Characterization of NIH-3T3 Mouse Fibroblast Cells Transfected with the Somatostatin Receptor Subtype 2 and the Protein Tyrosine Phosphatase Shp-1

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CHARACTERIZATION OF NIH-3T3 MOUSE FIBROBLAST CELLS TRANSFECTED WITH THE SOMATOSTATIN RECEPTOR SUBTYPE 2 AND THE PROTEIN TYROSINE PHOSPHATASE SHP-1

by

Scott Bradford Cross

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
June 1999
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Scott Bradford Cross
1999
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Scott Bradford Cross

ff
The hormone Somatostatin (SMS) and its analogs have been shown to mediate a variety of cellular actions including inhibition of cellular growth and differentiation of endocrine tumors such as insulinomas and glucagonomas. The mechanisms by which SMS exerts its antitumorigenic effects are poorly understood but are thought to include one of the five Somatostatin receptors (SSTR1-5), and the protein tyrosine phosphatase SHP-1. To determine if the antiproliferative effects of SMS were due to the presence of the SSTR2 receptor or the stimulation of SHP-1, we introduced cDNA's of the SMS receptor SSTR2, SHP-1, and a dominant negative mutant of SHP-1 into NIH-3T3 cells by lipofection. Our research has shown that the expression of the SSTR2 receptor and the SHP-1 gene product controls cellular proliferation. We have also shown that both the SSTR2 receptor and the SHP-1 gene product can control activation of MAPK proteins. Our results also demonstrate that the SMS analog Sandostatin can control MAPK activation in cells that express the SSTR2 receptor. We therefore conclude that SMS and its analog Sandostatin have different modes of action within the NIH-3T3 mouse fibroblast cell line.
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INTRODUCTION

Cancer is primarily caused by somatic mutations and it is an accumulation of somatic mutations which creates cells that proliferate at rates well above their norms and causes cells to lose the ability to control their own proliferation. Cancers are established through mutations that lead either to the inactivation of tumor suppressor genes such as p53 or the Retinoblastoma gene product Rb, or mutations that result in a gain of function as in the oncogenes Ras or Raf. Usually it is a combination of mutations, oncogenic events, and the inactivation of tumor suppressor genes that leads to a loss of homeostasis, as most cells in vivo require more than one or two separate mutational events to generate a malignant phenotype (Alison, 1997).

One form of cancer believed to be caused by separate mutational events is pancreatic cancer. Forty percent of all patients diagnosed with pancreatic adenocarcinomas have point mutations in the \( p16 \) tumor suppressor gene (Alison, 1997), and a loss of expression of the somatostatin receptor subtype 2 in exocrine pancreatic carcinomas (Buscail et al., 1996). Pancreatic cancer is a debilitating disease that generally is undetected until it is well advanced and not amenable to treatment. It ranks 11th in incidence of all cancers within the United States yet, it is our fifth leading cause of cancer deaths (Falk et al., 1988). Worldwide, pancreatic affects slightly more men than women, and occurs more frequently in urban
areas than rural settings (Parkin and Muir, 1992). Environmental factors are thought to play major roles in the etiology of the disease, however smoking has continued to be the only proven risk factor, increasing the risk by two fold (NCI website, January 1999).

The pancreas functions in producing enzymes that break down proteins and fats as well as producing and secreting the hormones insulin, glucagon, and somatostatin. Somatostatin (SMS) is a neuropeptide that was initially isolated as an inhibitor of growth hormone and purified from sheep hypothalamus. Cells producing SMS are found throughout the body in the central nervous system, the peripheral nervous system, the gut and pancreas, as well as the thyroid, adrenals, kidneys, and other essential organs (reviewed in Patel, 1995). It is known to inhibit endocrine and exocrine secretions, modulate other neuropeptide and neurotransmitter systems, and effect gastrointestinal motility, vascular smooth muscle tone, intestinal absorption of nutrients and ions, and inhibit cellular growth.

SMS exists as two isoforms, one with 14 amino acids, SST-14, and its N-terminal extended form SST-28, both of which are cyclic (Figure 1) and coded for by the same gene. A common precursor is produced and is differentially processed to form the two isoforms that are associated with the same function yet are differentially expressed in the tissues.

SMS has a short half-life of three minutes within the body and is therefore not stable enough to treat a variety of neuroendocrine tumors and gastrointestinal disorders. To improve upon the stability of SMS, analogs have been developed that
have longer half-lives yet still maintain the same effects as SMS. Most analogs are designed as hexapeptides or octapeptides, but all maintain two essential amino acids--number 8 and 9 (Tryptophan and Lysine)--which are necessary for biological activity (Raynor et al., 1993). Most analogs also preserve amino acids number 7 (Tyrosine) and 10, which can be either a Threonine or a Valine (Table 1).

**SMS-14:** Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp

  Cys-Ser-Thr-Phe-Thr-Lys

**SMS-28:** Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys

  Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp

  Cys-Ser-Thr-Phe-Thr-Lys

Figure 1. Amino Acid Sequence of SMS-14 and SMS-28.

One of the analogs which has shown promise as a chemotherapeutic agent in the treatment of cancers is SMS 201-995 (tradename Sandostatin or Octreotide). Sandostatin is an eight amino acid derivative of SMS (Table 1) which has a Ki of 2.1nM for the SSTR2 receptor (Patel et al., 1994; Table 2), reaches peak plasma concentrations after 30 minutes, and has a half-life of 90 to 115 minutes (Waxman et al., 1996).

Sandostatin has been shown to mediate the same wide variety of effects as SMS (see below), and it possesses the ability to reduce tumor size either alone in
patients with gastroenteropancreatic tumors and breast tumors (Weckbecker et al., 1992) or in conjunction with other therapies such as Tamoxifen (Weckbecker et al., 1994). However, it is now believed that it can only cause a reduction in tumors that express the SMS receptor subtype 2 (SSTR2) (Buscail et al., 1994; Fisher et al., 1996).

All five receptors belong to the G protein coupled receptor family and exhibit the typical seven hydrophobic membrane spanning regions (Figure 2). They are coded for by five genes located on separate chromosomes and are intronless with the exception of the SSTR2 gene, which can give rise to the spliced variants SSTR2-A and SSTR2-B in mice.

Table 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Tradename</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMS 201-995:</td>
<td>Sandostatin</td>
<td>Dphe-Cys-Phe-DTrp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thr(ol)-Cys-Thr-Lys</td>
</tr>
<tr>
<td>BIM 23014:</td>
<td>Somatuline</td>
<td>DβNal-Cys-Tyr-DTrp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thr-Cys-Val-Lys</td>
</tr>
<tr>
<td>RC-160:</td>
<td></td>
<td>Dphe-Cys-Tyr-DTrp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trp-Cys-Val-Lys</td>
</tr>
<tr>
<td>MK678:</td>
<td>Seglitide</td>
<td>(N-Me)-Tyr-DTrp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phe-Val-Lys</td>
</tr>
</tbody>
</table>

D, the D-isoform of amino acids; Nal, β-(2-nalphey)Alanine
### Table 2

Peptide Selective Binding Towards Human SMS Receptors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SSTR1</th>
<th>SSTR2</th>
<th>SSTR3</th>
<th>SSTR4</th>
<th>SSTR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMS-14</td>
<td>1.1</td>
<td>1.3</td>
<td>1.6</td>
<td>0.53</td>
<td>0.9</td>
</tr>
<tr>
<td>SMS-28</td>
<td>2.2</td>
<td>4.1</td>
<td>6.1</td>
<td>1.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Sandostatin</td>
<td>&gt;1000</td>
<td>2.1</td>
<td>4.4</td>
<td>&gt;1000</td>
<td>5.6</td>
</tr>
<tr>
<td>Somatuline</td>
<td>&gt;1000</td>
<td>1.8</td>
<td>43</td>
<td>66</td>
<td>0.62</td>
</tr>
<tr>
<td>Seglitide</td>
<td>&gt;1000</td>
<td>1.5</td>
<td>27</td>
<td>127</td>
<td>2</td>
</tr>
</tbody>
</table>

adapted from Patel, 1994

The Somatostatin receptors (SSTR’s) do not share a high degree of sequence specificity with other receptors except the opioid receptor family, for which there is a 40% amino acid sequence similarity (Reisine, 1993). However, within themselves, the five SSTR’s do share a fairly high degree of specificity ranging from 55% to 70% amino acid homology (Patel et al., 1995). Between species only the SSTR2 receptor shows a high degree of sequence specificity of 93% to 98% for human, pig, mouse, rat, and cow.

Sandostatin activity has been correlated with the high expression of the SSTR2 receptor (Fuji et al., 1994). A serine (Ser305), located within transmembrane domain 7, and a glutamine (Gln291), located within the 3rd extracellular loop of the SSTR2 receptor, have been shown to confer the specificity of Sandostatin for SSTR2 (Kaupman et al., 1995). Therefore, any cells, especially tumors, shown to express the SSTR2 receptor might be a potential target for treatment with the Sandostatin analog.
Figure 2. A Schematic of the SSTR2 Receptor Based Upon Its Predicted Amino Acid Sequence. Inset: predicted location of transmembrane-spanning regions viewed from the extracellular surface of the receptor. CHO, Asparagine bound carbohydrate. Darkened circles represent regions of the SSTR2 involved with binding the SMS analog MK-678. Used with permission of Brenda Rauner, Publications Manager and Executive Editor, The American Physiological Society, 4-30-99.

