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Phosphotyrosine Phosphatases of the AtT-20 Murine Corticotroph Cell Line

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ABSTRACT

Somatostatin (somatotropin release inhibitory factor; SRIF) is a peptide-signaling molecule that activates a family of heterotrimeric guanine nucleotide binding protein (G-protein) coupled receptors (sst_1-sst_5). SRIF receptors control essential intracellular signaling events and, by reducing cyclic nucleotide levels, ion concentrations and protein phosphorylation, ultimately controlling cell proliferation and secretion. In the current study, we investigated the intracellular phosphatase activity present in the AtT-20 cell, as well as whether these enzymes were under direct SRIF receptor control. AtT-20 cells retain many of the properties of anterior pituitary corticotrophs, yet are an established cell line that expresses at least two SRIF receptor subtypes (sst_2 and sst_5). Both SRIF receptor subtypes potently suppress hormonally induced adrenocorticotropic hormone (ACTH) secretion from AtT-20 cells. Since intracellular protein kinase activation plays a crucial role in ACTH release from AtT-20 cells, identifying the corresponding protein phosphatases will provide valuable information on corticotroph function. The novel fluorescent phosphatase substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiF-MUP) was employed to identify soluble or membrane-associated phosphatase activities. Treatment with a selective tyrosine phosphatase inhibitor (sodium vanadate; Na_3VO_4) attenuated DiF-MUP phosphatase activity by 75%, suggesting that the dominant activity detected in AtT-20 cells is a tyrosine phosphatase. Na_3VO4 inhibition was potent as concentration response studies demonstrated an inhibitory concentration for 50% activity (IC_{50}) of 90 nM. A serine/threonine phosphatase inhibitor failed todecrease phosphatase activity, indicating that the dominant phosphatase activity present is due to protein

tyrosine phosphatases. Immunoprecipitation studies, in conjunction with sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and immunoblotting identified two intracellular phosphotyrosine phosphatases: Src homology phosphatase-1 (SHP-1) and Src homology phosphotyrosine phosphatase-2 (SHP-2). Immunoblotting with phospho-specific antisera confirmed the presence of SHP-1 and SHP-2, while also demonstrating that neither enzyme appears to be under SRIF control in AtT-20 cells. Together, these results show that AtT-20 murine corticotrophs contain sodium vanadate sensitive, soluble tyrosine phosphatase activity that is independent of SRIF control.

INTRODUCTION

Protein phosphatases are ubiquitous enzymes responsible for the dephosphorylation of intracellular proteins. These enzymes antagonize the effects of protein kinases, which phosphorylate proteins predominately on serine, threonine and tyrosine residues. Three major categories of protein phosphatases have been identified based on their preference for dephosphorylating the amino acid residues tyrosine, serine/threonine, or both tyrosine and threonine.

Protein tyrosine phosphatases (PTPs) are categorized into groups that either contain or lack a transmembrane domain, and are further subdivided into 17 groups (Andersen *et al*, 2001). PTPs, such as SHP-2 (SH2 containing protein tyrosine phosphatase 2), contain a src homology 2 (SH2) domain composed of approximately 100 amino acids, including a tyrosine residue that undergoes phosphorylation, followed by Glu-Glu-Ile, leading to specificity in signaling control. PTPs regulate phosphorylated tyrosine residues, thereby controlling cell growth and differentiation (reviewed in Tonks and Neel, 1996). A second type of PTP, SHP-1, is responsible for dephosphorylating signaling molecules such as p27 (Kip1), leading to cell cycle arrest (Pages *et al*, 1999). Serine/threonine phosphatases regulate diverse cellular mechanisms including glycogen metabolism, DNA replication, and exocytosis (Sim *et al*, 2003). Threonine/tyrosine phosphatases, known as dual specificity MAPK phosphatases (DS-MKPs) regulate the mitogen-activated protein kinases (MAPKs), which are critical protein kinases that regulate diverse physiological events, such as long-term potentiation, and inflammation (Wera and Hemmings, 1995; Farooq and Zhou, 2004). Dual-specificity phosphatases

(DSPs) aid in controlling the cell cycle by interacting with both MAPKs and stress activated kinases (Keyse, 1999).

Somatostatin (somatotropin release inhibitory factor; SRIF) was originally identified in the central nervous system as a hypothalamic inhibitor of growth hormone release (Brazeau *et al*, 1973). SRIF exists in two distinct forms, SRIF-14 and SRIF-28, both originating from the SRIF-prepropeptide. SRIF is distributed throughout the entire human body, with SRIF-28 predominately localized in the gastrointestinal tract and SRIF-14 localized to the nervous system (Reisine, 1995). SRIF peptides regulate a spectrum of endocrine effects, including thyroid-stimulating hormone (Singh *et al*, 2000), modulating gastric acid secretion (Larsson *et al*, 1979), and inhibiting pancreatic insulin and glucagon secretion (Strowski *et al*, 2000).

