Receptor-mediated Internalization Is Critical for the Inhibition of the Expression of Growth Hormone by Somatostatin in the Pituitary Cell Line AtT-20*

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Philipppe Sarret‡, Dominique Nouel§, Claude Dal Farra‡, Jean-Pierre Vincent‡, Alain Beaudet§, and Jean Mazella¶

From the ‡Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, UPR 411, 660 Route des Lucioles, 06560 Valbonne, France and the ¶Montreal Neurological Institute, McGill University, Montréal, Québec H3A 2B4, Canada

The inhibitory effect of the neuropeptide somatostatin on the expression of growth hormone was measured by quantitative polymerase chain reaction in the pituitary cell line AtT-20. We demonstrate that this effect is dependent on the internalization of somatostatin-receptor complexes and that it is totally independent from the peptide-induced inhibition of adenylate cyclase. Indeed, the inhibitory effect of the peptide on growth hormone mRNA levels was totally insensitive to pertussis toxin treatment but was totally abolished under conditions which block somatostatin receptor internalization. Comparative confocal microscopic imaging of fluorescent somatostatin sequestration and fluorescence immunolabeling of sst1, sst2A, and sst5 receptors suggests that sst2A is most probably responsible of the inhibitory effect of somatostatin on growth hormone expression.

The neuropeptide somatostatin (somatotrophin release inhibitory factor; SRIF) is known to play a critical role for the regulation of hormone secretion by the anterior pituitary and peripheral glands as well as to act as neuromediator in the central nervous system. Biological actions of SRIF are exerted through multiple receptors subtypes, which have recently been cloned and are referred to as sst1, sst2, sst3, sst4, and sst5 (for review, see Refs. 1 and 2). These receptors are widely expressed in the anterior pituitary, peripheral tissues, and brain (3, 4). In the pituitary, the predominant effects of SRIF are the inhibition of the secretion of growth hormone (GH) and thyroid-stimulating hormone, although inhibitory effects of SRIF on the release of luteinizing hormone and prolactin have also been documented (5, 6). GH secretion is inhibited by SRIF through both cAMP-dependent and cAMP-independent pathways (7). In both cases, these effects have been described as being pertussin toxin-sensitive, suggesting the implication of a SRIF receptor coupled to Gi or Gs (8). SRIF has also been shown to reduce transcription of the GH gene in vivo (9). However, this effect was interpreted as being indirect, involving central inhibition of growth hormone-releasing hormone release (10–12). There has been no evidence thus far that SRIF may directly inhibit GH expression.

Recent studies have shown that interactions of SRIF with its receptors resulted in a temperature- and receptor-dependent internalization of receptor-ligand complexes (13, 14). The efficacy of this internalization process, as well as the pattern of intracellular trafficking of internalized ligand and receptors, were shown to vary according to the receptor subtypes involved (13–16). As for other protein-coupled receptors, ligand-induced ast receptor internalization has been proposed to play a role in receptor desensitization through cell surface down-regulation (17, 18). Early observations of nuclear translocation of internalized 125I-SRIF in AtT-20 cells have also raised the possibility that internalization may affect transcriptional activity in this cell line (19). The possibility that internalization of receptor-ligand complexes may play a role in transmembrane signaling has so far mainly been explored for growth factor and cytokine receptors (for a review, see Ref. 20). Recent studies, however, have suggested that internalization of G protein-coupled receptors may also be mandatory for certain types of cell signaling. Thus, changes in the duration of inositol phosphate accumulation and associated calcium responses (21, 22) or in the transcription of receptor mRNA (23) have been reported in target cells under conditions of impaired receptor internalization. Furthermore, ligand-induced receptor internalization of β-adrenergic receptors was recently shown to be directly involved in the activation of mitogen-activated protein kinase pathway (24).

The mouse AtT-20 pituitary cell line has been widely used as a model to study the physiological, pharmacological, and biochemical properties of SRIF receptors in anterior pituitary cells (25–27). Molecular biological and biochemical studies have shown that AtT-20 cells express four of the five cloned SRIF receptors subtypes (sst1, sst2, sst4, and sst5) (28) and internalize radioactive SRIF with high efficiency (29). Although AtT-20 cells are mainly documented to express POMC and to secrete ACTH (30, 31), preliminary studies from our laboratory suggest that AtT-20 cells may also express GH.2 The aim of the present study was to confirm that AtT-20 cells truly express GH, to determine whether this expression is regulated by SRIF, and to investigate the role played by receptor/ligand internalization in this regulatory function.

