

**IDENTIFICATION OF STEROID-SENSITIVE GENE-1
AS A NOVEL JAK2 BINDING PROTEIN AND THE
PHYSIOLOGICAL CONSEQUENCES
OF THIS INTERACTION**

by

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Dominoes for smart people!



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To my parents, Terrence and Jan O’Leary, who taught me never to give up.

And to my sister, Meghan O’Leary, who describes what I do as “dominoes for smart people”.

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TABLE OF CONTENTS

Dedication.....	ii
Acknowledgements.....	iii
List of Figures.....	viii
Abstract.....	x
CHAPTER	
I. INTRODUCTION	1
Ligand-stimulated kinase signal transduction.....	1
JAK family of kinases.....	1
Cytokine superfamily of receptors.....	2
Physiological role for JAKs revealed by knockout animals	5
Dysregulation of JAK signaling.....	6
JAK2	7
Signal transducers and activators of transcription	12
Physiological relevance of Stat3.....	16
Physiological function of Stat5.....	19
Post-translational modifications regulate Stat activity	21
Growth hormone activates the JAK-Stat pathway.....	25
Growth hormone activates other signaling pathways	27
GH-responsive genes	28
Steroid-Sensitive Gene-1	29
Aims of this thesis.....	35
References.....	38
II. IDENTIFICATION OF STEROID-SENSITIVE GENE-1 AS A NOVEL JAK2 BINDING PROTEIN AND THE SRPX5 DOMAIN AS A NOVEL PHOSPHOTYROSINE BINDING DOMAIN.....	62
Abstract.....	62
Introduction.....	63
Materials and Methods.....	65
Results.....	70
Discussion.....	86
References.....	91
III. PHYSIOLOGICAL CONSEQUENCES OF THE INTERACTION OF THE INTERACTION BETWEEN JAK2 AND SSG1	97
Abstract.....	97
Introduction.....	98
Materials and Methods.....	101
Results.....	108

	Discussion.....	130
	References.....	138
IV.	CONCLUSIONS & FUTURE DIRECTIONS.....	145
	Summary.....	145
	Future Directions.....	151
	Conclusion.....	165
	References.....	168

LIST OF FIGURES

Figure

1.1	Schematic representation of JAK domain structure.....	3
1.2	The cytokine/hematopoietin receptor superfamily.	4
1.3	Schematic representation of the domain structure of Stat proteins.....	13
1.4	Schematic representation of GH signal transduction.....	26
1.5	Schematic representation of the domain structure of SSG1	34
2.1	Identification of SSG1 as a binding partner for JAK2 in the yeast two-hybrid system.....	71
2.2	SSG1 (477-949) associates preferentially with and is phosphorylated by kinase-active JAK2 in mammalian cells	74
2.3	SSG1 (477-949) interacts with the kinase domain of JAK2.....	76
2.4	Identification of the SRPX5 domain within SSG1 as a region of interaction with JAK2.....	77
2.5	All three SRPX5 domains of SSG1 interact with kinase-active JAK2.....	79
2.6	The SRPX5 domains of SSG1 interact with the kinase-active kinase domain of JAK2.....	80
2.7	Full-length SSG1 interacts with full-length, kinase-active JAK2	82
2.8	SSG1 interacts with full-length JAK2 better than JH1.....	83
2.9	SRPX2, another SRPX5 domain-containing protein, binds kinase-active JAK2	85
3.1	The effect of SSG1 on JAK2 kinase activity	109
3.2	The effect of SSG1 on tyrosyl phosphorylation of Stat5b.....	110
3.3	SSG1 stimulates the tyrosyl phosphorylation of Stat3	112
3.4	The effect of alkaline phosphatase on Stat3	114
3.5	The effect of λ phosphatase on Stat3	115

3.6	SSG1 negatively regulates the GH-responsive gene <i>c-fos</i>	118
3.7A-I	Localization of SSG1.....	120
3.7J-P	Localization of SSG1.....	121
3.8	SSG1 is a secreted protein.	123
3.9	SSG1 secretion is enhanced by kinase-active JAK2.....	125
3.10	Localization of SSG1 in the presence of wild-type JAK2.....	128
3.10	Localization of SSG1 in the presence of wild-type JAK2.....	129
4.1	Schematic representation of SSG1 as a JAK2 interacting protein.....	147
4.2	Schematic representation of SSG1 as a regulator of Stat signaling.....	149
4.3	Schematic representation of SSG1 as a secreted protein.....	152
4.4	Model of SSG1 in GH Signal Transduction.....	166

ABSTRACT

Growth hormone (GH) is an important regulator of body growth and metabolism. GH binding to its receptor activates the receptor-associated tyrosine kinase, JAK2, which in turn, initiates multiple signaling cascades, including activation of Stats 1, 3, 5a, and 5b. JAK2 is an important signaling molecule activated by GH. However, very few proteins have been identified as binding partners for active JAK2. We identified steroid-sensitive gene-1 (SSG1) as a JAK2 binding protein that binds to the active form of JAK2 in a yeast two-hybrid screen of a rat adipocyte cDNA library. Full-length SSG1 binds to the kinase-active form of JAK2 and its kinase (JH1) domain, but not kinase-inactive JAK2 (K882E) or JH1 (K882E). Full-length SSG1 binds JAK2 better than the JH1 domain suggesting that there are at least two sites of interaction for SSG1 in JAK2. SSG1 contains three SRPX5 (sushi-repeat-containing protein, X-linked, domain 5) domains. All three of these SRPX5 domains interact preferentially with the kinase-active form of JAK2. Our results suggest that the SRPX5 domain is a novel protein-protein interaction domain, most likely a phosphotyrosine binding domain. To determine the physiological consequences of the interaction between SSG1 and JAK2, we show that SSG1 does not alter JAK2 kinase activity and only modestly increases GH-dependent phosphorylation of Stat5b on Tyr694. In contrast, SSG1 enhances phosphorylation of Stat3 on Tyr705 in the presence and absence of GH. Phosphatase experiments suggest that SSG1 enhances GH-independent phosphorylation of a different, currently unidentified amino acid in Stat3, and negatively regulates the GH-responsive *c-fos* promoter construct. SSG1 co-localizes with markers for both the endoplasmic reticulum and Golgi. By confocal microscopy, I show that a portion of JAK2 co-localizes with SSG1 in these subcellular compartments. SSG1, which contains a signal peptide, is a secreted protein. Secretion is blocked by Brefeldin A. JAK2 enhances both the secretion and cleavage of SSG1, assessed by TCA precipitation of medium and immunofluorescence. Together, my results show that SSG1

interacts with JAK2 and regulates downstream signaling of JAK2. JAK2 enhances the secretion and potentially extracellular cleavage of SSG1.

CHAPTER I

INTRODUCTION

Ligand-stimulated kinase signal transduction

Polypeptide ligand binding to extracellular cell surface receptors initiates intracellular signaling cascades. In many cases, this intracellular signaling cascade is a relay of reversible phosphorylation events. Kinases play a critical role in signaling events by phosphorylating other components in the cascade. Phosphorylation oftentimes, but not always, leads to activation of the target protein. There are three classes of kinases: serine/threonine kinases, tyrosine kinases, and dual specificity kinases which phosphorylate substrates on serine, threonine, and tyrosine residues. Sequencing has revealed that serine/threonine kinases make up the majority of protein kinases, with tyrosine kinases accounting for roughly 2% of all kinases (1). Once a protein has been phosphorylated by a kinase, it can then trigger other downstream events including binding to other proteins, including adaptor proteins; phosphorylating other proteins, including proteins with and without enzymatic activity; binding DNA and initiating gene transcription.

JAK family of kinases

The kinase family implicated in signaling events for the cytokine family of receptors is the JAK family of tyrosine kinases. There are four members of the mammalian Janus kinase (JAK) family of non-receptor tyrosine kinases: JAK1, JAK2, JAK3, and Tyrosine kinase 2 (Tyk2) (2-6). JAK1, JAK2, and Tyk2 have ubiquitous tissue expression; however, JAK3 expression is limited to myeloid and lymphoid cells (7,

8). The JAK proteins contain seven JAK homology domains, termed JH domains (Fig. 1.1). The JH1 domain is at the C-terminus of the protein and encodes the kinase. Each JAK family member contains a pair of conserved tyrosine residues in the activation loop. Phosphorylation on one or both of these activating tyrosines causes a conformational change, which allows substrate binding. Immediately N-terminal to the JH1 domain is the JH2 domain which resembles the kinase domain except it lacks kinase activity and is referred to as a pseudokinase domain. Evidence suggests that the pseudokinase domain regulates the activity of the kinase since deletion of this region in JAK2 results in kinase hyperactivity. JAKs also contains a region (JH3 and a part of JH4 domain) that resembles an SH2 domain; however, this region fails to bind phospho-tyrosine containing proteins (9). The JH4-JH7 domains compose a FERM (four point one, ezrin, radixin and moesin) domain and are necessary for the association with cell surface receptors (10-15). This amino terminal region is the least conserved among the JAK family; however, the differences lead to receptor binding specificity.

Cytokine superfamily of receptors

The cytokine/hematopoietin superfamily of receptors is a large family of receptors consisting of receptors for growth hormone (GH), prolactin (PRL), leptin, erythropoietin (EPO), most interleukins (ILs), and the interferons (IFNs). While these receptors share low amino acid identity, they share several conserved motifs (Fig. 1.2). Two fibronectin-type III domains are located in the extracellular region of these receptors (16, 17). Within this fibronectin domain is a WSXWS motif, which has been implicated in ligand binding to the receptor (18-21). The GH receptor contains a WSXWS-like motif, which has conservative substitutions: Y/F-G/S-E-F-S. The cytoplasmic region of these receptors contains a proline-rich region, termed Box 1 (22). Box1 is positioned adjacent to the plasma membrane and is required for interaction with the JAK tyrosine kinase family (13, 22-26). The cytoplasmic region of these receptors also contains a hydrophobic stretch of amino acids termed Box2. Mutational analyses of both of these regions in the receptors are consistent with these regions being critical for ligand-stimulated responses (22, 27-31).

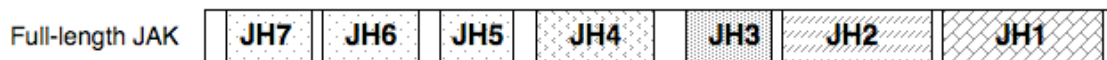


Figure 1.1 Schematic representation of JAK domain structure.

JAK family members are composed of seven JAK homology (JH) regions. JH1 is the kinase domain. JH2 is the pseudokinase domain. JH3 and part of JH4 resemble an SH2 domain. The rest of JH4-JH7 compose a four point one, ezrin, radixin and moesin (FERM) domain, which is necessary to interact with the cytokine receptors.

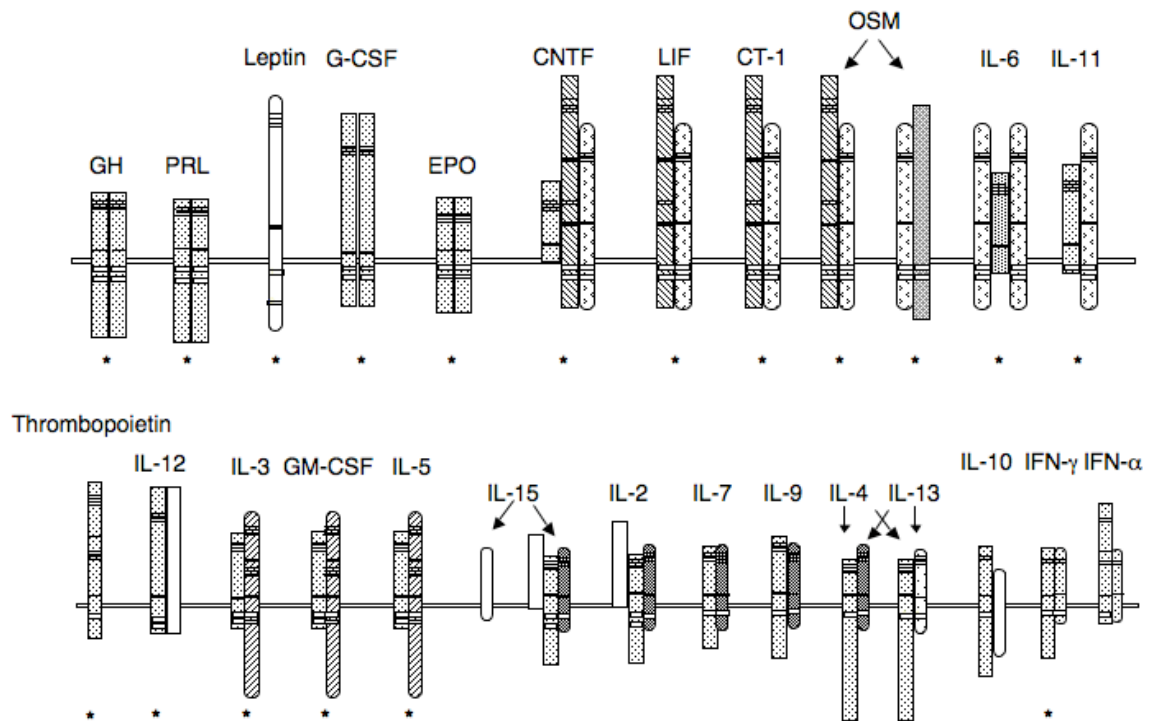


Figure 1.2 The cytokine/hematopoietin receptor superfamily.

Schematic representation of receptors of the cytokine superfamily including receptors for growth hormone (GH), prolactin (PRL), leptin, granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), ciliary neurotrophic factor (CNTF), leukemia inhibitor factor (LIF), oncostatin M (OSM), thrombopoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (ILs), and interferon (IFN)- γ and - α . The extracellular portion of these receptors contain conserved cysteine residues (four thin lines) as well as the WSXWS motif implicated in ligand binding (black box). The cytoplasmic tail of these receptors contain the proline-rich Box 1 important for interaction with JAK kinases and hydrophobic rich Box 2 (white boxes). Asterisks (*) denote receptors that activate JAK2. Adapted from (32).

The GH receptor, along with several other receptors in this family (e.g. prolactin, erythropoietin, and granulocyte colony stimulating factor receptors), consist of homodimers of identical subunits. Other receptors are heterodimers/trimer/tetramers composed of shared subunits (e.g. gp130, leukemia inhibitory factor (LIF) receptor- β , IL-3 β chain, IL-2 γ chain receptors) and ligand-specific subunits (e.g. ciliary neurotrophic factor (CNTF) and oncostatin M (OSM) receptors).

Members of the cytokine superfamily of receptors lack intrinsic kinase activity; therefore, they rely on intracellular tyrosine kinases to transmit extracellular signals to the internal cellular environment. The Janus family tyrosine kinases are activated upon cytokine binding to its receptor. JAKs are associated with the cytoplasmic region of the cell surface receptors. Ligand binding to what is thought to be pre-formed multimerized receptors induces a conformational change in the cytoplasmic portion of the receptor which brings two associated JAK molecules into close proximity of each other (33-38). Once the two JAK molecules are in close proximity and in the right conformation, they are able to trans-phosphorylate each other (37). The activated JAKs then phosphorylate tyrosines in their associated receptors as well as in the JAKs themselves. These phosphorylated tyrosine residues serve as docking sites for downstream signaling molecules, including the signal transducers and activators of transcription (Stat) proteins, which will be discussed below.

Physiological role for JAKs revealed by knockout animals

JAKs have important physiological roles since they mediate the response of a wide variety of cytokine receptors including IFNs, most ILs, GH, PRL, and EPO. JAKs mediate immune responses, growth, differentiation, lactation, and blood cell development and genetic loss of any of the JAK family members elicits a unique physiological outcome.