SRIF carries out its actions by interacting with a group of five highly homologous SRIF receptors subtypes (sst_1 – sst_5), including the sst_2 receptor splice-variants (sst_{2A} and sst_{2B}). This receptor family exists in an overlapping expression pattern in many tissue types throughout the body (Reisine, 1995; Vanetti *et al*, 1992). The SRIF membrane receptors are seven transmembrane spanning proteins that couple to intracellular heterotrimeric guanine nucleotide-regulated proteins (G proteins) (Reisine, 1995). Through G protein activation and appropriate effector stimulation, SRIF receptors regulate cyclic nucleotide levels, membrane ion conductances, and cellular protein phosphorylation levels (Florio and Schettini, 1996).

AtT-20 cells are a transformed mouse corticotroph cell line which constitutively express a number of neuronal features, including cytoplasmic processes that resemble

neuronal growth cones, neurofilament polypeptides, as well as neuron-specific phosphoprotein synapsin 1 (Tooze *et al*, 1989). In addition, and of importance in the current study, the AtT-20 cells endogenously co-express the $sst_{2A, 2B,}$ and sst_{5}, but not $sst_{1, 3, 4}$ SRIF receptor subtypes (Strowski *et al*, 2002; Cervia *et al*, 2003). AtT-20 cells retain the important property of primary corticotrophs, such as the hormonally regulated secretion of adrenocorticotropic hormone (ACTH) (Gumbiner and Kelly, 1981; reviewed in Antoni, 2000). Both corticotropin-releasing factor (CRF) and leukemia inhibitory factor (LIF) serve as potent ACTH secretagogues in corticotrophs and AtT-20 cells, making the AtT-20 cell an attractive model for studying ACTH stimulus-secretion coupling. (Vale *et al*, 1983; Auernhammer, 1998). SRIF treatment inhibits ACTH secretion by reducing intracellular 3', 5' cyclic adenosine monophosphate (cAMP) levels and suppressing calcium ion influx (Heisler *et al*, 1982; Richardson,1983).

Mitogen-activated protein kinases (MAPKs) and signal transducers and activators of transcription (STATs) have also been implicated in ACTH secretion by AtT-20 cells. Phosphotyrosine phosphatases may dephosphorylate the activated kinases, leading to a halt in ACTH release. SRIF's ability to inhibit these intracellular signaling pathways may be due to the recruitment of protein phosphatases, capable of dephosphorylating the activated kinases. In murine NIH 3T3 cells, SRIF regulates dephosphorylation of the tyrosine residue of Raf-1, a mitogen-activated kinase kinase kinase (MAPKKK), by stimulating protein tyrosine phosphatase activity (Reardon *et al*, 1996). SRIF's inhibitory effects on AtT-20 cellular function, including the reduction of both cAMP and

phosphorylated MAPK levels, led us to investigate and identify the possible protein phosphatase activity within this cell line.

In the current study we have characterized the endogenous protein phosphatase activity of the AtT-20 murine corticotroph. Using a fluorescent enzymatic (6,8-difluoro-4-methylumberiferyll phosphate; DiF-MUP) assay in conjunction with enzymatic phosphatase inhibitors, immunoprecipitation and immunoblotting we have established the presence of soluble phosphotyrosine phosphatase activity. Our results suggest the presence of a soluble tyrosine phosphatase activity that is independent of SRIF regulation.

MATERIALS AND METHODS

Cell culture

The AtT-20 cell line used in this study was a gift from Dr. Terry Reisine (Los Angeles, CA). Cell culture dishes and flasks were purchased from Corning (Corning, NY). Cell culture growth medium and supplements were purchased from Invitrogen (Carlsbad, CA). AtT-20 cells were cultured in Dulbecco's modified Eagle's medium, DMEM, (with GlutaMAXTH, high glucose, 110mg/L sodium pyruvate and pyridoxine-HCL, Invitrogen, Carlsbad, CA) containing 100 U ml^{-1} of penicillin, 100µg ml^{-1} streptomycin, and 10% (v/v) fetal calf serum. Cultures were maintained in 5% CO_2 at 37°C. Cell monolayers were grown in T-75cm^2 flasks and passaged when 75% confluence was reached. Cell suspensions were transferred to a new T-75cm^2 flask at a 1:10 dilution. Cells were given 48 hours to recover prior to experimentation.

Tyrosine phosphatase assay

AtT-20 cultures were incubated in the presence or absence of 100nM SRIF-14 (Peninsula Labs, Belmont, CA) for 30 min. Cell cultures were washed in PBS and then lysed in a 50mM HEPES buffer containing 0.5% Triton X-100, 10% glycerol (GibcoBRL), 2.5µM pepstatin A, 12.5µM leupeptin, 12.5µM aprotinin, 12.5µM bacitracin and 200nM polymethylsulfonyl fluoride. Lysates were then centrifuged at 13,500 x g for 15 min at 6°C and the supernatant retained.