EXPERIMENTAL PROCEDURES

Materials

Mouse AtT-20/D16–16 tumor cell line was a gift from Dr. Nabil Seidah (Clinical Research Institute, Montréal, Québec, Canada).

‡ P. Sarret, D. Nouel, C. Dal Farra, J.-P. Vincent, A. Beaudet, and J. Mazella, unpublished results.

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¶ To whom correspondence should be addressed: Institut de Pharmacologie Moleculaire et Cellulaire, CNRS, UPR 411, 660 Route des Lucioles, 06560 Valbonne, France. Tel.: 33-4-93-95-77-61; Fax: 33-4-93-95-77-08; E-mail: mazella@ipmc.cnrs.fr.
Dulbecco’s modified Eagle’s medium and gentamycin were purchased from Life Technologies Inc., fetal calf serum and 1,10-phenanthroline from Roche Molecular Biochemicals, and horse serum from Boehringer Ingelheim. The polyclonal rabbit anti-sst5 antibody was kindly provided by Dr. Hans-Jürgen Kreienkamp (Institut für Zellbiochemie und Klinische Neurobiologie, Universität Hamburg, Hamburg, Germany) and polyclonal rabbit anti-sst1 and -sst2A antibodies were gifts from Agnes Schonbrunn (Department of Integrative Biology and Pharmacology, University of Texas, Houston, TX). Other products were from the following sources: Texas Red-conjugated goat anti-rabbit antibody, Jackson Immunoresearch Laboratory Inc. (West Grove, PA); forskolin, isobutylmethylxanthine, and pertussis toxin (Sigma, Belgium); rat stomach cAMP and polylysine enzyme immunosassay system, Amersham Pharmacia Biotech; Erase-a-Base and Reverse Transcription Systems, Promega; TA cloning vector, Invitrogen; RNAble kit, Eurobio; Opti-Prime PCR optimization kit, Stratagene. Tyr$^3$-[D-Trp]$^8$SRIF-14 was iodinated and purified as described previously (32). Bodipy-$^5$-[D-Trp]$^8$SRIF-14 (fluo-SRIF) was synthesized and purified as published (14).

Cell Culture

Mouse AtT-20/D16–16 tumor cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 10% horse serum supplemented with gentamycin (50 μg/ml). Cells were plated in 12-mm multi-well dishes for binding experiments, in 60-mm culture dishes for cAMP measurements, and in 100-mm culture dishes for GH mRNA detection.

Binding and Internalization of $^{125}$I-[D-Trp]$^8$SRIF and Fluo-SRIF on AtT-20 Cells

Cell Pretreatments—The culture medium was removed, and cells were equilibrated with 500 μl of Earle’s Tris-Hepes buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 3.6 mM MgCl$_2$, supplemented with 0.1% glucose and 1% bovine serum albumin (binding buffer) in the presence or in the absence of 0.45 μM sucrose for 30 min or of 100 μM pertussis toxin for 15 h.

Equilibrium Binding Experiments—Saturation experiments were performed by incubating pretreated cells with increasing concentrations of $^{125}$I-[D-Trp]$^8$SRIF ($10^{-12}$ M–$10^{-7}$ M), or with 100 nM fluorescein-diacetate and varying concentrations of unlabeled [D-Trp]$^8$SRIF every 2 min with 500 μl of binding buffer.

Internalization of Fluo-SRIF in AtT-20 Cells—AtT-20 cells were grown on 12-mm glass coverslips coated with polylysine (10 μg/ml). Fluo-SRIF (20 nM) was added on preincubated cells for the indicated times, and cells were washed twice with 500 μl of binding buffer. Nonspecific binding was determined in parallel by carrying out the incubation in the presence of 1 μM unlabeled [D-Trp]$^8$SRIF and represented less than 5% of the total binding.

GH mRNA Measurement in AtT-20 Cells

Cell Pretreatments—Cells were preincubated in the culture medium either alone or in the presence of 100 ng/ml pertussis toxin, or with 0.45 μM sucrose in Earle’s buffer for 30 min to inhibit internalization. The culture medium was then removed, and cells were incubated with 100 nM [D-Trp]$^8$SRIF-14 for 60 min at 37 °C or 4 °C. Cells were then washed twice with TBS, and total RNAs were prepared at various times.

