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Somatostatin Is Expressed in FRTL-5 Thyroid Cells and Prevents Thyrotropin-Mediated Down-Regulation of the Cyclin-Dependent Kinase Inhibitor $p27^{kip1}$*

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ABSTRACT

Using RT and amplification, we have detected specific RNA transcripts encoding somatostatin in FRTL-5 thyroid cells. This observation indicates that within the thyroid context, expression of somatostatin is not restricted to the parafollicular C cells. Transfection of FRTL-5 cells with constructs containing either the complete somatostatin gene promoter or deletions carrying the cAMP response element-binding site allowed us to demonstrate that transcription of the somatostatin gene is hormonally regulated by TSH. Blockage of somatostatin by specific antibodies resulted in an increased capacity of TSH-induced FRTL-5 cell-conditioned medium to promote cell proliferation, demonstrating that under physiological conditions, somatostatin exerts a cytostatic effect on FRTL-5 cells growth. Somatostatin treatment of FRTL-5 cells resulted in a growth retardation, caused by a dose-response delay in the G_1 phase of the cell cycle. This effect appears to be mediated by the cyclin-dependent kinase inhibitor $p27^{kip1}$, which is clearly down-regulated in FRTL-5 cells treated with TSH and whose expression is reestablished by somatostatin in a dose-dependent manner. Participation of somatostatin in the control of FRTL-5 cell proliferation is in agreement with the detection of specific somatostatin receptor type 2. Flow cytometric assays reveal that FRTL-5 cells transformed with the K-*ras* oncogene are still sensitive to somatostatin treatment, whereas fully neoplastic FRT cells no longer respond to this peptide. Taking together, the results demonstrate the participation of an autocrine loop in the control of thyroid cell proliferation, and the possibility that this mechanism could be altered in the process of thyroid carcinogenesis. (*Endocrinology* **140:** 87–95, 1999)

THE PRECISE control of cell proliferation requires the balance between positive effectors of cell growth and cytostatic signals that allow the maintenance of the differentiated phenotype. In the follicular thyroid cell, the transition from quiescent to proliferating cells is mainly mediated by the actions of TSH and insulin-like growth factor I (IGF-I) (1, 2) and involves the activity of the tissue-specific transcription factors TTF-1 and Pax-8 (3). Less is known about the antimitogenic signals affecting thyroid cell proliferation.

Somatostatin was initially identified as a potent GH inhibitor in the hypothalamus and soon after was found in many tissues and cell lines, exerting different biological actions, including inhibition of hormone secretion processes, modulation of cardiovascular activity, control of neural cognitive and locomotor functions, and regulation of cell proliferation (4, 5). Immunoreactive somatostatin has also been found in the thyroid, more precisely limited to the parafollicular C cells, leading to the speculation that this peptide could elicit local effects on thyroid hormone release acting locally, in a paracrine fashion (6). In the differentiated thyroid cell line FRTL-5, somatostatin inhibits TSH- and IGF-I-mediated cell proliferation (7). More recently, the effect of somatostatin on cell proliferation has also been characterized in the thyroid cell line PC Cl3. In these cells, somatostatin is able to inhibit insulin-dependent and insulin-plus TSH-dependent cell growth, causing a delay block in the G_1-S transition of the cell cycle (8).

Somatostatin action is mediated by specific receptors, and to date, six different subtypes (designated SSTR-1, -2A, -2B, -3, -4, and -5) have been cloned and functionally characterized in various cell systems, including pancreas, adrenal cortex, and brain tissue (9). They belong to the seven-transmembrane domain superfamily and are coupled to different G proteins that link SSTR to distinct cellular effector systems, such as adenyl cyclase, K^+, and Ca^{2+} channels. Different receptors may regulate a variety of cellular functions, thus providing a broad spectrum of somatostatin action. SSTR2 along with SSTR1 and -5 are responsible for the antiproliferative effect of somatostatin and are expressed in many tumor types (10, 11). Currently, the design of synthetic somatostatin analogs with high affinity for these SSTR is a clinical approach for a variety of tumor diseases.

Several reports indicate that the mechanisms responsible for the antiproliferative effect of somatostatin are related to its ability to modulate phosphotyrosine phosphatase activity (8, 10, 12, 13). In this study we show that somatostatin action is mediated by the cyclin-dependent kinase inhibitor $p27^{kip1}$. This protein, initially identified by the ability to bind the complexes cyclin E-cyclin-dependent kinase-2 (cyclin E-CDK2) (14) and cyclin D-CDK4 (15), belongs to the Cip/Kip family of CDK inhibitors and plays a critical role in the regulation of G_1-S transition (16).

In this report we provide evidence that somatostatin is

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expressed in FRTL-5 cells. Furthermore, transcription of the somatostatin gene is under hormonal control exerted by TSH, as determined by transient transfection assays. Experiments with conditioned medium from TSH-treated FRTL-5 cells and somatostatin antibodies revealed that this peptide exerts a cytostatic effect on cell growth. This circumstance is further supported by cytometric assays, showing that somatostatin blocks FRTL-5 cell cycle in the G_1 phase. As mentioned above, we demonstrate that this effect is mediated by the capacity of somatostatin to reestablish the expression of the CDK inhibitor $p27^{kip1}$, which is down-regulated by TSH to promote proliferative signals. In thyroid tumor and transformed cells, the ability of somatostatin to modulate cell growth is altered. The results suggest that within the thyroid follicular cell, somatostatin expression is used as a precise, local mechanism to regulate cell growth in an autocrine fashion.