Protein phosphatase assays were performed in a clear bottom 96 well plate (Corning, Corning, NY). AtT-20 cell lysate was incubated with varying concentrations of the fluorescent phosphatase substrate, 6,8-difluoro-4-methylumbelliferyll phosphate

(DiF-MUP; Gee *et al*, 1999; Molecular Probes, Eugene, OR) up to 30 min at 37°C with intermittent mixing in a CytoFluor Series 4000 fluorescent plate reader (Applied Biosystems, CA), with 360nm excitation and 460nm emission filters. Parallel assays were conducted in the presence of 10μM Na_3VO_4, an inhibitor of tyrosine phosphatase activity and a commercial 10 mM serine/threonine phosphatase inhibitor cocktail (Sigma, MO). The resulting data were analyzed with GraphPad Prism 4.0.

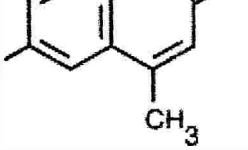
Immunoprecipitation

AtT-20 cells were cultured in T-75cm^2 flasks with DMEM and then incubated for 15 minutes with 100nM SRIF-14. Cells were washed with 5 mL PBS (with MgCl and CaCl, Invitrogen, Carlsbad, CA) and lysed with 500 μL of 50mM HEPES buffer. Supernatants were obtained and incubated overnight with phospho-SHP-1 or SHP-2 antibody (Cell Signaling, MA). Protein G agarose beads (450 mg/mL, Sigma, St. Louis, MO) were added and pellets were obtained and washed with 50 μL of 50mM HEPES buffer and resuspended in 1X NuPAGE LDS sample buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

AtT-20 cells were cultured in 12-well plates with medium (DMEM) for 48 h and then incubated in the presence or absence of 100nM SRIF-14 for 20 min at 37°C. AtT-20 cells were then lysed using 1X NuPAGE LDS sample buffer. Cell lysates were sonicated and heated to 70°C for 10 min, resolved on NuPAGE 10% Bis-Tris gels (Invitrogen Life Technologies, CA) electroblotted onto polyvinyldifluoride (PVDF) membranes and treated for 45 min in a blocking buffer containing 5% (w/v) of Carnation non-fat

dehydrated milk. Phospho-SHP-1 and SHP-2 were then immunodetected with a 1:1000 dilution of anti phospho-SHP-1 and anti-SHP-2 antibodies, respectively. The PVDF membrane associated immunoreactivity were then washed and incubated for 1 hour in a 1:2000 dilution of a horseradish peroxidase-conjugated anti-rabbit secondary antibody and detected using ECL chemiluminescence (Amersham, NJ). The resulting autoradiograph was scanned and integrated pixel density was obtained using NIH Scion Imaging software.



RESULTS

Tyrosine Phosphatase Activity in AtT-20 cell lysate

To identify a possible role of SRIF-14 in controlling AtT-20 cellular phosphatase activity, experiments using increasing concentrations of the fluorescent substrate DiF-MUP were performed with AtT-20 cell lysates prepared from untreated and treated (100 nM SRIF-14) cultured cells. DiF-MUP hydrolysis provides a fluorescent measure of protein phosphatase activity (Gee *et al*, 1999). Cleavage of the phosphate group results in the product 6,8-difluoro-4-methylumbelliferone, a fluorescent molecule capable of emitting high levels of fluorescence.

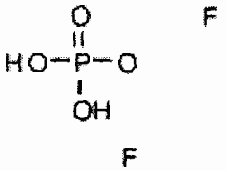


Figure 1. Chemical structure of 6,8-difluoro-4-methylumbelliferryl phosphate (DiF-MUP). Gee *et al*, 1999; Molecular Probes, Eugene, OR.

A concentration-response relationship for DiF-MUP was established by incubating increasing concentrations of DiF-MUP with a soluble fraction from the AtT-20 cell lysate (Figure 2). Non-linear regression analysis of the fluorescence results revealed an approximate effective concentration for 50% activity (EC50) of approximately 50 µM. However, as shown in Figure 2, it should be noted that the lysate enzyme saturation could not be achieved, even at the highest DIF-MUP concentrations used in this study. In addition, the concentration-response curve indicates that no

significant difference exists between SRIF-14 treated and non-treated samples (Figure 2). Experiments performed with AtT-20 cell membranes prepared from cultured cells demonstrated similar results, indicating that SRIF-14 had no significant effect on membrane protein phosphatase activity (data not shown).