Optimistic PCR—The method of analysis was based on the competition between known amounts of a cDNA competitor and the GH cDNA to be measured for the annealing with common primers during the PCR.

Preparation of the cDNA Competitor—The cDNA competitor was prepared from the TA-cloning plasmid containing the wild-type GH cDNA. The cDNA encoding GH was obtained by reverse transcription of the mRNA isolated from AtT-20 cells. Primers were designed on the 3′ and 5′ ends of the open reading frame of GH cDNA by PCR using the ABI Prism DNA sequencing kit (Promega). The PCR product (355 bp) was then analyzed by 2% agarose gel and subcloned into the TA-cloning vector. The plasmid was linearized with BamI, which cleaves the GH cDNA. After partial digestion with exonuclease III (Erase-a-Base) and treatment with nuclease S1, extremities were blunt-ended with the Klenow fragment of polymerase I and ligated. We selected a 384-bp fragment that has been used as the competitor in the RT-PCR quantitative experiments.

GH mRNA Content of Cultured Tumor Cells—Total RNAs were extracted from AtT-20 cells following various experimental conditions using the RNAble kit (Eurobio). The first cDNA strand was obtained from RNAs (6 μg) using the Reverse Transcription System kit (Promega). After cDNA synthesis for 1 h at 42 °C, samples were denatured for 5 min at 99 °C and chilled on ice. Before analysis of GH mRNA content from AtT-20 cells submitted to various effectors, PCR conditions were optimized with the Opti-Prime PCR kit (Promega). The PCR buffer was then: 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl$_2$, 75 mM KCl.

One tenth of the first-strand cDNA was amplified in a volume of 50 μl with 200 ng of each primer, 2 μl of Taq polymerase (0.5 unit/μl) (Appligene), and decreasing concentrations of the cDNA competitor. Amplification was carried out with a first cycle at 94 °C for 3 min, 55 °C for 2 min, 72 °C for 1 min 30 s, followed by 34 cycles, 94 °C for 45 s, 55 °C for 1 min, 72 °C for 1 min 30 s, and a final extension step at 72 °C for 8 min. PCR products were analyzed on a 2% agarose gel. As controls, each RT sample was submitted to PCR either with a sense or a reverse primer. In some cases, the PCR was carried out without the competitor cDNA, or without the RT product.

PCR band intensities detected with ethidium bromide were analyzed by laser densitometry. When the ratio between the density of the cDNA competitor band and that of the GH band was expressed as a function of the amount of competitor, the representation was linear (Fig. 6) (36, 37).

Measurement of CAMP Content in AtT-20 Cells

AtT-20 cells were preincubated with 1 mM isobutylmethylxanthine for 10 min in culture medium under the various experimental conditions (control, incubation in the presence of hypotonic sucrose, or pretreatment with 100 ng/ml pertussis toxin; see above). Cells were then incubated in culture medium containing 1 mM isobutylmethylxanthine with or without 0.1 μM [D-Trp]$^8$SRIF-14 or 10 μM forskolin. The reaction was terminated by removing the medium and scraping off cells in 1.5 ml of 65% ethanol. After centrifugation, the dried pellet was dissolved in 0.05 mM sodium acetate buffer, pH 5.8, containing 0.02% Triton X-100 and incubated overnight at 4 °C in TBS containing 0.05% Triton X-100 and one of the following: 1) a 1:50 dilution of a rabbit polyclonal sst5 antibody, 2) a 1:2500 dilution of a rabbit polyclonal sst2A antibody, 3) a 1:2000 dilution of a rabbit polyclonal sst1 antibody. The specificity of each of these receptor antibodies has been described previously (15, 18, 33, 34). Cells were then rinsed two times for 10 min each in TBS, incubated with a Texas Red-conjugated goat anti-rabbit antibody diluted 1:100 in TBS for 45 min, rinsed in TBS (two 10-min rinses), and mounted on glass slides with Aquamount for confocal microscopic examination. Images were acquired, stored, and archived as described above.
bovine serum albumin and 0.01% preservative. The cAMP content was then measured using an enzyme immunoassay kit according to the manufacturer’s recommendations.