Materials and Methods

Cell culture and somatostatin treatment

FRTL-5 thyroid cells (17) (American Type Culture Collection, Manassas, VA; CRL 8305) were grown in Coon's modified Ham's F-12 medium (Sigma Chemical Co.) supplemented with 5% donor calf serum (Life Technologies, Gaithersburg, MD) and a hormone mixture including 10 ng/ml glycyl-L-histidyl-L-lysine, 5 μg/ml transferrin, 10 nM hydrocortisone, 10 ng/ml somatostatin, 10 μg/ml insulin, and 1 nM TSH. K-*ras*-transformed FRTL-5 (18) and the thyroid tumor cell line FRT (19) cells were maintained in the same conditions. All of the hormones were purchased from Sigma Chemical Co. (St. Louis, MO). The human thyroid carcinoma cell line ARO (20), the rat pancreatic-derived cell line RIN (21), and the human medullary carcinoma cell line MTT (22) were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 mg/ml sodium pyruvate, and antibiotics. Before treatment, cell cultures were maintained for 72 h in a 3H medium (basal Coon's supplemented with 0.2% FCS without insulin, TSH, and somatostatin).

RT-PCR amplification

Total RNA was isolated as previously described (23). For first strand complementary DNA synthesis, total RNA (1 μg) was treated with deoxyribonuclease and reversely transcribed at 42 C for 30 min using Moloney murine leukemia virus reverse transcriptase (Pharmacia Biotech, Piscataway, NJ). Aliquots of the first strand reactions (10 μl) were used as templates for subsequent PCR using *Taq* polymerase (Perkin-Elmer, Norwalk, CT). For detection of somatostatin transcripts, the following primers were used: forward, 5′-CAGACTCCGTCAGTTTCTGCA-3′; and reverse, 5′-GCTAACAGGATGTGAATGTC-3′. Amplification was performed in 35 cycles of 30 sec (2 min for the first cycle) at 95 C, 30 s at 55 C, and 1 min at 72 C (5 min for the last cycle). For the different somatostatin receptors (24–27), the primers for forward and reverse annealing were: SSTR-1, 5′-GCTACGTGCTCATCATTGCTA-3′ and 5′-GGACTCCAGGTTCTCAGGTTC-3′; SSTR-2, 5′-TTGGTACACAGGGTTCACAT-3′ and 5′-GTCTCCGTGGTCTCATT CAGC-3′; SSTR-3, 5′-TCATCTGCCTCTGCT ACCTA-3′ and 5′-GAGCCCAAAGAAGGCAGGCT-3′; SSTR-4, 5′-ATCTTCGCA GACACCAGACC-3′ and 5′-ATCAAGGCTGGTCACGACGA-3′; and SSTR-5, 5′-GCCGGCCTCTACTTCTTCGTG-3′ and 5′-CCGTGGCGTCAGCGTCCTTGG-3′. Conditions for PCR have been recently described (28). PCR products were visualized in 2% agarose gels, and the identities of positive bands were confirmed by direct sequencing of the PCR products (automated DNA sequencer, Applied Biosystems, Foster City, CA).

Plasmids and transfections

The reporter construct containing the complete somatostatin gene promoter (SMS900), deleted constructs containing the cAMP response element (CRE)-binding site with or without the upstream enhancer promoter (SMS120 and SMS65, respectively), and the construct containing the region of the TATA box (SMS42) have been used in this study and were described previously (29). These constructs were transiently transfected by the calcium phosphate precipitation technique (30) into confluent FRTL-5 cells and then maintained in basal medium (3H) or treated for 24 h with 1 nM TSH. The plasmid CMV-Luc was used to correct for transfection efficiency. Forty-eight hours after transfection, cell extracts were prepared, and chloramphenicol acetyl transferase and luciferase activities were determined (30, 31).

Cell proliferation and flow cytometric analysis

Conditioned medium from FRTL-5 cells maintained in basal conditions (3H) and treated with TSH (3H+TSH) was collected after 72 h for functional assays. The mitogenic activity of the medium was tested on quiescent FRTL-5, by cell counting after 24, 48, and 72 h. To test any potential role of somatostatin, this conditioned medium was preincubated with a specific somatostatin antibody (4 μg/ml) (32) for 1 h at room temperature before addition to the FRTL-5 cultures. Growth curves in the presence of increasing concentrations of somatostatin were also performed. FRTL-5 cells (2×10^4) were maintained in basal medium for 72 h and then treated with 1 nM TSH, somatostatin, or TSH plus somatostatin (10 nM, 1 μM, and 2 μM). The number of viable cells was determined by cell counting every 24 h for 4 consecutive days, and the average of three independent experiments is represented. Cell cycle distribution from propidium iodide-stained samples was performed as previously described (33), using a FACScan flow cytometer (Becton Dickinson Co., Mountain View, CA). At least 10,000 events were collected and analyzed. Data were integrated and plotted with the software CellQuest.

Immunoblotting analysis

Total protein extracts (40 μg) were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) in a buffer containing 25 mM Tris, 200 mM glycine, and 20% methanol. After blocking the membranes with 10% low fat dried milk in Tris-buffered saline containing 0.05% Tween-20, immunodetection was performed using 5 μg/ml of a commercial antibody for $p27^{kip1}$ and cyclin D1 (Oncogene Science, Inc., Cambridge, MA). After probing with the antibodies, membranes were incubated with a streptavidin-conjugated antirabbit specific secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminiscence (Amersham, Arlington Heights, IL). To assess equal loading of the samples, the same blots were reprobed with a specific antiactin antibody (Calbiochem, La Jolla, CA).

Statistical analysis

Statistical significance between different treatments was determined using Student's t test. Differences are considered significant at $P < 0.05$.

Results

Somatostatin- and SSTR2-specific transcripts are detected in thyroid FRTL-5 cells

In search of regulatory mechanisms responsible for the control of thyroid cell proliferation, we evaluated whether somatostatin could be expressed and secreted by the follicular thyroid cell in an autocrine fashion. To address this question, we isolated total RNA from FRTL-5 cells and performed RT-PCR reactions in the conditions described *in Materials and Methods*. Forward primer was designed from exon 1 sequences, whereas reverse primer was located in exon 2. This way, any potential contamination of DNA would render a larger PCR fragment containing intronic sequences. PCR reactions were resolved in agarose gels and in these conditions, a 271-bp DNA band was reproducibly amplified from FRTL-5 retrotranscribed RNA (Fig. 1A). In control experiments, the same band was obtained from RNA samples extracted from the human medullary carcinoma cell line

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MTT and the rat pancreas-derived cell line RIN, whereas in agreement with previous reports (34, 35), somatostatin transcripts were absent in RNA extracted from liver tissue. The integrity of the RNA preparations was confirmed with specific primers for β-actin (Fig. 1A). To further assure that amplified DNA contained somatostatin related sequences, the 271-bp PCR product was purified and subjected to direct DNA sequencing. The nucleotide sequence obtained was identical to that previously described for the rat somatostatin gene (36).