All of the SSTR's except SSTR1 are coupled to G proteins (Bell and Reisine, 1993; Rens-Domiano et al., 1992). G proteins are heterotrimers composed of α, β, and γ subunits that mediate a wide variety of cellular actions. The Gα subunit binds GTP upon receptor ligand binding and becomes activated. Once activated, the heterotrimeric complex dissociates from the receptor and the Gβγ complex stimulates its own set of downstream effectors. It is not clear as to
whether the G protein complex separates upon dissociation from the membrane (Clampham and Neer, 1993), however, it is known that both Ga and Gβγ subunits remain active as long as GTP is bound to Ga. It is this cyclic association of GTP, its hydrolysis to GDP and Pi, and the exchange of GDP which regulate the functions of G proteins.

G proteins are classified based upon their activities and the relatedness of their Ga subunits amino acid sequence. The Ga subunit possesses a GTPase activity and contains a high affinity binding site for guanine nucleotides. Both the Gβ and Gγ subunits have multiple isoforms which, depending upon the complex, can be interchanged with each other (Schmidt et al., 1992; Pronin and Gautam, 1992; Spring and Neer, 1994). The Gβ subunits, of which there are three, appear to be highly conserved (Simon et al., 1991), but the Gγ subunits, of which there are at least eight, show a range of sequence similarity of 35% to 76% (Ray, 1995). It is this heterogeneity that may contribute to the functional differences between different Gβγ complexes.

Upon stimulation G proteins, and the receptors they are coupled to, have been shown to mediate and/or stimulate signal transduction events which can act as secondary messengers within the cell and control crucial cellular parameters. SMS receptors have been shown to mediate membrane potential, cytosolic Ca^{2+} levels, cellular differentiation, and cellular proliferation.

One of the most common pathways associated with cellular proliferation and differentiation is the MAPK pathway (reviewed in Seger, 1995). MAPK's are
serine/threonine kinases which are part of a large family of proteins that include the extracellular receptor kinases (ERK's), Jun N-terminal kinases (JNK's), stress-activated protein kinases (SAPK's) and p38 reactivating kinases (RK's). The MAPK's become activated once they are phosphorylated at tyrosine and threonine residues located within the kinase domain. Upon phosphorylation MAPK is typically translocated to the nucleus where it can then phosphorylate its target transcription factors.

Not much is known about how G protein coupled receptors activate MAPK's but the process is believed to be similar to the receptor tyrosine kinases (RTK's). It is thought that once a G protein coupled receptor binds to its ligand it activates specific Gβγ complexes. Gβγ complexes are known to stimulate the tyrosine phosphorylation of adapter proteins such as Src and Pyk2 which can then bind and stimulate the proteins Shc, Grb2 and mSOS, which is a guanine nucleotide releasing protein (GNRP) (van Biesen et al., 1995). The Grb2/mSOS complex induces GDP-GTP exchange of Ras probably through plextrin homology domains (Crespo et al., 1994), and then associates with the serine/threonine kinase Raf or MAPKK, phosphorylating it and thus activating it. Raf is known to activate another serine/threonine kinase termed MEK, or MAPKK, which goes on to phosphorylate the MAPK proteins (Figure 3). However, other pathways which stimulate MAPK have been established. Pumigilia et al. (1995) has shown a direct interaction between Gβγ subunits and the Raf-1 serine-threonine protein kinase, and Kranenberg et al., (1997) has shown that the G_{i}-mediated activation of the
Ras/MAPK pathway involves a 100 kd tyrosine phosphorylated Grb2-SH3 binding protein and not Src or Shc.

Figure 3. A Possible Mechanism for MAPK Activation via G-Protein Coupled Receptors.

Although stimulation of the MAPK pathway can be correlated with cellular proliferation two groups have been able to show that SMS and its analogs, which also stimulate the MAPK pathway, inhibit cellular proliferation. Bito et al., (1994) have shown that SMS is capable of transiently increasing MAPK activity in Chinese hamster ovary (CHO) cells transfected with the SSTR4 receptor, and that this effect was mediated by a G protein due to its repression by pertussis toxin. Yoshitomi et al., (1997) have been able to show a transient pertussis toxin insensitive increase in MAPK activity in Min6 cells expressing the SSTR2 receptor. SMS mediates its diverse effects by controlling the inhibition of adenylate cyclase.
(Liapi et al., 1986; Bito et al., 1994), hormone secretion, cellular proliferation (Schalley, 1988; Viguerie et al., 1989), Ca\(^{2+}\) influx (Lewis et al., 1986; Fujii et al., 1994), activation of protein tyrosine phosphatases (Liebow et al., 1989; Todisco et al., 1995; Reardon et al., 1996), Na\(^+\)/H\(^+\) exchangers (Hou et al., 1994), K\(^+\) channels (Yamashita et al., 1987; Fosset et al., 1988), arachidonate release (Bito et al., 1994), c-fos activity (Todisco et al., 1994) and mitogen activated protein kinase (MAPK) activity (Bito et al., 1994 and Yoshitomi et al., 1997).

One possible way that SMS might be mediating its effects is through the activation of one or more protein tyrosine phosphatases (PTPase). Liebow et al., (1989) have been able to show that some of the SMS analogs, RC-160 and RC-121, are able to stimulate PTPase activity in the human pancreatic cell line Mia PaCa-2. In contrast, the Sandostatin analog did not appear to be able to stimulate PTPase activity or cause growth inhibition in this cell line. Buscail et al. (1994) have shown that in NIH-3T3 cells as well as COS-7 cells Sandostatin does stimulate PTPase activity as well as inhibit serum stimulated cellular proliferation when cells express the SSTR2 receptor, an effect that dissipates when vanadate, a PTPase inhibitor, is added.

To determine if a PTPase plays an active role in the regulation of the SMS signal through the SSTR2 receptor the SSTR2 gene from rat genomic DNA was cloned and expressed in NIH-3T3 mouse fibroblast cells, which do not normally express any of the SSTR receptors. cDNA's of the PTPase SHP-1, or a dominant
negative mutant SHP-1 (C453S), were then transfected into NIH-3T3 cells which expressed the SSTR2 receptor.

Once both cDNAs were shown to be expressed, the effects of the mutant SHP-1 gene product on cellular proliferation were measured. This same experiment was also performed by stimulating the clones with the Sandostatin analog.

MAPK activity was also determined to see if SMS stimulation was affecting MAPK activity and if the signal was being regulated through the SHP-1 gene product.
MATERIALS AND METHODS

General

All primers were synthesized and purchased from Only DNA. All restriction endonucleases and enzymes were purchased from Life Technologies or New England Biolabs unless otherwise stated. NIH-3T3 mouse fibroblast cells were purchased from the American Type Culture Collection. SMS 201-995 (Sandostatin) was a generous gift from the Sandoz Pharmaceuticals Corporation.

Cloning of the SSTR2 Receptor

The SSTR2 receptor was cloned from Rat liver genomic DNA by the polymerase chain reaction (PCR) using primers complementary to the 5' and 3' ends of the SSTR2 receptor. These primers encompassed the whole coding region and amplified a fragment of the predicted size, 1119 bp. This fragment was ligated overnight at 14°C to the pCR2.1 cloning vector (Invitrogen), which contained the ampicillin resistance marker. The clones were then selected for by ampicillin resistance and whether or not they developed a blue color in the presence of 40mg/ml X-gal, and digested with restriction endonucleases to ensure the ligated fragment had inserted into the plasmid in the correct orientation. This fragment was then sequenced using a dideoxy method (T7 Sequenase version 2.0, Amersham) to
ensure that the PCR amplification steps did not cause any mutations within the coding sequence.

The SSTR2 receptor was then separated from the pCR2.1 vector by digestion with the restriction endonuclease EcoRI, and electrophoresis through a low-melting point (LMP) agarose gel. The expected 1134 bp fragment was then excised from the LMP gel, heated 65°C for 15 minutes and purified by phenol/chloroform extraction, and ethanol (EtOH) precipitated using 0.1M NaCl. After digestion with EcoRI, the 5.7 kb pTracer-CMV vector (Invitrogen) was used for ligation to the SSTR2 receptor fragment using T4 DNA Ligase (Invitrogen) overnight at 14°C.

Cloning of the SHP-1 and SHP-1 Dominant Negative Mutant PTPase

The SHP-1 cDNA and the SHP-1 dominant negative mutant (C453S) cDNA were the kind gifts from Dr. Mathew Thomas, of Washington University in St. Louis, Missouri. The SHP-1 dominant negative mutant (C453S) which has the amino acid serine number 453 replaced with a cysteine within the catalytic domain is catalytically inactive. The SHP-1 cDNA clone, its mutant, and the 5.4 kb pcDNA3.1(+) vector (Invitrogen) were all digested with the restriction endonuclease EcoRI giving a 2207 bp SHP-1 fragment, a 2207 bp mutant SHP-1 fragment, and a linearized pcDNA3.1(+) vector. These fragments were then electrophoresed through a LMP agarose gel, excised from the LMP gel heated at 65°C for 15 minutes, purified by phenol-chloroform extraction, and EtOH
precipitated using a 0.1M NaCl. Both SHP-1 fragments were ligated to the linear 
pcDNA3.1(+) vector using T4 DNA Ligase (Invitrogen) and overnight ligation at 
14°C.

