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# Somatostatin Increases Mitogen-Induced IL-2 Secretion and Proliferation of Human Jurkat T Cells via sst3 Receptor Isotype

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**Abstract** The neuropeptide somatostatin (SRIF) modulates normal and leukemia T cell proliferation. However, neither molecular isotypes of receptors nor mechanisms involved in these somatostatin actions have been elucidated as yet. Here we show by using RT-PCR approach that mitogen-activated leukemia T cells (Jurkat) express mRNA for a single somatostatin receptor, sst3. This mRNA is apparently translated into protein since specific somatostatin binding sites ($K_{I1}$ = 78 ± 3 pM) were detected in semipurified plasma membrane preparations by using $^{125}$I-Tyr$^{1}$-SRIF14 as a radioligand. Moreover, somatostatin inhibits adenylyl cyclase activity with similar efficiency ($IC_{50}$ = 23 ± 4 pM) thus strongly suggesting a functional coupling of sst3 receptor to this transduction pathway. The involvement of sst3 receptor in immuno-modulatory actions of somatostatin was assessed by analysis of neuropeptide effects on IL-2 secretion and on proliferation of mitogen-activated Jurkat cells. Our data show that in the concentrations comprised between 10 pM and 10 nM, somatostatin potentiates IL-2 secretion. This effect is correlated with somatostatin-dependent increase of Jurkat cell proliferation since the $EC_{50}$ concentrations for both actions were almost identical ($EC_{50}$ = 22 ± 9 pM and $EC_{50}$ = 12 ± 1 pM for IL-2 secretion and proliferation, respectively). Altogether, these data strongly suggest that in mitogen-activated Jurkat cells, somatostatin increases cell proliferation through the increase of IL-2 secretion *via* a functional sst3 receptor negatively coupled to the adenylyl cyclase pathway. J. Cell. Biochem. 68:62–73, 1998. © 1998 Wiley-Liss, Inc.

**Key words:** somatostatin; receptor isotypes; adenylyl cyclase; Interleukin-2 (IL-2); proliferation; Jurkat cells

Somatostatin (Somatotrope Release Inhibiting Factor, SRIF) is a potent inhibitor of numerous endocrine and exocrine secretions. Major endocrine targets of SRIF are pituitary and pancreas where it inhibits growth hormone/thyrotropin/prolactin and insulin/glucagon secretion, respectively. The best documented exocrine role of SRIF is inhibition of gastric acid release in stomach. In the central nervous system, SRIF acts as a neurotransmitter/neuromodulator activating hyperpolarizing $K^+$ current and inhibiting $Ca^{2+}$ influx; it is believed to be involved in the central regulation of locomotor activity and cognitive functions. Two naturally occuring SRIF isoforms (SRIF14 and SRIF28) are underlying these biological actions of SRIF. SRIF14 and SRIF28 isoforms consist of 14 and 28 amino acids, respectively, and the entire SRIF14 sequence is present in the C-terminus of SRIF28. The predominant biologically isoforme is SRIF14 [for review, see Reisine and Bell, 1995].

During the last decade a substantial body of evidence has been compiled on the bidirectional relationship between immune and neuroendocrine systems. Molecular and cellular mechanisms underlying these interactions involve the sharing of chemical messengers and their receptors [Felten et al., 1985]. A pertinent example of such neuro-immuno-endocrine interactions is a recently reported stimulatory action of growth hormone and prolactin on T lymphocyte response to mitogen- and antigen-stimulation [Postel-Vinay et al., 1997]. Indeed, effector T cells bear receptors for these two particular

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hormones [Postel-Vinay et al., 1997] as well as for all major neurotransmitters and neurohormones yet assessed [Stanisz et al., 1987]. Consequently, T lymphocyte function may be modulated by neuropeptides circulating in the blood as hormones or liberated as neurotransmitters at the nerve endings innervating lymphoid organs [Elliott et al., 1992; Felten et al., 1985].

In the immune system, SRIF14 is localized in the lymphoid organs [Aguila et al., 1991] including the proximity of sensory nerve terminals [Felten et al., 1985]. In particular, it is also produced, stored, and probably secreted by a subpopulation of T cells [Aguila et al., 1991]. SRIF14-specific binding has been detected on the surface of human T lymphocytes and T cell lines [Blum et al., 1992; Hiruma et al., 1990; Sreedharan et al., 1989; Stanisz et al., 1987]. Physiological roles of SRIF14 in the modulation of T cell activity include SRIF14 inhibition of some cytokine (e.g., IFNγ) secretion by $CD4^+$ T cells [Hinterberger et al., 1978].

In addition, SRIF14 modulates the proliferation of normal T cells and leukemia T cells [Nio et al., 1993; Pawlikowski et al., 1985; Payan et al., 1984]. According to the radioligand binding studies, it appears that the SRIF14 actions on T cells proliferation involve heterogenous binding sites [Sreedharan et al., 1989]. The heterogeneity of relevant SRIF14 receptors has been supported by the observed multimodal (inhibitory and stimulatory) and complex (depending on the neuropeptide concentration) SRIF14 effects on T cell proliferation. Indeed, in addition to the well-established inhibitory actions [Nio et al., 1993; Pawlikowski et al., 1985; Payan et al., 1984], reported also in other cell types [for review, see Reisine and Bell, 1995], SRIF14 specifically enhances the proliferative response of T cells [Nordlind and Mutt, 1986]. However, these opposite SRIF14 effects may be explained by the heterogeneity of T cell populations used in these studies. Otherwise, they might be explained by different SRIF14 effects on IL-2 secretion (IL-2 is the major lymphocyte growth factor necessary to trigger T cell proliferation [Taniguchi and Minami, 1993]). Indeed, SRIF14 is able to modulate IL-2 secretion by human colonic lamina propria mononuclear cells [Nio et al., 1993; Payan et al., 1984]. Precise molecular characteristics of SRIF14 receptors mediating these neuropeptide effects are not known.