The results unambiguously demonstrate that somatostatin transcripts are expressed in FRTL-5 cells, so we next determined the presence of specific somatostatin receptors. Specific pairs of primers for SSTR-1, -2, -3, -4, and -5 were used in RT-PCR reactions. The assay revealed that FRTL-5 cells exclusively express SSTR-2 (Fig. 1B), failing to detect the expression of SSTR-1, -3, -4, and -5. We also observed expression of SSTR-2 in MTT cells, RIN cells, and the human thyroid carcinoma cell line ARO, which also expressed SSTR-1. Positive amplification of SSTR-3, SSTR-4, and SSTR-5 was found in the human medullary carcinoma cell line MTT.

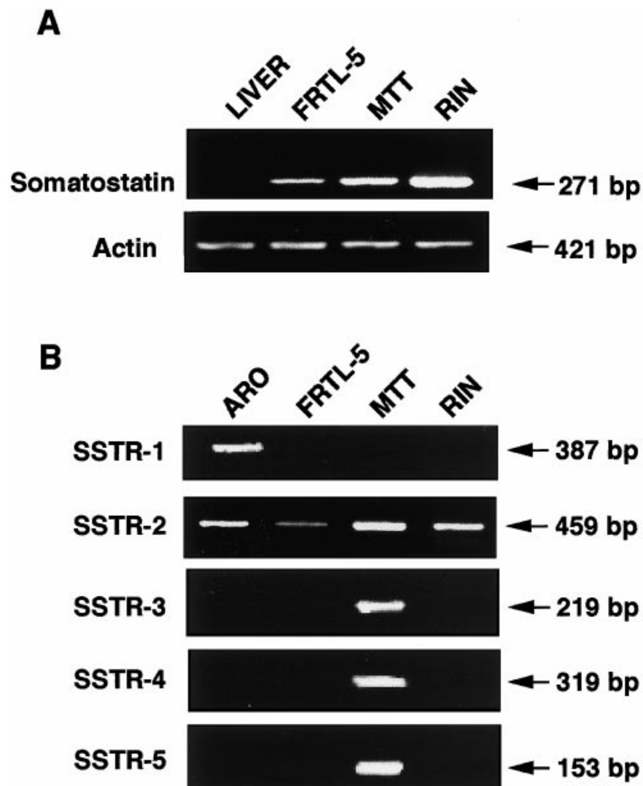

FIG. 1. Detection of somatostatin and SSTR2-specific transcripts in FRTL-5 cells. A, Total RNA extracted from rat liver and FRTL-5, MTT, and RIN cells was subjected to RT and amplification using specific primers for somatostatin. To assess the integrity of the RNA preparations, β-actin was amplified from the same samples. B, Detection of different SSTRs from ARO, FRTL-5, MTT, and RIN cells was also performed by RT and amplification. PCR products were resolved by electrophoresis, and DNA fragments were visualized in agarose gels stained with ethidium bromide. The sizes of the PCR fragments are indicated.

Transcription of the somatostatin gene in FRTL-5 cells is hormonally regulated by TSH

The detection of somatostatin transcripts in FRTL-5 cells prompted us to analyze whether transcription of this gene is hormonally regulated by TSH, one of the major inductors of thyroid cell proliferation. Due to the low levels of somatostatin produced by FRTL-5 cells and the difficulty in determining precisely by RT-PCR messenger RNA variations in response to TSH, an indirect transfection approach was used. For that purpose, we determined the ability of TSH to promote transcription of a reporter construct containing the complete somatostatin gene promoter (designated SMS900). Also, considering that somatostatin expression in other cell systems is mainly controlled at the transcriptional level by CRE (29), the participation of CRE in TSH-mediated somatostatin gene expression was evaluated by using deletion constructs carrying a CRE-binding site with or without the upstream enhancer element (SMS120 and SMS65, respectively). These constructs, along with a negative TATA control vector (SMS42) were transiently transfected in FRTL-5 cells, using the plasmid CMV-Luc to correct for transfection efficiency. After transfection, cells were maintained in 3H medium and then treated with TSH for 24 h. Control assays without TSH were carried out in parallel. Results showed a significant increase in transcription activity from the complete somatostatin promoter (SMS900) in the presence of TSH (Fig. 2). To a minor extent, transcription was also increased with the SMS120 construct, whereas SMS65, which contains a unique CRE-binding site, yielded a weak activation of gene transcription. When the same set of experiments were performed in the presence of the cAMP analog forskolin, we

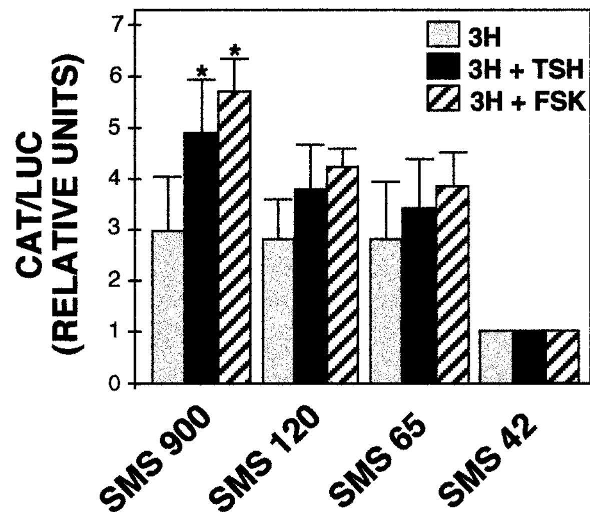

FIG. 2. Transcription of somatostatin in FRTL-5 cells is regulated by TSH. Equal numbers of cells (2×10^6) were transfected with reporter construct containing the complete somatostatin promoter (SMS900) or deletions containing a CRE element with or without the upstream enhancer element (SMS120 and SMS65, respectively). A control vector containing the TATA box (SMS42) was also used. Plates were then treated with either TSH (10^{-9} U/ml) or forskolin (10 μg/ml) for 24 h. Induction of the somatostatin gene was determined as percentage of acetylation. Values were corrected for transfection efficiency with luciferase activity. Values represent the average of three independent experiments. *, Significant differences *vs.* 3H ($P < 0.01$).