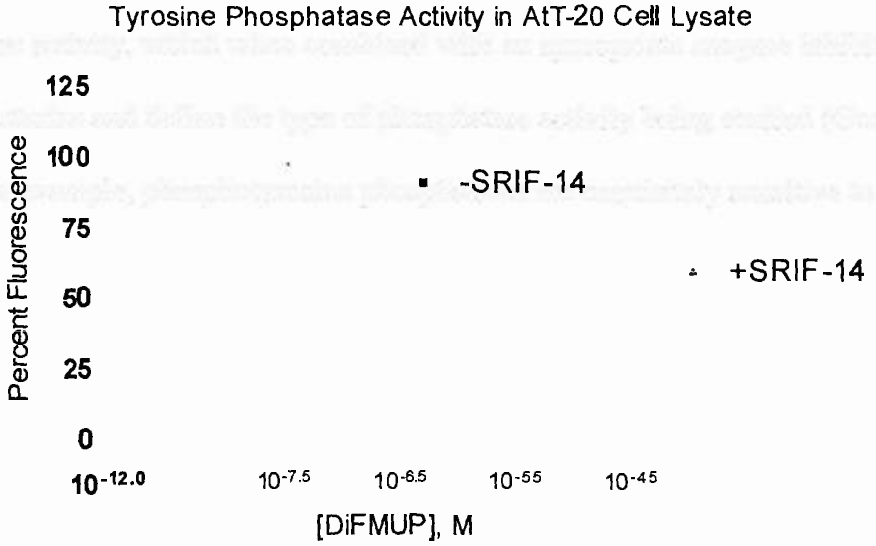
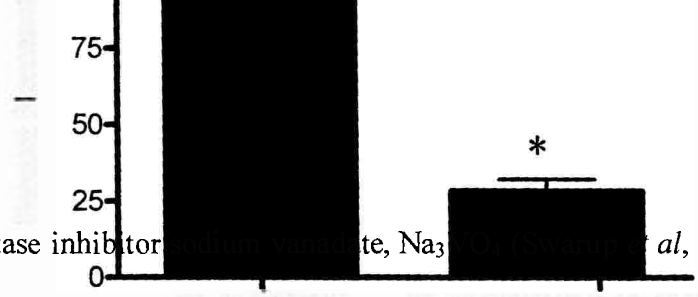


Figure 2. Tyrosine phosphatase activity in AtT-20 cell lysate. Cell monolayers were incubated in the presence or absence of 100 nM SRIF-14 for 15 min at 37°C. A soluble cell lysate was prepared by lysis of the AtT-20 cell monolayers, and fluorescent activity was assessed in a 96-well using a Cytofluor 4000 fluorescent plate reader (Applied Biosystems). Assays were conducted in triplicate and the data mean± SEM of twelve separate experiments are shown.

AtT-20 lysate tyrosine phosphatase inhibition by sodium vanadate (Na_3VO_4)

DiF-MUP provides a convenient and robust fluorescent measure of protein phosphatase activity, which when combined with an appropriate enzyme inhibitor, can help characterize and define the type of phosphatase activity being studied (Gee *et al*, 1999). For example, phosphotyrosine phosphatases are exquisitely sensitive to the potent



tyrosine phosphatase inhibitor sodium vanadate, Na_3VO_4 (Swarup *et al*, 1982). Vanadate-mediated enzyme inhibition serves as a readily available marker for establishing the amount of tyrosine phosphatase activity present in a heterogeneous cell lysate. To establish the level of tyrosine phosphatase activity in the AtT-20 cell lysates, approximately 50 µg of AtT-20 cell lysate was incubated for 15 min with 50 µM DiF-MUP in the presence or absence of 10 µM Na_3VO_4. DiF-MUP activity was reduced in AtT-20 cell lysate samples approximately 75% by Na_3VO_4 (Figure 3; * $p < 0.05$ vehicle

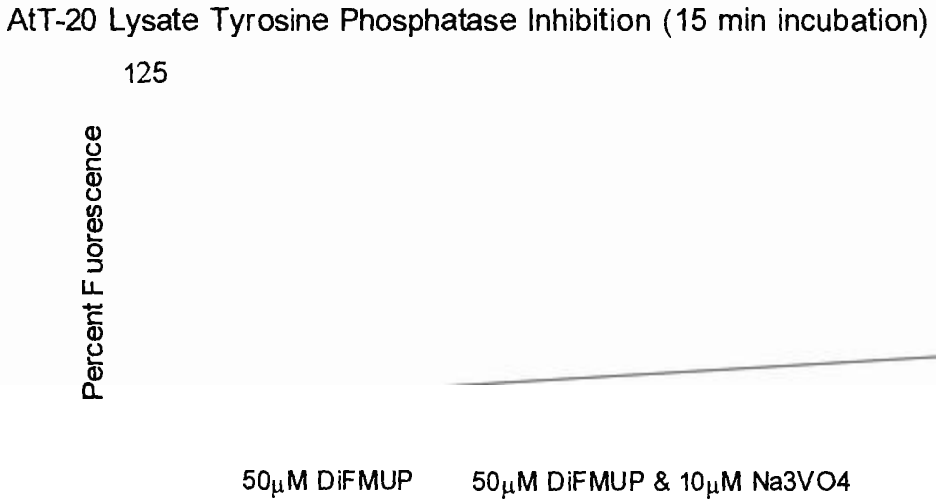


Figure 3. Sodium vanadate attenuates protein phosphatases activity in AtT-20 cell lysate. AtT-20 cell monolayers were prepared in cell lysis buffer and lysates were incubated 15 minutes with 50 µM DiF-MUP in the presence or absence of 10 µM Na_3VO_4. Fluorescence was measured using a Cytofluor 4000 plate reader and data were analyzed in GraphPad Prism 4.0. One way ANOVA followed by Dunn's non-parametric *post hoc* test,* $p < 0.05$ compared vehicle to Na_3VO_4 treated AtT-20 cells.