RESULTS

Binding and Internalization of sst Receptors in AtT20 Cells—In order to characterize the properties of SRIF internalization in AtT-20 cells and to define optimal conditions for blocking the internalization process, we performed association kinetics of $^{125}$I-Tyr$^0$-[D-Trp$^8$]SRIF-14 ($^{125}$I-SRIF) on whole AtT-20 cells under various experimental conditions. In the absence of any pretreatment, $^{125}$I-SRIF bound specifically to AtT-20 cells at 37 °C in a time-dependent manner (Fig. 1A). The radioactivity associated with the cells reached a plateau within 20 min. Removal of surface-bound radioactivity by acid-NaCl wash revealed that 80% of the total $^{125}$I-SRIF bound at this time was intracellular (Fig. 1A). By comparison, when the incubation was carried out at 4 °C, maximal binding reached a plateau somewhat later (at approximately 40 min). The bulk of this binding (85% of total at 60 min) was acid-washable at all times, indicating that the ligand had not been internalized into the cells (Fig. 1B). In the presence of 0.45 M sucrose, the association of $^{125}$I-SRIF was similar to that observed at 37 °C (plateau value obtained at 20 min; Fig. 1C). However, no remaining radioactivity was detected following acid-NaCl wash, indicating that hyperosmolar sucrose efficiently blocked internalization of the bound ligand (Fig. 1C). Finally, when cells were preincubated with pertussis toxin, the amount of $^{125}$I-SRIF sequestered into the cells (i.e. acid wash-resistant) was similar to that seen in controls, indicating that pertussis toxin had no effect on the internalization process (Fig. 1D).

In order to verify that the parameters of $^{125}$I-SRIF association with AtT-20 cells were not modified by the addition of hyperosmolar sucrose or by preincubation with pertussis toxin, saturation experiments were performed at 37 °C under equilibrium conditions. As illustrated by the Scatchard plot in Fig. 2, the maximal amount of cell-associated $^{125}$I-SRIF was affected neither by hyperosmolar sucrose nor by pertussis toxin treatment. However, the addition of 0.45 M sucrose decreased the EC$_{50}$ value toward $^{125}$I-SRIF (4.42 ± 0.38 nM, n = 2) as compared with control experiments (1.49 ± 0.21 nM, n = 4) or to experiments performed in the presence of pertussis toxin (1.34 ± 0.17 nM, n = 3) (Fig. 2). These data clearly indicate that the maximal amount of $^{125}$I-SRIF associated with AtT-20 cells is not affected by sucrose or pertussis toxin treatment.

Confocal Microscopic Visualization of Internalized SRIF Molecules and sst Receptors—To visualize the intracellular trafficking of internalized SRIF, AtT-20 cells were incubated at 37 °C with 20 nM Fluo-SRIF and cell surface labeling was stripped off with hypertonic acid wash. After 5 min of incubation, the internalized ligand formed small intracellular fluorescent hot spots distributed throughout the cytoplasm (Fig. 3). By 30 min, these hot spots had increased in number and intensity and were heavily clustered next to the nucleus (Fig. 3). In cells incubated in the presence of 0.45 M sucrose, the fluorescent...
label remained sequestered at the periphery of the cells at all times (Fig. 4A). This labeling was exclusively surface-bound since it was entirely strippable by hypertonic acid wash (Fig. 4B).

Immunohistochemistry revealed very distinct distributional patterns for sst1, sst2A, and sst5 receptors. In the absence of SRIF, all of these receptors were essentially localized at the cell surface (Fig. 5). Following 5 or 20 min of incubation with SRIF, both sst1 and sst5 immunolabeling remained confined to the periphery of the cells, where it formed a more or less continuous ring (Fig. 5, left and right). By contrast, after 5 min of incubation with SRIF, sst2A immunolabeling was clearly translocated from the cell surface to intracellular endosome-like compartments (Fig. 5, center). After 20 min of incubation with SRIF, sst2A-immunoreactive receptors formed a single concentrated hot spot in the perinuclear region (Fig. 5). By that time, sst2 cell surface labeling had almost completely disappeared (Fig. 5).

