this cell line still needs external stimulatory signals to proliferate.

In PC Cl3 cells, somatostatin significantly inhibited the induction of DNA synthesis stimulated by insulin and the synergetic activation induced by insulin and TSH. FACS analysis demonstrated that somatostatin exerted its antiproliferative effects by slowing down the G2/S progression. This cytostatic effect was temporary, because after 32 h of treatment there was no significant difference between PC Cl3 cells treated with insulin or insulin + TSH, in the absence or the presence of somatostatin. Similar results were recently demonstrated in the GH3 pituitary cell line where somatostatin induced antiproliferative effects via a partial G1/S block (33). The lack of a prolonged inhibitory effect of somatostatin may be due to either

**FIG. 5.** Effect of vanadate pretreatment on somatostatin (SOM) inhibition of insulin and insulin + TSH stimulation of DNA synthesis in PC Cl3 cells. Vanadate potentiated basal, insulin, and insulin + TSH stimulation of $[^{3}H]$thymidine incorporation. The blockade of PTPases by vanadate pretreatment completely abolished somatostatin inhibition of insulin-dependent DNA synthesis and greatly reduced the effects of somatostatin on the stimulatory activity of the insulin + TSH treatment. *, p < 0.05; **, p < 0.01 versus respective treatment in the absence of somatostatin.

**FIG. 6.** Effect of vanadate pretreatment on somatostatin’s effects on insulin and insulin + TSH stimulation of DNA synthesis in PC E1A cells. Vanadate potentiated basal, insulin, and insulin + TSH stimulation of $[^{3}H]$thymidine incorporation. Somatostatin (SOM) treatment did not modify both insulin and insulin + TSH stimulation of DNA synthesis neither in control cells nor after vanadate pretreatment.

**TABLE IV**

Effect of somatostatin on basal and TSH-stimulated cAMP accumulation in PC Cl3 and PC E1A cells

|        | PC Cl3 | PC E1A |
|--------|--------|--------|
| Basal  | 1.2 ± 0.1 | 0.95 ± 0.05 |
| Somatostatin (1 mM) | 1.1 ± 0.07 | 0.92 ± 0.07 |
| TSH (100 nM) | 5.7 ± 0.2a | 5.1 ± 0.7a |
| TSH + somatostatin | 2.95 ± 0.5b | 2.0 ± 0.5b |

*a p < 0.01 versus basal values.

*b p < 0.01 versus TSH.
SSTR down-regulation after prolonged somatostatin stimulation or degradation of the peptide. This effect was completely reverted by pretreatment of PC Cl3 cells with the PTPase inhibitor vanadate, suggesting that somatostatin may exert direct antiproliferative effects through the activation of PTPases.

Vanadate has been reported to affect also other intracellular effectors, such as Na\(^+\)/K\(^-\)ATPases, that cannot be completely excluded as mediators of somatostatin effects. However, the direct measurement of somatostatin stimulation of PTPase activity strongly supports the involvement of this class of enzymes in the inhibition of growth induced by somatostatin.

Interestingly, vanadate did not completely abolish the effects of somatostatin on the insulin + TSH-stimulated DNA synthesis. This observation may be ascribed to the somatostatinergic inhibition of cAMP formation that is not blocked by vanadate. Thus, somatostatin antiproliferative activity seems to be mediated by a simultaneous modulation of both PTPase and adenylyl cyclase activities, although the former event seems to be essential for the induction of the somatostatin-dependent growth arrest.

On the contrary, in PC E1A, somatostatin was completely ineffective in inhibiting insulin-stimulated DNA synthesis, being only able to slightly reduce \[^3\]H\)-thymidine incorporation induced by the simultaneous treatment with insulin and TSH. This observation suggests that although PC E1A cells are comparable with the normal cells regarding their responsivity to stimulatory factors, they lack some component of the inhibitory pathways modulated by somatostatin. In particular, our observation that following the expression of the E1A oncogene, the somatostatinergic inhibition of adenylyl cyclase is still present but that a marked impairment of the stimulatory effects of somatostatin on PTPase activity occurred may represent a good biochemical correlate to explain the complete inefficacy of somatostatin on insulin-stimulated DNA synthesis, whereas a low level of inhibition persist in conditions of stimulation with insulin + TSH in these cells.

Previous studies demonstrated that although all of the five SSTR subtypes are able to inhibit adenylyl cyclase activity (34), only SSTR1 and SSTR4, which show the highest structural homology among the SSTRs (35), seem to mediate the effects of somatostatin on PTPase activity. In fact, the activation of SSTR1, transfected in heterologous cells, greatly increased PTPase activity (12, 9), and SSTR4 was reported to be the only SSTR subtype expressed in the Mia PaCa2 cell line (35), where initially the coupling between SSTRs and PTPases was discovered (11, 8). On the contrary, controversial data have been reported on the role of SSTR2 on this parameter (12, 9). Our study confirms these observations, because SSTR4 is the only SSTR expressed in PC Cl3 cells in which somatostatin is able to stimulate PTPase activity. In our experimental model, the expression of E1A oncoprotein did not alter the pattern of expres-
sion for the SSTRs. The activation of this receptor inhibited TSH-stimulated cAMP accumulation in both cell lines, confirming that both in normal and in E1A-expressing cells, SSTRs are present on the membranes and regularly coupled to adenyl cyclase. Conversely, alterations of the somatostatin signal transduction, induced by E1A expression in thyroid cells, were observed in the coupling of SSTRs with PTPases. In fact, in these cells, although PTPases seem to be regularly active in basal conditions, as revealed by the PTPase assay or by the stimulatory effects induced by vanadate on DNA synthesis, there was no stimulation of PTPase activity after somatostatin treatment. The nature of this alteration is still to be determined, although it is possible that it may involve the activity and/or the expression of adaptor molecules or possibly GTP-binding proteins that were previously reported to couple SSTRs to PTPases (8). Because E1A activity is exerted at the level of the G1/S transition, likely bypassing the retinoblastoma gene product-dependent check point (19), and somatostatin exerted its cytostatic effects by blocking the entry in the S phase (this paper and Ref. 33), the expression of E1A could be able to overcome this checkpoint by abolishing the somatostatinergic modulation of PTPase activity. Interestingly, in another PC 13-derived cell line, expressing both E1A and polyomavirus middle T oncogenes, a completely malignant phenotype was induced (21), and basal and somatostatin-stimulated PTPase activity was almost completely abolished (2), further supporting the hypothesis that a hormonal-regulated subclass of PTPase may be responsible for the control of cell proliferation.

In conclusion, our data demonstrate that: 1) in the PC 13 thyroid cell line, somatostatin, through its receptor subtype SSTR4, causes antiproliferative effects inducing a partial block in G0/G1 phases; 2) this effect may be, at least in part, due to the stimulation of PTPase activity, which causes dephosphorylation of specific substrates; and 3) the expression of E1A oncogene in these cells abolishes the somatostatinergic negative control of cell proliferation, likely by impairing somatostatin effects on PTPases.

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Somatostatin Inhibits PC Cl3 Thyroid Cell Proliferation through the Modulation of Phosphotyrosine Phosphatase Activity: IMPAIRMENT OF THE SOMATOSTATINERGIC EFFECTS BY STABLE EXPRESSION OF E1A VIRAL ONCOGENE

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