To determine the potency of Na_3VO_4 on tyrosine phosphatase activity, increasing concentrations of sodium vanadate were added to cell lysates. The DiF-MUP activity

found in the cell lysate exhibits a potent sensitivity to Na_3VO_4, as non-linear regression analysis of the DiF-MUP inhibition revealed a 50% inhibitory concentration (IC_{50}) of approximately 90 nM (Figure 4).

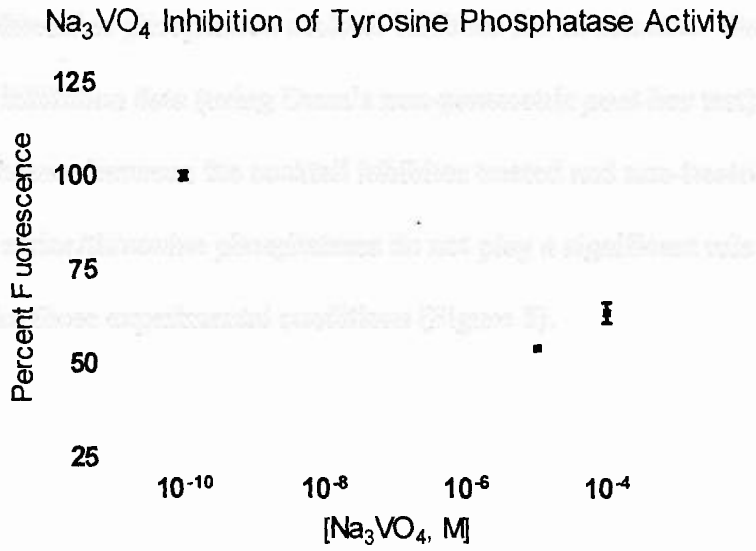


Figure 4. Sodium vanadate inhibition of DiF-MUP activity in AtT-20 cell lysate. AtT-20 cell lysates were prepared in the cell lysis buffer and incubated 15 minutes with 50μM DiF-MUP and Na_3VO_4 at concentrations ranging from 10^{-9}M to 10^{-4}M. The IC_{50} value in control cell lysates was 90nM.

AtT-20 cell lysate phosphatase inhibition with serine/threonine cocktail inhibitor

To determine if DiF-MUP hydrolysis is also sensitive to serine/threonine phosphatase activity, AtT-20 cell lysates were co-incubated with 50 μM DiF-MUP and 10 mM serine/threonine phosphatase cocktail inhibitor for 15 minutes. Statistical analysis of the inhibition data (using Dunn's non-parametric *post-hoc* test) shows no significant difference between the cocktail inhibitor treated and non-treated samples, indicating that serine/threonine phosphatases do not play a significant role in DiF-MUP hydrolysis under these experimental conditions (Figure 5).

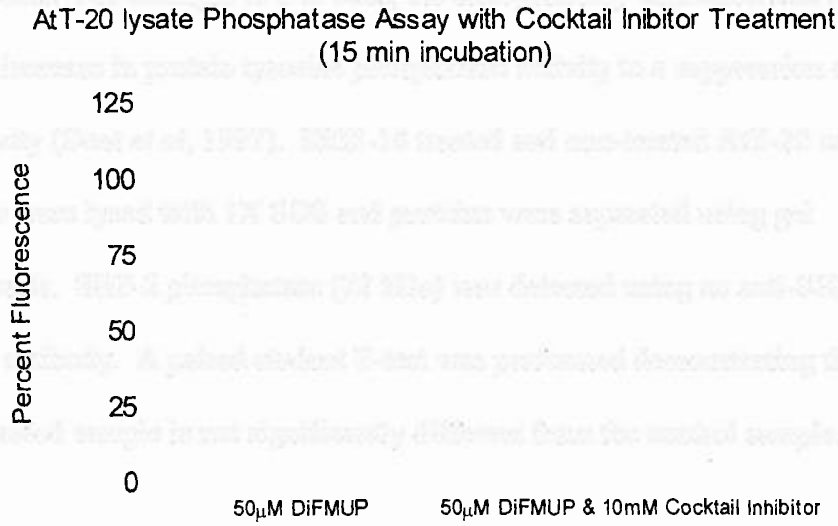


Figure 5. Phosphatase inhibitor cocktail effect on DiF-MUP activity in AtT-20 cell lysate. AtT-20 cell lysates were prepared in the cell lysis buffer and incubated 15 minutes with 50μM DiF-MUP and 10mM of the phosphatase inhibitor cocktail. Fluorescence was measured using a Cytofluor 4000 plate reader and data were analyzed in GraphPad Prism 4.0. One-way ANOVA, followed by Dunn's non-parametric *post hoc* test, shows no significant difference.