Tyk2 has been demonstrated to play a role in signaling in response to IFN- α , IL-6, IL-10, and IL-12. Mice lacking Tyk2 have a modest reaction to viral infection, and macrophages lacking Tyk2 have an impaired response to LPS (39, 40). JAK3 expression is limited to hematopoietic tissues (7, 8). JAK3 is important for lymphoid development;

however, mice lacking JAK3 (and lymphocytes) can survive normally in a pathogen-free environment (41-43). Consistent with defects in the development of lymphoid cells, mice lacking JAK3 have impaired responses to IL2-, IL-4, and IL-7 (43). JAK3 deficient mice have a phenotype similar to severe combined immunodeficiency (SCID) seen in humans suggesting that JAK3 plays a critical role in the immune response.

JAK1 and JAK2 have ubiquitous tissue expression; therefore, loss of either of these JAK family members results in a more severe phenotype than Tyk2 or JAK3 deficient mice. JAK1 signaling is important for many of the ILs and IFNs. JAK1 deficient mice die post-natally (44) which was attributed to a neurological defect resulting in a failure to suckle. Additionally, JAK1 deficient mice have impaired thymocyte and B cell production and diminished responses to a number of cytokines including LIF, IL-6, IL-7, IL-10 and IFNs (45). JAK2 deficient mice are embryonic lethal with death attributed to a failure of blood cell development demonstrating that JAK2 plays an essential role in EPO signaling (46). JAK2 deficient cells fail to respond to EPO, Tpo, IL-3, granulocyte/macrophage colony-stimulating factor, IFN- γ , PRL and GH (47, 48); however, JAK2 deficient cells are able to respond to granulocyte specific colony-stimulating factor, IFN- α , IFN- β , and IL-6 (47).

Dysregulation of JAK signaling

In the absence of ligand, JAKs are thought to be kept in an inactive state in close proximity to the receptors. Constitutive activation of JAK signaling results in constitutive activation of the Stat transcription factors. Dysregulation of JAK-Stat signaling in these cells and tissues has been associated with immune disorders and a variety of cancers.

The homolog for JAK in *Drosophila melanogaster*, Hopscotch (Hop), has been studied, and mutations in *Hop* lead to developmental abnormalities (49). In *Drosophila*, *Tum-l* was shown to be an overactive mutation of *Hop* (50). This mutant displayed genetically determined characteristics of leukemia in the fly (51). Hyperactivation of the JAK-Stat (Hop-Stat) pathway results in an alteration of the chromatin structure such that

more genes may be activated (52). Conversely, a loss-of-function mutation in *Hop* results in a condensation of chromatin thereby inactivating gene transcription.

Several JAK mutations have been discovered in human cancers. These mutant, constitutively active JAK proteins result in aberrant Stat signaling promoting oncogenesis in myeloproliferative disorders. The most studied of these JAK2 mutants is JAK2 V617F (53-56). The JAK2 V617F mutation is related to polycythemia vera (PV) as demonstrated in mouse studies where the JAK2 V617F induced a PV phenotype. Indeed, greater than 80% of patients with PV have the JAK2 V617F mutation. JAK2 V617F was also identified in 50% of patients with thrombocythemia (ET) and idiopathic myelofibrosis (IMF), two other myeloproliferative diseases. JAK2 V617F is constitutively phosphorylated on the activating tyrosine, tyrosine 1007, with downstream signaling comparable to cytokine-induced signaling. This elevated downstream signaling is confirmed in patients with PV who demonstrated elevated Stat5 and Stat3 activation. Recently, other human mutations in JAK2 have been identified: K539L (PV), T875N (acute megakaryoblastic myeloid leukemia), and deletion of 682-686 (acute lymphoblastic leukemia) (reviewed in (57)). The role they play in oncogenesis is not fully elucidated.

As mentioned above, JAK3 deficiency results in SCID consistent with JAK3 playing a key role in the immune response. Mutations in JAK3 have also been identified with patients presenting of a wide variety of immunodeficiency.

JAK2

JAK2 is activated by nearly two-thirds of the cytokine receptors including the receptors for GH, PRL, EPO, leptin, many of the ILs and IFNs (32, 58) (Fig. 1.2). Consistent with JAK2 playing a significant physiological role, JAK2 knockout mice were embryonic lethal.

Murine JAK2 consists of 1132 amino acids and has a molecular weight of ~120,000. Murine JAK2 contains 49 tyrosine residues. Ligand binding to receptor causes a conformational change (35), allowing the associated JAK2 molecules to auto-activate (59, 60). At least ten sites of autophosphorylation have been identified in JAK2:

tyrosines 119, 221, 570, 637, 813, 868, 913, 966, 972, 1007, and 1008 (59-67). Phosphorylation of tyrosines 1007/1008, 221, and 570 regulate JAK2 kinase activity. Tyrosine 1007 is the activating tyrosine in JAK2, and phosphorylation of this tyrosine is thought to stabilize JAK2 in an active conformation (62, 68). Mutation of Tyr1007 to phenylalanine almost completely abolishes the kinase activity of JAK2 (62).

When either Tyr221 or Tyr570 was mutated to phenylalanine, JAK2 kinase activity was altered, suggesting that these sites regulate JAK2 kinase activity (59, 61). Argesting et al. (59) propose that pTyr221 increases the kinase activity of JAK2. In contrast, phosphorylation of Tyr570 appears to decrease the kinase activity of JAK2 (59, 61). Additionally, the Y570F mutant prolonged JAK2 activation by EPO binding to an EPO-leptin chimeric receptor (EPO ligand binding and transmembrane domains fused to the intracellular leptin receptor) (61). Phosphorylated Tyr813 interacts with SH2B1, which dramatically enhances JAK2 kinase activity (60). 2D peptide mapping combined with LC-MS/MS revealed that tyrosines 868, 966, and 972 within the kinase domain of JAK2 are sites for autophosphorylation and are required for JAK2 kinase activity (69). Argetsinger et al. (69) proposes that phosphorylation of tyrosines 868, 966, and 972 in JAK2 may result in a conformational change which would open up the ATP and substrate binding sites and increase catalytic activity. Mass spectrometry (70), X-ray crystallography (68), 2D phosphopeptide mapping, and/or high pressure liquid chromatography (62, 65) indicate that tyrosines 918, 931, 934, 940, 956, 966, 972, and 1008 may also be phosphorylated.

In addition to phosphorylated tyrosine residues, JAK2 also contains phosphorylated serines. The MAPK pathway is thought to phosphorylate Ser523 of JAK2 since phosphorylation is blocked with a MEK inhibitor. Phosphorylated Ser523 inhibits JAK2 kinase activity (71, 72). Ishida-Takahashi et al. (72) demonstrates that a Ser523 to Ala mutant has prolonged JAK2 signaling consistent with phosphorylation of this serine playing an inhibitory role. Further, Ser523 of JAK2 may regulate the duration of kinase activity.

Phosphorylated tyrosine residues play other important roles in addition to regulating JAK2 kinase activity. Some phosphorylated tyrosines serve as docking sites for downstream signaling molecules after ligand stimulation. Proteins containing Src

homology 2 (SH2) or phosphotyrosine binding (PTB) domains are recruited to the phosphorylated GH receptor-JAK2 complex. Surprisingly, only a few proteins have been identified as proteins directly interacting with phosphorylated tyrosines within JAK2. Negative regulators of JAK2 signaling, suppressor of cytokine signaling (SOCS)-1 (73) and SOCS-3 (74), and protein tyrosine phosphatase (PTP)-1B (75) all have been reported to interact with the phosphorylated form of the activating tyrosine, tyrosine 1007, of JAK2. Several signaling proteins interact with a peptide containing phosphorylated tyrosine 966 including signal transducers and activators of transcription (Stat) 5a and 5b, phospholipase C (PLC)- γ 1, PLC- γ 2, SHC, and the p85 α , p85 β and p110 δ subunits of phosphatidylinositol (PI) 3-kinase, and Sts-1 (76-78). However, it is unclear whether any of these proteins are recruited to pTyr966 of JAK2. Only the interaction with Sts-1 has been verified for binding pTyr966. Sts-1 is a tyrosine phosphatase, which regulates Zap-70 activity (76-78). Phosphorylated tyrosine 813 binds SH2B1 (60, 79) and its family member, SH2B2 (79). Interaction with SH2B1 and SH2B2 enhances JAK2 kinase activity, potentially by stabilizing the active JAK2 dimer (79) as discussed in more detail below. Finally, previous work by our lab (80) demonstrated that a C-terminal fragment of steroid-sensitive gene-1 (SSG1) interacts preferentially with kinase-active JAK2.

Positive regulation of JAK2

Two proteins, SH2B1 and SH2B2, have been shown to positively regulate JAK2 (79, 81). SH2B1 was first identified as an adaptor protein, which interacts with tyrosine, phosphorylated JAK2 in a yeast two-hybrid assay screening a rat adipocyte cDNA library with JAK2 as bait (82). Subsequent work showed that SH2B can also interact with JAK2 in a non-phosphotyrosine dependent manner (83) and is phosphorylated by JAK2 (82). Phosphorylated Tyr813 in JAK2 has been shown to bind SH2B1 (60). SH2B1 binding to pTyr813 enhances JAK2 kinase activity. One group proposes SH2B1 and SH2B2 enhances JAK2 activity by forming homodimers and inducing JAK2 dimer formation (84) while others provide evidence that SH2B1 and SH2B2 stabilize active JAK2 (79). SH2B2 also binds to pTyr813 and enhances JAK2 kinase activity (79).

Negative regulation of JAK2

Negative regulatory pathways are important mechanisms used by the cell to terminate cytokine-induced signals. Termination of these signals is critical as exemplified by the observation that mutations that cause JAK2 to be constitutively active can lead to cancer ((53-56, 85-88) and reviewed in (57)). JAK2-Stat signaling is thought to be negatively regulated by at least two mechanisms, including SOCS proteins via a classical negative feedback loop and phosphatases which dephosphorylate tyrosines in JAK2 as well as GH receptor and Stat proteins.

Suppressor of cytokine signaling (SOCS) proteins play a critical role in the negative feedback loop induced by cytokine stimulation. SOCS gene transcription is induced by cytokine binding to cytokine receptor, activating JAKs and is mediated, at least in part, by the Stat family of transcription factors (see below) which are activated by JAKs. As in all negative feedback loops, SOCS proteins act to inhibit further signaling initiated by the receptor-JAK complex and activated Stats (89-91). GH induces the expression of the negative regulators SOCS-1, -2, -3 and CIS (92-94). SOCS-2 protein expression increases with time, but the expression of the other SOCS family members is more transient (95, 96). The SH2 domain within SOCS-1 interacts with phosphorylated Tyr1007, the activating tyrosine in the kinase domain of JAK2 (73). SOCS-1 binding to JAK2 inhibits JAK2 kinase activity, resulting in decreased phosphorylation of Stat5b and decreased transcription of Stat5b target genes (92, 93, 97). SOCS-3 can also bind the activating tyrosine in JAK2 and inhibit tyrosine kinase activity via the N-terminal kinase inhibitory region of SOC-3 (74, 98). However, inhibition by SOCS-3 of GH-stimulated Stat signaling (97) is thought to be dependent on SOCS-3 binding to GH receptor and inhibiting JAK2 kinase activity.

SOCS-2 plays a role in JAK2-Stat signaling as suggested by gigantism in SOCS-2 null mice, as a consequence of upregulated GH-Stat5b signaling (99). Intriguingly, SOCS-2 overexpression results in a slight increase in growth (100). SOCS-2 has been shown to interact with Tyr487 and Tyr595 (100-102) in the GH receptor which are different than the phosphotyrosines that Stat5b is recruited to (pY534, pY566, and pY627 in rabbit GH receptor; pY577, pY606, and pY639 in mouse GH receptor) (101). Thus, SOCS-2 does not appear to inhibit GH receptor-JAK2-Stat signaling by competing for

Stat5 binding sites within the activated receptor (101). Rather, SOCS-2 may downregulate GH signaling by binding to the activated GH receptor, recruiting an E3 ubiquitin ligase complex thus targeting the receptor for degradation (93, 102-105). SOCS-2 may also target SOCS-1 and SOCS-3 for degradation, prolonging GH signals (106). Recently, it was shown that CIS also binds the GH receptor at Tyr487 and Tyr595 suggesting that CIS binding to the GH receptor does not block Stat5 signaling by blocking recruitment to the GH receptor (101).

Phosphatases also play an essential role in shutting off GH signals. Several protein tyrosine phosphatases (PTP) have been implicated in the downregulation of GH receptor-JAK2 signaling including PTP-1B, PTP-H1, TC-PTP, and SHP-1, and SHP-2 (reviewed in (107)). SHP-1 and SHP-2 are SH2 domain-containing phosphatases (108). GH induces the expression of SHP-1 (109). In addition, GH induces the nuclear translocation of SHP-1, which is then able to dephosphorylate Stat5b, thus terminating Stat5b-mediated gene transcription (109, 110). In support of this, hepatic cells from mice deficient of SHP-1 have prolonged GH-dependent tyrosyl phosphorylation of JAK2 and Stat5b activation (111). SHP-2 has also been reported to dephosphorylate Stat5b in the cytosol (110). SHP-2 can also bind pTyr595 and pTyr487 in the GH receptor with pTyr595 being the major site for interaction (112). Stofega et al. (113) demonstrated that GH stimulates the tyrosine phosphorylation of SIRP- α (signal regulatory protein alpha). These pTyr in SIRP- α recruit SHP-2. Mutating the SHP-2 binding sites in SIRP α enhances GH receptor-JAK2 signaling (113). Mutations in GH receptor, which prevent SHP-2 from binding to GH receptor, result in prolonged activation of JAK2 and Stat5b (112).

Several PTPs have been identified as phosphatases that dephosphorylate tyrosines within the GH receptor including PTP-H1, PTP1, TC-PTP, and PTP-1B (114). Evidence that PTP-1B is important for GH signaling is provided by PTP-1B knockout mice, which exhibit increased GH-stimulated phosphorylation of JAK2, Stat5, and Stat3 (115). Additionally, PLC γ 1 has been shown to form a complex with PTP-1B and JAK2. This complex is able to attenuate GH-induced phosphorylation of both JAK2 and Stat5, which suggests that this complex is a negative regulator of GH signaling (116). Finally, JAK2

has also been shown to interact with a substrate-trapping mutant of PTP-1B and to be a substrate of PTP-1B (75).

The GH-induced JAK2 signal may also be terminated by receptor-mediated endocytosis. By decreasing the number of receptors available on the cell surface, fewer JAK molecules are activated, thus lessening overall GH signal transduction. Govers et al. (117) reported a motif in the cytosolic portion of the GH receptor that recruits the ubiquitin conjugation machinery to the GH receptor. Their data suggest that GH receptor may be internalized and degraded in an ubiquitin-dependent manner (118, 119). Finally, in addition to degradation of the receptor, degradation of JAK2 or the Stat proteins may also play a critical role in silencing GH-induced JAK-Stat signaling. To support this hypothesis, Wang et al. (120) identified a region in Stat5b, which mediates proteasome-targeted degradation.

Signal transducers and activators of transcription

Signal transducers and activators of transcription (Stat) proteins were initially identified as components of interferon (IFN) signaling (121, 122). Subsequently, other Stat family members were identified as transcription factors induced by other polypeptides resulting in distinct and important physiological outcomes.

The Stat family consists of seven proteins: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. The Stat family genes are localized to three chromosomal regions: murine chromosomes 1, 10, and 11; human chromosomes 2q12-33, 12q13-14.1 and 17q11.2-22. An invertebrate Stat protein was identified in *Drosophila*, Stat92E (123, 124). A Stat protein was also identified in *Dictyostelium discoideum* (125) suggesting that Stat proteins have an important evolutionary role. The Stat proteins are remarkably similar in most of their protein composition; however they contain unique C-termini, which contain critical and conserved residues and elements for activation and function. Stat proteins contain a conserved structural arrangement of their conserved motifs, including an N-terminus that is important in the formation of Stat dimers, a coiled-coil domain, a DNA binding domain, and an SH2 domain (Fig. 1.3).