Bacterial Transformation

Ligation reactions were mixed with DH5αF' chemically competent cells 
(Life Technologies) and incubated on ice for 30 minutes. The mixture was heat 
shocked for 30 seconds at 42°C and placed on ice for 2 minutes before 450µl of 
SOC medium was added. The mixtures were then incubated at 37°C for 60 minutes 
in a shaking incubator. After incubation the transformed cells were plated onto LB 
agar which contained 10g/L Bacto Tryptone, 5g/L Yeast Extract, 5g/L NaCl, 15g/L 
agar, and 50mg/L ampicillin (Sigma). These plates were incubated overnight at 
37°C. Individual colonies were used to inoculate LB broth supplemented with 
50mg/L ampicillin at 37°C in a shaking incubator overnight.

Plasmid Purification

Small scale plasmid preparations were performed to purify the plasmids for 
analysis by restriction endonuclease digestion. A 1.5ml bacterial broth culture was 
grown overnight at 37°C in a shaking incubator and pelleted by centrifugation at 
14,000 rpm for 1 minute. The supernatant was discarded and the pellet was 
resuspended in residual medium and 350µl of microscreening buffer that contained 
an isosmotic solution of 8% sucrose, 50mM EDTA, 0.5% Triton X-100, and 10mM
Tris-HCl (pH 8.0). This mixture was then vortexed for 20 seconds and 35µl of 10mg/ml lysozyme (Sigma) was added to the mixture before vortexing briefly and incubating at 37°C. After the solution became viscous tubes were placed in a boiling water bath for 1 minute and pelleted by centrifugation at 14,000 rpm for 5 minutes in order to pellet out the membranes and the genomic DNA. To recover the plasmid 30µl of 3M sodium acetate was then added to the supernatant and vortexed briefly. This was followed by the addition of 420µl of isopropanol and incubation at -20°C for 30 minutes. Centrifugation at 14,000 rpm for 5 minutes was used to pellet the DNA, and the pellet was then washed once with 100% EtOH and allowed to air dry. Once dry, the pellet was resuspended in 200µl of water to which 200µl of 3M sodium acetate was then added. The solution was vortexed briefly and 500µl of 100% EtOH was added and again vortexed briefly. The mixture was then incubated at -20°C for 30 minutes, centrifuged at 14,000 rpm for 3 minutes, and washed with 100% EtOH and allowed to air dry. The pellet was then resuspended in water and the plasmid was digested with different restriction endonucleases to determine the presence of the appropriate DNA fragment and its orientation.

Large scale plasmid preparations were performed in order to obtain quantities of purified DNA for cellular transfections and probes for Northern blot analysis. To begin, bacteria were grown in LB broth, see above, in baffled flasks for 24-48 hours. The bacteria were then centrifuged at 4000 rpm for 10 minutes and resuspended in a solution that contained 50mM glucose, 25mM Tris-HCl, and
10mM EDTA. A 1/8th volume of 0.2M NaOH/1% sodium decodyl sulfate (SDS) solution was then added and the contents were mixed and allowed to incubate at room temperature for 5 minutes. A 4% volume of 3M potassium acetate and 2M glacial acetic acid solution was then added to precipitate protein, membrane complexes, and genomic DNA. The sample was then vortexed and incubated on ice for 10 minutes. The sample was then centrifuged at 4000 rpm for 15 minutes and the supernatent was filtered. A 60% volume of isopropanol was added, and the sample was mixed and incubated at room temperature for 10 minutes. The sample was then centrifuged at 5000 rpm for 15 minutes and the pellet was rinsed with 70% EtOH and resuspended in water. A 33% volume of 5M lithium chloride was then added and the sample was centrifuged at 10,000 rpm for 10 minutes. An equal volume of isopropanol was then added to the supernatant and the sample was centrifuged for another 10 minutes at 10,000 rpm. The pellet was then rinsed with 70% EtOH and resuspended in 100µl water. A 4µl aliquot of RNAse A (Sigma) was then added and the sample was allowed to incubate at room temperature for 30 minutes. A 5µl aliquot of a 5% Cetyltrimethylammonium bromide (CTAB) (Sigma) solution was then added and the mixture was allowed to incubate for 3 minutes before it was centrifuged for 5 minutes at 14,000 rpm. The pellet was then resuspended in 300µl of 1.2M sodium chloride and 750µl of 100% EtOH was then added. The sample was then placed at -20°C for 30 minutes before it was centrifuged for 5 minutes at 14,000 rpm. The pellet was then rinsed in 70% EtOH and allowed to air dry before being resuspended in water.
The Wizard Plus minipreps and midipreps DNA purification systems (Promega) were also used to purify plasmid DNA as per the instructions of the manufacturer. Briefly, a bacterial culture was grown in 3-100ml of LB broth that contained 50µg/ml ampicillin by shaking at 37°C. The broth culture was then centrifuged to pellet the bacteria and resuspended in Cell Resuspension Solution (50mM Tris-HCl (pH 7.5), 10mM EDTA, and 100µg/ml Rnase A). The cells were then lysed by the addition of the Cell Lysis Solution containing 0.2M NaOH and 1% SDS and briefly inverting the tubes several times. A Neutralization Solution containing 1.32M potassium acetate was then added and also mixed by inversion several times. The lysate was then centrifuged at 10,000 rpm for 5 minutes to pellet bacterial proteins and membranes, and the supernatent was then mixed with a Column Wash Solution (80mM potassium acetate, 8.3mM Tris-HCl (pH 7.5), 40µM EDTA, and 55% EtOH) and Resuspension Resin and passed through a purification column. Purified DNA was then removed from the column by applying 50µl of water and briefly centrifuging.

Sequencing

Double stranded plasmid DNA was purified as described above and determined to have a 260/280 ratio of greater than 1.7. A 20µl aliquot of 2µg DNA was then denatured by adding 0.1 volumes of 2M NaOH and 2mM EDTA and incubation for 30 minutes at 37°C. The mixture was then neutralized by the
addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and EtOH precipitated by increasing the salt concentration to 0.1M NaCl and adding two volumes of EtOH.

Sequencing was performed by using the Fidelilty DNA Sequencing System (S7600-KIT, Oncor) according to the manufactures specifications. A total of 5µg of DNA was added to a mixture containing 2µl Annealing Buffer (125mM Tris pH 8.5, 100mM MgCl₂, and 250mM NaCl), 1pmol of primer, and water to a final volume of 10µl. The primer and template were then annealed by heating for 2 minutes at 65°C, cooled to 35°C, and chilled on ice. A 3µl aliquot of Reaction Buffer (0.4M Tris pH 8.5, 40mM MgCl₂, 40mM DTT, 0.4 mg/ml acetylated BSA, 4mM ATP, 1.5µM dTTP, 1.5µM dGTP, 1.5µM dCTP), T4 DNA polymerase, and [α-³⁵S]-dATP (Dupont NEN Life Sciences) were then added to the annealing mixture and water was used to make the final volume 18µl. The mixture was then at 40°C for 15 minutes and cooled on ice before 6µl of T4 Accessory Mix was added and aliquots of 5.5µl of the labeling reactions were then added to the appropriate termination mixtures (C,A,T, and G), mixed, and incubated for 5 minutes at 40°C. The reactions were then stopped by addition of Stop Solution, the Proteinase K solution, and incubation for another 15 minutes at 40°C. The samples were then heated to 80°C for 5 minutes and loaded onto a 6% polyacrylamide glycerol-tolerant buffered gel that contained 4.8g/ml urea and electrophoresed at 65 watts until the bromphenol blue indicator had been electrophoresed off the gel. The gel was then fixed for 15 minutes in a 5% acetic acid and 15% methanol solution, dried onto 3mm paper, and exposed to film.
Transfection

NIH-3T3 cells were seeded onto 60mm tissue culture dishes of $2 \times 10^5$ cells/dish and allowed to grow overnight in Dulbecco's Modified Eagles Medium (DMEM, Life Technologies) supplemented with 10% calf serum (CS, Life Technologies). To prepare a total of 2µg of DNA was then added to 100µl of Opti-MEM I Reduced Serum Medium (Life Technologies). A solution containing 10µl of Lipofectamine reagent (Life Technologies) and 100µl of Opti-MEM I Reduced Serum Medium was prepared and combined with the DNA/Opti-MEM I complex. While incubating at room temperature the cells were washed once in a phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3 mM Na$_2$HPO$_4$·7H$_2$O, and 1.4 mM KH$_2$PO$_4$) and then with Opti-MEM I Reduced Serum Medium. After a 45 minute incubation an 800µl aliquot was used to dilute the DNA/lipofectamine complex. This was then placed onto the cells and allowed to stand overnight at 37°C. The medium was then replaced with DMEM supplemented with 10% CS for 48 hours. After 48 hours, the respective antibiotics (400µg/ml G418 sulfate, Life Technologies for selection of pcDNA3.1 containing clones and 400µg/ml Zeocin, Invitrogen, for selection of the pTracer containing clones) were added to the medium for selection every 48 hours for two to three weeks until only antibiotic resistant colonies were seen growing on the plates. Once established, the selected clones were taken off antibiotic selection and grown in DMEM supplemented with 10% CS. Antibiotic selection was occasionally reapplied to ensure that
contamination of the cell lines was not occurring. Confirmation that clones which survived the selection process expressed the inserted genes was performed by northern blot analysis, and reverse transcription polymerase chain reaction (RT-PCR).