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found a similar patter of induction of gene transcription. The results demonstrated that the somatostatin gene promoter is functional in FRTL-5 cells, and although moderate, its expression is positively regulated by TSH through the cAMP signaling pathway.

Biological effects of somatostatin expression in FRTL-5 cells

The detection of somatostatin transcripts, the presence of somatostatin receptors, and the fact that transcription of the gene is positively regulated by TSH prompted us to analyze whether an autocrine loop of somatostatin could be functionally established in FRTL-5 cells. The contribution of autocrine production of somatostatin to FRTL-5 cell growth was analyzed by testing the mitogenic activity of the conditioned medium from cells maintained in basal medium (3H) and treated with TSH (3H+TSH), where expression of somatostatin should be induced. Conditioned medium was collected and added to quiescent FRTL-5 cells, and cell number was determined after 24, 48, and 72 h. Results are summarized in Table 1. 3H-conditioned medium did not have any significant effect on FRTL-5 cell growth. As expected, conditioned medium from TSH-stimulated FRTL-5 clearly induced cell proliferation due to the presence of TSH in the conditioned medium. These effects were evident after 24 h of the addition of conditioned medium.

To search for any specific effect mediated by somatostatin, presumably produced by FRTL-5 after TSH stimulation, conditioned medium from TSH-stimulated cells was preincubated with an antisomatostatin antibody (32) before the addition to quiescent FRTL-5 cells. Cell number was also determined at the same time points (24, 48, and 72 h), and cell number was determined. Although no significant differences were found after 24 and 48 h, cell number was always higher when somatostatin was specifically blocked from the FRTL-5 culture medium. These results were significant after 72 h and indicate that although other growth factors secreted by TSH-induced cells could mask somatostatin action, this peptide exerts a cytostatic effect on FRTL-5 cell growth. When the same set of experiments was performed in the presence of an irrelevant IgG instead of the somatostatin antibodies, cell number was similar to that obtained with 3H+TSH-conditioned medium (data not shown).

Somatostatin exerts an inhibitory effect of TSH-mediated cell proliferation resulting in cellular accumulation in the G_1 phase of the cell cycle

After demonstrating a biological role for somatostatin in the FRTL-5 conditioned medium, the effects of the peptide on cell growth and cell cycle distribution were analyzed in more detail. Growth curve profiles of depleted cells treated with TSH (1 nM), somatostatin, and the combination of TSH and somatostatin (10 nM, 1 μM, and 2 μM) were determined every 24 h for 4 consecutive days. Control cells maintained in 3H medium were also included in the assay (Fig. 3). In the latter, the number of viable cells remained unaltered throughout the assay. TSH rapidly increased cell number, with the induction of cell proliferation more evident within the first 24 h of TSH exposure. To a minor extent, proliferation was detected up to 72 h. When TSH was added in combination with somatostatin, a detectable inhibition of cell proliferation was observed even at a dose of 10 nM. Higher concentrations of somatostatin increased growth inhibition, and at 1 μM, reduction of TSH-induced proliferation was nearly 40% after 24 h. Similar results were obtained at 2 μM, suggesting that although the cellular response is dose dependent, a partial desensitization in response to somatostatin is observed at high doses. It is important to remark that whereas cell number stabilized after 72 h of TSH treatment, the presence of somatostatin in the medium decreased cell viability after 3 days in culture. We also found that somatostatin requires the presence of the positive effector to alter cell proliferation, as in the absence of TSH, somatostatin did not exert any effect on FRTL-5 cell growth, demonstrating the connection between somatostatin and TSH signaling pathways.

Cell cycle distribution in the same experimental conditions were performed by flow cytometric assays from propidium iodide-stained samples (Fig. 4). Cells maintained in 3H medium for 72 h were stimulated with TSH and treated with different concentrations of somatostatin. After 16 and 24 h, samples were collected, fixed in ethanol, and stained with propidium iodide for cell cycle analysis. Results are summarized in Table 2. Cell cycle distribution of 3H cells remained unaltered throughout the assay, with S phase values approximately 4% and G_2-M values 16% of the total cell population. It is important to mention that although these values seem to indicate cellular transit through the cell cycle, growth curves indicate that FRTL-5 cells do not proliferate under these conditions (Fig. 3). As it will be discussed below,

TABLE 1. Effect of FRTL-5 conditioned medium on cell growth

	Cell no. ($\times 10^4$)		
	24 h	48 h	72 h
Basal (3H)	38.7 ± 3.2	40.75 ± 10.1	51 ± 14.3
+TSH	84.8 ± 4.3	100.37 ± 13.1	125.1 ± 19.1
+TSH + anti-SS	88.2 ± 3.9	106.75 ± 12.7	189.6 ± 18.2[a]

Cells were maintained in 3H for 96 h, conditioned medium was collected and added to depleted FRTL-5 cells in culture (2×10^5 cells/dish). Viable cell number was then determined after 24, 48, and 72 h. Somatostatin antibody was used at a dilution of 4 μg/ml.
[a] $P < 0.01$ *vs.* 3H + TSH.