Recent molecular characterization of rodent and human SRIF receptors indicated that all five cloned receptors (sst1–sst5) belong to the superfamily of G protein-coupled receptors with seven transmembrane domains. They are encoded by five different genes and are highly conserved between species (e.g., sst1 of mouse and human share 99% of the amino acid sequence) and within the same species (different SRIF receptors present 45–61% identity). The known SRIF receptors are coupled to multiple signaling pathways. After expression of the cloned receptors in heterologous cell lines, all of them (sst1–sst5) inhibit the adenylyl cyclase activity but in parallel also selectively activate other transduction effectors such as PLCβ, tyrosine phosphatase, and ionic channels [for review, see Reisine and Bell, 1995].

The expression of mRNA for the five cloned receptors is tissue- and cell type-specific. For example, sst1, sst2, sst3, and sst5 mRNAs are expressed in adult human pituitary [Miller et al., 1995; Panetta and Patel, 1994]. All five receptor isotype mRNAs are expressed in the adult rat brain but with distinct regional patterns of distribution [see for example Beaudet et al., 1995; Pérez and Hoyer, 1995]. Data on SRIF14 receptor isotype expression in the immune system are very scarce. It has been reported that rat spleen cells express the mRNAs specific for all five receptor isotypes [Bruno et al., 1993]. In addition, it has been shown that murine T lymphocytes from hepatic granulomas of *Schistosome*-infected animals display only sst2 receptor [Elliott et al., 1994].

In the present study we analyzed the molecular isotypes of SRIF receptors expressed in the homogeneous human T cell line, Jurkat. Our RT-PCR data show that Jurkat cells express exclusively mRNA specific for sst3 receptor. The expression and function of these receptors on Jurkat cells were confirmed by the observed SRIF14-dependent inhibition of $^{125}$I-Tyr$^{1}$-SRIF14 specific binding and adenylyl cyclase activity in semipurified membrane preparations. The assessment of SRIF14 effects on IL-2 secretion and proliferation strongly suggests the involvement of sst3 receptors in modulation of these cellular responses by the neuropeptide.

## METHODS

### Cell Culture and Mitogen Stimulation

Jurkat cells (clone J77.6.8) were grown in RPMI 1640 medium (Gibco, Cergy-Pontoise, France) supplemented with 10% (v/v) heat inactivated FCS (Boehringer Meylan, France), 2 mM

L-glutamine, 100 U/ml gentamycin in a 5% $CO_2$ humidified atmosphere at 37°C.

In order to test the effects of SRIF14 (Peninsula Laboratories, Belmont, CA) on IL-2 secretion, we treated Jurkat cell suspensions ($0.5 \times 10^6$ cell/ml) with a combination of phorbol ester, TPA (10 ng/ml, Sigma, L'Isle d'Abeau, France) and lectin PHA-L (2 μg/ml, Sigma, L'Isle d'Abeau). The mitogen-driven IL-2 production by Jurkat cells increased up to 24 h of culture and then reached a plateau; the half-time of maximal stimulation was estimated to be 16 h (data not shown). For further experiments, Jurkat cells were therefore activated for overnight (16 h) period.

### RT-PCR Analysis of Jurkat T Cell SRIF Receptors

Total cellular RNA was extracted from Jurkat cell pellets by using the RNA$^{NOW}$ (Biogenetex, Ozyme, Montigny-le-Bretonneux, France) according to the manufacturer's instructions. To exclude any genomic DNA contamination of RNA samples, an aliquot (10 μg/5 μl) of each sample was treated with 0.1 μl (0.75 U) of DNA-se (Pharmacia Biotech, Uppsala, Sweden) for 10 min at 37°C. DNA-se was then inactivated at 75°C (10 min) and RT-PCR performed as follows. Cellular RNA (10 μg) was reversely transcribed (1 h, 42°C) with Moloney monkey leukemia virus reverse transcriptase (400 U) by using an 18-mer of oligo-dT (20 μM) as a primer in the reaction mixture containing 1 μl of 10 mM dNTP (Pharmacia Biotech), 2 μl of 0.1 M DTT (Gibco-BRL, Cergy Pontoise, France), 1 μl of RNAsin (Promega, Charbonnière, France) in a final buffer (Gibco-BRL) volume of 20 μl as previously described [Frohman, et al., 1988]. To ascertain that cDNA was not contaminated by genomic DNA, reverse transcription was also performed in the absence of Moloney monkey leukemia virus reverse transcriptase.

One-tenth (2 μl) of the first strand cDNA synthesis reaction mixture was added to PCR buffer (100 mM Tris) containing 0.2 mM dNTP, 1.25 U Taq DNA polymerase (Promega) in a total volume of 50 μl. Concentration of $MgCl_2$ in the reaction mixture was 2.5, 4, 1.5, 1.5, 1, and 4 mM for sst1–sst5 and β-actin, respectively. Primers for SRIF receptors were added in a final concentration 0.4 μM and were as follows: for sst1, the sense primer was 5′-GGAACTCTATGGTCATCTAC-3′ and the antisense primer was 5′-GCTGAGCACAGTCAGACAGT-3′ [Kubota et al., 1994]; for sst2, the sense primer was 5′-TCATCAAGGTGAAGTCCTCTGG-3′ and the antisense primer was 5′-AGATACTGGTTTGGAGGTCTCCA-3′ [Rohrer et al., 1993]; for sst3, the sense primer was 5′-TCATCTGCCTCTGCTACC-3′ and the antisense primer was 5′-GAGCCCAAAGAAGGCAGCT-3′ [Miller et al., 1995]; for sst4, the sense primer was 5′-ATCTTCGCAGACACCAGACC-3′ and the antisense primer was 5′-ATCAAGGCTGGTCACGACGA-3′ [Miller et al., 1995]; for sst5, the sense primer was 5′-GCCGGCCTCTACTTCTTCGTG-3′ and the antisense primer was 5′-CCGTGGCGTCAGCGTCCTTGG-3′ [Miller et al., 1995]. Amplification of cDNA encoding human β-actin by using the sense primer 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ and the antisense primer 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (Stratagene, Cambridge, UK) was used as a positive control of reverse transcription. Expected amplification products were of the following lengths (bp): 233, 414, 222, 321, 154, and 661 for sst1–sst5 receptors and human β-actin. After initial denaturation (150 s, 94°C), the samples were subjected to 40 cycles (for sst1–sst5 receptors) or 25 cycles (for human β-actin) of amplification including denaturation (60 s, 94°C), hybridization (60 s, 65°C, 60°C, 65°C, 65°C, 60°C, and 67°C for sst1–sst5 receptors and β-actin, respectively) and elongation (75 s, 72°C). Final elongation was achieved at 72°C for 5 min. The absence of reactive contamination by DNA was checked by PCR amplifications systematically carried out in parallel by replacing cDNA by water.