RNA Isolation and Northern Blot Analysis

Cells were grown in 60mm tissue culture plates until they were 80% - 90% confluent. The cells were then washed in PBS and 2ml of TRIZOL Reagent (Life Technologies) was added to the culture dish and the cells were allowed to incubate for 5 minutes at room temperature. The cell lysate was then transferred to a 1.6ml centrifuge tube and 400µl of chloroform was added to the tube. The tubes were then shaken and incubated at room temperature for 3 minutes before being centrifuged at 12,000 rpm for 15 minutes at 4°C. The upper aqueous phase was then removed and mixed with 1ml isopropanol, incubated at room temperature for 10 minutes, and centrifuged at 12,000 rpm for 10 minutes at 4°C. The pellet was suspended in 2ml of 75% EtOH, mixed, and centrifuged at 7000 rpm for 5 minutes at 4°C. After partial drying the pellet was dissolved in 20µl diethylpyrocarbonate (DEPC) treated water (Form 3796, Life Technologies, February 1997) and allowed to incubate for 10 minutes at 55°C. The optical density (OD) of an aliquot of the sample was then measured using spectrophotometric methods in order to determine RNA concentration and purity.
Five μg of RNA was glyoxylated by adding 20μl of a solution containing 342μl deionized glyoxal, 30 μl of 1M NaPO₄ (pH 6.7), 1.5ml dimethylsulfoxide (DMSO), and 30μl of 10% SDS. The solution was then incubated at 50°C for 30 minutes. It was then loaded onto a 1% agarose GTG (FMC) gel containing a 1/50th volume of 1M sodium phosphate (pH 6.8) and electrophoresed at 70 watts. The gel was then soaked in water containing ethidium bromide, photographed, and the RNA was transferred to a Durlon membrane (Stratagene) using a Rapid Downward Transfer Turboblotter (Schleicher & Schuell).

Labeling of the probe was accomplished by placing 50ng of the purified fragment in a tube. Five microliters of [α-32P]dCTP (50uCi) was added to the reaction. The volume was then adjusted to 50μl with water. This mixture was then added to a Ready-To-Go DNA labeling Bead (Pharmacia Biotech) and incubated for 30 minutes at 37°C. Following incubation the reaction mixture was purified by a Centricon filter (Micron Separations) to separate DNA with incorporated 32P from unincorporated nucleotides. This was accomplished by adding the mixture to the column and spinning the column for 5 to 15 minutes at 10,000 rpm. The purified DNA was then washed three times with TE buffer (10mM Tris-Cl pH 7.4, and 1 mM EDTA pH 8.0), and centrifuged briefly. The labeled probe(s) were then analyzed by a liquid scintillation counter to determine their specific activity.

Prehybridization of the Duralon membrane was performed at 68°C in a rolling Hybaid oven using a QuikHyb Hybridization Solution (Stratagene) for 1-2 hours. At least 1 x 10⁶ cpm of labeled probe was denatured by boiling in a water
bath for 3 minutes along with 100µl of 10mg/ml sonicated salmon sperm (Sigma). The mixture was then added to the prehybridization solution and incubated at 68°C for at least 1 hour. The hybridization solution was disposed of and the membrane was washed twice with a 2X SSC (0.2M C₆H₅Na₃O₇, 3M NaCl), and 0.1% SDS solution for 15 minutes each time. The membrane was then washed with a 0.1X SSC, 0.1% SDS solution at least once for 15 minutes. The membrane was then allowed to air dry before wrapping in Saran Wrap (Dow) and autoradiographed (Kodak XAR 5 film) for at least 24 hours at -80°C. The film was then developed in Developing Solution (Kodak) for 5 minutes, rinsed with cold water for 30 seconds, fixed in Fixing Solution (Kodak) for 10 minutes, rinsed in cold water for 10 minutes, and allowed to air dry.

Reverse Transcription Polymerase Chain Reaction

A set of primers was created that were homologous to the 5' and 3' ends of the SSTR2 receptor and the SHP-1 cDNA's to amplify the mRNA's of interest. RNA was isolated as previously stated. In order to reverse transcribe the RNA into cDNA 5µg of total cellular RNA was diluted into 18µl of water. It was then heated at 90°C for 5 minutes in order to denature any secondary structure associated with the RNA. It was then centrifuged briefly at 14,000 rpm and incubated on ice. A cocktail consisting of 30U/µl AMV Reverse Transcriptase (Life Technologies), 1X AMV Reverse Transcriptase Buffer (Life Technologies), 40U/µl RNasin (Promega), 100µg/ml random hexamers (Life Technologies), 40mM dNTP's (Life
Technologies), and water was added to the denatured RNA and the mixture was allowed to incubate at 23°C for 10 minutes. The samples were then incubated at 42°C for 1 hour, 95°C for 10 minutes, and then chilled on ice.

PCR of the cDNA was optimized using the Opti-Prime PCR Optimization Kit (Stratagene) which use 12 buffers, which vary in pH, magnesium concentration, and potassium chloride concentration. These were placed into individual centrifuge tubes along with 2µl of cDNA, and 45µl of a cocktail containing 400µM Master Mix buffer (Stratagene), 10mM dNTPs, 2.5µg upstream primer, 2.5µg downstream primer, and 30U of Taq DNA polymerase (Life Technologies). The PCR conditions were 94°C for 3 minutes, 50°C for 2 minutes and allowed to proceed for 1 cycle, then PCR conditions of 72°C for 1.5 minutes, 94°C for 1 minute, and 50°C for 1 minute was allowed to proceed for 30 cycles. To ensure non templated addition of a polyadenylase tail an adenine nucleotide extension cycle of 72°C for 10 minutes was performed. The samples were then electrophoresed through a 1% agarose gel.

Mitogen Activated Protein Kinase Assay

Cells were grown in 60mm tissue culture plates until they were 60-80% confluent. Serum depleted medium (DMEM supplemented with 1% CS) was then added overnight and the cells were then incubated overnight at 37°C. The cells were then treated with a 1 x 10^{-6}M concentration of Sandostatin (Sandoz
Laboratories), a concentration which has been shown to be an effective dose in NIH-3T3 cells, or PBS for 5 minutes.

The p44/42 MAP Kinase Assay Kit (New England Biolabs) was used to analyze all MAPK activation according to manufactures instructions. Briefly, all cells were first washed once with ice-cold PBS before treatment with 0.5ml of 1X Cell Lysis Buffer (20mM Tris HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM Na₃VO₄, 1µg/ml leupeptin, and 1mM PMSF and incubated on ice for 5 minutes. The cells were then scraped with a rubber policeman into 1.6ml centrifuge tubes and sonicated 4 times for 5 seconds each time at power level 5 with a sonic dismembraner (Fisher Scientific). Lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was then stored at -80°C.

Total cellular protein was quantitated using the Bradford microassay procedure (BioRad). Protein standards were made in 800µl of water and consisted of 1-10µg/ml bovine serum albumin (BSA, Sigma). Aliquots of 2-5µl of cellular lysate were diluted to a final volume of 800µl and 200µl of Protein Assay reagent was added to the standards and samples, mixed, and allowed to incubate for 30 minutes. The standards and the samples were then aliquoted in triplicate onto a 96-well plate and analyzed on a spectrophotometric plate reader at an absorbance of 595nm. A standard curve was constructed and sample concentrations were determined by comparison to the curve.
A standard amount of total protein (200µg) was incubated with 15µl of resuspended Immobilized Phospho-p44/42 MAP Kinase (Thr202/Tyr204) Monoclonal Antibody overnight at 4°C. The samples were then centrifuged for 30 seconds at 4°C and the pellet was washed twice with 1X Lysis Buffer and twice with 1X Kinase Buffer (25mM Tris–HCl, pH 7.5, 5mM β-glycerolphosphate, 2mM DTT, 0.1mM Na₃VO₄, and 10mM MgCl₂).

Aliquots of 50µl containing 1X kinase buffer, 100µM ATP and 2µg Elk-1 fusion protein were then added to the sample and allowed to incubate for 30 minutes at 30°C. The reaction was terminated with 25µl of 3X SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50mM DTT, and 0.1% bromphenol blue), and boiled for 5 minutes, mixed, and centrifuged for 2 minutes at 14,000 rpm. The samples were then loaded onto a 6% SDS-PAGE gel and electrophoresed at 100 watts until the dye marker was centered in the gel.

Protein was then transferred to a polyvinylidene flouride (PVDF) membrane (Millipore) using Ideal turboblotter at 6 volts for 2-3 hours. The membrane was then washed with 25ml 1X tris-buffered saline (TBS) for 5 minutes at room temperature. The membrane was then incubated overnight in 25ml blocking buffer (1X TBS, 0.1% Tween-20, and 5% nonfat dry milk), at 4°C. A PhosphoElk-1 Antibody (New England Biolabs) was added at a 1:1000 dilution to 10ml of Antibody Dilution Buffer (1X TBS, 0.05% Tween-20, and 5% BSA) and incubated with the membrane overnight at 4°C. The membrane was then washed 3 times in 15ml of TBST (1X TBS and 0.05% Tween-20) for 5 minutes each time. It was then
incubated with an HRP-conjugated anti-rabbit secondary antibody (1:2000, New England Biolabs) and an HRP-conjugated anti-biotin antibody (1:1000, New England Biolabs) in 10 ml of blocking buffer for 1 hour at room temperature. The membrane was then washed 3 times in 15 ml of TBST for 5 minutes each.

In order to detect the proteins the membrane was incubated in 10 ml of 1X LumiGLO reagent (New England Biolabs) for 1 minute at room temperature. The membrane was then wrapped in Saran Wrap and exposed to film on three different times ranging from 10 seconds to 5 minutes before being developed (see above).