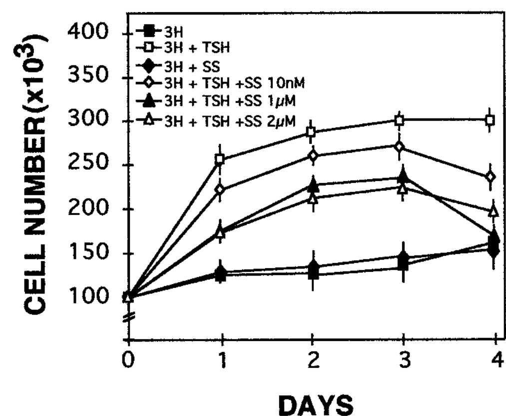

FIG. 3. Effect of somatostatin on TSH-mediated cell proliferation. Cells maintained in basal medium for 72 h were treated with somatostatin and TSH alone or in combination. Cell number was monitored every 24 h for 4 consecutive days, and viable cell number is represented. The data are the mean ± SD of three independent experiments.

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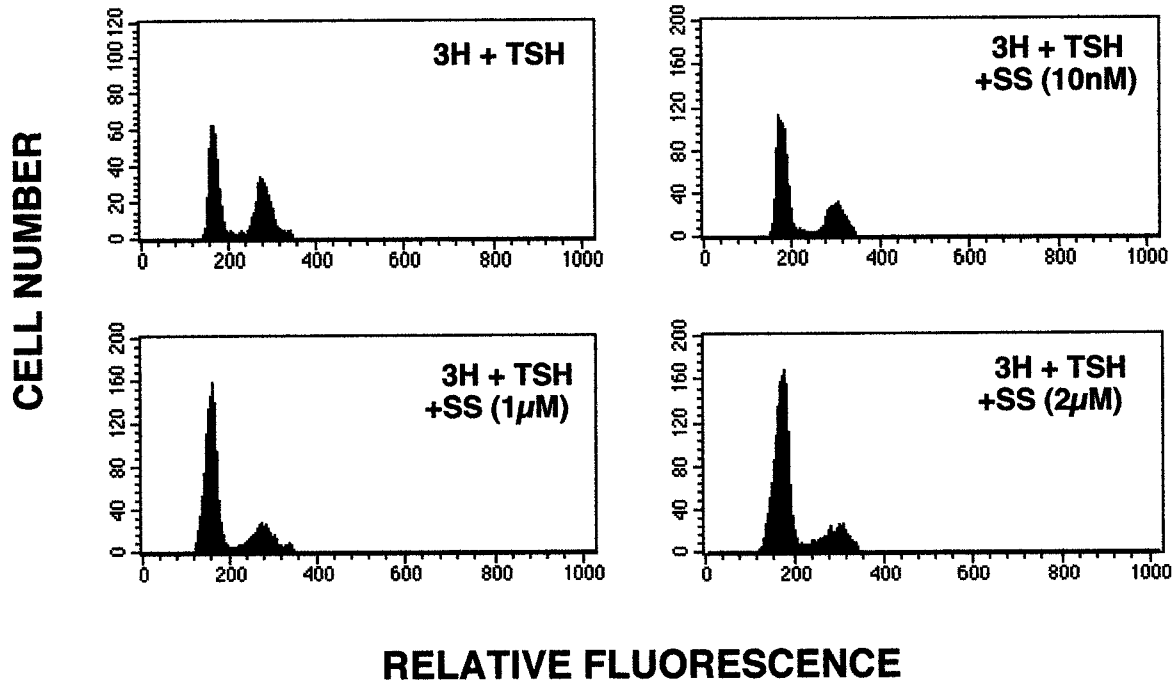

FIG. 4. Effect of somatostatin treatment on cell cycle distribution of FRTL-5 cells. Flow cytometric histograms of FRTL-5 cells maintained in 3H medium for 72 h, stimulated with TSH, and treated with different concentrations of somatostatin are represented. Samples were collected after 24 h of treatment for FACS analysis. The intensity of the propidium iodide staining *vs.* cell number is represented. The percentage of the cell cycle distribution is shown in Table 2.

TABLE 2. Cell cycle progression of FRTL-5 cells stimulated with TSH and treated with different concentrations of somatostatin

	16 h			24 h		
	G0/G1	S	G2/M	G0/G1	S	G2/M
3H	79.9 ± 1.5	4.1 ± 0.08	16.0 ± 0.16	78.9 ± 0.08	4.0 ± 0.08	17.1 ± 0.08
+TSH	76.7 ± 0.16	3.4 ± 0.08	19.9 ± 0.21	51.0 ± 0.24	6.2 ± 0.08	42.8 ± 0.24
+TSH + SS (10 nM)	74.9 ± 0.16	7.8 ± 0.16	17.3 ± 0.24	65.5 ± 0.16[a]	4.4 ± 0.16	30.1 ± 0.16
+TSH + SS (1 μM)	78.6 ± 0.32	4.3 ± 0.08	17.1 ± 0.16	77.7 ± 0.24[a]	5.6 ± 0.17	16.7 ± 0.24
+TSH + SS (2 μM)	77.1 ± 0.16	4.6 ± 0.09	18.3 ± 0.16	80.5 ± 0.4[a]	3.8 ± 0.16	15.7 ± 0.26

Samples were collected after 16 h and 24 h of TSH with or without somatostatin treatment, and cell cycle was analyzed by FACScan. Values are expressed as percentages.

[a] $P < 0.01$ *vs.* 3H + TSH.

we believe that this circumstance could be due to the absence of somatostatin and the presence of 0.2% serum in the basal medium. TSH treatment of depleted FRTL-5 cells clearly promoted G_1 transition to S phase, reaching maximum accumulation in G_2-M (42.8%) after 24 h. However, when the cells were treated with TSH and somatostatin, a dose-dependent accumulation in G_1 was detected, and at 2 μM, cell cycle profiles were similar to those obtained in cells maintained in 3H medium (Fig. 4).