### $^{125}$I-Tyr$^1$-SRIF14 Binding Assay

Mitogen-activated Jurkat cells were sedimented by quick centrifugation (1,000*g*, 5 min, 25°C) and washed three times in cell culture medium. After the last wash, the cell pellets were resuspended in a small volume of 50 mM Tris-HCl buffer, pH = 7.4, transferred to a glass-glass homogenizer and broken by 20 hand strokes. Homogenates were centrifuged for 3 min at 1,800*g* (4°C); pellets were discarded while supernatants were recentrifuged for 15 min at 28,000*g* (4°C). Resulting pellets were resuspended in an incubation buffer consisting of 50 mM Tris-HCl buffer, pH = 7.4 supplemented with 5 mM $MgCl_2$, 0.2% (w/v) of BSA and 0.05% (w/v) bacitracin. Aliquots of these semipurified plasma membrane preparations were frozen and kept at −80°C until used.

$^{125}$I-Tyr$^{1}$-SRIF14 binding assays were performed as previously described [Krantic et al., 1992]. In brief, 50 µl aliquots of semi-purified membrane preparations (40–80 µg of protein equivalent) were incubated with 25 µl of $^{125}$I-Tyr$^{1}$-SRIF14 in a final volume of 150 µl for 90 min at 25°C. Non-specific component of total binding was determined in the presence of 1 µM SRIF14. Membrane-bound radioligand was separated from free one by rapid filtration under reduced pressure through Whatman GF/C glass fiber filters (pre-soaked for 2 h in incubation buffer). After the wash (3 × 5 ml of 50 mM Tris-HCl, pH = 7.4), radioactivity retained by filters was determined in LKB Rackgamma counter with 72% of efficiency.

Results were expressed in fmol of $^{125}$I-Tyr$^{1}$-SRIF14 bound per mg of protein. Protein concentration was determined according to the method of Lowry and coworkers [1951].

### Adenylyl Cyclase Assay

Semipurified plasma membrane fractions for adenylyl cyclase assay were prepared as described for binding assays except that cells were homogenized by 10 hand strokes in 1 mM Tris-HCl buffer, pH = 7.2 supplemented with 10 mM EGTA and 1% (w/v) sucrose and frozen in the same buffer containing 10 mM EGTA and 10% (w/v) sucrose.

Adenylyl cyclase activities were measured by conversion of $\alpha^{32}$P-ATP to $^{32}$P-cAMP [Enjalbert et al., 1986]. The final incubation medium (50 µl) contained 50 mM Tris-maleate buffer (pH = 7.2), 1.5 mM MgSO4, 1 mM cAMP, 5 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.15 mM ATP, 0.01 mM GTP, 10 mM theophyline, 1 µCi $\alpha^{32}$P-ATP and 0.001 µCi of $^{3}$H-cAMP. The reaction was initiated by addition of 10 µl homogenate. Incubation was performed under conditions of linear $^{32}$P-cAMP production (37°C for 30 min). $^{32}$P-cAMP was separated from $\alpha^{32}$P-ATP by two-step elution as previously reported [Enjalbert et al., 1986]. Recovery of $^{3}$H-cAMP on individual columns varied from 70–80%. Adenylyl cyclase activity was expressed in pmol of $^{32}$P-cAMP (corrected for elution efficiency of each individual column (determined on the basis of $^{3}$H-cAMP recovery)) formed per mg protein over 30 min.

The basal adenylyl cyclase activity measured in these conditions was at the limit of the method sensitivity (i.e., it equaled two- to three-fold blank values). We therefore used a plant alkaloid, forskolin, to increase the enzyme activity by targeting directly its catalytic domain [Seamon et al., 1981]. Indeed, forskolin (10 µM) increased the adenylyl cyclase activity three- to four-fold from basal values. This increase is in perfect agreement with those reported previously [Seamon et al., 1981]. The effects of SRIF on adenylyl cyclase activity were therefore assessed in the presence of 10 µM forskolin in all further experiments.

### HPLC Assessment of SRIF Stability

During initial experiments, a trace amount (1.5 µl corresponding to 100,000 cpm, specific activity, 2,200 Ci/mmol) of $^{125}$I-Tyr$^{1}$-SRIF14 (NEN Dupont de Nemours) was added to an aliquot (1 ml) of culture supernatants obtained both at the beginning and after 4 h incubation of mitogen-activated Jurkat cells in the presence of the highest SRIF14 concentration tested (1 µM). These aliquots were diluted (1:1, v/v) in water solution of acetonitrile (48%, v/v) containing 0.1% (v/v) of trifluoroacetic acid (pH = 2). The intact peptide and its degradation products were separated by HPLC using C18 µBondapak (3.9 × 300 mm, Waters) column with a gradient of 24–60% acetonitrile. Elution (flux = 1 ml/min) was accomplished between 24 and 36 min. The radioactivity in collected (1 ml) fractions was determined using a LKB Rackgamma counter with 72% of efficiency.

### IL-2 Assays

We first assured that biological assay of IL-2 (based on the measurement of survival of IL-2-dependent CTLL-2 cells [Mosman, 1983]) can be used for determination of IL-2 content in the culture supernatants of Jurkat cells previously incubated in the presence of SRIF14. To do that we studied the effects of SRIF14 on CTLL-2 survival. Our results show that in conditions in which SRIF degradation was minimized (see SRIF stability assays), SRIF14 (in the concentration range from 1 pM to 1 µM) has any obvious effect on CTLL-2 survival (Table I). This was evidenced both in the absence and in the presence of exogenously added human IL-2 (Table I) thus indicating that somatostatin could neither support CTLL-2 survival in the absence of exogenously added recombinant IL-2 (Boehringer-Mannheim, Germany) nor could alter CTLL-2 viability in the presence of exogenous IL-2.