Figure 1.3 Schematic representation of the domain structure of Stat proteins.

Stat proteins contain an N-terminal coiled-coiled (CC) domain, a DNA binding domain (DBD) and a Src Homology 2 (SH2) domain as well as a conserved tyrosine (Y) which is phosphorylated in response to ligand and important for dimerization. The Stat proteins contain a unique C-terminal transactivation domain (TAD).

The C-terminus contains the tyrosine critical for the interaction with other Stat proteins (126-128). Further, the unique C-terminus of the Stat proteins contains the transcription activation domain (TAD). Stat proteins lacking the TAD can form dimers with activated Stats and serve as dominant negative proteins (129).

Stats are latent cytoplasmic transcription factors that are transiently activated upon ligand stimulation and serve as messengers inside the cell. Stat proteins are tyrosyl phosphorylated, often by a receptor associated JAK kinase. This phosphorylation leads to homo- or heterodimerization by reciprocal SH2 domain-phosphotyrosine binding of the Stat proteins. The Stat dimers translocate to the nucleus where they bind to DNA (130) and serve as transcription factors for target genes. Because the C-termini of the Stat proteins differ, the Stats have different DNA binding specificities. This binding specificity combined with the ability of different cytokines to stimulate different Stats and form homo- or heterodimers upon ligand stimulation provides specificity in this signal transduction cascade.

This model of cytosolic Stat activation, dimerization, and nuclear translocation leading to Stat-mediated transcription is widely accepted. However, others have shown that Stat dimers exist in the absence of ligand stimulation (126, 131-137). Novak et al. (131) showed that Stat3 homodimer formation was independent of tyrosine phosphorylation but dependent on the presence of divalent cations. Kretzschmar et al. (133) showed by FRET analysis that Stat3 homodimers exist prior to cytokine stimulation. Dimer formation was dependent on an intact SH2 domain; however, ligand activation increased the FRET signal indicating a ligand-dependent increase in dimer formation or conformational change of the preformed dimer yielding a higher FRET efficiency (133). Ndubuisi et al. (132) reported that only a small percentage of Stat3 monomers are present in the cytosol. The majority of Stat3 was found in a high molecular weight complex that the authors termed a “statosome” (132).

Stat nuclear localization can be constitutive (ligand-independent) or conditional (ligand-dependent). Nuclear accumulation of tyrosine phosphorylated Stat1 is in part due to the interaction of Stat1 with the proteins in the nuclear pore complex, specifically importin- α 5 (138, 139). Tyrosine phosphorylation of Stat1 was found to be essential for

nuclear import as was the GTP-ase, Ran, as demonstrated by the failure of mutant proteins to translocate to the nucleus after IFN- γ stimulation (139).

Additionally, it has been shown that Stat proteins are present in the nucleus independent of ligand stimulation (133, 137, 140-146). This is not entirely surprising since Stat proteins contain both a nuclear localization signal (NLS) and a nuclear export signal (NES). Consistent with this, using fluorescence localization after photobleaching, Pranada et al. (146) demonstrated that Stat3 constitutively shuttles between the cytoplasm and nucleus independent of ligand stimulation or tyrosine phosphorylation. Liu et al. (140) reported that Stat3 nuclear import by importin- α 3 was independent of tyrosine phosphorylation. Stat1 also shuttles between the nucleus and the cytoplasm independent of ligand-stimulated tyrosine phosphorylation (142, 143). Unphosphorylated Stat5b also shuttles into and out of the nucleus (144). However, tyrosyl phosphorylated Stat5b accumulates to a greater extent in the nucleus than non-tyrosyl phosphorylated Stat5b in response to GH (147). Non-phosphorylated nuclear Stats can regulate gene expression in unstimulated cells (reviewed in (148)); however, the genes that are regulated by unphosphorylated Stat are different from those regulated by phosphorylated Stat dimers (reviewed in (148)).

Nuclear import of Stat proteins is balanced by nuclear export. Stat proteins also contain a nuclear export sequence, which facilitates their exit from the nucleus. Nuclear export of Stat proteins helps maintain the cytoplasmic pool of Stats for ligand activation as well as contributes to silencing of the target genes activated by these transcription factors. McBride et al. (149) suggest that nuclear tyrosine dephosphorylation of Stat1 is necessary for Stat1 to disassociate from DNA, thus unmasking the NES and allowing for CRM1-mediated nuclear export. Bhattacharya & Schindler (141) reported that two of three Stat3 NES were important regulators of basal Stat3 nuclear export. According to Pranada et al. (146), a Stat3 Y705F mutant was able to cycle between the cytoplasm and nucleus suggesting that tyrosine phosphorylation/dephosphorylation is not necessary for import or export of Stat3. Nuclear export of Stat5b is also CRM-1 dependent (144). The same may be true for other Stat proteins, which undergo nucleo-cytoplasmic shuttling. Taken together, the shuttling of Stats into and out of the nucleus suggests that a pool of cytosolic Stats must be maintained for ligand-dependent signaling.

Stat proteins can interact with other transcription factors or coactivators to regulate gene expression (reviewed in (148)). Both unphosphorylated (150, 151) and phosphorylated (152-154) Stat3 protein form dimers with NF- κ B and regulate NF- κ B responsive genes. Unphosphorylated Stat3 interacts with the unphosphorylated p65 subunit of NF- κ B to regulate RANTES gene expression (150). Hagihara et al. (153) demonstrated that IL-1 stimulates a complex formation of Stat3, NF- κ B, and p300 on the serum amyloid promoter, resulting in enhanced transcription of serum amyloid A. Yu et al. (154) demonstrated a complex between Stat3 and the p65 subunit of NF- κ B, which inhibits the transcription of *iNOS*. Stat1 interacting with NF- κ B p65 alters p65 DNA binding, ultimately decreasing the expression of NF- κ B anti-apoptotic target genes (155). Unphosphorylated Stat6 has been shown to interact with the coactivator, p300, resulting in constitutive expression of the COX-2 gene in non-small cell lung cancer (156).

Physiological relevance of Stat3

Stat3 was first identified as acute phase response factor (APRF) in response to IL-6, a transcription factor important in the acute phase inflammatory response (157). Stat3 is expressed in most tissues. Subsequent work has shown that a number of cytokines and growth factors, including multiple ILs, GH, CNTF, OSM, LIF, leptin, EGF, and PDGF, activate Stat3. Stat3 regulates expression of a subset of genes important in the cell cycle progression, including *cyclinD1*, *p21*, and *c-myc*, as well as genes important in apoptosis, including *Bcl-x_L*.

Consistent with Stat3 being expressed in most tissues, the lack of Stat3 results in embryonic lethality (158). Therefore, to gain a better understanding of the cellular function of Stat3, tissue-specific targeting of Stat3 has been done in various cell lineages. When Stat3 is conditionally knocked out of T cells, mice exhibit a marked reduction in IL-6 responsiveness and T-cell proliferation (159). This result supports a role for Stat3 in regulating anti-apoptotic gene expression. Genetic deletion of Stat3 in macrophages highlighted the role Stat3 plays in anti-inflammation and the immune response. Mice lacking Stat3 in macrophages have elevated production of inflammatory cytokines resulting in enterocolitis in adult mice (160). Stat3 deletion in the mammary gland

resulted in a decrease in apoptosis of mammary epithelial cells and a delay of involution (161). Stat3-deficient hepatocytes fail to induce acute phase response genes in response to IL-6 (162). Stat3 has also been deleted in the skin resulting in delayed wound healing as a result of impaired growth factor-dependent (EGF, HGF, TGF- α , and IL-6) migration of epidermal cells (163). Mice lacking Stat3 in the skin also exhibited impairment in the hair cycle, including sparse hair with age, development of ulcers with age, and hyperplasia of the epidermis (163). In total, these tissue-specific Stat3 deficient mice reveal that Stat3 plays a variety of physiological roles including both pro- and anti-apoptotic roles, inflammation, roles in the immune response, cell growth and motility.

In humans, some patients with idiopathic short stature exhibit defective activation of Stat3 (164). Fibroblasts from these patients revealed impaired GH-induced Stat3 activation. Concomitant with these findings, Stat3 regulated gene expression of cyclins was reduced while p21 was increased (164). This phenotype was reversed with administration of exogenous GH (164). It remains unclear whether impaired Stat3 activation and/or elevated p21 levels play a role in short stature.

Dysregulation of Stat3

Several lines of evidence suggest that Stat3 participates in oncogenesis. Several studies have shown that Stat3 is constitutively activated in a number of tumors and tumor models, promoting cell proliferation and cell survival ((165), reviewed in (166, 167)). Stat3 is constitutively phosphorylated on both Tyr705 and Ser727 in anaplastic lymphoma kinase (ALK) positive tumor cells (165). Constitutively phosphorylated Stat3 enhances *Bcl-x_L* gene transcription and promoted cell survival of human U266 myeloma cells (165). Others have shown that dominant negative forms of Stat3 can suppress features of a transformed phenotype. In one such example, Bowman et al. (129) demonstrated that Stat3 β , which lacks the C-terminal transactivation domain, acts as a dominant negative by suppressing expression of *c-myc*. Further, *c-myc* expression is required for v-Src transformation, and Stat3 β was able to inhibit Src transformation (129).

Bromberg et al. (168) showed that a dominant active form of Stat3 was capable of transforming fibroblasts, suggesting that Stat3 is oncogenic. A gain of function mutation in which Stat3 is constitutively dimerized is able to bind DNA and constitutively drive gene transcription, resulting in a transformed cell phenotype (168). Bromberg et al. (168) also demonstrated that this constitutively dimerized Stat3 was tumorigenic in nude mice, apparent after 2 weeks of injection. It should be noted, however, that thus far, constitutively activated Stats in cancer are usually a result of hyperactive kinase activity. It will be interesting to determine if constitutive dimers are found in vivo with the same oncogenic phenotype described by Bromberg et al. (168).

Several reports have established that constitutively seryl phosphorylated Stat3 is present in a number of tumors, consistent with serine phosphorylation promoting tumorigenesis (169-171). Yang et al. (169) demonstrated a correlation between phosphorylated Ser727 levels of Stat3 with cervical intraepithelial neoplasia. This is not entirely surprising since phosphorylated Ser727 has been linked to increased transcriptional activity (172) and increased transcription can result in enhanced cell proliferation, thus promoting transformation. Constitutive pSer727 Stat3 may result in cancer progression; therefore Yang et al. (169) hypothesized that pSer727 Stat3 may be a useful biomarker to monitor the progression of cervical lesions.

Stat3 gene expression is positively regulated by tyrosyl phosphorylated Stat3 dimers. In addition to phosphorylated Stat3 dimers, nonphosphorylated Stat3 can participate in regulating gene expression of a different subset of genes, including anti-apoptotic genes and oncogenes (150, 173). Yang et al. (150) demonstrated that unphosphorylated Stat3 Y705F interacts with the unphosphorylated p65 subunit of NF- κ B, which can then serve as a transcriptional activator of RANTES (Regulated on Activation, Normal T-Expressed and Secreted). It is possible that in cancer, constitutively activated Stat3 proteins increase the amount of Stat3. The increase in Stat3 then allows for the expression of more Stat3-mediated genes, modulated by both phosphorylated and unphosphorylated Stat3 dimers, leading to the oncogenic phenotype.

Stat3 regulates gene transcription for several proteins important for cell survival, including Bcl-2, Bcl-xL, and Fas as well as for proteins important in the cell cycle including cyclin D1 and p21. Therefore, constitutively active Stat3 may promote cell

survival in cancer cells. In addition to regulating gene expression, Niu et al. (174) demonstrated that Stat3 can act as a transcriptional repressor of p53 expression. In cancer cells expressing constitutively active Stat3, repression of p53 promotes oncogenic tumor growth, and blocking Stat3 in these cells induces p53-mediated apoptosis of tumor cells (174).

Physiological function of Stat5

Stat5, specifically Stat5a, was initially identified as a prolactin-responsive transcription factor in mammary tissue (175, 176). Stat5a and Stat5b are encoded by two linked genes. The protein products share 96% amino acid identity with the divergence localized to the C-terminus. Many studies have revealed that Stat5 has an ubiquitous tissue expression. Subsequent work has revealed that Stat5 proteins are activated by both a variety of cytokines including many of the ILs, EPO, and GH, and by receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) (177-179).

Several studies have shown that GH-induced Stat5 activation is important in physiological functions of GH including metabolism, body growth, and sexual dimorphic liver gene expression. Since Stat5a and Stat5b are highly similar, it is not surprising that Stat5a and Stat5b display functional redundancies (180, 181). Stat5a and Stat5b also display some functions that are unique to the individual transcription factor (181).

Genetic models of Stat5a and Stat5b have given much insight into the function of these Stats and have revealed distinct phenotypes for the single Stat5a and Stat5b knockout mice. Stat5a activated by PRL is important for mammary gland development and lactation (181, 182). Genetic loss of Stat5a in females resulted in the inability to lactate although milk proteins were expressed (at lower levels) and secreted in Stat5a-deficient animals (181, 182).

The phenotype of Stat5b-deficient mice is similar to that of GH receptor-deficient mice (181, Udy, 1997 #2046, 183, 184). Stat5b-deficient male mice were smaller in size and displayed a loss of expression of male liver-specific gene expression (184, 185). GH pulses are important in maintaining male liver-specific gene expression, such that loss of GH signaling in Stat5b deficient mice revealed female levels of P450 and major urinary

protein (MUP) (185). Stat5a knockout mice also demonstrated loss of some female-specific, GH-regulated liver gene expression suggesting that Stat5a/b heterodimers are important in sex-dependent gene expression (186). Serum IGF-1 levels were reduced in male Stat5b knockout animals relative to controls but were unaffected in female knockouts (181). Recently, Stat5b binding sites in the IGF-1 promoter and in the second intron of IGF-1 were established (187, 188), further supporting Stat5b regulating IGF-1 transcription in a GH-dependent manner, thereby contributing to overall body growth (188).

Stat5a/b double knockout mice reveal the importance these transcription factors as deletion of Stat5a/b results in few viable mice that die within 6 weeks after birth (189-191). Most Stat5a/b double deficient mice are perinatally lethal (189, 191). Surviving mutant mice have smaller thymi, spleen, and lymph nodes (190) indicative of the role Stat5 plays in lymphoid development. Analysis of embryonic day 18.5 fetuses of Stat5a/b double knockout mice confirms that Stat5a/b play a critical role in the development of the lymphoid cells resulting in severe combined immunodeficiency (191) similar to the phenotype of JAK3 deficient mice. Stat5a/b double deficient mice also displayed impaired hematopoietic stem cells, which fail to generate T and B cells (191). Conditional Stat5a/b knockout mice have also been generated to determine the function these transcription factors play in various tissues. Of particular importance is the hepatocyte-specific deletion of Stat5a/b, which reveals reduced circulating IGF-1 levels and stunted growth in both males and females weeks after birth (192) consistent with Stat5 playing a critical role in pos-natal body growth. However, Cui et al. (193) also deleted Stat5a/b specifically in the liver. Their liver-deficient Stat5a/b mice did not have a reduction in body size, but rather mice developed fatty livers and had increased GH levels.

Stat5 and Disease

Like Stat3, Stat5 may play a role in cancer. An activated form of Stat5 has been described in chronic myelogenous leukemia. Stat5 activation by a TEL-JAK2 fusion variant has also been implicated in myeloproliferative and lymphoproliferative diseases

(194). Interestingly, Stat5 tetramers were found in leukemia cells from patients. It is not clear whether these tetramers promote gene transcription.