Identification of SHP-2 and Phospho-SHP1 in AtT-20 cells

The tyrosine phosphatase SHP-2 has been implicated in SRIF actions in several cellular models. For example in S49 cells, the endogenously expressed sst2 receptor couples an increase in protein tyrosine phosphatase activity to a suppression of MAP kinase activity (Dent *et al*, 1997). SRIF-14 treated and non-treated AtT-20 cell monolayers were lysed with 1X SDS and proteins were separated using gel electrophoresis. SHP-2 phosphatase (72 kDa) was detected using an anti-SHP-2 polyclonal antibody. A paired student T-test was performed demonstrating that the SRIF-14 treated sample is not significantly different from the control sample.

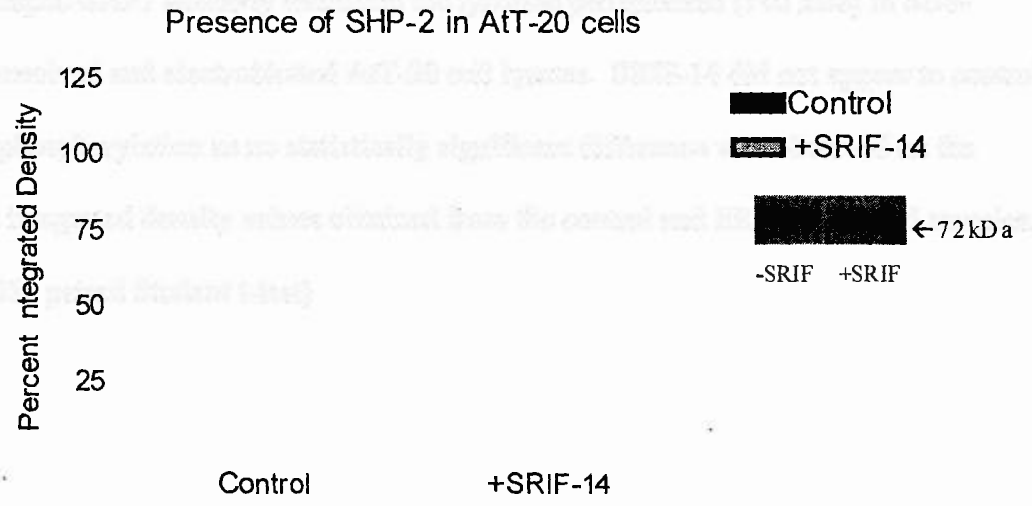


Figure 6. Detection of SHP-2 in the AtT-20 cell. Parallel cultures of AtT-20 cells were incubated in the presence or absence of 100 nM SRIF-14 for 20 minutes. Cell lysates were separated by SDS-PAGE, electroblotted, and incubated with anti-SHP-2 antibody. Autoradiographs were quantified using NIH Scion image. The results represent the mean +SEM of three separate experiments. The paired student T-test shows no significant difference between the vehicle and SRIF-14 treated samples. The inset shows a representative autoradiograph.

The SHP-1 phosphatase plays a role in regulating intracellular signaling events leading to cell cycle arrest and cell growth inhibition (Ferjoux *et al*, 2003). A polyclonal anti-Phospho-SHPl antibody identified the tyrosine phosphatase (140 kDa) in SDS-PAGE resolved and electroblotted AtT-20 cell lysates. SRIF-14 did not appear to control SHP-1 phosphorylation as no statistically significant difference was observed on the percent integrated density values obtained from the control and SRIF-14 treated samples. ($p > 0.05$; paired Student t-test)