**TABLE I. Somatostatin Effects on CTL-L2 Cell Proliferation**

| SRIF concentration (M) | SRIF 14 | |
|---|---|---|
| | +IL-2[a] | −IL-2 |
| 0 | 1.075 ± 0.007[b] | 0.102 ± 0.002 |
| $10^{-12}$ | 0.919 ± 0.027 | 0.126 ± 0.012 |
| $10^{-11}$ | 0.945 ± 0.026 | 0.103 ± 0.002 |
| $10^{-10}$ | 1.055 ± 0.070 | 0.134 ± 0.022 |
| $10^{-9}$ | 0.923 ± 0.075 | 0.107 ± 0.001 |
| $10^{-8}$ | 0.967 ± 0.003 | 0.097 ± 0.001 |
| $10^{-7}$ | 0.948 ± 0.051 | 0.113 ± 0.009 |
| $10^{-6}$ | 0.937 ± 0.008 | 0.115 ± 0.002 |

[a]The concentration of IL-2 added was 5 U/ml.
[b]Values correspond to O.D. at 490/650 nm.

For quantification of somatostatin effects on IL-2 secretion, mitogen-activated Jurkat cells were first washed twice in RPMI 1640 medium (0% FCS) and distributed in 24 well plates (Costar, Cambridge, MA) as a suspension containing $0.5 \times 10^6$ cells per well. They were then incubated in the same medium supplemented with 0.5 g/l bacitracin (see SRIF stability assays). After a 4 h incubation in the absence or in the presence of SRIF14 in the concentration range 1 pM to 1 μM, culture supernatants were collected and frozen at −20°C until assayed.

To increase the accuracy of the measurement, the Jurkat cell supernatants were serially diluted. IL-2 content was quantified in 100 μl of each dilution by the assessment of CTLL-2 survival ($2 \times 10^4$ cells per well) in 96 well flat-bottomed microplates over 19 h incubation. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, L'Isle d'Abeau, France) solution (7.5 mg/ml, 20 μl per well) was then added for 4 h. Crystal products of MTT, formed by enzymatic conversion in surviving cells, were dissolved by adding 200 μl 0.04 M HCl in DMSO to each well and the absorbance determined at 490/650 nm. Measured O.D. values were plotted against dilution for each initial Jurkat cell culture supernatant (corresponding to the cultures incubated in the absence or in the presence of somatostatin in the concentration from 1 pM to 1 μM) in order to determine the dilution range in which the absorbance at 490/650 nm is a linear function of the IL-2 content [Mosman, 1983]. For the dilution 1/9 all curves obtained for initial Jurkat cell supernatants satisfied this criterium: corresponding O.D. values were therefore taken as a reference value. Since these absolute O.D. values fluctuated in different experiments, they were normalized against the O.D. value measured for the culture supernatants obtained after Jurkat cell incubation in the absence of SRIF14 in each individual experiment.

### Cell Proliferation Assay

Jurkat cells were either mitogen-activated (10 ng/ml TPA and 2 μg/ml PHA-L for 16 h) or left untreated. They were then cultured in 96-well plaques in 200 μl final volume ($2 \times 10^5$ cells per well) of the complete, 10% FCS supplemented RPMI medium in the absence or in the presence of SRIF14 (1 pM to 1 μM). $^3$H-CH3-thymidine (Dupont-NEN, Les Ulis, France; specific activity: 88.7 Ci/mmol) was added at the rate 1 μCi per well at the same time as SRIF14 and incubation was carried out for 5 h. The cultures were stopped by culture filtration on cell harvester (Tomtec) and the filters were counted in a liquid scintillation beta counter (Wallac 1450 Microbeta Trilux).

### Data Analysis

Variance existing between individual experiments, as well as differences between the effects of SRIF14 concentrations studied on IL-2 secretion and proliferation of mitogen-activated Jurkat cells were statistically evaluated by using an analysis of variance (ANOVA). The effects of the experimental factors on the observed differences were considered significant for the *P*-value (probability) of F-statistics less than 0.05.

Pharmacological parameters (concentration necessary to inhibit 50% of maximal response, $IC_{50}$; concentration necessary to trigger 50% of maximal response, $EC_{50}$) were estimated by computer-assisted non-linear regression analysis using McPherson's modified method [McPherson, 1983].

## RESULTS

### sst3 Receptor Isotype Expression by Mitogen-Activated Jurkat Cells

In order to assess the expression of SRIF receptors, we performed the analysis of cDNA reversely transcribed from mRNAs isolated from mitogen-activated Jurkat cells. When primers specific for sst3 receptor were used, the expected 222-base pair fragments were detected (Fig. 1). As sst3 receptor gene contain no introns [Reisine and Bell, 1995] their size was