Recently, Rosenfeld and colleagues (195) identified a patient with a mutation in Stat5b. This was the first patient studied with a mutation in Stat5b. The female patient presented with severe growth retardation and immunodeficiency (195). By RT-PCR, Rosenfeld et al. (195) identified a missense mutation, which resulted in a proline substitution in the SH2 domain of Stat5b just upstream of the critical tyrosine. GH and IFN- γ failed to stimulate the tyrosyl phosphorylation of Stat5b A630P, which suggests that this missense mutation disrupts dimerization and gene transcription. Importantly, this patient's serum IGF-1 levels were significantly low (195). Rosenfeld et al. point out that this human patient had a different phenotype than female mice deficient in Stat5b; she had both severe growth retardation and low serum IGF-1 levels (195). While Stat5b deficient female mice have lower IGF-1 serum concentrations compared to wild-type mice, they were not smaller (i.e. female Stat5b knockouts were the same size as male Stat5b knockouts which were both smaller than male wild-type mice) (184). To date, five different mutations in Stat5b have been identified in six patients with growth retardation (reviewed in (196)). Five of these six patients were female, suggesting that Stat5b has an important role in body growth in both human males and females, unlike mice where Stat5b has an important role in body growth of male mice but not female mice.

Post-translational modifications regulate Stat activity

Post-translational modifications play a critical role in signaling cascades. Indeed, various post-translational modifications have been shown to regulate the activity of Stat proteins. As already mentioned above, tyrosine phosphorylation is important for the overall activity of Stat proteins. It has also been reported that Stats are phosphorylated on serine residues, acetylated, and methylated.

Tyrosine phosphorylation of Stats

All Stat proteins are phosphorylated on a critical tyrosine residue in their C-terminus(197, 198). This phosphorylated tyrosine residue interacts with the SH2 domain of another Stat protein to form Stat dimers. Following dimer formation, Stats translocate to the nucleus and regulate gene transcription. Mutations of the key tyrosine have shown that phosphorylation of this residue is critical for Stat function, specifically binding DNA.

Serine phosphorylation of Stats

Stats 1, 3, 4, 5a, and 5b contain a conserved serine residue in their C-termini. For Stats 1 (Ser727), 3 (Ser727), and 4 (Ser721), this serine is in a PMSP motif (172); for Stats 5a (Ser725) and 5b (Ser730), the serine is in a PSP motif (199). This motif suggests that a proline-directed kinase is responsible for the phosphorylation of this conserved serine in these Stats. In fact, several groups have shown that MAPKs (200), including ERK1/2 (201-204), p38 (205-207), and JNK (205, 208) are, in part, responsible for phosphorylation of this serine both *in vitro* and *in vivo* (reviewed in (209)). Further support that a MAPK may be responsible for Stat serine phosphorylation comes from a study by David et al. (202) which demonstrated that Stat1 was able to co-immunoprecipitate with MAPK (probably ERK1/2, 42kDa) after IFN- β stimulation. Lim et al. (208) demonstrated that JNK phosphorylates Stat3 on Ser727, which negatively regulates the tyrosine phosphorylation of Stat3. This, in turn, inhibits Stat3 DNA binding and transcriptional activation (208).

In addition to the MAPK being activated by cytokine stimulation, the PKC pathway is also activated by a number of cytokines, including PRL and ILs. PRL-induced PKC was shown to increase Stat5a mediated β -casein gene expression (210). Jain et al. (211) demonstrated that a number of ligands (e.g. IL-6, OSM, LIF, EGF) activate PKC δ which stimulates the association with Stat3 in a number of cell types. Ligand-stimulated PKC δ can also phosphorylate Ser727 in Stat3 *in vivo* (211). It is likely that several signaling pathways result in phosphorylation of this serine residue. It is also likely that this serine phosphorylation is ligand- or cell-specific which adds another level of regulation to Stat-mediated responses.

The role of Stat serine phosphorylation is unclear. There are several reports in the literature where serine phosphorylation on this conserved residue in the C-terminus of Stat proteins enhance gene transcription (172, 212-214), whereas others (211) demonstrate that serine phosphorylation of this site in Stat proteins negatively regulates the ability of Stat to bind to DNA. Mutation of the key serine, Ser727, of Stat1 to alanine (S727A) resulted in approximately 80% decreased gene transcription of an INF- γ responsive luciferase promoter as well as decreased levels of mRNA of an endogenous IFN- γ responsive gene, IRF-1, suggesting that maximal transcription requires phosphorylation of both Tyr701 and Ser727 of Stat1 (172). Further support that phosphorylation of Ser727 is required for maximal transcription came from studies where IFN- γ stimulation resulted in serine phosphorylation of Stat1 and increased gene expression (207, 215-218). The S727A mutation in Stat3 did not affect DNA binding to the m67 SIE probe in an electrophoretic mobility shift assay (172); however, maximal luciferase gene transcription was not achieved with the Stat3 S727A mutant (172). In contrast, Jain et al. (211) showed that Ser727 phosphorylation by PKC δ negatively affected the ability of Stat3 to bind DNA and induce gene expression of a CAT reporter. It is possible that serine phosphorylation of the Stat proteins is necessary for Stat to bind to low-affinity DNA binding sites (219, 220). While it is controversial whether serine phosphorylation of Stats affects DNA binding, it is possible that the effect is promoter or cell-context specific with serine phosphorylation playing an inhibitory role in some cases and stimulatory in others. Several groups have performed large scale mass spectroscopy revealing the likely presence of numerous additional sites of phosphorylation within Stat3: Thr268 (221), Ser269 (221), Ser273 (221), Tyr539 (70, 222), Tyr686 (70, 223), and Ser691 (224). However, it is not yet known whether phosphorylation of these sites in Stat3 is stimulatory or inhibitory in function.

Stat5 has been shown to be phosphorylated on serine residues in response to GH (212, 225-228) as well as PRL (199, 229, 230) and IL-2 (231). Stat5a is serine phosphorylated on at least two sites: Ser725 and Ser779 (232, 233). GH-induced gene transcription was only decreased when both Ser779 and Ser725 were mutated, but not when Ser725 alone was mutated, suggesting that phosphorylation of Ser725 does not regulate transcription (212). It has also been reported that seryl phosphorylation of Stat5

results in increased gene transcription of a CAT reporter (231). However, Yamashita et al. (199) reported that serine phosphorylation of Stats 5a and 5b was not essential for DNA binding or for activation of a β -casein reporter gene suggesting that the requirement for serine phosphorylation of Stat5 differs from that of Stats 1 and 3. Stat4 is also reported to be phosphorylated on Ser721 resulting in an enhancement of transcriptional activity (234).

Acetylation of Stats

In addition to being phosphorylated, Stats are also acetylated. This is not completely surprising since Stats can associate with the acetyltransferases CREB binding protein (CBP) and p300 (235-238). This association with CBP/p300 can increase Stat-mediated gene transcription (235, 236). OSM stimulated acetylation of Stat3 in MCF-7 breast cancers cells while IFN- α stimulated acetylation of K685 in Stat3 in HeLa cells (237). Acetylation of K685 in Stat3 by p300 has been shown to be critical for dimerization, DNA binding, and gene transcription (237). In addition, Wang et al. (239) showed that acetylation of K685 by p300/CBP increases nuclear accumulation of Stat3 which is consistent with increased Stat3 DNA binding and increased transactivation by Stat3. Acetylated Stat1 has been shown to interact with NF- κ B p65 resulting in decreased expression of anti-apoptotic genes (155). Stat6 is acetylated in response to IL-4 (240).

Methylation of Stats

Stat proteins have been reported to undergo methylation (241), although Komyod et al. (242) and Meissner et al. (243) argue that Stats are not methylated. Basal levels of methylation were detected for Stat1, and methylation increased upon further IFN- α treatment (241). Mowen et al. (241) also showed that Stat1 associates with the protein arginine methyl-transferase PRMT1 and that PRMT1 methylated Stat1 on the highly conserved Arg31. Methylation of this residue was found to be important in regulating transcriptional activation (241). Stat6 is reported to be methylated on Arg27 (244).

When Arg27 was mutated to Ala, Stat6 was not maximally phosphorylated in response to IL-4, which in turn, reduced nuclear translocation and gene expression (244). Stat3 is recognized by an anti-methyl antibody (245); however, it is unknown if Stat3 is methylated. Methylation has not been reported for Stat5a or Stat5b.

Growth hormone activates the JAK-Stat pathway

One of the earliest characterized cytokine receptors is the receptor for GH (reviewed in (196, 246)). GH is an important regulator of body growth and metabolism. GH is a 191 amino acid, 22kDa polypeptide hormone that is secreted in a pulsatile manner from the anterior pituitary and enters the blood. GH binds to its receptors in target tissues, eliciting a transient response. In addition to being an important hormone for longitudinal bone growth, GH also has important metabolic actions in muscle and fat. GH promotes a decrease in body fat, which is counterbalanced by an increase in lean muscle. GH exerts these effects, at least in part, by inhibiting lipogenesis, promoting lipolysis, and stimulating protein synthesis (247). GH is also important in cardiac and immune function and aging.

GH binding to its receptor in target tissues rapidly activates JAK2. Current evidence suggests that one GH molecule binds to a pre-formed GH receptor dimer (34, 35, 38, 248) causing a conformational change (35, 248) that allows the associated JAK2 molecules to autophosphorylate (59-67, 249). Once activated, the JAK2 molecules phosphorylate tyrosines on the GH receptor as well as themselves (59, 60, 250). These phosphorylated residues serve as docking sites for a variety of signaling proteins.

One class of proteins activated by JAK2 upon GH stimulation are the Stat proteins (Fig. 1.4). GH activates Stats 1, 3, 5a, and 5b ((251-255) and reviewed in (256-258)). Activation of these Stat proteins requires that the GH receptor-JAK2 complex be tyrosyl phosphorylated. Stats 5a and 5b are recruited to phosphorylated tyrosine residues located in the cytoplasmic tail of the GH receptor (250, 259-262), whereas Stats 1 and 3 interact with phosphorylated tyrosines within activated JAK2 (48, 261-264). Stats 1 and 3 can homo- or heterodimerize and bind to the sis-inducible elements (SIE), such as the

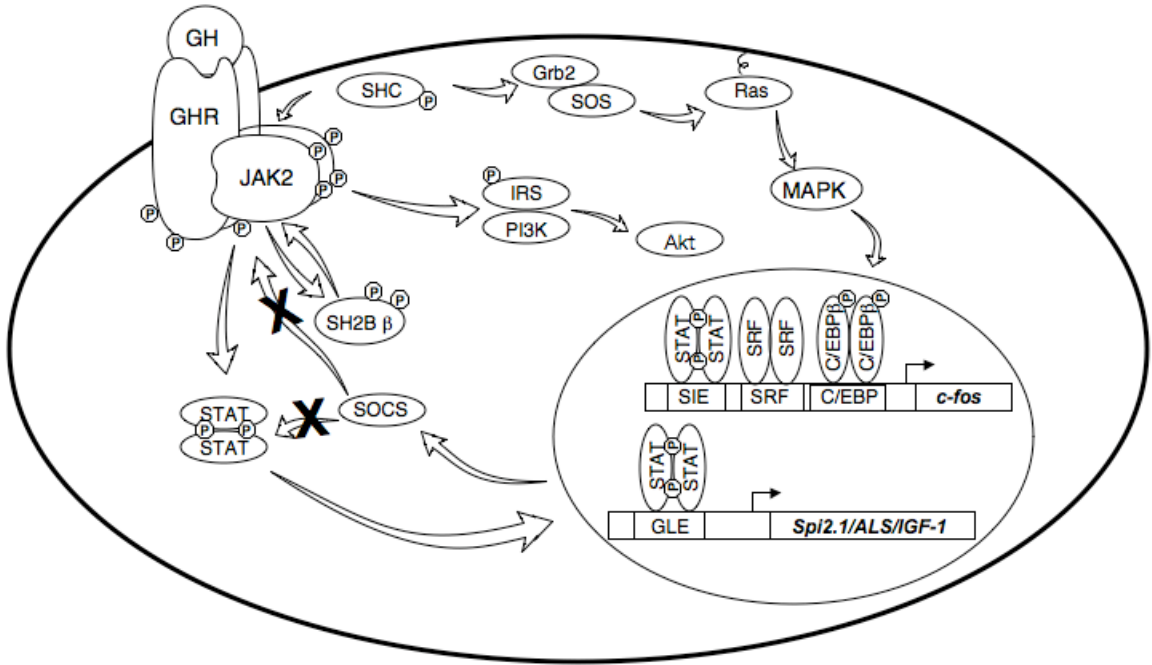


Figure 1.4 Schematic representation of GH Signal Transduction.

Growth hormone (GH) binding to its receptor activates a number of downstream signaling pathways, including the JAK2-Stat pathway, Ras-MAPK pathway, and the PI-3K-Akt pathway. Activation of these pathways result in the initiation of gene transcription. Arrows indicate pathways regulated by GH; Xs indicate regulation by negative feedback.

one found in the *c-fos* gene promoter. Homo- and heterodimerized Stats 5a and 5b bind to IFN γ -activated sequences (GAS)-like elements (GLEs). Binding of the Stat dimers to these elements in DNA, along with other transcription factors and transcriptional co-activators, regulate gene transcription. GH-induced Stat5 dimers promote gene transcription of *liver-specific serine protease inhibitor 2.1 (Spi2.1)* (265, 266), *insulin-like growth factor-1 (IGF-1)* (188, 266), *acid labile subunit (ALS)* (267), and *insulin* (268, 269). Stat5b also regulates GH-stimulated transcription of the negative regulators, *SOCS-2* and *SOCS-3* (270).

Growth hormone activates other signaling pathways

In addition to the JAK-Stat pathway, GH also activates the Ras/MAPK pathway (Fig. 1.4) (reviewed in (258)). The activated GH receptor-JAK2 complex recruits the adaptor molecule Shc which is then tyrosyl phosphorylated. Phosphorylated Shc then binds Grb2 and SOS, a guanine nucleotide exchange factor (GEF) for Ras. GTP-bound Ras triggers the activation of Raf, which in turn activates mitogen-activated protein kinase (MEK) leading to the activation of extracellular-regulated protein kinases (ERKs) 1 and 2. The MAPK pathway may also be activated by cross-talk between the GH receptor and EGFR. GH-activation has been shown to promote phosphorylation of the Grb2 binding site the EGFR thus activating the MAPK pathway (271). GH also activates the PKC pathway (32). Activation of ERKs promotes the activation of several downstream proteins (reviewed in (272)). In GH signaling, GH-activated ERKs have been shown to phosphorylate C/EBP β (273, 274), a transcription factor important in initiating gene transcription in the adipocyte differentiation program (275). GH-induced ERK activity may also regulate the expression of CYP2C11 (276), a sexually dimorphic, liver-specific gene that is regulated by Stat5b (251).

GH also phosphorylates the insulin receptor substrate (IRS) proteins, IRS-1, IRS-2, and IRS-3 (Fig. 1.4) (reviewed in (258)). Once phosphorylated, IRS proteins can bind to the p85 regulatory subunit of PI-3 kinase (PI-3K), which activates the PI-3K pathway, which is upstream of the Akt pathway. GH-activated Akt, which inhibits GSK-3

(glycogen synthase kinase-3), has been implicated in promoting cell survival, possibly by inhibiting caspase-3 (277).

GH has also been shown to be important in regulating cell motility implicating that GH promotes actin rearrangement. Indeed, GH stimulation rapidly promotes depolymerization of actin stress fibers which was followed by repolymerization of localized actin complexes (278). Further, GH promotes the phosphorylation of several proteins resulting in the formation of a large multiprotein complex including, but not limited to, p130 (Cas), CrkII, c-Src, c-Fyn, the p85 subunit of PI-3K, paxillin, tensin, and IRS-1 (279). Formation of this complex may regulate GH-induced actin dynamics. Evidence from our lab demonstrates that GH-induced JAK2 activation of SH2B1 is involved in regulating the actin cytoskeleton (280, 281).