Cell Growth Curves

Cells were initially plated at a density of $5 \times 10^4$ cells/well in a 12-well tissue culture plate containing 1 ml/well serum depleted medium (DMEM supplemented with 1% CS) and incubated over night at 37°C. The cells were fed within 24 hours with either serum depleted medium or serum depleted medium containing $1 \times 10^{-6}$ M Sandostatin. At 48 hours post plating the wells from each plate were washed once with 1 ml 1X PBS and then trypsinized with 1 ml of Trypsin-EDTA (Life Technologies). The cells were centrifuged for 3 minutes at 800 rpm and then resuspended in DMEM. The cells were then counted using a hemocytometer to determine the number of cells per ml. These experiments were repeated three times. For each experiment three wells were counted per sample. A total of 9 wells were counted per sample.
Statistics

Statistics for the cell counts included determination of analysis of variance (ANOVA), and a comparisons of individual clones by a Tukey test (α=0.05).

Statistics for the MAPK activation assays included an ANOVA, a split-split replicated block, and student T-tests.

For the cell counts, the ANOVA was calculated using the Microsoft Excel statistical analysis program. Because these results were not amenable to parametric analysis the Tukey's test, a non-parametric statistical test, was performed as described (Zar, 1984). All statistical analysis used an α value of 0.05.

For the MAPK activation assays, the ANOVA was also calculated using the Microsoft Excel statistical analysis program. This data was also not amenable to parametric analysis. Comparisons between treatment and non-treatment of the clones was performed using a split-split replicated block analysis to determine if there were differences between the clones and the Sandostatin treatment. Once a difference was established a students T-test was run on the means of the clones to determine exactly which clones were different from their controls. All statistical analysis again used an α value of 0.05.
RESULTS

Cloning of the SSTR2 Receptor

To clone the SSTR2 receptor we used a PCR reaction to amplify the gene from rat genomic DNA by utilizing two PCR primers which were identical to the 5' and 3' ends of the coding sequence of the rat SSTR2. Amplification using these two primers resulted in a 1119 bp fragment which was ligated to the pCR2.1 vector. The ligation mixtures were used to transform chemically competent *Escherichia coli* DH5α cells (see Materials and Methods). Restriction endonuclease digests of the plasmids showed that two clones contained inserts of the correct length and orientation (data not shown). These two clones were sequenced (see Materials and Methods) and a clone that did not contain mutations within the coding sequence of the SSTR2 receptor was selected and used in all future experiments.

To clone the SSTR2 receptor it was separated from the pCR2.1 vector by digestion of the plasmid with the restriction endonuclease *EcoRI*, gel purification, and ligation into *EcoR1* digested pTracer vector. This ligation mixture was then used to transform chemically competent DH5α cells. Restriction endonuclease digests of a selected clone demonstrated that this clone did contain an insert of the correct length and orientation (Figure 4).
Cloning of the SHP-1 Gene

The 2207 bp protein tyrosine phosphatase SHP-1 cDNA and the mutant SHP-1 (C453S) cDNA were ligated separately into the pcDNA3.1(+) vector at the EcoR1 site located within the pcDNA3.1 multiple cloning site (MCS). These plasmids were then transformed into chemically competent DH5α cells. Restriction endonuclease digests of DNA from one clone showed that it contained an insert of the correct length and orientation (Figure 4). One of the dominant negative mutant clones was also sequenced to ensure that the clone contained the C453S mutation (data not shown).

Figure 4. Restriction Endonuclease Digestions of the pTracer Vector + SSTR2 and the pcDNA3.1(+) Vector + SHP-1. 20µg of pTracer + SSTR2 DNA or pcDNA3.1(+) + SHP-1 was digested with the restriction endonuclease EcoRI for 60 minutes at 37°C. 20µl of diluted DNA was loaded into each lane and electrophoresed on a 1% agarose gel.
Transfections of NIH-3T3 Mouse Fibroblasts

Transfections were accomplished using a liposome technique as described above. The plasmid DNA was allowed to incubate with the Lipofectamine reagent to form micelles and then incubated with the cells in a serum free medium overnight. After media replacement the cells were allowed to grow for 48 hours before media containing serum and antibiotics were added for the selection process. G418 Sulfate was the antibiotic used for clones which had been transfected with the plasmid derived from the pcDNA3.1(+) vector, and Zeocin was the antibiotic used for cells that were transfected with the plasmid derived from the pTracer-CMV vector (see materials and methods). Cells that were transfected with both vectors received a combination of both G418 Sulfate and Zeocin for selection.

The cells were allowed to grow in the medium containing serum and antibiotics until several colonies appeared to be well established (approximately two to three weeks). These colonies were then trypsinized off the plates using cloning cylinders, and plated onto tissue culture plates. Table 3 describes all the clones that were used and which cDNA’s were transfected into them.

Characterization of the Transfected NIH-3T3 Cells

To determine if antibiotic resistant clones were expressing the genes of interest, RNA produced from these constructs was analyzed by Northern blotting. Hybridization with \(^{32}\text{P}\)-labelled SSTR2 and SHP-1 cDNA fragments were performed to determine the relative expression levels for these genes, which show a
range of expression levels. Autoradiographs show that clones 4B and 4C both express the SSTR2 cDNA and the mutant SHP-1 cDNA. Clone 4B expresses the mutant SHP-1 at a much higher level than does clone 4C (Figure 5). Clone 4B also shows a higher level of expression of the SSTR2 cDNA than does clone 4C (Figure 6). Clone 5A shows a slight expression of the wild-type SHP-1 protein (Figure 7) and a high level of expression of the SSTR2 receptor (Figure 8).

Table 3

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Transfected cDNA(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSTR2 ligated to the pTracer vector</td>
</tr>
<tr>
<td>2</td>
<td>Dominant Negative Mutant (DNM) SHP-1 Phosphatase ligated to the pcDNA3.1(+) vector</td>
</tr>
<tr>
<td>3</td>
<td>Wild-Type SHP-1 Phosphatase ligated to the pcDNA3.1(+) vector</td>
</tr>
<tr>
<td>4B</td>
<td>SSTR2 ligated to the pTracer vector and DNM SHP-1 Phosphatase ligated to the pcDNA3.1(+) vector</td>
</tr>
<tr>
<td>4C</td>
<td>SSTR2 ligated to the pTracer vector and DNM SHP-1 Phosphatase ligated to the pcDNA3.1(+) vector</td>
</tr>
<tr>
<td>5</td>
<td>SSTR2 ligated to the pTracer vector and Wild-Type SHP-1 Phosphatase ligated to the pcDNA3.1(+) vector</td>
</tr>
<tr>
<td>6</td>
<td>pTracer vector only</td>
</tr>
<tr>
<td>7</td>
<td>pcDNA3.1(+) vector only</td>
</tr>
<tr>
<td>8</td>
<td>pTracer vector and pcDNA3.1(+) vector</td>
</tr>
</tbody>
</table>
SHP-1 (2207bp) fragment

Figure 5. Autoradiograph of Northern Blot Probed With SHP-1 cDNA. Lanes 1-4 are clones 4B, C, D, and E which were transfected with both the SSTR2 receptor and the dominant negative mutant (DNM) SHP-1 phosphatase cDNA's. 5µg of total RNA was glyoxylated and electrophoresed through a 1% GTG-agarose gel and transferred to Duralon. The Northern blot was then probed with 1x10^6 counts/ml 32P-labelled SHP-1 cDNA and washed under stringent conditions. The autoradiogram was exposed for 24 hours at -80°C.

Analysis of Cellular Proliferation

To determine what affect the SSTR2 receptor and the SHP-1 protein would have on the growth rates of the different clones, cells were grown in 12-well tissue culture plates and counted as described in Material and Methods. The clone that was transfected with only the SSTR2 receptor cDNA (clone 1) showed a growth
Figure 6. Autoradiograph of Northern Blot Probed With SSTR2. Lanes 1-4 are clones 4A, B, C, and D which were transfected with the SSTR2 receptor and the dominant negative mutant (DNM) SHP-1 phosphatase cDNA’s. 5µg of total RNA was glyoxylated and electrophoresed through a 1% GTG-agarose gel and transferred to Duralon. The Northern blot was then probed with 1x10^6 counts/ml 32P-labelled SHP-1 cDNA and washed under stringent conditions. The autoradiogram was exposed for 24 hours at -80°C.

rate that was 32% of the control NIH-3T3 cell line (Figure 9), and was significantly different from the control cell line as the calculated q-value of 9.57 was greater than the critical q-value of 4.735 with an α-value of 0.05. Clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA showed a growth rate of 62% of the NIH-3T3 control cells and was significantly different
Figure 7. Autoradiograph of Northern Blot Probed With Wild-Type SHP-1 Phosphatase. Lane 1 is clone 4A, which was transfected with the SSTR2 receptor and the dominant negative mutant (DNM) SHP-1 phosphatase cDNA, and lane 2 is clone 5A, which was transfected with the SSTR2 receptor and the wild-type SHP-1 phosphatase cDNA. 5μg of total RNA was glyoxylated and electrophoresed through a 1% GTG-agarose gel and transferred to Duralon. The Northern blot was then probed with 1x10^6 counts/ml 32P-labelled SHP-1 cDNA and washed under stringent conditions. The autoradiogram was exposed for 24 hours at -80°C.

from the control cells as the calculated q-value of 5.37 was greater than the critical q-value of 4.735 with an α-value of 0.05. Clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA showed a growth rate of 124% of the NIH-3T3 control cells and therefore showed no effect on cellular proliferation as the calculated q-value was less than the critical q-value. The clones that expressed both the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA (clones 4B and 4C) showed decreased growth rates when compared to the control cells. Clone 4B, which expressed the SSTR2 receptor at higher levels, grew at rates just greater than 43% of the controls while clone 4C, which expressed the
Figure 8. Autoradiograph of Northern Blot Probed With the SSTR2 cDNA. Lane 1 is clone 1 which was only transfected with the SSTR2 receptor cDNA. Lane 2 is clone 2 which was only transfected with the dominant negative mutant (DNM) SHP-1 phosphatase cDNA. Lane 3 is clone 3 which was transfected with the wild-type SHP-1 phosphatase cDNA. Lane 4 is clone 4 which was transfected with both the SSTR2 receptor and the dominant negative mutant (DNM) SHP-1 cDNA’s. Lane 5 is clone 5 which was transfected with both the SSTR2 receptor and the wild-type SHP-1 cDNA’s. Lanes 6, 7, and 8 are clones 6, 7, and 8 respectively. Clone 6 was only transfected with the pTracer vector. Clone 7 was only transfected with the pcDNA3.1(+) vector, and clone 8 was transfected with both the pTracer and the pcDNA3.1(+) vectors. 5µg of total RNA was glyoxylated and electrophoresed through a 1% GTG-agarose gel and transferred to Duralon. The Northern blot was then probed with 1x10⁶ counts/ml 32P-labelled SSTR2 cDNA and washed under stringent conditions. The autoradiogram was exposed for 24 hours at -80°C.