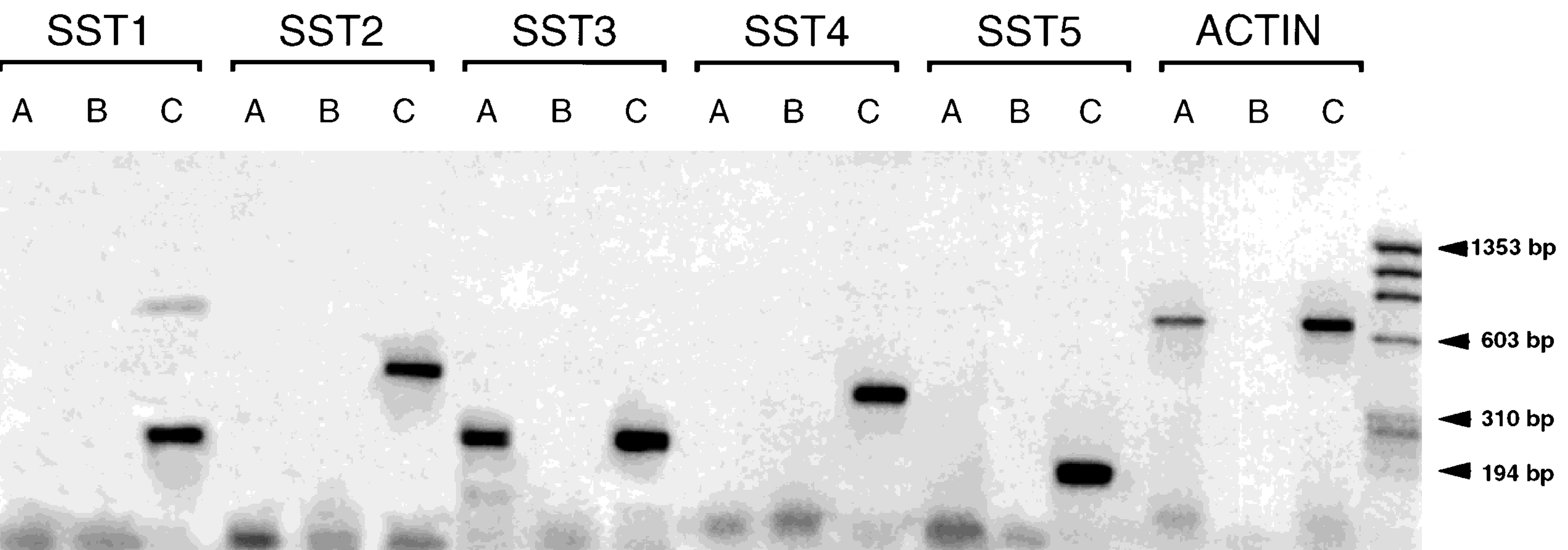


**Fig. 1.** RT-PCR analysis of SRIF receptor expression in mitogen-activated Jurkat cells. Complementary DNA was prepared from RNA isolated from Jurkat cells in the presence (**A**) or in the absence (**B**) of reverse transcriptase as described in Methods. PCR amplification was performed with primers specific for sst1-sst5 somatostatin receptors and β-actin and products of amplification were stained with ethidium bromide. Genomic DNA (**C**) was amplified in parallel to serve as a positive control for the size of amplified fragments.

expected to be identical to the size of the fragments amplified from genomic DNA of Jurkat cells (Fig. 1). However, no amplification product was obtained from cDNA used as a template with primers specific for sst1, sst2, sst4, and sst5 receptors in any sample of Jurkat RNA tested. The same primers amplified the fragments of the expected size from genomic DNA (Fig. 1). In addition, the primers specific for sst1, sst2, sst3, sst4, and sst5 (sst1–sst5) receptors used in our study could amplify the fragments of expected size for all five receptors when the mRNA from either fetal (Clonotech, Palo Alto, CA) or adult (Clonotech) human brain was used as a template for reverse transcription (data not shown). Both fetal and adult human brain have been previously described to contain sst1–sst5 receptors [Panetta and Patel, 1994; Rohrer et al., 1993]. In contrast, no amplification occured when RT-PCR was performed by omitting Moloney monkey leukemia virus reverse transcriptase (Fig. 1).

### Functional Expression of sst3 Receptor: SRIF14 Effects on $^{125}$I-Tyr$^{1}$-SRIF14 Binding and Adenylyl Cyclase Activity

In the absence of mitogen stimulation, Jurkat cells express a putative SRIF14 receptor at their surface [Sreedharan et al., 1989]. We first assessed whether the mitogen activation alters the characteristics of these receptors by analysing the competition between $^{125}$I-Tyr$^{1}$-SRIF14 and nonradioactive SRIF14 for binding on plasma membrane preparations obtained from mitogen-activated Jurkat cells. This radioligand was chosen since it binds with subnanomolar affinity all five cloned SRIF receptors (such a choice was obvious given that sst3-selective ligand is still lacking). The analysis of SRIF14 inhibition of $^{125}$I-Tyr$^{1}$-SRIF14 specific binding yielded two binding sites characterized with $K_{I1}$ of 78 ± 3 pM and $K_{I2}$ of 12.4 ± 3.9 nM (Fig. 2A). One of these two binding sites, having the high-affinity for SRIF (i.e., the site characterized by $K_{I1}$ of 78 ± 3 pM) corresponds to the cloned SRIF receptors as estimated by its affinity for non-selective ligand. The molecular identity of the low-affinity site (i.e., the site characterized by $K_{I2}$ of 12.4 ± 3.9 nM) remains unknown.

In addition, SRIF14 inhibits adenylyl cyclase activity of mitogen-activated Jurkat cells in a monophasic manner with an $IC_{50}$ value of 23 ± 4 pM (Fig. 2B) thus suggesting that at least the high-affinity binding site is functionally coupled to the adenylyl cyclase. However, the SRIF14-dependent inhibition of adenylyl cyclase was moderate ($I_{max}$ = 18 ± 6%): it was achieved at 1 nM concentration and remained unchanged up to 1 μM (Fig. 2B).