GH-responsive genes

GH promotes the expression of a variety of proteins important in cell growth and differentiation. Microarray analysis on 3T3-F442A adipocytes revealed that 561 genes are regulated temporally by GH-stimulation (282). GH stimulates the expression of early response genes, including *c-fos* (283, 284), *c-jun* (284, 285), and *c-myc* (286) as well as the expression of many other genes including the genes for SOCs proteins, IGF-1, and ALS. The early response genes *c-fos*, *c-jun*, and *c-myc* encode transcription factors that in turn regulate the expression of other target genes.

In addition to the growth promoting effects by GH, GH induces the expression of IGF-1 from the liver, bone, and muscle (287). *IGF-1* expression is mediated by GH-induced Stat5b (181, 266, 288, 289). IGF-1 produced in the liver is released into the blood, and this GH-dependent circulating IGF-1 plays an essential role in somatic growth (288). IGF-1 and IGF-1 receptor knockout mice exhibit impaired growth supporting that IGF-1 is important in the effects mediated by GH (290). Human patients with short stature from a mutation in Stat5b display low serum concentrations of IGF-1 (reviewed in (196)) lending further support that IGF-1 is critical in body growth. Stat5b has been implicated in regulating GH-induced *IGF-1* expression (266), and recent work has

established GH-stimulated Stat5b binding sites in the promoter of *IGF-1* as well as in the second intron (188).

Of the GH-regulated genes, *c-fos* is one of the most widely studied because it is induced by GH 15-30 minutes post-stimulation in a variety of cell lines, making it a good candidate to study the earliest responses elicited by GH (283, 284). The promoter region of *c-fos* contains several binding sites for proteins regulated by GH including an SIE which binds the GH-induced phosphorylated Stat1 and Stat3 homo- or heterodimers (252, 291), an SRE (serum response element) which binds SRF dimers (serum response factors), and a C/EBP β binding site (292). C/EBP β contains a known MAPK consensus phosphorylation site and has been shown to be phosphorylated in response to GH on Thr188 (liver activating protein, LAP) or Thr37 (liver inhibitory protein, LIP) by a kinase in the MAPK pathway (273). When this site was mutated, transcription of *c-fos* was inhibited, suggesting that phosphorylation of C/EBP β on this site is required for maximal transcription factor activity (273). GH also induces the dephosphorylation of C/EBP β , which allows C/EBP β to bind to the C/EBP β site in the *c-fos* promoter (293). Piwien-Pilipuk et al. (293) suggest that the GH-activated PI3K/Akt pathway inhibits GSK-3 which regulates the phosphorylation of C/EBP β . Taken together, the GH-activated Stat, MAPK, and PI-3K pathways converge on transcription factors that regulate *c-fos* gene transcription.

Steroid-Sensitive Gene-1

Identification of SSG1

SSG1 was originally identified as a 17 β -estradiol (E_2) responsive gene by Marcantonio et al. (294) using differential display of RNA samples from uteri from ovariectomized or ovariectomized plus E_2 -treated rats. SSG1 mRNA levels were found to be high in the uteri of ovariectomized rats, but was significantly reduced in the uteri of ovariectomized E_2 -treated rats (294). In this initial paper, it was reported that SSG1 mRNA had a broad tissue distribution with varying levels of mRNA expression detected in the ovary, uterus, mammary gland, lung, spleen, kidney, bladder, and skeletal muscle (294). Since then, it has been reported that SSG1 has a wide tissue distribution.

Importantly, SSG1 was expressed in GH-responsive tissues including the liver, heart, and brain (294). In contrast to the downregulation seen in E₂-treated uterine tissues, SSG1 mRNA was unchanged in control, E₂-treated, or tamoxifen-treated mammary samples (294). In a follow-up study, Marcantonio et al. (295) reported that SSG1 mRNA was significantly increased in the prostates of rats following castration compared to the prostates of sham-operated rats. It remains unclear if androgens and/or estrogen are able to regulate SSG1 protein expression.

The size and sequence of SSG1 were incorrectly published in the first report of SSG1. Full-length SSG1 has subsequently been identified by several groups using a variety of different screening techniques, which suggests that SSG1 may have a variety of functions, some of which are tissue-specific (Table 1.1).

SSG1 has been subsequently, independently cloned as Urb, CL2, DRO1, and Equarin. Murine SSG1 (Urb, upregulated in BRS-3-deficient mice) was initially identified in bombesin-receptor-subtype-3 (BRS-3)-deficient mice as a gene whose expression was increased four-fold in brown adipose tissue compared to control mice at eight months of age (296). In mice seven weeks old, there was no difference in SSG1 gene expression in either brown or white adipose tissue comparing BRS-3-deficient mice and wild-type mice (296). BRS-3-deficient mice demonstrate mild late-onset obesity (297). Because SSG1 was upregulated at eight months of age, SSG1 may play an important role in regulating body weight and energy metabolism in adult mice. *In situ* hybridization of brain tissue showed SSG1 mRNA in the anterior olfactory nucleus, the dorsal endopiriform nucleus and the choroid plexus (296). The gene expression pattern of murine SSG1 in the choroid plexus is similar to that of the leptin receptor consistent with SSG1 playing a role in energy metabolism.

Recently, Okada et al. (298) demonstrated that mouse SSG1 was highly expressed in white adipose tissue of 7 week old mice and that SSG1 mRNA levels were lower in adipose tissue in genetic mouse models of obesity as well as in mice on a high fat diet. Further, SSG1 mRNA increased two-fold when preadipocytes were differentiated into adipocytes (298). However, when adipocytes were treated with hormones or stressors, which are upregulated in obesity, SSG1 mRNA expression was decreased to varying

Table 1.1 Identification and proposed functions for SSG1

Gene	Identification	Proposed Function	Author
rat SSG1	Differential display as estrogen responsive in uterine tissues	n/a	Marcantonio (294)
murine Urb	Differential display of adipose tissue in BRS-3-deficient mice	energy metabolism	Aoki (296)
rat CL2	Differential display of E1A responsiveness in thyroid	tumor suppressor	Visconti (299)
rat DRO1	Subtractive suppression hybridization of an immortalized rat kidney epithelial cell line	tumor suppressor	Bommer (300)
murine Urb	Differential display of bone marrow vs. osteoblasts	bone formation	Liu (301)
human Urb	Microarray comparison of dermal fibroblasts vs. papilla	hair growth in response to androgen	Cha (302)
chicken equarin	Signal sequence trap of chick lens cDNA library	eye formation	Mu (303)
murine Urb	Novel adipocytokines that are upregulated in adipose tissue	role in obesity	Okada (298)

extents (298). Taken together, these results suggest that SSG1 may have a function in obesity.

Human SSG1 (Urb) was identified in a differential display of undifferentiated bone marrow stromal cells (BMSC) and BMSC-derived osteoblasts as a gene that is downregulated during osteoblast differentiation of BMSC (301). Liu et al. (301) showed the spatial and temporal expression profile for SSG1 in the developing cartilage during embryogenesis by *in situ* hybridization and immunohistochemistry. SSG1 expression in the developing chondrocytes, but absence in osteoblasts, is consistent with SSG1 functioning in skeletal development in the embryo. Microarray analysis revealed that human SSG1 was higher in dermal papilla cells compared to dermal fibroblasts (302). Human SSG1 mRNA expression was induced in response to dihydrotestosterone suggesting that SSG1 expression may be androgen sensitive (302) in contrast to the negative regulation by androgens of SSG1 proposed by Marcantonio et al. (295).

Rat SSG1 was independently cloned as CL2 (299) and as DRO1 (down-regulated by oncogenes-1) (300). SSG1/CL2 was identified using differential display as a gene induced by the expression of E1A 13S in infected rat thyroid PC Cl3 cells (299). The mRNA expression of SSG1 was downregulated in PC E1A cells infected with v-raf, but not changed by the oncogenes polyoma middle-T or v-src (299). SSG1 gene expression was low or absent in human thyroid carcinoma cell lines. SSG1/DRO1 was identified using a subtractive suppression hybridization technique as a gene downregulated in RK3E rat epithelial cells neoplastically transformed by β -catenin (300). In these studies, SSG1 mRNA expression was reduced in colon and pancreatic cancer cell lines and human colorectal cancer samples (300). Ectopic expression of SSG1 in cells lacking endogenous SSG1 was able to reduce colony formation in two pancreatic cancer cell lines (300). Taken together, these results are consistent with SSG1 acting as a tumor suppressor.

A chicken ortholog of SSG1, equarin, was cloned from a chick E6 lens library using a signal sequence trap screen (303). Alternative splicing of the equarin gene results in two transcripts, equarin-S and equarin-L (303). The resultant protein products of these transcripts is identical in the first 592 amino acids with the longer transcript encoding an additional 366 amino acids at the C-terminus. Equarin-L shares high homology with

mouse, rat, and human SSG1. Equarin is expressed in the lens during embryonic development suggesting that equarin plays a role in eye formation (303).

SSG1 protein domain structure

Rat SSG1 is a 949 amino acid protein with a molecular weight of ~110,000. The gene for SSG1 has been mapped to rat chromosome 11q21 (300) and human chromosome 3q13.2-3q13.3 (299, 300). In both human (299-301) and chicken (303), there are two reported transcripts for SSG1, whereas there is only one reported transcript in mouse (296, 299) and rat (299, 300). By motif scans and sequence analysis, SSG1 contains a putative signal peptide, five putative bipartite nuclear localization sequences (NLS), and three conserved domains which we denote SRPX5 because of their similarity to the fifth domain in sushi repeat containing protein, X-linked (SRPX) (Fig. 1.5). SSG1 contains several predicted N-glycosylation sites (296, 300, 301, 303): (murine) Asn467, Asn666, and Asn834. Mu et al. (303) predicts three shared N-glycosylation sites for Equarin-L at Asn474, Asn675, and Asn843 with two additional sites unique for Equarin-L, Asn58 and Asn488. Consistent with a protein containing a signal peptide, Liu et al. (301) demonstrated that SSG1 is secreted, though three groups were unable to detect secreted SSG1 in the culture medium (300, 302, 303). One group claimed that secreted SSG1 was seen in the extracellular matrix of bone tissue at high magnification (301). The reports in the literature on the secretion of SSG1 are controversial; therefore, it remains unclear whether SSG1 is a secreted protein.

Thus far, two other proteins also contain an SRPX5 domain: SRPX and SRPX2 (SRPUL). SRPX and SRPX2 are members of the selectin family, which includes SRPX, SRPX2, selectin P precursor (SELP), selectin E precursor (SELE), and selectin-like protein (SVEP1) (304). The selectin family of proteins also contain a signal peptide, sushi domains, and a hyalin repeat (305). The sushi domains and hyalin repeat have been implicated in cell adhesion (306, 307). SRPX and SRPX2 also contain one uncharacterized fifth domain which is found in SSG1. For this reason, we designated this domain in SSG1 SRPX5 for similar to the fifth domain in SRPX. The three SRPX5 domains in SSG1 have 40-50% amino acid similarity to each other and to the single domains in SRPX and SRPX2.

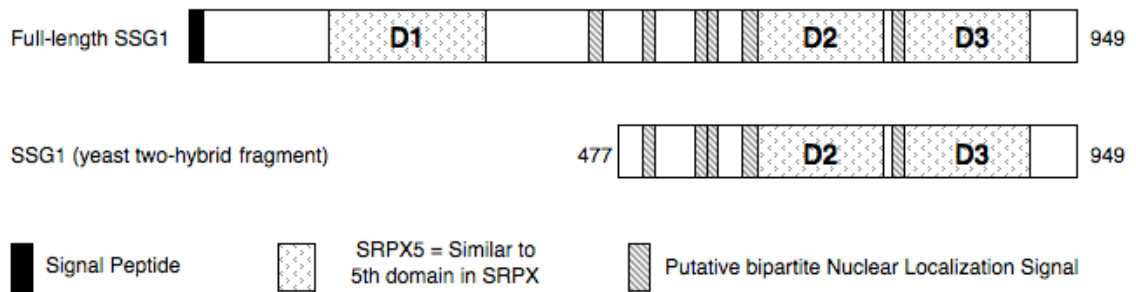


Figure 1.5 Schematic representation of the domain structure of SSG1.

SSG1 contains a putative signal peptide at the N-terminus (black box), three consensus repeat regions (D1, D2, and D3) which we denote SRPX5 domains, and five putative bipartite nuclear localization sequences (NLS, hatched boxes).

The Carter-Su lab identified SSG1 as a protein which interacts with kinase-active JAK2 in a yeast two-hybrid screen of a rat adipocyte library. The yeast two-hybrid screen identified the C-terminal half of SSG1 as a binding partner for JAK2. This yeast two-hybrid fragment, SSG1 (477-949), preferentially interacted with kinase-active JAK2 versus kinase-inactive JAK2 (K882E) in both the yeast two-hybrid assay and in a mammalian system. Further, it was demonstrated that SSG1 (472-788) was a substrate for JAK2 overexpression. Much of this early work done was using the yeast two-hybrid clone and not full-length SSG1. Based on the current literature, it is unclear how SSG1 functions at the cellular level. To follow up on these earlier studies, I investigated the interaction of SSG1 with JAK2, the role SSG1 plays in GH signal transduction, and whether SSG1 is secreted.

Aims of this thesis

The hypothesis of this thesis project was that SSG1 is a multi-functional protein having a synergistic relationship with JAK2. SSG1 regulates GH signaling by interacting with JAK2 thus affecting downstream GH-stimulated signaling molecules and gene expression. Conversely, JAK2 regulates the function of and cellular trafficking of SSG1.

The first aim of my thesis was to characterize the regions of interaction between SSG1 and JAK2 by confirming preliminary findings. Previous work using the yeast two-hybrid fragment of SSG1 revealed that SSG1 (477-949) interacts preferentially with kinase-active JAK2 versus kinase-dead JAK2. The region of interaction in JAK2 for the yeast two-hybrid clone of SSG1 was shown to be the JH1 kinase domain. In Chapter II of this thesis, I show that full-length SSG1 also interacts preferentially with kinase-active JAK2. I also demonstrate that full-length SSG1 binds to full-length JAK2 better than to the JH1 kinase domain of JAK2 alone suggesting that there are two regions of interaction in JAK2, one within the JH1 domain and one outside of the JH1 domain. SSG1 does not contain the traditional phosphotyrosine binding motifs, SH2 or PTB domains. SSG1 does contain three consensus SRPX5 domains. An interaction between the second SRPX5 domain of SSG1 and JAK2 had been previously shown by the Carter-Su lab; I tested the interaction of all three of these domains of SSG1 with JAK2. As predicted, all three

SRPX5 domains in SSG1 interact preferentially with full-length tyrosyl phosphorylated, kinase-active versus inactive JAK2 (K882E) and with kinase-active JH1 versus kinase-inactive JH1 (K882E). Based on truncation experiments, these SRPX5 domains are both necessary and sufficient for binding JAK2. I propose that the SRPX5 domains are novel phosphotyrosine binding domains. Further support for the SRPX5 domain being a phosphotyrosine binding domain is obtained from experiments in which JAK2 was shown to co-immunoprecipitate with another SRPX5 domain-containing protein, SRPX2. Like the SSG1-JAK2 interaction, SRPX2 binds preferentially to the kinase-active JAK2 versus kinase-dead JAK2 (K882E).