**GH mRNA Measurement**—The amount of GH mRNA present in AtT-20 cells following incubation with SRIF under various experimental conditions was determined by quantitative RT-PCR using competing primers against the target cDNA and a competitor DNA. An example is given in Fig. 6A, in which the amount of the target GH cDNA (579 bp) was determined in AtT-20 cells in the absence of SRIF (control) and after 90 min of incubation with the peptide. This amount of GH mRNA was measured in a series of PCR runs containing various amounts of the competitor DNA (384 bp). All PCR products were quantified by laser scanning, and the ratios of competitor over target band densities were plotted for each PCR condition as a function of the amount of competitor DNA (Fig. 6B). The amount of...
GH cDNA was then extrapolated from the straight line for a ratio of 1 and expressed in attomoles (amol/μg) of total RNA taking into account that the DNA competitor was double-stranded and that 1/10th of the RT product was used for PCR.

Once the method had been validated, the amount of GH mRNA was measured in AtT-20 cells at various times following incubation with SRIF in conditions of unimpaired (controls and pertussis toxin-treated) and impaired (incubation at 4 °C or in the presence of hyperosmolar sucrose or of pertussis toxin). Incubation of AtT-20 cells with 10 μM forskolin increased the intracellular content of cAMP from 0.4 pmol/10^5 cells to 2.2 pmol/10^5 cells within 75 min (Fig. 8A). SRIF-14 (100 nM) inhibited this forskolin-stimulated cAMP content after 20 min and up to 75 min of incubation with the peptide at 37 °C (Fig. 8A). There also was an inhibitory effect of SRIF on forskolin-stimulated cAMP formation when the experiments were carried out at 4 °C, although the level of stimulation by forskolin was weaker than at 37 °C (Fig. 8B). When the incubations were carried out at 37 °C but in the presence of hyperosmolar sucrose, the inhibitory effect of SRIF was similar to that observed in the absence of sucrose (Fig. 8C). By contrast, when cells were preincubated with pertussis toxin, SRIF was without effect on the stimulatory action of forskolin (Fig. 8D). These results demonstrate that the inhibitory action of SRIF on adenylate cyclase is blocked by preincubation with pertussis toxin but not by co-incubation with hyperosmolar sucrose and that the effect of SRIF on adenylate cyclase activity is independent of internalization.

**DISCUSSION**

The present study provides the first demonstration that SRIF can directly decrease GH mRNA levels in anterior pituitary cells. It also demonstrates that this inhibitory effect is independent from the inhibition of adenylate cyclase also produced by SRIF in these cells but that it is dependent upon the internalization of SRIF/sst receptor complexes.

The parameters of ^125^I-Tyr^5^-[D-Trp^8^]SRIF-14 (^125^I-SRIF) binding to AtT-20 cells at 37 °C were comparable to those reported previously in this cell line using different SRIF agonists (19, 29). As documented previously for other SRIF analogs in these cells (19, 29), ^125^I-[D-Trp^8^]SRIF-14 was internalized in a specific time- and temperature-dependent manner. However, in contrast to what was reported for ^125^I-Tyr^3^-octreotide inter-
mediated signaling in the case of m2 muscarinic and NT1 linked, mechanisms, an interpretation consistent with previous ing and endocytic processes are two independent, albeit inter- 14 internalization was totally insensitive to pretreatment of Cells were incubated with solvent ( AtT-20 cells. ) FIG.8 . Effect of SRIF on forskolin-stimulated cAMP levels in AtT-20 cells. Cells were incubated with solvent (hatched bars), with 10 mM forskolin (white bars), or with forskolin and 10^{-7} M SRIF (black bars) for 2, 20, and 75 min. Experiments were carried out at 37 °C (A, C, and D) or at 4 °C (B). All values are the mean ± S.E. of triplicate determinations from two independent experiments.

The internalization of both 125I-SRIF and fluo-SRIF in AtT-20 cells was inhibited by hyperosmolar sucrose, suggesting that the internalization process is initiated at the level of clathrin-coated pits. These results are congruent with those obtained previously in other cell lines naturally expressing SST receptors and/or of sequences thereof into the nucleus (49, 50). Either of these mechanisms could theoretically be involved in the recent demonstration of abundant sst receptor recycling in both naturally expressing and transfected cell lines (16, 41).

A major finding of the present study is the observation that SRIF directly inhibits the expression of GH mRNA in AtT-20 cells. This finding is novel on two counts. First, it demonstrates that GH mRNA is expressed in AtT-20 cells, contrary to the common belief that the GH gene is silent in this cell line (44). Second, it demonstrates that SRIF may directly regulate GH gene transcription, and not merely indirectly, through central inhibitions of growth hormone-releasing hormone, again contrary to current assumptions (9).