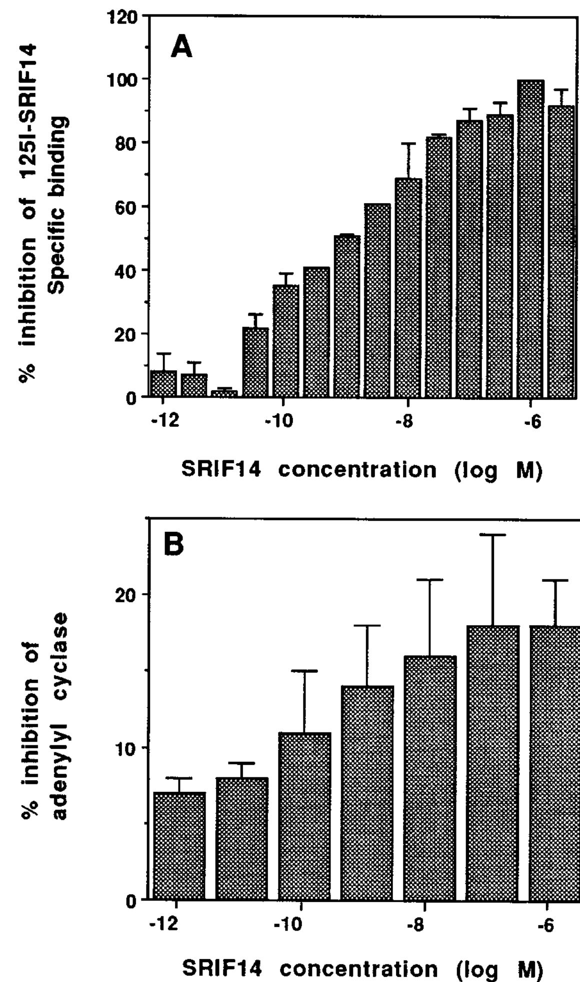


Fig. 2. sst3 receptor is functionally expressed at the mitogen-activated Jurkat cell plasma membrane. SRIF14 inhibits both $^{125}$I-Tyr$^{1}$-SRIF14 specific binding (**A**) and adenylyl cyclase activity (**B**) in a dose-dependent manner. Each depicted value represents the mean ± S.D. and is expressed as % of inhibition calculated against the reference value taken as 100%. These reference values correspond to $^{125}$I-Tyr$^{1}$-SRIF14 specific binding (A) and adenylyl cyclase activity (B) measured in the absence of SRIF14. They were 21 ± 3 fmol/mg protein and 126 ± 7 pmol/mg protein/30 min in (A) and (B), respectively.

### SRIF Stability Assays

Given the instability of SRIF14 in the presence of serum proteases (the half-life of SRIF14 in circulation is few min [McMartin and Purdon, 1978; Sheppard et al., 1979]), to study its effects on IL-2 secretion, we first extensively washed mitogen-activated Jurkat cells in cul-

ture medium without FCS and then incubated them in fresh serum-free medium without or in the presence of maximal SRIF14 concentration tested (1 μM). The assays were carried out for different time periods (1–5 h) in order to establish the incubation time necessary to allow IL-2 accumulation in the culture medium sufficient for detection by bioassay. This criterium was satisfied for incubation times longer than three hours (data not shown). We therefore chose 4 h incubation periods for further experiments.

HPLC verification of SRIF14 stability indicated however that only 26% of the initially added SRIF14 was not degraded after 4 h incubation of mitogen-activated Jurkat cells in culture medium without FCS (Fig. 3C). An endopeptidase inhibitor, bacitracin (0.5 g/l) was therefore included in the culture medium (trypan blue exclusion assays showed that in the concentration used, bacitracin did not alter the cell viability, data not shown). In these experimental conditions, 82% of initially added SRIF14 was preserved after a 4 h incubation (Fig. 3B).

### SRIF14 Effects on IL-2 Secretion

Jurkat cells used in the present study are not spontaneously producing IL-2 but can be triggered to do so after mitogenic stimulation as previously described [Manger et al., 1985; Weiss et al., 1984]. SRIF14 (in the concentration range from 1 pM to 1 μM) could not induce detectable IL-2 secretion from nonactivated Jurkat cells thus excluding any agonist action of SRIF14 on IL-2 release (data not shown). In contrast, the presence of SRIF14 increased IL-2 secretion by mitogen-activated Jurkat cells with an $EC_{50}$ concentration of 22 ± 9 pM and a peak of secretion comprised between 0.1 and 1 nM concentrations (Fig. 4). This SRIF14-dependent increase of IL-2 secretion was modest, but significant ($P < 0.05$). In the concentration range from 10 nM to 1 μM, SRIF14 had no effect on IL-2 production (Fig. 4).

### SRIF14 Effects on Cell Proliferation

The basal rate of $^{3}$H-CH3-thymidine incorporation of Jurkat cells (i.e., in the absence of IL-2) was of 148185 ± 7563 cpm. Mitogen stimulation (10 ng/ml TPA and 2 μg/ml PHA-L for 16 h) did not significantly alter the incorporation of $^{3}$H-CH3-thymidine (155,263 ± 2,221 cpm) as expected for the IL-2-independent proliferation of these cells [Manger et al., 1985; Weiss et al.,