The second aim of my thesis was to examine the role SSG1 plays in GH signaling. In Chapter III of this thesis, I demonstrate that SSG1 does not alter the kinase activity of JAK2. Further, SSG1 only modestly increased the GH-dependent tyrosyl phosphorylation of the downstream GH signaling molecule, Stat5b. Surprisingly, SSG1 enhanced the phosphorylation of Stat3 on Tyr705 and appeared to promote the phosphorylation of an unknown residue in Stat3. SSG1 did not alter phosphorylation of Ser727 of Stat3. Because SSG1 appears to enhance Stat3 phosphorylation on Tyr705, we hypothesized that Stat3-mediated gene transcription would be enhanced. However, in Chapter III, I confirmed previous data from the Carter-Su lab that SSG1 negatively regulates transcription of a *c-fos* reporter construct which contains a SIE which binds Stat1 and Stat3 homo- and heterodimers.

The third aim of my thesis was to determine the subcellular localization of SSG1. SSG1 contains several putative nuclear localization motifs as well as a putative signal peptide. In Chapter III, I show that SSG1 is localized to the ER and Golgi, consistent with it being in secretory vesicles and indicative of a secreted protein. When SSG1 is co-expressed with GFP-JAK2, a portion of JAK2 is colocalized with SSG1 in the region surrounding the nucleus. Importantly, no cells expressed nuclear SSG1 suggesting that the nuclear localization signals in SSG1 are non-functional or that SSG1 does not accumulate in the nucleus under normal culture conditions.

The final aim of my thesis was to determine whether SSG1 is a secreted protein and whether secretion is regulated by JAK2. Reports in the literature are controversial as to whether SSG1 is a secreted protein. Initial findings in our lab revealed that SSG1 was

not secreted, but rather got inserted into the ER membrane. In Chapter III of this thesis, I demonstrate that SSG1 is a secreted protein found in the culture medium of transfected cells. This secretion was blocked with Brefelin A, which suggests that SSG1 is in the constitutive secretory pathway. When SSG1 was co-expressed with wild-type JAK2, SSG1 secretion was enhanced. It was not enhanced with co-expressed with kinase-dead JAK2 (K882E). Further, SSG1 cleavage products are prominently detected in the culture medium with co-expression of wild-type JAK2 but not JAK2 (K882E). This suggests that JAK2 enhances the secretion and/or processing of SSG1.

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CHAPTER II

IDENTIFICATION OF STEROID-SENSITIVE GENE-1 AS A NOVEL JAK2 BINDING PROTEIN AND THE SRPX5 DOMAIN AS A NOVEL PHOSPHOTYROSINE BINDING DOMAIN

Abstract

Janus kinase 2 (JAK2) is the receptor-associated tyrosine kinase activated upon growth hormone (GH) stimulation. JAK2 is an important signaling molecule activated by many of the cytokine receptors, however very few proteins have been identified as binding partners for JAK2. We identified steroid-sensitive gene-1 (SSG1) as a JAK2 binding protein that binds to the active, phosphorylated, form of JAK2 in a yeast two-hybrid screen of an adipocyte cDNA library. Full-length SSG1 is a 110 kDa protein that contains several protein motifs including three SRPX5 (sushi-repeat-containing protein, X-linked, domain 5) domains, which we characterize in this study. We confirmed that SSG1 interacts with and is tyrosyl phosphorylated by JAK2 in a mammalian system. We show that full-length SSG1 binds to the active, tyrosyl phosphorylated form of JAK2 and the kinase-active JH1 domain, but not JAK2 (K882E) or JH1 (K882E). Full-length SSG1 binds full-length JAK2 better than the JH1 kinase domain. All three myc-tagged SRPX5 domains of SSG1 were found to co-immunoprecipitate with full-length, wild-type JAK2 as well as with the kinase domain, JH1, but not with kinase-inactive JAK2 (K882E) or with JH1 (K882E) suggesting that all three SRPX5 domains interact preferentially with the tyrosyl phosphorylated, kinase-active form of JAK2. The second SRPX5 domain had the strongest interaction with wild-type JH1 and the first SRPX5 domain had the weakest. Another SRPX5 domain-containing protein, SRPX2, also binds JAK2 in a phosphotyrosine-dependent manner. These results suggest that the SRPX5 domain is a novel protein-protein interaction domain, most likely a phosphotyrosine binding domain.

Introduction

The Janus family of tyrosine kinases, which includes JAK1¹, JAK2, JAK3 and Tyk2, plays a critical role in signaling by members of the cytokine/hematopoietin superfamily of receptors. JAK2 is activated by two-thirds of these receptors including the receptors for growth hormone (GH), prolactin, erythropoietin, leptin, leukemia inhibitory factor and multiple interleukins and interferons (1). JAK2 promotes the growth, proliferation and/or differentiation of many cell types (2, 3) and dysregulation of JAK has been linked various forms of cancer (4-10).

Despite the fact that JAK2 is necessary for signaling by multiple cytokines, hormones, and growth factors, and is the most studied of the JAKs, only a handful of proteins have been identified thus far that bind directly to JAK2. The negative regulators SOCS-1 (11), SOCS-3 (12), and protein tyrosine phosphatase (PTP)-1B (13) have all been shown to bind to phosphorylated Tyr 1007, the critical tyrosine for activation of JAK2. Several known signaling proteins bound to a phosphorylated peptide containing Tyr 966 of JAK2 including Stats 5a and b, phospholipase C (PLC)- γ 1, PLC- γ 2, the adapter molecule SHC, and the p85 α , p85 β , and p110 δ subunits of phosphatidylinositol (PI) 3-kinase, and a novel protein, p70, which was later renamed and characterized as suppressor of *T* cell receptor signaling-1 (Sts-1), a tyrosine phosphatase which regulates Zap-70 activation (14-16). However, it is unclear whether any of these signaling molecules are recruited to phosphorylated Tyr 966 in activated JAK2. Finally, phosphorylated Tyr813 binds SH2B1 (SH2-B β , PMS) (17), an adapter protein and enhancer of JAK2 kinase activity (18, 19), and its family member SH2B2 (formerly APS) (20, 21). The identification of additional signaling proteins that specifically bind to JAK2 is essential for expanding our understanding of signaling by GH and other cytokines that activate JAK2.

In this study, we identify a novel signaling protein, steroid-sensitive gene-1 (SSG1), as a JAK2 binding protein that binds preferentially to the active, phosphorylated, form of JAK2 in a yeast two-hybrid screen of an adipocyte cDNA library. SSG1 was originally characterized as an estrogen-regulated gene based on its cloning using differential display of uterine tissues from ovariectomized and ovariectomized/estrogen-

treated rats (22). Rat SSG1 was independently recloned both as CL2, a protein upregulated in E1A 13S infected rat thyroid PC Cl3 cells (23), and as DRO1 (downregulated by oncogenes -1), a protein downregulated in RK3E rat epithelial cells neoplastically transformed by β -catenin (24). Murine SSG1 was cloned as Urb, a protein upregulated in brown adipose tissue of bombesin receptor subtype-3-deficient mice (25). A chicken ortholog, Equarin-L, was cloned from a chick E6 lens cDNA library using a signal sequence trap screen and named for its expression in the equatorial region of the chick lens (26). Rat, murine, and human SSG1 have been shown to have a wide tissue distribution (e.g. fat, lung, ovary, uterus, mammary gland, testis, liver, spleen, pancreas, kidney, heart, stomach, bladder, skeletal muscle, skin and brain) (22-25, 27, 28). Roles for SSG1 in tumor suppression (23, 24), energy metabolism (25, 28), embryonic development and skeletal formation (27) have been suggested. However, little to nothing is known about the cellular regulation and function of SSG1.

We show here that SSG1 interacts with and is phosphorylated by JAK2. SSG1 binds to the active, tyrosyl phosphorylated form of JAK2 but not the inactive form of JAK2. The SRPX5 domains in SSG1 are both necessary and sufficient for binding to JAK2. The SRPX5 domain in SSG1 has 30% amino acid identity to the uncharacterized fifth region in SRPX (sushi-repeat containing protein, X-linked) (29). This domain is common to a newly emerging group of proteins that includes rat, murine and human SSG1 (22-25, 27); chicken Equarin, an ortholog of SSG1 (26); SRPX/downregulated by Src (drs) (rat)/ETX1 (human) (30-32), and SRPX2 (also known as SRPUL for sushi repeat protein upregulated in leukemia) (33). Finally, we show that SRPX2 also binds JAK2 in a phosphotyrosine-dependent manner. These results suggest that the SRPX5 domain is a JAK2 binding domain, most likely a phosphotyrosine binding domain.

Materials and Methods

Cells and reagents.

Sacharomyces cerevisiae EGY48 (MAT α trp 1 ura3-52 his3 leu) and all yeast expression plasmids came from Dr. R. Brent (The Molecular Sciences Institute, Berkeley, CA) (34-36). 293T and COS-7 cells were from American Type Culture Collection. Recombinant protein A-agarose was from Repligen. Bovine serum albumin (CRG-7) was from Intergen. Dulbecco's Modified Eagle Medium (DMEM) was from Invitrogen. Aprotinin, leupeptin, and Triton X-100 were from Roche. The enhanced chemiluminescence detection system and nitrocellulose paper were from Amersham Pharmacia Biotech. X-ray film was from Kodak. The QuickChange Site-Directed Mutagenesis Kit was from Stratagene. Polyvinylpyrrolidone (PVP-360) was from Sigma.

Antibodies.

Polyclonal antibody to JAK2 (α JAK2) and monoclonal anti-phosphotyrosine antibody (α PY; 4G10) were used at a dilution of 1:7,500 for immunoblotting and were from Millipore. Monoclonal antibody to influenza virus hemagglutinin (HA) tag (α HA; HA.11) was from Covance and used at a dilution of 1:100 for immunoprecipitation and 1:10,000 for immunoblotting. Monoclonal antibody against Myc-tag (α myc; 9E10) was used at a dilution of 1:100 for immunoprecipitation and 1:10,000 for immunoblotting and polyclonal antibody against myc-tag (α myc; A14) used at a dilution of 1:1000 were from Santa Cruz Biotechnology, Inc. Monoclonal anti-FLAG M2 antibody (α FLAG) used at a dilution of 1:1000, anti-FLAG M2 antibody (α FLAG) conjugated to agarose beads were from Sigma. Monoclonal anti-GFP antibody (α GFP; JL-8) from BD Biosciences was used at a dilution of 1:100 for immunoprecipitation and 1:5,000 for immunoblotting. Polyclonal anti-GFP antibody (α GFP) from Clontech was used at a dilution of 1:100 for immunoprecipitation. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Santa Cruz and used at a dilution of 1:7,500. IRDyes 680 or 800 conjugated to either affinity purified anti-mouse IgG or anti-rabbit IgG antibodies from Rockland and Licor were used at a dilution of 1:20,000.

Two hybrid library screening and cloning of full-length SSG1.

Screening of the yeast two-hybrid rat adipose cDNA library (gift of Dr. T. A. Gustafson, University of Maryland) (18) was conducted as described previously (35, 36). Briefly, yeast (EGY48) cells were grown at 30°C in YPD medium (1% yeast extract, 2% polypeptone, 2% dextrose). Yeast cells were then sequentially transformed with the *lexAop-lacZ* reporter plasmid, pEG202-JAK2, and rat adipose cDNA library prey plasmids (18) by the lithium acetate method (37). Triple transformants were grown for 4 h in liquid yeast dropout medium lacking Trp, Ura, and His and plated on the same dropout medium with the addition of 15% agar. The yeast cells were grown for 4 days at 30°C. Colonies were then collected and replated on yeast dropout medium lacking Trp, Ura, His and Leu to select for Leu prototrophy. Dextrose was substituted with galactose to induce expression of the activation domain hybrids. After 5 days, colonies were subjected to the filter lift color assay to test for β -galactosidase activity (38, 39). Positive clones were selected, and prey plasmids containing library cDNA inserts were isolated and transformed into DH5 α bacterial strain for amplification. The insertion cDNAs were subjected to DNA sequence analysis.

Plasmids.

The cDNA encoding the carboxyl-terminal portion of rat SSG1 (amino acids 477-949) was cut from the yeast two-hybrid prey plasmid using EcoRI and XhoI and subcloned in-frame into the pcDNA3 mammalian expression vector (Invitrogen) with an amino terminal HA tag (40) and into the prk5 expression vector (BD Bioscience) containing a Myc tag at the amino terminus. PCR based mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit from Stratagene. All PCR products and junctions were verified by sequencing. SSG1 truncation mutants were created by introducing stop codons into HA-SSG1 (477-949) using the following primers (mutated bases are in lower case and the introduced stop codons and restriction sites are underlined): (stop @ 609: 5'-CCGAGTCCCAGGtAGTCAGTGGCC-3'; stop @ 663: 5'-GGTACTATCTTTGaCCTGTCAACAACAGC-3'; stop @ 689: 5'-CGTGTGGT GGATGACtAGGACTTGGTAGAC-3'; stop @ 724: 5'-GGTCTCAGAGTCAAGtAAT

ACTACGAAGTGC-3'; stop @ 763: 5'-ACCTGCAAGGAGGACtAGAGGCAGTCC-3'). Myc-SSG1 (472-788), encoding amino acids 472-788, was generated from full-length SSG1 cDNA using customized primers (5'-tctatcccgagactcgaattcACCATGGAACATGGCCATCAGGACCCAAATGTGGTG-3'; 5'-gatccgctcgagcggtctagagcaTCATTGGGAGCAGAGATCACCAGCAACCGCC-3') to introduce EcoRI and XhoI sites at the 5' and 3' ends, respectively, and subcloning the fragment in-frame into prk5 expression vector containing an amino terminal myc tag.

The cDNA for SSG1 (accession number AF223577) (22) was resequenced. Clustal analysis indicated that the sequence of rat SSG1 was identical to rat DRO1 (accession number AY548105) (24) having only one mismatch between nucleotides 1 and 3596 of DRO1, with no mismatches in the predicted coding sequence between nucleotides 389-3238 of DRO1. Therefore, SSG1 and DRO1 are likely to be the same gene. The coding sequence codes for a 949 amino acid protein. Therefore, amino acids in SSG1 will be numbered according to the accession number for rat DRO1 (AAS66670). Full-length SSG1 with a myc tag at the C-terminus was created using the following primers: 5'-tctatcccgatgatagatctaggaattccaccATGACGTGGAAAATGGGACCCCACTTCACC-3' and 5'-gatccggaattccccgaagcttgggttacaggtcctcctcctcgtgatcagcttctgctccattccGTAAGGGTATCCATGGTGGTAACTTTCATGATG-3', myc tag and restriction sites are underlined, and the stop codon is in italics. SSG1 was cloned in-frame into the prk5 expression vector.

To introduce an internal HA tag after the signal peptide in SSG1-myc, a Sall restriction site was introduced after amino acid 30 of SSG1-myc using the following primer: 5'-CAGTCTTCTGCCTTGGATgtCGAcGGCCGCCAGG-3' (mutations lower case, Sall site underlined). Primers containing the sequence for the HA tag (bold) as well as the Sall overhang (underlined): 5' TCGAGGATGTCGACGCCTACCCATACGATGTTCCTGACTATGCGGTCGACGGC-3' and 5'-TCGAAGCCCGTCGACCGCATAGTCAGGAACATCGTATGGGTAGGCGTCGACCTA-3' were annealed, labeled with T4 nucleotide kinase, and inserted into the newly generated Sall site in SSG1-myc to create SSG1-HA30-myc.