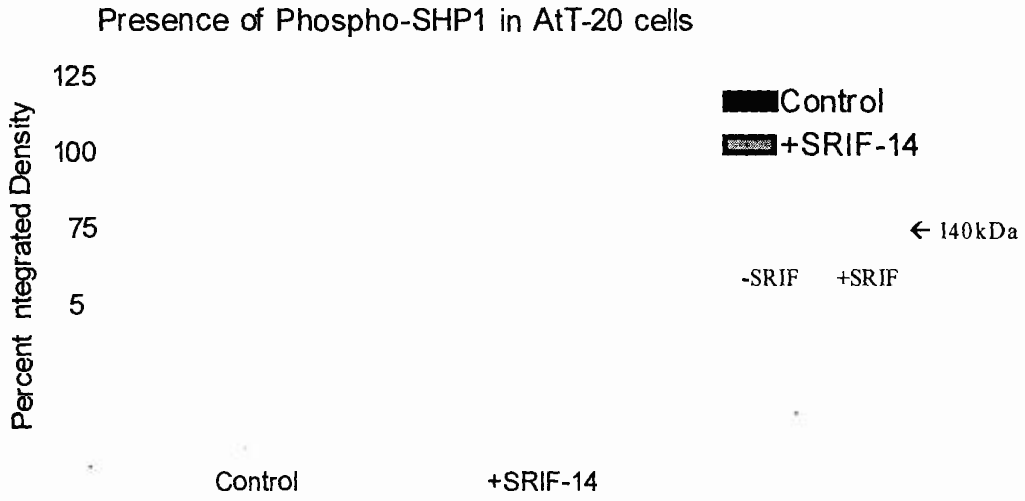


Figure 7. Detection of Phospho-SHP1 in AtT-20 cells. AtT-20 cell lysates were separated by SDS-PAGE, electroblotted and incubated with anti-Phosph-SHP1 primary antibody. Images were quantified using NIH Scion Image. The results represent the mean +SEM of three separate experiments. The paired student T-test shows no significant difference between the vehicle and SRIF-14 treated samples. The inset shows a representative autoradiograph.

DISCUSSION

SRIF regulates multiple intracellular signaling pathways such as intracellular ion concentrations, cyclic nucleotide levels and protein phosphorylation (Blake *et al*, 2004; Olias *et al*, 2004). Protein phosphorylation controlled by SRIF includes the activation of phosphoprotein phosphatases (Olias *et al*, 2004) that are responsible for regulating crucial cellular events, including cell growth and proliferation, differentiation, and cell cycle progression.

SRIF receptor subtypes are well documented in the AtT-20 cell, controlling cyclic nucleotide metabolism (Strowski *et al*, 2002; Cervia *et al*, 2003) and hormonal secretion (Strowski *et al*, 2002). In the AtT-20 cell model, SRIF inhibits ACTH release via both cAMP independent and dependent mechanisms, suggesting that multiple intracellular events may be under SRIF control (Litvin *et al*, 1986, Reisine *et al*, 1988; McFerran and Guild, 1996). However, whether SRIF events outside of intracellular ion concentrations or cyclic nucleotide metabolism are affected remains unknown. In the current study, we sought to establish and identify the protein phosphatase family of enzymes in the AtT-20 cell, which may serve to inactivate the protein kinases responsible for ACTH secretion.

Within the AtT-20 cell, we have identified at least two cytosolic protein tyrosine phosphatases, SHP-1 and SHP-2. We used the fluorescent substrate difluoro-4-methylumberiferyll phosphate (DiF-MUP) in combination with sodium vanadate to measure the relative fluorescence of PTPs upon SRIF-treatment, as a means of indirectly determining phosphatase activity. Our results demonstrate that basal phosphatase activity

was not affected by SRIF incubation, suggesting that within AtT-20 cells, PTPs, including SHP-1 and SHP-2, act independently of SRIF activation.

Both SHP-1 and SHP-2 have been identified in previous studies, but this experiment provides the first indication of the presence of these protein tyrosine phosphatases within a corticotroph cell line, such as the AtT-20 cell. SHP-1 plays a largely inhibitory role within a cell, negatively regulating both cytokine and growth receptors, as well as intracellular signaling molecules such as Janus activating kinase 2 (Jak2) (Tonks and Neel, 2001; Wu *et al*, 2000). In the AtT-20 cell, SHP-1 function may dephosphorylate both growth and cytokine receptors, the CRF- and LIF-receptors, respectively. The negative regulation of both G protein coupled receptors and an interleukin-6 cytokine receptor would prove important in the overall control of ACTH secretion.

SHP-2 function in a cell demonstrates both negative and positive regulatory roles. SHP-2 activity involves inactivation and dephosphorylation of both JAK and STAT in rat brain microglial cells, but it functions as a positive regulator of receptor tyrosine kinase signaling (Kim *et al*, 2003; Keyse, 2000). Within the AtT-20 cell, SHP-2 may co-regulate ACTH secretion with SHP-1 via a cytokine-induced pathway leading to the activation of JAK-STAT signaling proteins. In rat pancreatic cancer cells, both SHP-1 and SHP-2 have been found to work in conjunction with one another by coupling to sst_2 (Ferjoux *et al*, 2003), indicating a potential mechanism for SRIF control of these PTPs. Our results indicate that within the AtT-20 cell, both SHP-1 and SHP-2 are independent

of SRIF function, suggesting that neither PTP is docked to the sst_2 receptor under basal conditions.