SSTR2 receptor at much lower levels, had a growth rate of 97% compared to NIH-3T3 control cells. Clone 4B was statistically different from the control cells as the calculated q-value of 8.02 was greater than the critical q-value of 4.735 with an α-value of 0.05. Clone 4C was found to have no effect on cellular proliferation.
when compared to the untreated NIH-3T3 control cells as the calculated q-value was less than the critical q-value with an $\alpha$-value of 0.05.

![Cellular Proliferation](image)

**Figure 9.** Cellular Proliferation of Clones. Reported as Percent of Control. Cellular proliferation assays were replicated three times. Cells were plated at initial densities of 50,000 cells/well and counted at 48 hours post plating using a hemocytometer. Cell number represents an average of three replicates. Blue bars represent clones that were not treated with Sandostatin. Red bars represent cells that were treated with Sandostatin (1x $10^{-6}$M).

Clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA, showed a growth rate equal to 36% of the control NIH-3T3 cells, and was significantly different from control cells as the calculated q-value of 8.94 was greater than the critical q-value of 4.735 with an $\alpha$-value of 0.05. Clone 6, which was transfected with only the pTracer vector, and
clone 8, which was transfected with both the pTracer and pcDNA3.1(+) vectors, both showed reduced growth rates of 48% and 44% respectively when compared to the control NIH-3T3 cells, and were both found to be significantly different from the control cells as their calculated q-values of 7.34 and 5.34 respectively were both greater than the critical q-value of 4.735 with an $\alpha$-value of 0.05. This is in sharp contrast to clone 7 which was transfected with only the pcDNA3.1(+) vector and showed a growth rate equal to 84% of the control NIH 3T3 cells. Clone 7 was found to have no effect on cellular proliferation when compared to the NIH-3T3 control cells as its calculated q-value was less than its critical q-value.

Treatment of the cell lines with the analog Sandostatin showed no affect on the growth rates of any of the clones. However, clone 1, which was transfected with only the SSTR2 receptor cDNA, and clone 5, which was transfected with the SSTR2 receptor and the wild-type SHP-1 phosphatase cDNA’s, were both found to be significantly different from the NIH-3T3 control cells as their calculated q-values of 5.91 and 5.93 respectively were greater than the critical q-value of 4.735 with an $\alpha$-value of 0.05.

**Determination of MAPK Activation**

To determine if activation of MAPK was affected by any of the clones or treatments, MAPK activation assays were performed as described in the Materials and Methods. The cells were either not treated or treated with Sandostatin (Figures 10 and 11). In the untreated cells (Figures 10 and 12) clone 1, which was only
Figure 10. Western Blot of MAP Kinase Activation of Untreated Total Cellular Protein. 200µg total cellular protein was analyzed for MAPK activation using the p44/42 MAP Kinase Assay Kit (New England Biolabs) and electrophoresed through a 6% SDS-PAGE and transferred to a PVDF membrane. Proteins were detected using chemiluminescence. Densitometry was performed on a Kodak imaging system.

Figure 11. Western Blot of MAP Kinase Activation of Total Cellular Protein Treated With Sandostatin (1x10^-7M). 200µg total cellular protein was analyzed for MAPK activation using the p44/42 MAP Kinase Assay Kit (New England Biolabs) and electrophoresed through a 6% SDS-PAGE and transferred to a PVDF membrane. Proteins were detected using chemiluminescence. Densitometry was performed on a Kodak imaging system.
Figure 12. MAP Kinase Activity for Untreated Cells. Net Intensities are reported as Percent of Control of NIH-3T3 cells. Percent control represents an average of 3 replicates.

transfected with the SSTR2 receptor cDNA, depleted MAPK activity to below background levels and was therefore undetectable and significantly different than the untreated NIH-3T3 control cells as it had a p-value of 0.009 with an α-value of 0.05. Clone 2, which was transfected with only the dominant negative mutant SHP-1 phosphatase cDNA, decreased MAPK activity to approximately 62% of the control cells and was found to have no effect on MAPK activation as its p-value was 0.1 with an α-value of 0.05. Clone 3, which was transfected with only the
wild-type SHP-1 phosphatase cDNA, showed a slight increase in MAPK activity to 106% of the untreated NIH-3T3 control cells and was determined to have no effect on MAPK activation as the p-value was 0.8 with an α-value of 0.05.

Both of the clones which were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA, clones 4B and 4C, had decreased MAP kinase activity, but to different levels. Clone 4B decreased MAPK activity to approximately 26% of the untreated NIH-3T3 control cells and was found to be significantly different with a p-value of 0.0447 at an α-value of 0.05. Clone 4C, which expressed the SSTR2 receptor at lower levels than did clone 4B, decreased MAPK activity to 53% of the untreated NIH-3T3 control cells and was determined to have no effect on MAPK activation as its p-value was 0.096 with an α-value of 0.05. Clone 5A, which was transfected with both the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA, showed a decrease in MAPK activity equal to 86% of the untreated NIH-3T3 control cells and was found to have no effect on MAPK activation as its p-value was 0.09 at an α-value of 0.05. The only other clone that was shown to decrease MAPK activation was clone 6 which was transfected with only the pTracer vector. This clone showed a decrease in MAPK activity that was slightly greater than 53% of the untreated NIH-3T3 control cells and was found to be significantly different than the control cells as its p-value was 0.0221 at an α-value of 0.05. Clone 7, which was transfected with the pcDNA3.1(+) vector, and clone 8, which was transfected with the pTracer vector and the pcDNA3.1(+) vector, both showed activated MAPK levels that were
approximately 92% and 97% respectively of the untreated NIH-3T3 control cell line. Neither of these clones were found to have an effect on MAPK activation as their p-values were 0.585 and 0.812 respectively, at an α-value of 0.05.

Clones that were treated with the SMS analog Sandostatin showed different levels of MAPK activation than did the clones which were not treated with the analog (Figure 13). Clone 1, which was transfected with only the SSTR2 receptor cDNA, increased MAPK activity to 59% of the treated NIH-3T3 control cells.

Figure 13. MAP Kinase Activity of Cells Treated with Sandostatin. Cells were grown overnight in serum depleted medium and treated with $1 \times 10^{-6}$ M Sandostatin for 5 minutes. MAPK intensity is reported as Percent of Control intensity of band obtained with NIH-3T3 cells. Percent control represents an average of 3 replicates.
Clone 2, which was transfected with only the dominant negative mutant SHP-1 phosphatase cDNA, decreased MAPK activity to approximately 46% of the treated NIH-3T3 control cells. Overall, all other clones only decreased MAPK activity by 2-10% except for clone 8, which was transfected with both the pTracer vector and the pcDNA3.1(+) vector, which showed a decrease of MAPK activation equal to 55% of the treated NIH-3T3 control cells. All of the clones treated with Sandostatin were found to have no effect on MAPK activation.

Comparisons between cells that were transfected with cDNA inserts and the appropriate vector controls were also performed. As expected, when clone 1, which was transfected with the SSTR2 receptor cDNA is compared to clone 6, which was only transfected with the pTracer vector, it is evident that treatment of the cells with the Sandostatin analog increases MAPK activity in clone 1 (Figure 14).

![pTracer Expressing Clones](image)

**Figure 14.** pTracer Expressing Clones. This figure compares clone 1, which was transfected with the SSTR2 receptor and clone 6, which was transfected with only the pTracer vector, and is the vector control of clone 1, in both untreated and treated cell lines. Cells were grown overnight in serum depleted medium and treated with $1 \times 10^{-6}$M Sandostatin for 5 minutes before being lysed. MAPK intensity is reported as percent of control intensity of bands obtained with clone 8 cells. Percent control represents an average of 3 replicates.
When clones 4B and 4C, which were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA, and clone 5, which was transfected with the SSTR2 receptor and the wild-type SHP-1 phosphatase, are compared to clone 8, which was transfected with pTracer vector and the pcDNA3.1(+) vector, it also appears evident that treatment of the cells with the Sandostatin analog increases MAPK activity in all of the clones shown (Figure 15).