The SRPX5 domains of SSG1 were inserted into XhoI and EcoRI restriction sites in the pEGFP-C1 expression vector (Clontech) or prk5-myc vector to create tagged

fusion proteins using the following primers (restriction sites underlined): for GFP-SSG1 (615-756): (5'-atcgatcgattctcgagccgatTTGGGGTCTTTTCGAAGGCAAACGAAGACTC-3', 5'-atcgatcgatcgatcgatcccggaattcgctaGCCCTCCTTCTTCTGCTTTTCCATATC TTTGATTC-3'); for myc-SSG1 (137-281): (5'-atcgatcgattctcgaggccgatAGTTCTC CCAATATCCTGGCCAGCTTTGC -3'; 5'-atctgtcgacgaattcgctaACACTTTTGGGA CAAAACCCTTCTGCCTGATTTTCTC-3'); for myc-SSG1 (615-756): (5'-atcgatcga tctcgaggccgatTTGGGGTCTTTTCGAAGGCAAACGAAGACTC-3', 5'- atcgatcgatcgatcgatcccggaattcgctaGCCCTCCTTCTTCTGCTTTTCCATATCTTTGATT -3'); for myc-SSG1 (766-912): (5'- atcgatcgattctcgagccgatTCCCTGGAGAATTTCTAT CCAGGTTCC -3'; 5'- actgtcgacgaattcgctaACAGCGCATCCCAGTGACTGCTG-3').

cDNA encoding murine SRPX2 (SRPUL; accession number NM_026838) was obtained from OriGene. A myc-tag was inserted between the BamHI and HindIII sites in the prk5 vector using the following primer which included the BamHI site (underlined), a NotI site (underlined), the myc tag (bold), the stop codon (italics), and a HindIII site (underlined): 5'-GCTACGGGATCCCGCGCCGCAGAGCAGAAGCTGATCAGCGA GGAGGACCTGTAGAAGCTTACGCGT-3'. SRPX2 with a C-terminal myc tag was then created using PCR primers which introduced a BamHI restriction site (underlined), as well as a Kozak sequence (italics, ATG in bold) or a NotI site and no stop codon (5'-GCTACGGGATCCGCCACCATGATGACCAGTCCACTGACTCAGAGAGGTGC-3' and 5'-GCTACGCGGCCGCCCTCGCATAGGTCCCTCTGCTCCACTCTCC-3') were used to insert SRPX2 into the prk5-C myc vector between the BamHI and the Not I restriction sites.

The prk5 expression vector encoding wild-type murine JAK2 (41) or murine JAK2 (K882E) (42) in which the critical lysine in the ATP-binding domain is mutated to a glutamate were provided by Dr. J. Ihle (St. Jude Children's Research Hospital). PCMV tag 28 vectors encoding FLAG-tagged JAK2 (797-1132) or FLAG-tagged JAK2 (830-1132) were described previously (17). Carboxyl-terminally HA tagged wild-type JAK2 (JAK-HA), mutant JAK2 lacking amino acids 548-804 (Δ JH2-HA) and the JH1-JH2 fragment of JAK2 (535-1132) (JH1-2-HA) constructs were provided by Dr. O. Silvennoinen (University of Tampere, Finland) (43).

Cell culture and transfection.

293T and COS-7 cells were grown in DMEM supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and either 8% calf serum (293T) or 8% fetal bovine serum (COS-7). Both 293T and COS-7 cells were transiently transfected by calcium phosphate precipitation (44) and assayed 24 or 48 h after transfection, respectively.

Immunoprecipitation and immunoblotting.

Cells were washed three times with chilled PBSV (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na₃VO₄, pH 7.4) and solubilized in lysis buffer (50mM Tris, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na₃VO₄, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Lysates were centrifuged at 16,750 xg for 10 min at 4°C. The supernatant (cell lysate) was incubated with the indicated antibodies on ice for 2 h. The immune complexes were collected on protein A-agarose for 1 h at 4°C. The beads were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl, 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, pH 6.8). To reduce non-specific binding in some experiments, cells were lysed in lysis buffer supplemented with 0.1% Triton X-100 plus 1% SDS. Protein A agarose beads were incubated with 0.5% polyvinylpyrrolidone (PVP) in 100 mM acetic acid (pH 6.2) for 1 hour at 4°C prior to incubation with the cell lysate. After incubation with cell lysates, protein A agarose beads were washed in 500 mM NaCl two times, then with lysis buffer two times. Proteins were then boiled for 5 min in a mixture (80:20) of lysis buffer. The solubilized proteins were separated on a 5-12% SDS-PAGE gradient gel. Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with the indicated antibody using enhanced chemiluminescence (Fig. 2A, 3, 4) or Odyssey Infrared Imaging System (LI-COR Biosciences) (Fig. 2B, 5-9).

Results

Identification of SSG1 as a binding partner for JAK2 in the yeast two-hybrid system.

In an effort to identify novel cytokine receptor effector proteins that are recruited to the activated form of JAK2, full-length JAK2 was fused to the DNA binding portion of LexA and used to screen for JAK2 interacting proteins in a yeast two-hybrid system. Previous work showed that LexA-JAK2 expresses in yeast, is active as a kinase, is tyrosyl phosphorylated, and does not autoactivate the system (18).

An adipose tissue library was used because GH and a number of other cytokines [e.g. IL-6, IL-11, LIF, leptin, IFN α , INF γ , oncostatin M] elicit multiple effects in adipocytes (45-50). Activation of two reporter genes, *Leu* and *lacZ*, was used to indicate protein-protein interactions. Over 110 positive clones were isolated from 11 x 10⁶ yeast transformants. Three clones, out of the 9 first sequenced, encoded a protein that closely corresponded to the carboxyl-terminal portion of rat SSG1. SSG1 was initially cloned from rat uterus (GenBank accession number AF223677) and predicted to be a 385 amino acid, 41.8 kDa protein (22). The presence of multiple mismatches between the sequence of our yeast two-hybrid clone and the published sequence for SSG1 (22) led us to resequence the full-length SSG1 cDNA of Marcantonio et al. (22). Resequencing revealed that in contrast to what was reported initially (22), the cloned rat SSG1 cDNA predicts a 110 kDa protein of 949 amino acids. The sequence of our yeast two-hybrid clone corresponds to the C-terminal portion, amino acids 477-949, of the full-length clone (Fig. 1A). The sequence of rat SSG1 is identical to the subsequently cloned rat CL2 (23) and rat DRO1 (GenBank accession no. AY548105) (24). The amino acid sequence of rat SSG1 shares high identity with murine Urb (94%) (GenBank accession no. AB075019), also cloned from adipose tissue (25), and human Urb (84%) (GenBank accession no. AF506819). SSG1 also shares considerable amino acid sequence identity (69%) with chicken Equarin-L (GenBank accession no. AB086824).

Sequence analysis indicates that SSG1 contains a signal peptide (amino acids 1-24), a glycine rich region (amino acids 285-307), a proline rich region (amino acids 322-353), a threonine rich region (amino acids 332-386), a lysine rich region (amino acids

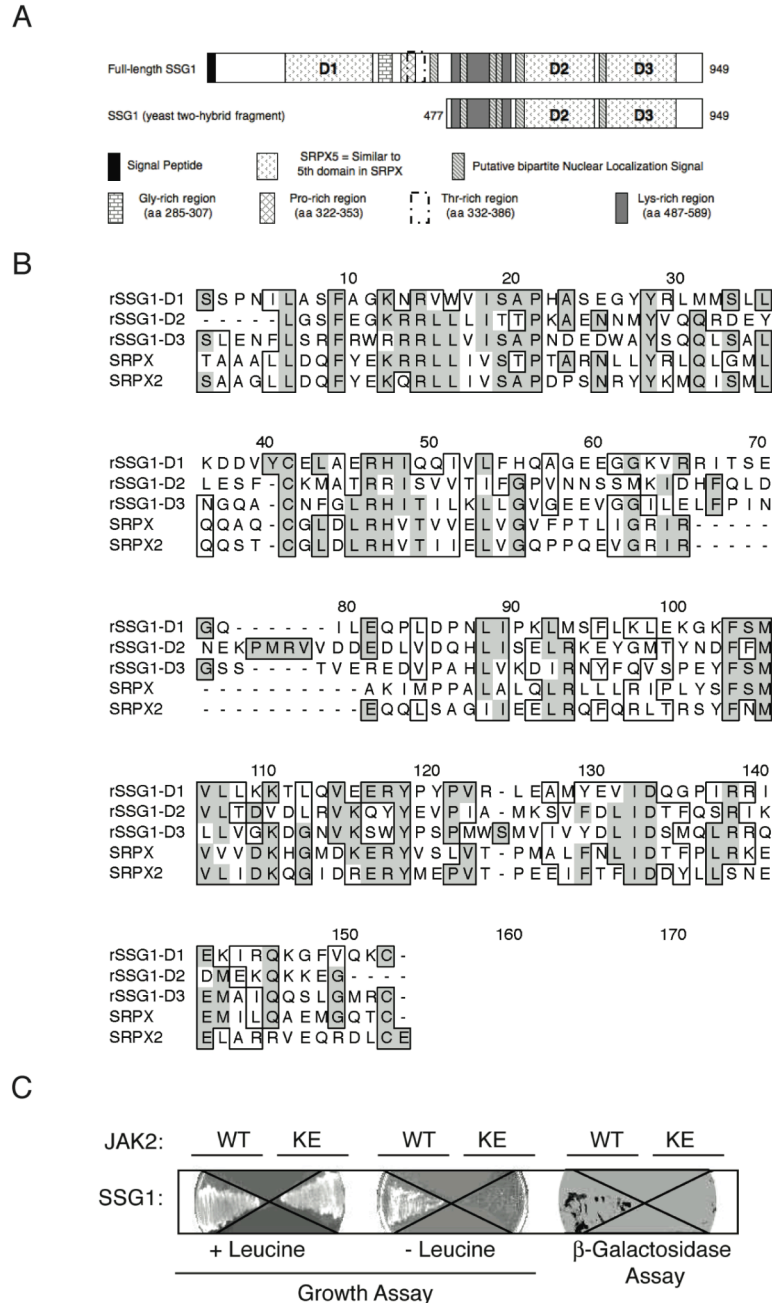


Figure 2.1 Identification of SSG1 as a binding partner for JAK2 in the yeast two-hybrid system.

(A) Domain structures for full-length SSG1 and for the yeast two-hybrid fragment of SSG1, SSG1 (477-949), are shown. The shaded boxes represent the different domains within SSG1. The SRPX5 domains, *D1*, *D2*, *D3*, are noted. (B) Clustal W plot of the three SRPX5 domains (*D1*, *D2*, and *D3*) in SSG1 along with the fifth domains of the SRPX/*drs*/ETX1 and SRPX2/SRPUL proteins. Identical amino acids are shaded in grey; similar amino acids are boxed. (C) Yeast cells transformed with cDNA encoding SSG1 (477-949) in the prey vector with cDNA encoding either JAK2 (WT) or JAK2 (K882E) (KE) fused to a LexA DNA binding domain were streaked onto 15% agar-containing dropout medium in presence of leucine (+ leucine) or the absence (- leucine) or with galactose to induce β -galactosidase activity.

487-589) which contains 2 out of a total of 5 putative bipartite nuclear localization signals, and three consensus repeat domains (amino acids 137-281 [designated domain 1 or D1], 615-756 [D2], and 766-912 [D3]) (Fig. 1A). The consensus repeat domains display approximately 30% amino acid identity and 40-50% amino acid similarity to each other (Fig. 1B) and to the fifth domain of murine SRPX (GenBank accession no. CAM18748) (29). We have designated these domains SRPX5 domains. An SRPX5 domain is also present in murine SRPX2 (GenBank accession no. NP_081114.1). The SRPX5 domains of SSG1 are highly conserved between species, with rat and mouse SSG1 showing 95%, 94%, 97% amino acid identity for D1, D2 and D3 respectively; rat and human SSG1 showing 98%, 94%, 97% amino acid identity, and rat SSG1 and chicken Equarin-L showing 88%, 85%, 89% amino acid identity for D1, D2 and D3 respectively.

Interaction of SSG1 with JAK2 requires JAK2 kinase activity in a yeast system.

To determine whether JAK2 kinase activity and/or tyrosyl phosphorylation of JAK2 is required for the interaction of JAK2 with SSG1 (477-949), the SSG1 (477-949) prey plasmid was retransformed into yeast along with either LexA-JAK2 or the catalytically inactive LexA-JAK2 (K882E) in which a critical lysine in the ATP binding loop is mutated to glutamate. As judged by the ability to activate the *Leu* and *lacZ* reporter genes embedded in the prey plasmid, SSG1 (477-949) bound LexA-JAK2 but not LexA-JAK2 (K882E) (Fig. 1C). This result suggested that SSG1 interacts preferentially with tyrosyl phosphorylated, kinase-active JAK2.

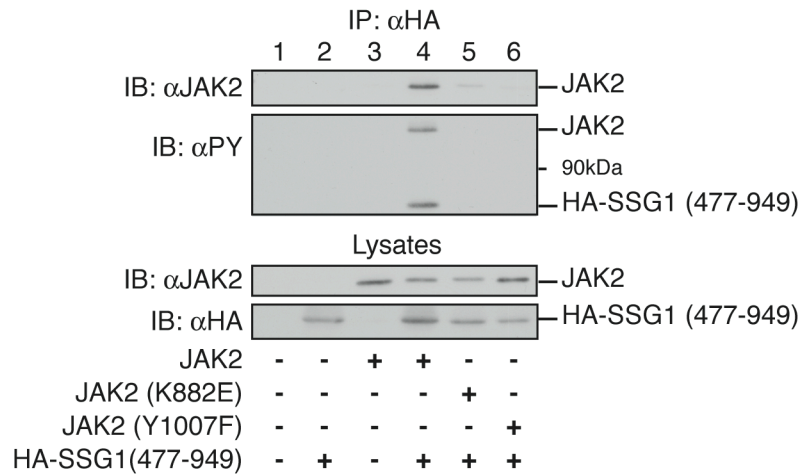
SSG1 (477-949) associates preferentially with and is phosphorylated by kinase-active JAK2 in mammalian cells.

To verify that SSG1 and JAK2 interact in mammalian cells, 293T cells were co-transfected with cDNA encoding vector alone or HA-SSG1 (477-949), and cDNA encoding wild-type JAK2, JAK2 (K882E), JAK2 (Y1007F), or empty vector. JAK2 (K882E) is completely inactive (42). In JAK2 (Y1007F), the activating tyrosine in the kinase domain of JAK2 is mutated to a phenylalanine. This mutation markedly reduces but does not completely inhibit the kinase activity of JAK2 (51). HA-SSG1 (477-949)

was immunoprecipitated using anti-HA antibody (α HA) and the immunoprecipitated proteins were immunoblotted with α JAK2 (Fig. 2A, top panel). HA-SSG1 (477-949) co-immunoprecipitated JAK2 to a much greater extent than either JAK2 (K882E) or JAK2 (Y1007F). Reprobing the blot with α PY (Fig. 2A, second panel) demonstrated that HA-SSG1 (477-949) and the co-precipitated JAK2 were both tyrosyl phosphorylated when SSG1 was co-expressed with JAK2 but not when SSG1 was co-expressed with either of the two kinase-inactive forms, JAK2 (K882E) or JAK2 (Y1007F). HA-SSG1 (477-949) migrated as a 60 kDa protein as predicted. Immunoblotting the lysates with α JAK2 and α HA (Fig. 2A, lower panels) shows that expression of HA-SSG1 (477-949) and the JAK proteins did not vary substantially for the different conditions.

Because the HA tag contains tyrosines, the assay was repeated with a myc-tagged version of SSG1, myc-SSG1 (472-788). The myc tag contains no tyrosines. 293T cells were co-transfected with cDNA encoding either wild-type JAK2 or JAK2 (Y1007F) and cDNA encoding myc-SSG1 (472-788). Proteins in the cell lysates were immunoprecipitated with α myc and the immunoprecipitated proteins were immunoblotted with α pY (Fig. 2B). Consistent with SSG1 forming a complex with JAK2, myc-SSG1 (472-788) co-immunoprecipitated with a tyrosyl phosphorylated protein with a molecular weight appropriate for JAK2 whereas there was no detectable phosphorylated protein corresponding to JAK2 (Y1007F). A tyrosyl phosphorylated protein with a molecular weight appropriate for myc-SSG1 (472-788) was observed when cells co-expressed wild-type JAK2, but not JAK2 (Y1007F) (Fig. 2B, second panel). Immunoblotting the lysates with α JAK2 and α myc (Fig. 2B, bottom two panels) shows that differences in expression of myc-SSG1 (472-788) and the JAK proteins could not explain the difference in degree of SSG1 phosphorylation detected. Together these findings indicate that SSG1 (477-949) interacts preferentially with tyrosyl phosphorylated, kinase-active JAK2, and that SSG1 is tyrosyl phosphorylated and most likely a substrate of JAK2.