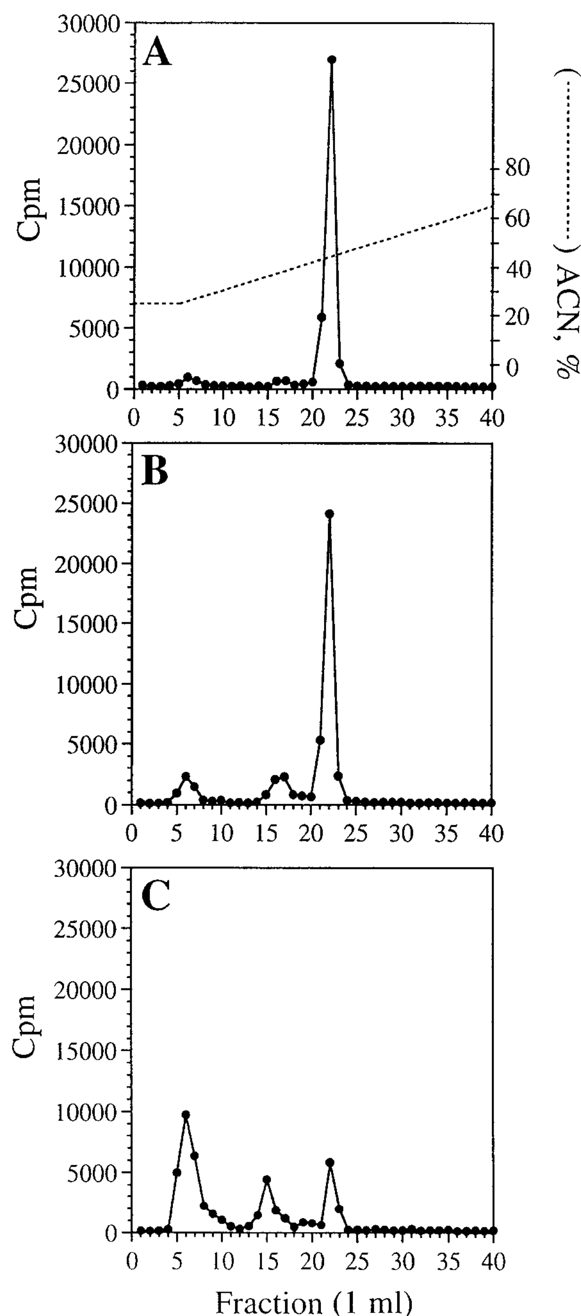


**Fig. 3.** HPLC assessment of SRIF stability in the supernatant of mitogen-activated Jurkat cells. **A:** SRIF elution profile of the Jurkat cell culture supernatant at the time 0 (i.e., time of the neuropeptide addition). Ninety-three percent of the initially added peptide (as determined by the comparison to the elution profile of $^{125}$I-Tyr$^{1}$-SRIF14 in 50 mM Tris-HCl, pH = 7.4) is intact. **B:** After 4 h of incubation in the presence of Jurkat cells and 0.5 g/l bacitracin, 82% of initially added SRIF is preserved. **C:** In the absence of bacitracin, only 26% of the initially added SRIF is not degraded after 4 h of incubation. Note the appearance of two additional peaks corresponding to the SRIF degradation products with smaller size (lower retention time). Acetonitrile (ACN) gradient is given by dashed line in (A).

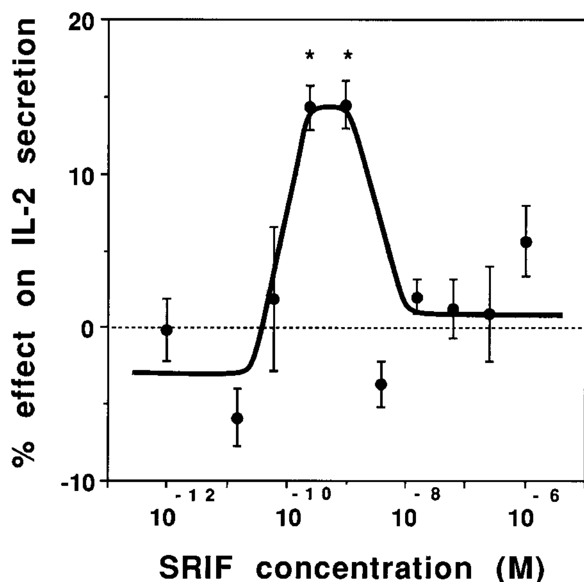


**Fig. 4.** Effects of SRIF14 on IL-2 secretion by mitogen-activated Jurkat cells. O.D. values (reflecting CTLL-2 survival, see Methods) for each individual experimental point obtained for SRIF14 effects on IL-2 secretion were normalized against O.D. values obtained in the absence of SRIF14. This reference O.D. value was 0.115 ± 0.006. Each point depicted is the mean ± S.D. obtained in three independent experiments for initial Jurkat culture supernatant dilutions 1/9 assayed in replicates. The asterisks point to the peptide concentrations for which a significant ($P < 0.05$) difference of IL-2 content was observed when compared to the IL-2 secretion measured in the absence of SRIF14.

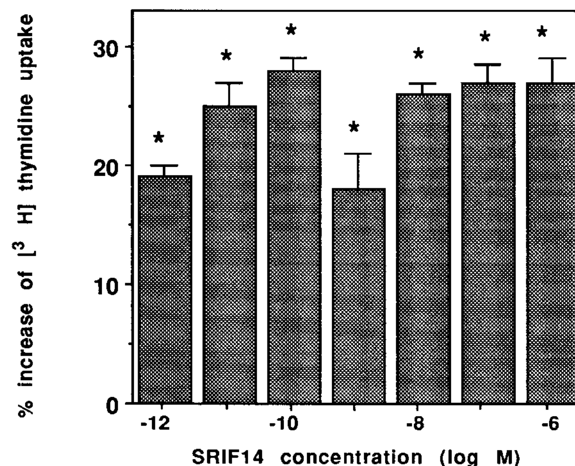


**Fig. 5.** Effects of SRIF14 on Jurkat cell proliferation. The incorporation of $^3$H-CH3-thymidine in the presence of increasing concentrations of SRIF14 is expressed as a percentage of increase of $^3$H-CH3-thymidine uptake measured in the absence of SRIF14 (this reference value was 155,263 ± 2,221 cpm per $2 \times 10^5$ cells). Data presented are the mean (±S.D.) of triplicate determinations obtained in four independent experiments. $^*P < 0.05$.

1984]. However, SRIF14 was able to increase the proliferation rate of mitogen-treated cells in a dose-dependent manner with an $EC_{50}$ concentration of 12 ± 1 pM. The maximal effect (30% increase) was achieved between 0, 1 and 10 nM SRIF14 concentrations and remained unchanged up to 1 μM (Fig. 5).

## DISCUSSION

In the present study we report for the first time the expression of SRIF receptor mRNA in the mitogen-activated human Jurkat T cell line. Our data show that, in these conditions, Jurkat T cells express one single (i.e., sst3) out of five cloned receptor isotypes. The experimental approach used was validated by the fact that the length of RT-PCR fragments obtained for the amplification by using the primers specific for sst3 receptor from either genomic or cDNA were identical in size as expected for intronless genes such as genes encoding SRIF receptors [for review, see Reisine and Bell, 1995].

sst3 receptor mRNA is apparently expressed as a protein at the surface of mitogen-activated Jurkat cells since specific binding sites for SRIF were attested in plasma membrane preparations by competitive inhibition of $^{125}$I-Tyr$^1$-SRIF14 specific binding in the presence of nonradioactive SRIF14. The subnanomolar affinity (as determined in $^{125}$I-Tyr$^1$-SRIF14 binding assays) of the sole SRIF14 receptor identified here by RT-PCR (i.e., sst3) is in perfect agreement with those reported previously for the high-affinity SRIF14 binding site of nonactivated Jurkat cells [Sreedharan et al., 1989]. As expected for the functional somatostatin receptor, it is negatively coupled to the adenylyl cyclase. Indeed, SRIF14 inhibits both $^{125}$I-Tyr$^1$-SRIF14 specific binding and adenylyl cyclase activity with similar efficiencies. However, nonselective radioligand used in our study recognizes an additional, low-(submicromolar) affinity receptor. The existence of such low-affinity SRIF receptor on Jurkat T cells [Sreedharan et al., 1989], on other T cells [Hiruma et al., 1990], and on T lymphocytes [Bhathena et al., 1981] has already been suggested. However, the precise molecular and pharmacological characteristics of the low-affinity receptor remain unknown.