Effect of somatostatin on cell cycle correlate with the ability to prevent TSH-mediated down-regulation of $p27^{kip1}$

To examine the mechanisms responsible for growth inhibition induced by somatostatin, and more precisely the G_1 block detected by flow cytometry, we investigated cell cycle-related genes that could be mediating somatostatin action. As the CDK-dependent kinase inhibitor $p27^{kip1}$ is one of the major regulators of the G_1-S transition, we analyzed the effect of TSH on $p27^{kip1}$ protein levels by Western blot in the absence or presence of somatostatin (Fig. 5). The results demonstrate that the ability of TSH to promote cell proliferation is associated with a strong inhibitory effect on $p27^{kip1}$, which becomes undetectable after 24 h of TSH treatment. Somatostatin was able to block TSH-mediated down-regulation of $p27^{kip1}$, returning protein levels to almost those levels observed in quiescent cells. The effect was dose dependent, and as shown in Fig. 5B, increasing the concentration of somatostatin resulted in higher levels of $p27^{kip1}$ protein. The results are in agreement with flow cytometric profiles, indicating that maximum G_1 arrest is achieved at a 2-mM dose of somatostatin. These results were already evident after 16 h of TSH treatment and extended up to 36 h (not shown).

To further confirm that the effect on $p27^{kip1}$ protein levels was specifically modulated by somatostatin, we analyzed other cell cycle regulatory genes that are also involved in the G_1-S transition. We reasoned that if somatostatin specifically targets the G_1 phase of the cell cycle acting through p27, other cell cycle regulatory proteins important for the G_1-S transi-

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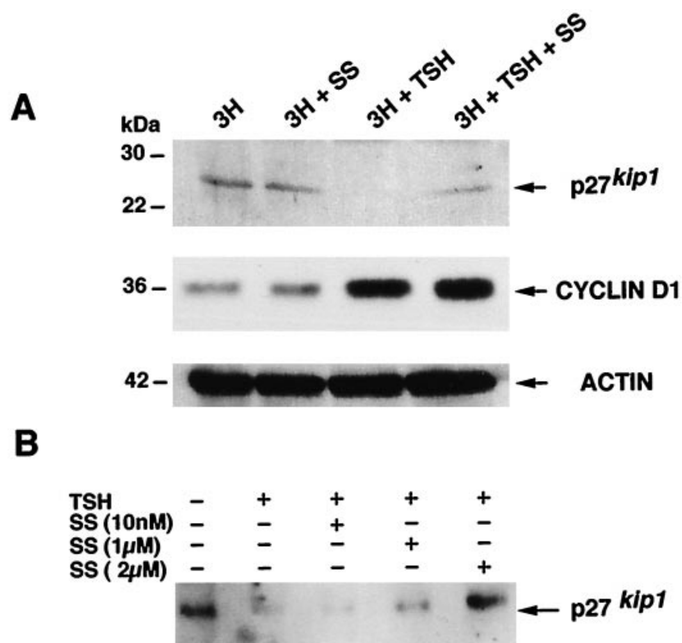

FIG. 5. Somatostatin prevents TSH-mediated down-regulation of the CDK-dependent inhibitor $p27^{kip1}$. A, FRTL-5 protein extracts from depleted (3H), somatostatin (3H+SS), and TSH-stimulated without (3H+TSH) or with somatostatin (3H+TSH+SS) were probed with specific antibodies for $p27^{kip1}$, cyclin D1, and actin. B, Dose response of $p27^{kip1}$ protein levels. Depleted FRTL-5 cells were treated with TSH in the absence or presence of increasing concentration of somatostatin for 24 h. After treatment, proteins were extracted and probed with a $p27^{kip1}$ antibody and revealed by chemiluminiscence.

tion, such as cyclin D1, would not be modulated by somatostatin. As expected, TSH treatment of quiescent cells increased protein levels of cyclin D1 (37), but in this case, somatostatin is not able to down-regulate cyclin D1 (Fig. 5A). This observation indicated that rather than decreasing the activity of the cyclin-CDK complexes, somatostatin causes cell cycle delay by increasing the level of the CDK inhibitor $p27^{kip1}$.

The ability of somatostatin to modulate cell growth is altered in transformed and tumor thyroid cells

The results presented indicate that somatostatin, secreted by FRTL-5 cells and acting through an autocrine loop, regulates thyroid cell growth. We analyze whether this potential regulatory pathway could be altered in the process of thyroid carcinogenesis, testing the cellular response to somatostatin in a transformed cell line (K-*ras*-FRTL-5) and a tumor-derived cell line (FRT). First, we analyzed whether these transformed and tumor cells express somatostatin receptors that could mediate a potential response to somatostatin. RT-PCR analysis reveals that as observed for FRTL-5 cells, K-*ras*-FRTL-5 cells and FRT cells express SSTR-2 receptor (Fig. 6).

We next determined the ability of somatostatin to modulate cell cycle in these cell lines. Because both cell lines are independent of TSH, quiescent cells were treated with serum in the present or absence of somatostatin. Results (Fig. 7) showed that K-*ras*-FRTL-5 cells are still sensitive to somatostatin, although higher doses (2 μM) are required to obtain a detectable increment in G_1 phase. Any dose over 2 μM did

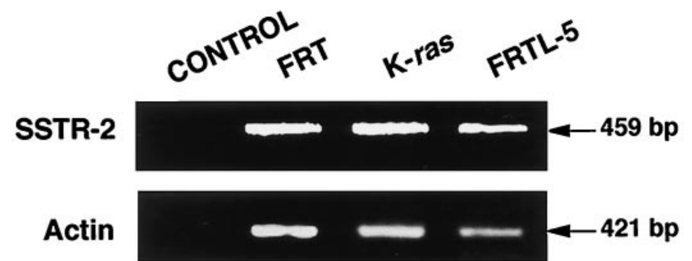

FIG. 6. Detection of SSTR-2 in transformed K-*ras*-FRTL-5 and tumor FRT cells. Total RNA was subjected to RT and amplification using specific primers for SSTR-2. PCR products were resolved by electrophoresis, and DNA fragments were visualized in agarose gels stained with ethidium bromide. β-Actin was amplified from the same samples. For the control reaction, the same RT and PCR reactions were performed in the absence of RNA. The sizes of the PCR fragments are indicated.

not result in greater inhibition of cell proliferation (Table 3). We also detected the presence of hypodiploid cells, presumably undergoing apoptosis, which are absent in control cells and FRTL-5 cells, although this observation requires further analysis. However, fully neoplastic thyroid FRT cells were no longer sensitive to somatostatin, as cell cycle distribution remained unchanged in the absence or presence of somatostatin.