AtT-20 cells endogenously express the SRIF receptor subtypes sst_2 and sst_5; however, sst_2 has been reported to be the dominant receptor subtype in the rodent pituitary corticotroph (Patel *et al*, 1996). Studies have shown that SHP-1 is constitutively associated with sst_2, rather than sst_5, and displays antiproliferative effects in CHO (Chinese hamster ovarian) cells when the stimulated sst_2 receptor activates the tyrosine phosphatase (Lopez *et al*, 1997). In contrast, CHO cells transfected with sst_2 do not recruit either protein tyrosine phosphates or serine/threonine phosphatase (Buscail *et al*, 1995). Provided with this information, we can speculate that the AtT-20 cell responds to SRIF-14 via sst_2 rather than sst_2 to regulate the activity of PTPs. The expression profile of $ssts_{2\&5}$ in the AtT-20 may explain the inability of SRIF-14 to significantly increase PTP levels. A novel approach to clarify the SRIF receptor pharmacology may involve profiling sst_2 selective agonist-induced PTP expression. Delineating SRIF's intracellular effects via different receptors could serve to answer the question of why multiple receptor subtypes are co-expressed in individual cells and tissues, a question that may have significant therapeutic implications for hormone secretion from SRIF sensitive tissues (Blake *et al*, 2004; van der Hoek *et al*, 2005).

While SRIF has been shown to have no direct effect on basal tyrosine phosphatase activity in AtT-20 cells, SRIF's effects on stimulated AtT-20 cells has not been examined. Clearly, future experiment measuring SRIF's actions on tyrosine phosphatase activity in LIF-stimulated AtT-20 cells may demonstrate an increase in

activity. Leukemia inhibitory factor (LIF), has been shown to increase ACTH secretion in AtT-20 cells (Auernhammer *et al*, 1996) through the JAK-STAT intracellular signaling pathway. Studies have shown that endogenously expressed LIF receptors stimulate Janus-activated kinases (JAK), a family of tyrosine kinases located within the AtT-20 cell membrane. (Stahl *et al*, 1994). Following activation, signal transduction and activation of transcription (STAT) proteins are phosphorylated on tyrosine residues and translocate to the nucleus to promote the expression of proopiomelanocortin (POMC), the precursor to ACTH. SRIF may recruit protein tyrosine phosphatases capable of dephosphorylating STAT3 on tyrosine residues, preventing nuclear localization and subsequent POMC gene expression. Recruitment of PTPs, such as SHP-1 and SHP-2, could occur by association with sst_2, based on the ability these phosphatases to interact with the SRIF receptor subtype.

The importance of delineating the regulatory mechanism of ACTH secretion from the murine corticotroph may provide a model relevant in understanding the aberrant secretory responses in Cushing's syndrome. Cushing's syndrome is a hormonal disorder caused by the hyperstimulation of the adrenal gland by ACTH, allowing dangerous levels of cortisol to be released. This can be caused by pituitary adenomas and impose detrimental effects on a patient, including high blood pressure, high blood glucose levels and obesity. Currently, research is being conducted on the SRIF pharmacological analogue, SOM230, and its ability to inhibit ACTH secretion from both human and mouse corticotroph adenomas (Hofland *et al*, 2005). Uncovering a potential role of protein tyrosine phosphatases in ACTH secretion could assist in understanding the

mechanism of SOM230 inhibition, solidifying its role as a potential therapeutic agent for Cushing's syndrome.

CONCLUSIONS

In summary, the activation of protein kinases within the AtT-20 cell regulates the cellular processes involved in ACTH secretion. SRIF has been shown to inhibit the secretion of ACTH in AtT-20 cells by regulating cyclic nucleotide levels, ion conductance, and protein phosphorylation. While the protein kinases involved in AtT-20 cellular mechanisms have been well characterized, protein phosphatases responsible for antagonizing protein kinase effects have yet to be determined.

Our results demonstrate that AtT-20 cells contain a soluble protein phosphatase activity capable of hydrolyzing the fluorescent substrate DiF-MUP. This protein phosphatase activity is independent of SRIF control, but is exquisitely sensitive to sodium vanadate, a potent tyrosine phosphatase inhibitor. The sensitivity of the enzymatic activity to sodium vanadate indicates that protein tyrosine phosphatases are present within the AtT-20 cell. Further inspection of this activity led to the identification of the protein tyrosine phosphatases SHP-2 and SHP-1 within the murine corticotroph, however neither phosphatase was under the control of SRIF.

AtT-20 cells endogenously co-express three SRIF receptors; sst_{2A}, sst_{2B}, and sst_{5}. Future studies, with receptor-selective agonists, should be used to delineate each receptor's influence on SRIF's effects on tyrosine phosphatase activity in AtT-20 cells. Further investigation of tyrosine phosphatase recruitment in AtT-20 cells stimulated by LIF should be performed in order to develop a possible regulatory mechanism of ACTH secretion. This information could lead to a better understanding of how ACTH secretion

controlled in AtT-20 cells and provide possible therapeutic implications in diseases such as Cushing's syndrome.

LITERATURE CITED

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