![Figure 15. Clones Expressing Both Vectors.](image)

Figure 15. Clones Expressing Both Vectors. This figure compares clones 4B and 4C, which were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA, and clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA, to clone 8, which was transfected with both the pTracer vector and the pcDNA3.1(+) vector, and is the vector control for clones 4B, 4C, and 5, in both untreated and treated cell lines. Cells were grown overnight in serum depleted medium and treated with 1 x 10^-6 M Sandostatin for 5 minutes before being lysed. MAPK intensity is reported as percent of control intensity of bands obtained with clone 8 cells. Percent control represents an average of 3 replicates.

When clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA, and clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA, are compared to clone 7, which was transfected with only the
pcDNA3.1(+) vector, there does not appear to be a difference between untreated MAPK levels and treated MAPK levels (Figure 16).

Figure 16. pcDNA3.1 Expressing Clones. This figure compares clones 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA, and clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA, to clone 7, which was transfected with the pcDNA3.1(+) vector, and is the vector control for both clones 2 and 3, in untreated and treated cell lines. Cells were grown overnight in serum depleted medium and treated with $1 \times 10^{-6}$M Sandostatin for 5 minutes before being lysed. MAPK intensity is reported as percent of control intensity of bands obtained with clone 8 cells. Percent control represents an average of 3 replicates.
DISCUSSION

SMS is a neuropeptide produced throughout the body. It has a variety of functions, but one of its most unique characteristics is its ability to inhibit the growth and secretion of compounds from cells that express its receptors. The antiproliferative effects of SMS make it a candidate for chemotherapeutic therapies in diseases such as cancer however, due to its extremely short half-life, its effectiveness is severely limited.

Agonsits of SMS have been designed to eclipse this problem, and are currently being used in the treatment of specific types of cancers. These agonsists are designed to maintain the biologically active amino acids tryptophan and lysine that are known to be necessary for binding to the SMS receptor. They are also designed to have characteristics of longer half-lifes and greater binding affinities towards specific receptors.

Exactly how SMS or any of its analogs work is yet to be determined, but it is known that one of the five G protein coupled SSTR's must be present on the cell surface Fugii et al., 1994). There is also evidence that the protein tyrosine phosphatase SHP-1 may play a vital role in deactivating secondary signaling molecules and their associated signal transduction pathways (Yi et al., 1993). What these signals are, and how they work, is as of today still largely unknown.
However, the SHP-1 protein has been shown to bind intracellularly to the SMS receptor SSTR2 (Lopez et al., 1994), and appears to be good candidate for controlling cellular proliferation and gastric secretions within the SMS signaling pathway as this phosphatase can act on a variety of growth factor receptors (Liebow et al., 1989). To determine if the wild-type SHP-1 protein plays a regulatory role in the signal transduction pathway of SMS and its analog Sandostatin, I cotransfected the SSTR2 receptor along with a dominant negative mutant SHP-1 phosphatase into wild-type NIH-3T3 cells, which do not normally express the SSTR2 receptor, and analysed the growth properties and state of MAPK activation of these cells.

Northern blot analyses of total cellular RNA demonstrated that clones transcribed the SSTR2 cDNA at differential levels. Some of these same clones were also shown to produce the wild-type SHP-1 phosphatase mRNA or the dominant negative mutant SHP-1 phosphatase mRNA. Clones 1, 4B, 4C, and 5 were all shown to be able to transcribe the transfected SSTR2 receptor cDNA. Clone 4B expressed the SSTR2 receptor at higher levels than did clone 4C. The differential expression of SSTR2 in clones 4B and 4C presented an opportunity to study the effects of high and low receptor expression on both cellular proliferation and MAPK activity.

To determine if the wild-type SHP-1 phosphatase gene product regulated cellular proliferation within the SMS pathway cells were grown in various treatments. Results showed that clone 1, which was transfected with the SSTR2 receptor cDNA, had a decreased growth rate that was equal to 32% of the control
cell line (Figure 9). This was statistically significant (q-calculated = 12.95, q-critical = 4.735, α = 0.05) and suggested that expression of the SSTR2 receptor alone can control cellular proliferation, possibly by stimulating the production of endogenous SMS. These results are in agreement with Buscail et al., (1996) who examined the differential mRNA expression of the five SSTR’s in primary tumors or metastases from colorectal or exocrine pancreatic cancer patients. They were able to demonstrate that the SSTR2 receptor was present in normal pancreatic tissues but was absent in exocrine pancreatic carcinomas and their metastases. Therefore, it appears that just the absence of the SSTR2 receptor can benefit cells in terms of proliferation as cell lines that express the SSTR2 receptor, such as clone 1, have significantly decreased growth rates.

Delesque et al., (1997) demonstrated that when human SSTR2 cDNA was transfected into pancreatic cancer cells (Capan-1 and BxPC-3), which do not express the SSTR2 receptor, cell growth was suppressed by up to 41% after 5 days. They went on to show that cells which were transfected with the SSTR2 receptor were able to increase SMS mRNA levels resulting in an increase in the production and secretion of SMS-14.

Clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA alone, and therefore should not stimulate SMS production, showed a decrease of 62% in cellular proliferation (q-calculated = 5.37, q-critical = 4.735, α = 0.05) and clone 3, which was transfected with only the wild-type SHP-1 phosphatase cDNA, showed an increase of 24% (q-critical > q-calculated)
when compared to control cells (Figure 9). The results with clone 2 were statistically significant and showed that cells needed the SHP-1 protein to proliferate normally, and the dominant negative mutant SHP-1 phosphatase protein decreased cell counts. This demonstrates that the SHP-1 protein does play an active role in transmitting the SMS signal.

Clone 4B, which showed a high level of expression of the SSTR2 receptor and also expressed the dominant negative mutant SHP-1 phosphatase, showed a growth rate equal to 43% of control cells (Figure 9) and was statistically significant (q-calculated=8.02, q-critical=4.735, \( \alpha = 0.05 \)). Clone 4C, which expressed the SSTR2 receptor at lower levels and also expressed the dominant negative mutant SHP-1 phosphatase protein, showed a growth rate almost equal to 97% of the control cells (Figure 9) and was shown to have no effect (q-critical > q-calculated) on cellular proliferation. These results demonstrate that coexpression of the SSTR2 receptor and the dominant negative mutant SHP-1 phosphatase does not have an additive effect upon cellular proliferation, as the expression of the SSTR2 receptor decreases cellular growth more than the coexpression of the two cDNA's together.

Clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA, decreased cellular proliferation to 36% of control cells (Figure 9) and was statistically significant (q-calculated=8.94, q-critical=4.735, \( \alpha = 0.05 \)). As was the case for clone 4B, which expressed the SSTR2 receptor at high levels along with the dominant negative mutant SHP-1 phosphatase protein, and clone 4C, which expressed the SSTR2 receptor at low levels along with
the dominant negative mutant SHP-1 phosphatase protein, clone 5 also does not show an additive effect as its cellular proliferation levels are almost equal to those of clone 1, which expressed the SSTR2 receptor (Figure 9). Overexpression of the wild-type SHP-1 protein does not appear to increase cell numbers within clone 5, and therefore, it appears that the SSTR2 receptor has a larger influence on cellular proliferation than does the wild-type SHP-1 protein.

The clones that were transfected with only one or both vectors, clones 6-8, also tend to reduce cell numbers (Figure 9). It is not understood what direct effect the vectors themselves were having upon cellular proliferation however, it was significant for clone 6, which was transfected with the pTracer vector (q-calculated=7.34, q-critical=4.735, α=0.05) and clone 8, which was transfected with the pTracer vector and the pcDNA3.1 vector (q-calculated=5.34, q-critical=4.735, α=0.05). It is possible that cellular proliferation was also dependent upon the location of incorporation of the plasmid(s) into the cell’s genome. Incorporation at specific sites may have accounted for the decreases in cellular proliferation seen within the clones. It is also possible, although not likely, that the vectors themselves were, in someway, directly controlling cellular proliferation. This seems improbable because these effects have not been seen in other cells transfected with these vectors (data not shown).

In an attempt to determine if the SMS analog Sandostatin could affect the growth rates of cells expressing the transfected genes, the cells were treated with 1 x 10^{-6} M Sandostatin for 5 minutes after growth overnight in serum depleted
medium. The results showed that the Sandostatin analog did not substantially affect the growth rates of these clones (Figure 9). This data is inconsistent with results published by Lopez et al., (1997). Their results showed that the analog did decrease cellular proliferation by up to 39% in CHO cells transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA when compared to controls. When cellular proliferation studies were performed using, DMEM supplemented with 10% CS, all clonal growth was similar to Lopez et al., (1997) (data not shown). The clones, which were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA, had higher cell counts (38%) when compared to clones which were transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA. These cells were initially treated with Sandostatin at the time of plating and were then trypsinized and subsequently counted at 72 and 120 hours. However, concern about effects caused by endogenous growth factors within the serum led me to perform these tests under serum depleted conditions. These results do agree with those of Liebow et al., (1989), who showed that Sandostatin does not have an effect on the growth inhibition of serum starved Mia PaCa-2 cells.

To determine if any of these clones have either a positive or negative effect upon MAPK activation MAPK phosphorylation assays were performed. Data from untreated cells showed that clone 1, which was transfected with the SSTR2 receptor cDNA, depleted activated MAPK to less than detectable levels, and was significantly different (p=0.009, α=0.05) from NIH-3T3 control cells (Figure 10).
Clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA, decreased MAPK activity to 62% of the NIH-3T3 control cells (Figure 10) and was shown to have no effect (p=0.1, α=0.05). Clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA, was also shown to have no effect (p=0.808, α=0.05) upon MAPK activity (Figure 10).