The fact that GH mRNA expression had previously been overlooked in AtT-20 cells is probably due to methodological differences between the present and earlier studies. Indeed, our results were obtained using RT-PCR, a method that is considerably more sensitive than the Northern blotting approach on which earlier results were based. The inhibitory effect of SRIF on the expression of GH is in keeping with the wide body of evidence for an inhibitory role of SRIF on the secretion of GH in vivo as well as in vitro (7). This effect of the peptide on hormonal secretion has been correlated with the coupling of sst receptors to Gi or Gs, and was shown to be pertussis toxin-sensitive, i.e. to be linked to the adenylate cyclase signaling cascade (8). By contrast, the present study clearly demonstrates that the inhibitory effects of SRIF on GH mRNA levels are totally insensitive to pertussis toxin treatment. They are, however, highly dependent on SRIF-induced internalization of sst receptors. Indeed, conditions that blocked sst receptor internalization also blocked the inhibitory effect of SRIF on GH mRNA levels. Furthermore, these procedures were ineffective against the effects of the peptide on adenylate cyclase activity, confirming that the expression and release of GH by SRIF are mediated by distinct regulatory pathways.

Until recently, ligand-induced endocytosis had been considered to mainly subserve receptor regulatory functions such as desensitization (45, 46) and desensitization (47, 48). Evidence has been accumulating, however, to suggest that this endocytic process may also be critical for transmembrane signaling. Most of the information available to date concerns growth factors and cytokine receptors, for which there is good evidence that ligand-induced internalization is essential for full signaling activity (20, 49). There is also evidence that in the case of G protein-coupled receptors, ligand-induced internalization may be mandatory for the expression of certain signaling functions ranging from the activation of mitogen-activated protein kinase (24) to the regulation of target genes (23). Proposed mechanisms for this type of G protein-independent signaling include mobilization of adaptor proteins such as β-arrestins (24), endo- somal signaling (21, 22), or nuclear translocation of internal- ized receptors and/or of sequences thereof into the nucleus (49, 50). Either of these mechanisms could theoretically be involved in mediating the effects of SRIF documented here on GH mRNA. One might even speculate that it involves interaction of the internalized ligand, provided that it remains intact, with the 86-kDa subunit of autoantigen Ku, which has been shown to correspond to an intracellular SRIF receptor regulating protein phosphatase 2A activity and has been postulated to play a role in gene transcription (51). It is unlikely that the internal- ization-dependent signaling cascade unraveled in the present study involves the pituitary-specific transcription factor GH factor-1 (GHF-1; Pit-1) since treatment of pituitary cells with

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SRIF for either 2 or 48 h failed to modify basal or growth hormone-releasing hormone-induced GHF-1 mRNA levels (52). It could, however, implicate one or more other putative-specific transcription factors yet to be identified (53).

As AtT-20 cells were demonstrated to express four (sst1, the two variants of sst2, sst4, and sst5) of the five cloned sst receptors (28, 54), it is impossible to precisely determine which receptor(s) is (are) involved in the internalization-dependent effects observed in the present study. However, several lines of evidence point to sst2A as playing a predominant role in this regard. Of all sst receptor subtypes, sst2A is the one that is most abundantly expressed in AtT-20 cells (28) or is found to internalize efficiently here by sst2 immunohistochemistry, is the one that most closely resembled that of the fluorescent ligand. Finally, in all cell lines in which sst receptor internalization has been studied to date, sst2A is the one that was found to be the most efficiently internalized (14, 55). Neither sst1 nor sst4 are likely to contribute significantly to the internalization of [α-Trp]SRIF-14 observed in the present study since neither is expressed very abundantly in AtT-20 cells (28) or is known to contribute significantly to the internalization of [α-Trp]SRIF for either 2 or 48 h failed to modify basal or growth hormone-releasing hormone-induced GHF-1 mRNA levels (52). It could, however, implicate one or more other putative-specific transcription factors yet to be identified (53).

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In conclusion, the present work demonstrates that SRIF inhibits GH mRNA transcription in AtT-20 cells and that this effect is mediated by internalization of receptor/ligand complexes, independently from activation of the adenylylate cyclase pathway. Further studies are in order to identify the receptor(s) responsible for this important effect and to unravel the signaling cascade implicated in its transduction.

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