The possibility that the regulatory mechanism mediated by $p27^{kip1}$ in differentiated thyroid cells could be also responsible for the cell cycle delay in K-*ras*-FRTL-5 was then analyzed. By Western analysis, we determined that K-*ras*-FRTL-5 express $p27^{kip1}$ in the absence of serum (Fig. 8). Unlike the addition of TSH to normal FRTL-5 cells, serum did not result in any significant decrease in $p27^{kip1}$. When serum-treated cells were exposed to somatostatin, $p27^{kip1}$ levels recovered, although these results were not significant either. These results indicate that in these transformed cells, the effect of somatostatin is rather weak, presumably indicating that within the process of carcinogenesis, the loss of somatostatin sensitivity is a late event and drives cells to a more undifferentiated status.

Discussion

The results presented in this paper provide strong evidence that somatostatin, produced by the differentiated thyroid cell and acting locally, may be responsible for a precise regulation of cell proliferation. The detection of somatostatin transcripts by means of PCR amplification in follicular thyroid cells revises classic studies in which expression of this peptide in the thyroid is limited to the parafollicular C cells (6) and suggest novel mechanisms of autocrine regulation of thyroid cell growth. This study also confirms previous observations that although somatostatin is mainly produced by central nervous structures and pancreatic cells (34), other sources may be found. In particular, in organs such as the pineal gland, a dual origin (neural and parenchymal) of somatostatin has been demonstrated (35).

Regulation of somatostatin transcription by TSH is specially relevant in FRTL-5 cells. First, it is important to remark that somatostatin expression is increased when FRTL-5 cells are treated with TSH. Second, the results obtained in our

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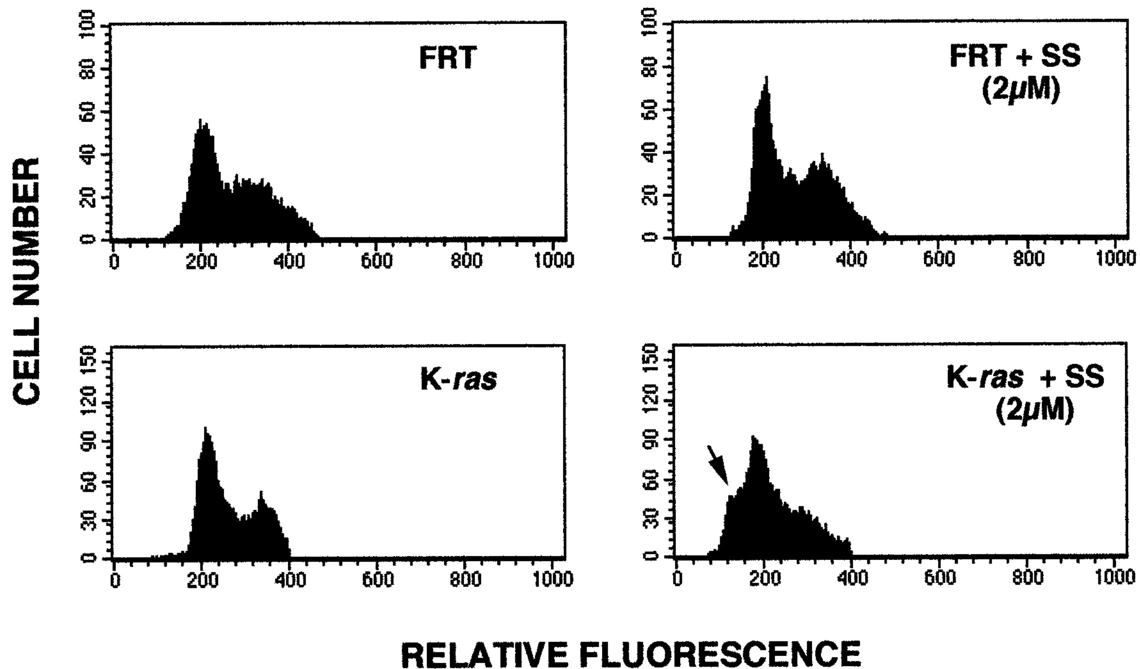

FIG. 7. Sensitivity of transformed and tumor cells to somatostatin. Exponentially growing rat thyroid carcinoma cells FRT and K-*ras*-transformed FRTL-5 cells were treated with 2 μM somatostatin for 24 h, and cell cycle distribution was analyzed (*right panels*). Untreated cells were used as a control (*left panels*). The percentage of the cell cycle distribution is shown in Table 3. The presence of hypodiploid nuclei after somatostatin treatment of K-*ras*-transformed FRTL-5 is shown by an *arrow*.

TABLE 3. Cell cycle progression of K-*ras*-FRTL-5 and FRT cells treated with different concentrations of somatostatin

	K-*ras*-FRTL-5			FRT		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	56.9 ± 0.08	24.7 ± 1.5	18.4 ± 0.48	50.7 ± 0.5	28.5 ± 0.48	20.8 ± 0.57
+SS (10 nM)	58.0 ± 0.8	24.4 ± 0.6	17.6 ± 0.58	52.9 ± 0.53	25.9 ± 1.63	21.1 ± 0.73
+SS (1 μM)	58.3 ± 0.4	24.6 ± 0.7	17.1 ± 0.97	54.0 ± 1.22	23.5 ± 0.5	22.5 ± 0.4
+SS (2 μM)	68.3 ± 1.14[a]	20.3 ± 1.3	11.4 ± 1.12	50.1 ± 0.27	22.5 ± 3.2	27.4 ± 1.87

Samples were collected after 24 h of TSH with or without somatostatin treatment, and cell cycle was analyzed by FACScan. Values are expressed as percentages.