This data correlated well with the cell count data. When the SSTR2 receptor cDNA was transfected alone into NIH-3T3 cells, cell counts decreased and MAPK activity fell to below detectable levels. The transfection of the dominant negative mutant SHP-1 phosphatase into NIH-3T3 cells was also capable of decreasing cell counts and MAPK activity, but not to the levels seen by transfection of the SSTR2 receptor alone. An overexpression of wild-type SHP-1 phosphatase in these clones increased cell counts and slightly increased MAPK activity.

When the SSTR2 receptor cDNA was transfected into NIH-3T3 cells along with the dominant negative mutant SHP-1 phosphatase the same effects occurred as in the cellular proliferation assays. Clone 4B, which was transfected with the SSTR2 receptor at high levels along with the mutant SHP-1 protein, reduced MAPK activity to levels that were 26% of the NIH-3T3 control cells (Figure 10) and showed significant difference (p=0.0447, α=0.05), while clone 4C, which expressed the SSTR2 receptor at low levels along with the mutant SHP-1 protein, only reduced MAPK activity to 53% of the NIH-3T3 control cells (Figure 10) and showed no effect (p=0.0957, α=0.05). These results however do appear to at least be partially additive, unlike the cell counts. MAPK activity was decreased to a
greater extent in cells that were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA than in cells that were transfected with the dominant negative mutant SHP-1 phosphatase cDNA. They are not however decreased to levels that are undetectable, as is the case for clone 1, which was transfected with the SSTR2 receptor cDNA. This suggests that the wild-type SHP-1 phosphatase played a role in the activation of MAPK by SMS, although we are not sure to what extent. It appears that the dominant negative mutant SHP-1 phosphatase is somehow interfering with the normal role of the SSTR2 receptor.

The transfection of the wild-type SHP-1 phosphatase cDNA, when transfected along with the SSTR2 receptor, as in clone 5, stimulated MAPK activity to levels that were 86% of the NIH-3T3 control cells (Figure 10) and had no effect ($p=0.0906$) on MAPK activity. This was interesting because as MAPK activity was increasing in this clone its cellular proliferation was decreasing to levels resembling clone 1, which was transfected with the SSTR2 receptor cDNA. This suggests that the overexpression of wild-type SHP-1 phosphatase inactivated some of the MAPK activity, and that this inactivation decreased cellular proliferation. This was also evident in clone 6, which was transfected with the pTracer vector, where the decrease in cellular proliferation (Figure 9) was associated with a decrease in MAPK activity (Figure 10) and was significantly different ($p=0.0221$, $\alpha=0.05$), not however to the extent seen in clone 1, which was transfected with the SSTR2 receptor cDNA, and 4B, which was transfected with the SSTR2 receptor cDNA along with the dominant negative mutant SHP-1 phosphatase cDNA. The reason
behind the clone 6 decrease in cellular proliferation and MAPK activity was not clear, and these correlations were not consistent with clone 7, which was transfected with the pcDNA3.1 vector, and clone 8, which was transfected with both the pTracer vector and the pcDNA3.1 vector. Both of these clones showed MAPK activity levels that were similar to the NIH-3T3 control cells (Figure 10) and statistically different (p=0.585, α=0.05 and p=0.812, α=0.05 respectively). Both, however, showed decreased cellular proliferation, especially clone 8.

When the clones were treated with the Sandostatin analog under serum depleted conditions the results seemed to be varied. The MAPK activity level of clone 1, which was transfected with the SSTR2 receptor cDNA, increased from below background levels in untreated cells to 58% of the treated NIH-3T3 control cells (Figure 11). Clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA, decreased MAPK activation levels to 44% of the treated NIH-3T3 control cells (Figure 11). Clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA, showed MAPK activation levels of 85% of the treated NIH-3T3 control cells (Figure 11). Clones 4B, and 4C, both of which were transfected with the SSTR2 receptor cDNA along with the dominant negative mutant SHP-1 phosphatase cDNA, showed MAPK activation levels equal to 80% and 92% of treated NIH-3T3 control cells respectively (Figure 11). Clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 phosphatase cDNA, showed MAPK activation levels equal to 79% of treated NIH-3T3 control cells (Figure 11). Clone 6, which was transfected with the pTracer
vector, showed MAPK activation levels equal to 87% of treated NIH-3T3 control cells (Figure 11). Clone 7, which was transfected with the pcDNA3.1 vector, and clone 8, which was transfected with both the pTracer vector and the pcDNA3.1(+) vector, showed MAPK activation levels equal to 54% and 55% of the treated NIH-3T3 control cells respectively (Figure 11). Overall, all of the clones treated with the Sandostatin analog were found to have no effect on MAPK activation levels ($p > 0.05$, $\alpha = 0.05$) when compared to the treated NIH-3T3 control cells. This correlates well with the cellular proliferation data which also showed that Sandostatin only significantly affected clone 1, which was transfected with the SSTR2 receptor cDNA, and clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 cDNA.

When the clones are compared to their respective control vectors a trend begins to appear. Treatment with the Sandostatin analog increases MAPK activity in clones that were transfected with the SSTR2 receptor cDNA (clones 1, 4B, 4C, and 5) (Figures 14 and 15) much more than it does in the clones which were not transfected with the SSTR2 receptor (clones 2 and 3) (Figure 16). Clone 1, which was transfected with the SSTR2 receptor cDNA, under no treatment was 0% of its control vector clone, clone 6, which was transfected with the pTracer vector. Upon treatment with the Sandostatin analog MAPK activity in clone 1 increased to 67% of its control vector clone 6. Clones 4B and 4C, which were transfected with the SSTR2 receptor cDNA and the dominant negative mutant SHP-1 phosphatase cDNA, increased their MAPK activity from 25% and 52% respectively to 147%
and 171% also respectively when treated with the Sandostatin analog. Clone 5, which was transfected with the SSTR2 receptor cDNA and the wild-type SHP-1 cDNA, also increased its MAPK activity from 88% to 147% when treated with the Sandostatin analog. Clone 2, which was transfected with the dominant negative mutant SHP-1 phosphatase cDNA, and clone 3, which was transfected with the wild-type SHP-1 phosphatase cDNA, decreased their MAPK activity from 65% and 113% respectively to 55% and 107% also respectively. This data shows that only the clones which were transfected with the SSTR2 receptor cDNA showed increases in MAPK activity. Therefore, it appears that the Sandostatin analog increases MAPK activity when the SSTR2 receptor is expressed, and tends to decrease it when the receptor is not expressed. Sandostatin does not appear to be able to affect MAPK activity through the wild-type SHP-1 phosphatase.

It is known that the SHP-1 protein is recruited to activated growth factor receptors and causes a transient dephosphorylation and inactivation of specifically associated signaling molecules Fugii et al., (1994), and it has been postulated by Lopez et al., (1997) that the rapid activation of the SHP-1 protein following SMS stimulation of cells is the initiating step for SSTR2 signal transduction that leads to growth inhibition. These data tend to support Lopez et al., (1997), however not directly, as they indicate SHP-1 works independently of the SSTR2 receptor in controlling cell growth as expression of the dominant negative mutant SHP-1 phosphatase protein can reduce cell growth on its own. Expression of the SSTR2 receptor, especially at high levels, however, appears to be the largest factor.
regulating cellular proliferation in the NIH-3T3 mouse fibroblast cells whether or not cells were stimulated with Sandostatin, but the SSTR2 receptor and the SHP-1 protein however, do appear to cooperate in regulating MAPK activation. Therefore, it appears that SMS can partially regulate cell growth through the SHP-1 protein, but that the SSTR2 receptor and the deactivation of MAPK have a greater effect. This data has also shown that the Sandostatin does not work through the exact same mechanism that SMS does. Instead, it appears that Sandostatin regulates its cellular proliferation through the SHP-1 protein.

In conclusion this data suggests that the SSTR2 receptor does control cellular proliferation, and that this control was somehow regulated through the MAPK pathway. The SHP-1 protein also appears to be involved in cellular proliferation as its inhibition by a dominant negative mutant SHP-1 phosphatase drastically reduced cell counts. This data also suggests that the SHP-1 protein is involved in signaling through the MAPK pathway as the dominant negative mutant SHP-1 protein was able to decrease MAPK activation. However, it does not appear that the SSTR2 receptor and the SHP-1 protein are able to cooperate in regulating cellular proliferation. This is shown when the SSTR2 receptor and the wild-type SHP-1 protein are cotransfected into NIH-3T3 cells. Here, cellular proliferation does not decrease to the levels seen in clones that only express one of the proteins. However, the two proteins do appear to cooperate, at least in part, to control MAPK activation.
This data also suggests that treatment of the clones with the SMS analog Sandostatin does not affect cellular proliferation in any of the clones. It does however appear to affect MAPK activation as clones that were transfected with the SSTR2 receptor cDNA, either alone or with the dominant negative mutant SHP-1 phosphatase cDNA, increased MAPK activation.

Future experiments to determine if the MAPK pathway is directly affected by SMS or Sandostatin is to look at the possible roles of the adapter protein Shc. It would also be of interest to determine if different expression levels of the SSTR2 receptor influence expression levels of SMS, and if the rate of SMS secretion can be manipulated by expressing the mutant SHP-1 protein.

It will be a long time before a good part of the signaling pathway of SMS and its analogs are well understood, however manipulation of this pathway offers a good possibility of alleviating some of the symptoms common in several major diseases like diabetes, and specific forms of cancer.
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