Our study provide further data on the mechanisms involved in neuroimmuno-modulatory effects of SRIF14. In order to study these effects, we took advantage of the homogeneity of the Jurkat T cell line. Moreover, the use of this cell

line as a model to study the modulation of cell proliferation vs. IL-2 secretion is of particular interest because in these cells, proliferation on one hand, and IL-2 secretion on the other hand, can be dissociated (proliferation of Jurkat cells is IL-2 independent). Additionally, previous to the assessment of neuroimmuno-modulatory actions of SRIF in Jurkat cells, we ensured that SRIF14 added to the culture medium was not degraded. Indeed, all previous studies addressed SRIF14 actions on T cells 24, 48, or 96 h after a single neuropeptide addition in the culture medium containing FCS [Nio et al., 1993; Scicchitano et al., 1988]. In these experimental conditions, all SRIF14 is proteolytically cleaved during the first few minutes after addition [S. Krantic and J.-P. Gautron, unpublished results] thus implying that the analysis of its effects a few days later is very difficult.

Our data show that the addition of SRIF14 subsequent to the mitogen-activation of Jurkat cells leads to an increased IL-2 secretion. This is in agreement with the previously reported data obtained on another type of immune cells [Nio et al., 1993]. The SRIF14 concentrations, efficient in the potentiation of IL-2 secretion reported here are well correlated with the affinities (Kd = 0, 1 to 1 nM) of cloned SRIF receptors (sst1–sst5) [Reisine and Bell, 1995]. Moreover, we were able to show that in this concentration range SRIF14 inhibits the production of cAMP by Jurkat cells (see above), a condition necessary to allow the increase in IL-2 production [Mary et al., 1987]. sst3 receptor appears thus to be functionally involved in the transduction of SRIF14 effects on IL-2 secretion since it is the only SRIF14 receptor that we could detect in mitogen-activated Jurkat cells by RT-PCR. However, the unequivocal demonstration of the involvement of sst3 receptor in the positive modulation of IL-2 secretion must await the development of sst3-selective agonists, antagonists, and antibodies.

In our experimental conditions, SRIF14 could not trigger IL-2 secretion in the absence of mitogens. This is in contrast with the reported stimulatory effects of another neuropeptide, Substance P, on IL-2 production in Jurkat cells [Calvo et al., 1992], colonic lamina propria mononuclear cells and human T cell clone [Nio et al., 1993] irrelevantly of whether these cells have been mitogen-stimulated or not. It appears thus that some neuropeptides (e.g., Substance P; luteinizing hormone-releasing hormone [Azad et al., 1997]) can both trigger and modulate the immune response while the action of others (e.g., SRIF14) is limited to the immune-modulation.

In accord with observed SRIF14-dependent enhancement of IL-2 secretion by Jurkat cells, we were able to evidence an increase in the cell proliferation rate in the presence of SRIF14. Indeed, it is currently well established that antigen- or mitogen-activation of a quiescent T cells renders the cells competent to proliferate in the presence of appropriate growth factors such as IL-2 [for review, see Taniguchi and Minami, 1993]. The induction of IL-2 secretion and T cell proliferation appears therefore regulated according to a coordinated pattern. Consistently, vasoactive intestinal peptide (VIP) inhibits IL-2 secretion by activated murine [Ganea and Sun, 1993; Sun and Ganea, 1993; Xin et al., 1994] and human [Nio et al., 1993] T cells and inhibits T cell proliferation [Boudard and Bastide, 1991; Xin et al., 1994]. However, it is worth noting that SRIF14 also enhances the Jurkat cell proliferation at higher (10 nM to 1 μM) concentrations which have no significant effect on IL-2 secretion. A considerable body of experimental evidence suggests the existence of additional, yet uncloned SRIF receptors [for review, see Reisine and Bell, 1995]. sst3 receptor isotype identified here by RT-PCR might therefore not be the only receptor mediating the observed SRIF14 actions on mitogen-activated Jurkat cell proliferation.

It is particularly intriguing that in colonic lamina propria mononuclear cells SRIF14 inhibits its proliferation whereas it stimulates IL-2 secretion [Nio et al., 1993]. The molecular isotypes of SRIF receptors expressed by these cells are currently unknown and further studies are needed to determine if sst3 receptor is expressed in these cells and if it is involved in the positive modulation of IL-2 secretion. However, an interesting hypothesis to explain the opposite SRIF14 actions on IL-2 secretion and cell proliferation in colonic lamina propria mononuclear cells in contrast to the positive modulation of both parameters in Jurkat cells would be that different T cells express different set of SRIF14 receptors. According to such a hypothesis, colonic lamina propria mononuclear cells would express, besides sst3, additional receptors mediating the inhibitory effects of SRIF14 on cell proliferation. Indeed, sst2 and sst5 receptors have been reported to inhibit the prolifera-

tion of other cell types [for review, see Reisine and Bell, 1995].

Altogether our results suggest that in mitogen-activated human Jurkat T cells, at least sst3 receptor isotype mediates SRIF14 stimulatory actions on IL-2 secretion and cell proliferation. However, our data do not allow to exclude the involvement of additional mechanisms in the regulation of IL-2 secretion and cell proliferation including those involving the transduction via yet unknown SRIF receptor(s). The data presented here bring therefore some new insights on the mechanisms underlying the neuro-immune-endocrine actions of SRIF. In the larger context, they further document the poorly understood positive regulation of cellular proliferation by this neuropeptide. Future studies using different cell types will show whether this positive regulation is confined to the particular immune cells and whether sst3 receptor is generally involved in the transduction of SRIF-mediated enhancement of proliferation.

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