[a] $P < 0.01$ *vs.* control.

transfection assays indicate that the somatostatin promoter is functional and positively regulated by TSH. The fact that a positive effector of cell growth such as TSH promotes transcription of an antiproliferative peptide is physiologically important, because the interaction and balance between both signals may be responsible for the precise growth rate of the thyroid cell. Somatostatin gene transcription has been well characterized in the pancreatic RIN cells (29, 38) and has been demonstrated to be mainly controlled at the transcriptional level by CRE, which is recognized by the CREB and several other nuclear proteins (36). In these cells, a CRE site and another element, designated UE (upstream enhancer), cooperate synergistically to promote transcription, and therefore, a deleted construct of the somatostatin promoter containing these two elements confers maximum activity to reporter constructs in the presence of cAMP activators (29, 38). With respect to FRTL-5 cells, we demonstrated that the somatostatin promoter is functional, and therefore, all the regulatory elements necessary for the transcription of this gene are present within these thyroid cells. It would be of interest to analyze which transcription factor(s) is involved in the control of somatostatin gene transcription and whether thyroid-specific transcription factors (39) play any role in this process.

It is generally accepted that TSH and IGF-I synergize to stimulate thyroid cell proliferation (1, 2). Whether TSH is sufficient to promote cell proliferation of FRTL-5 cells may depend on the culture conditions (37, 40). In our experiments, the addition of TSH to cells maintained in 3H medium clearly induces cell proliferation. The explanation for this different response of FRTL-5 cells to TSH may lie in the absence or presence of somatostatin. This peptide is routinely added to the FRTL-5 culture medium (17, 41, 42), whereas it is absent in our control assays. Somatostatin may be sufficient to keep cells arrested, and our cell cycle profiles in the absence or presence of somatostatin also support the idea that this peptide is a critical regulator of thyroid cell proliferation. These results are further supported by the fact that somatostatin, secreted by FRTL-5 cells in the presence of TSH, exert a cytostatic effect on thyroid cell growth.

The results presented indicate that the control of cell cycle regulation exerted by somatostatin specifically targets the G_1

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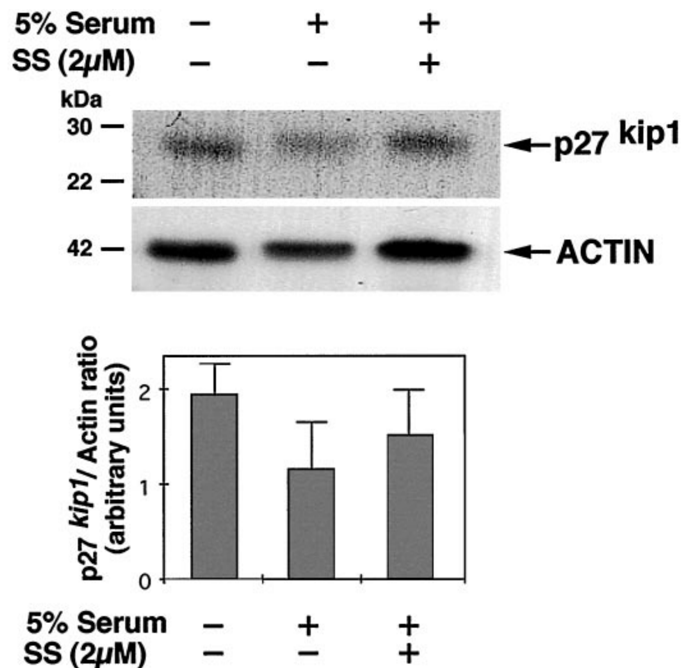

FIG. 8. Effect of somatostatin treatment on $p27^{kip1}$ protein levels in FRTL-5 cells transformed with K-*ras*. Cells were depleted for 36 h and then treated with serum in the absence or presence of somatostatin. After treatment, proteins were extracted and probed with a $p27^{kip1}$ antibody and revealed by chemiluminiscence. After exposure, the same membrane was stripped and reprobed with a actin antibody for loading control. Data obtained by the analysis of three autoradiographs are shown in the *lower panel* and represent the $p27^{kip1}$/actin ratio.

phase of the cell cycle. As described previously (43, 44), we also show that proliferative signals induced by TSH are associated with a down-regulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$. This down-regulation is prevented by somatostatin, which returns $p27^{kip1}$ protein levels to those observed in quiescent cells. Two pieces of evidence support the idea that this effect is specific rather than a consequence of differences in cell growth. First, the effect is dose dependent, and second, other cell cycle regulatory proteins important for G_1-S transition, such as cyclin D1, are not modified by somatostatin treatment. In this context, our results are in agreement with the description that TSH is able to up-regulate the expression of cyclin D1 (37) to favor cell proliferation. We are currently investigating possible links between changes in cell cycle and changes in tyrosine phosphatase activity, which is also mediated by somatostatin, as previously described in the literature (8, 10).

Our results indicate that the ability of some transformed and tumor thyroid cell lines to respond to somatostatin is altered. Moreover, as many of these cell lines proliferate independently of TSH, the idea that somatostatin requires the positive effect induced by TSH to modulate cell growth is further supported. Our observations are in agreement with those previously reported (8) showing that differentiated PC-Cl3 thyroid cells turned insensitive to somatostatin when transformed with the E1a oncogene. These cells retain the expression of SSTR4 (8), and in our assays we have detected the expression of SSTR2 in both FRT and K-*ras*-FRTL-5 cells. These data indicate that the expression of somatostatin receptors does not directly correlate with the capacity of a given cell line to respond to somatostatin. Rather, some other intracellular mechanism, currently under investigation, is responsible for the interference with the somatostatin effect in thyroid transformed and tumor cells.

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