A



B

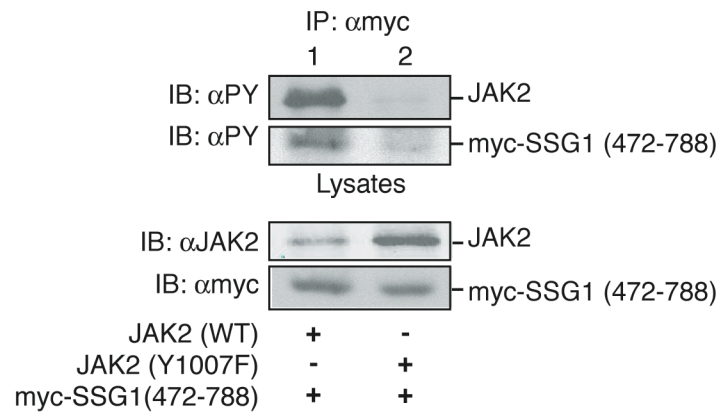


Figure 2.2 SSG1 (477-949) associates preferentially with and is phosphorylated by kinase-active JAK2 in mammalian cells.

(A) 293T cells were transfected with 3 μ g empty vector (lane 1 and 3) or cDNA encoding HA-SSG1 (477-949) (lanes 2, 4-6), in combination with 2 μ g of empty vector (lanes 1 and 2) or cDNA encoding JAK2 (lanes 3 and 4), JAK2 (K882E) (lane 5) or JAK2 (Y1007F) (lane 6). HA-SSG1 (477-949) was immunoprecipitated (IP) with α HA and immunoprecipitated proteins immunoblotted (IB) with α JAK2 (top panel) or α PY (second panel). Lysates from the transfected cells were immunoblotted with α JAK2 (third panel) and α HA (bottom panel). The migration of JAK2, HA-SSG1 (477-949), and a 90 kDa molecular weight marker is indicated. (B) 293T cells were transfected with cDNA encoding myc-SSG1 (472-788) and either JAK2 (lane 1) or JAK2 (Y1007F) (lane 2). myc-SSG1 (472-788) was immunoprecipitated (IP) with α myc and immunoblotted (IB) with α PY (top two panels). Lysates from the transfected cells were immunoblotted with α JAK2 (third panel) and α myc (bottom panel). The migration of JAK2 and myc-SSG1 (472-788) is indicated. n=2-5

SSG1 (477-949) interacts with the kinase domain of JAK2.

Because SSG1 binds to tyrosyl phosphorylated JAK2, we sought to define the region within JAK2 responsible for binding to SSG1 (477-949) by using JAK2 truncation and deletion mutants (Fig. 3A). Myc-SSG1 (477-949) was co-expressed with various HA-tagged truncation and deletion mutants of JAK2. Myc-SSG1 (477-949) was immunoprecipitated with α myc and precipitated proteins immunoblotted with α HA. Full length HA-JAK2, HA-JAK2 with the JH2 domain deleted [HA-JAK2 (Δ 549-803), designated HA- Δ JH2] and just the JH1 and JH2 domains of JAK2 [HA-JAK2 (535-1132), designated HA-JH1-2], all co-immunoprecipitated with SSG1 (477-949) (Fig. 3B), implicating the kinase domain of JAK2, JH1, in SSG1 binding.

To confirm the involvement of the JAK2 kinase domain in SSG1 binding, myc-SSG1 (477-949) was co-expressed with either FLAG-tagged JAK2 (797-1132) containing JH1 and a portion of the linker region between the JH1 and JH2 domains, or the just the JH1 domain [FLAG-JAK2 (830-1132)] (Fig. 3A). Myc-SSG1 (477-949) co-immunoprecipitated with both JAK2 (797-1132) and JAK2 (830-1132) (Fig. 3C), confirming that SSG1 (477-949) interacts with the kinase domain (JH1) of JAK2.

Identification of the SRPX5 domain within SSG1 as a region of interaction with JAK2.

We next determined what region of SSG1 binds JAK2. The yeast two-hybrid and co-immunoprecipitation experiments in 293T cells revealed that amino acids 477-949 in SSG1 are sufficient to bind to JAK2. cDNAs encoding a series of carboxyl-terminal truncation mutants of HA-SSG1 (477-949) were created (Fig. 4A) and the various mutants were co-expressed with JAK2 in 293T cells. JAK2 co-immunoprecipitated with all but the shortest SSG1 truncation mutant, HA-SSG1 (477-608) (Fig. 4B, top panel, lane 8), suggesting that the region in SSG1 between amino acids 609 and 662 comprising the N-terminal half of D2, the second SRPX5 domain, is sufficient for SSG1 binding to JAK2. As expected, none of the SSG1 truncation mutants bound to either of the kinase deficient JAK2 mutants, JAK2 (K882E) or JAK2 (Y1007F) (data not shown).

Having implicated that the N-terminal half of the D2 domain of SSG1 in JAK2 binding, we examined whether the isolated D2 is sufficient to mediate binding to JAK2.

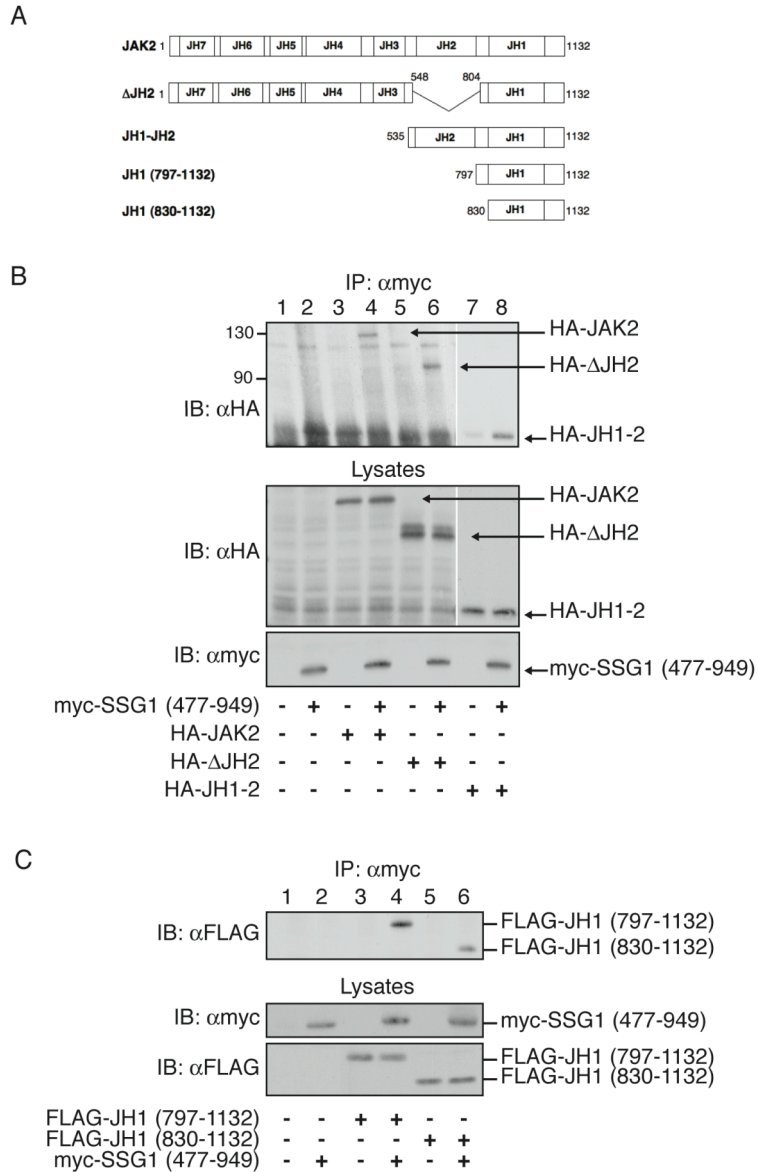


Figure 2.3 SSG1 (477-949) interacts with the kinase domain of JAK2.

(A) Domain structure and truncation mutants of JAK2 are shown. *JH*, JAK homology domain. (B) 293T cells were transfected with 4 μ g of vector alone (lanes 1 and 2) or cDNA encoding HA-JAK2 (lanes 3 and 4), HA- Δ JH2 (lanes 5 and 6) or HA-JH1-2 (lanes 7 and 8), with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) 2 μ g of cDNA encoding myc-SSG1 (477-949). Myc-SSG1 (477-949) was immunoprecipitated (IP) with α myc and immunoprecipitated proteins were immunoblotted (IB) with α HA (top panel). Lysates were immunoblotted with α HA (middle panel) and α myc (bottom panel). Due to a substantially higher expression of HA-JH1-2, lanes 7 and 8 were exposed for a shorter period of time. Migration of HA-JAK2, HA- Δ JH2, JH1-2 and myc-SSG1 (477-949) is indicated. (C) 293T cells were transfected with 3 μ g of vector (lanes 1 and 2) or cDNA encoding FLAG-JH1 (797-1132) (lanes 3 and 4) or FLAG-JH1 (830-1132) (lanes 5 and 6) and 2 μ g vector (lanes 1, 3, 5) or cDNA encoding myc-SSG1 (477-949) (lanes 2, 4, 6). myc-SSG1 (477-949) was immunoprecipitated (IP) with α myc and immunoprecipitated proteins immunoblotted (IB) with α FLAG (top panel). Lysates were immunoblotted with α myc (middle panel) and α FLAG (bottom panel). Migration of FLAG-JH1 (797-1132), FLAG-JH1 (830-1132), and SSG1-myc is indicated. n=3-5

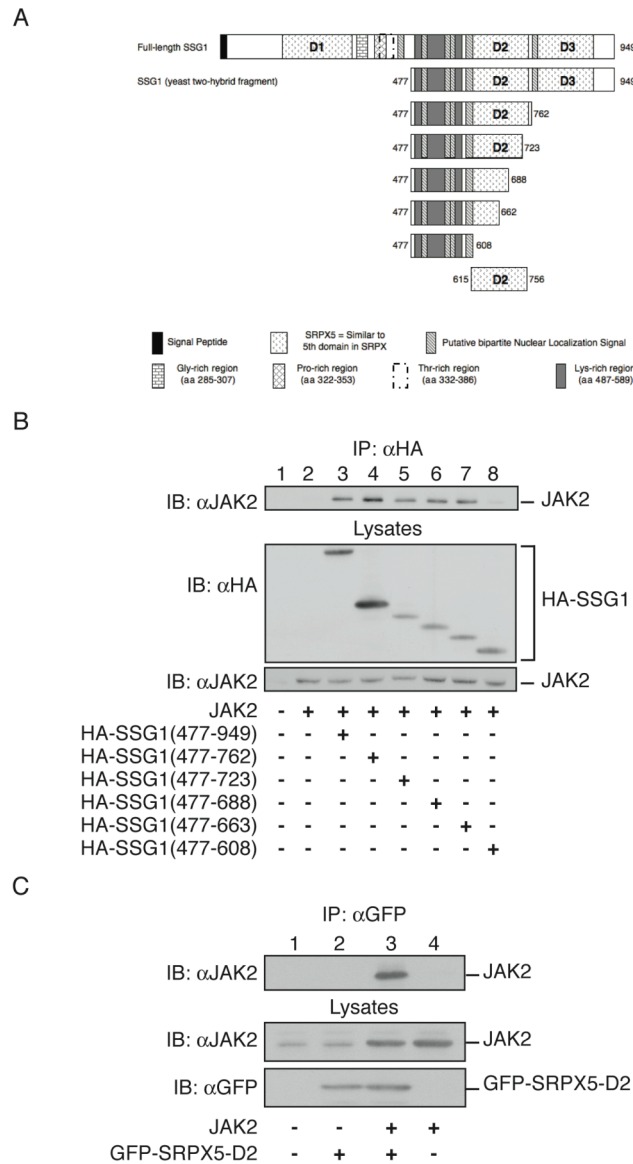


Figure 2.4 Identification of the SRPX5 domain within SSG1 as a region of interaction with JAK2.

(A) Domain structure for a series of carboxyl-terminal truncation mutants of HA-SSG1 (477-949). (B) 293T cells were transfected with 2 μ g of cDNA encoding JAK2 (lanes 2-8) and 2 μ g of cDNA encoding either HA-SSG1 or HA-SSG1 truncation mutant as noted. HA-SSG1 was immunoprecipitated (IP) with α HA and immunoprecipitated proteins immunoblotted (IB) with α JAK2 (top panel). Lysates were immunoblotted with α HA (middle panel) and α JAK2 (bottom panel) to assess levels of expression of the HA-SSG1 mutants and JAK2, respectively. (C) GFP-SSG1 (615-756) encoding the D2 domain of SSG1 (designated GFP-SRPX5-D2) (3 μ g cDNA) (lanes 2, 3) or vector alone (lanes 1, 4) was co-expressed without (lanes 1, 2) or with (lane 3, 4) JAK2 (2 μ g cDNA) in 293T cells. GFP-SSG1 (615-756) was immunoprecipitated (IP) with α GFP and immunoprecipitated proteins immunoblotted (IB) with α JAK2 (top panel). Lysates were immunoblotted with α JAK2 (middle panel) and α GFP (bottom panel) to assess levels of expression of JAK2 and GFP-SSG1 (615-756), respectively. The migration of JAK2 and GFP-SSG1 (615-756) is indicated. n=2

GFP-SSG1 (615-756) fusion protein, corresponding to D2 of SSG1 (Fig. 4A), was co-expressed with JAK2 in 293T cells. JAK2 clearly co-precipitated with GFP-SSG1 (615-756) when both were co-expressed (Fig. 4C). This finding suggests that an SRPX5 domain is not only necessary for JAK2 binding to SSG1 but also sufficient.

All three SRPX5 domains of SSG1 interact with kinase-active JAK2.

Since the second SRPX5 domain (D2) of SSG1 interacts with JAK2, we hypothesized that the other SRPX5 domains (D1 and D3) within SSG1 might also interact with JAK2. cDNAs encoding D1, D2, and D3 fused to the myc tag were transfected into 293T cells with cDNA encoding JAK2 or JAK2 (K882E). JAK2 co-immunoprecipitated with all three SRPX5 domains of SSG1 (Fig. 5, top panel) to a significantly greater extent than JAK2 (K882E). Similar results were obtained with GFP-tagged D1, D2, and D3 domains (data not shown).

The SRPX5 domains of SSG1 interact with the kinase-active kinase domain of JAK2.

The experiments in Figs. 3-5 indicate that the SRPX5 domains in SSG1 interact with the kinase domain (JH1) of JAK2. To test this more directly, myc-tagged D1, D2, and D3 of SSG1 were co-expressed in 293T cells with either kinase-active FLAG-JAK2 (797-1132) [designated FLAG-JH1 wt], or kinase-inactive FLAG-JAK2 (797-1132, K882E) [designated FLAG-JH1 (K882E)]. As predicted, the kinase-active JH1 domain of JAK2 co-precipitated with all three of the SRPX5 domains of SSG1 (Fig. 6, top panel, lanes 5, 8, and 11) while the kinase-inactive FLAG-JH1 (K882E) did not (Fig. 6, top panel, lanes 6, 9, and 12). The interaction of JH1 with D2 appeared to be the strongest and the interaction with D1 the weakest.

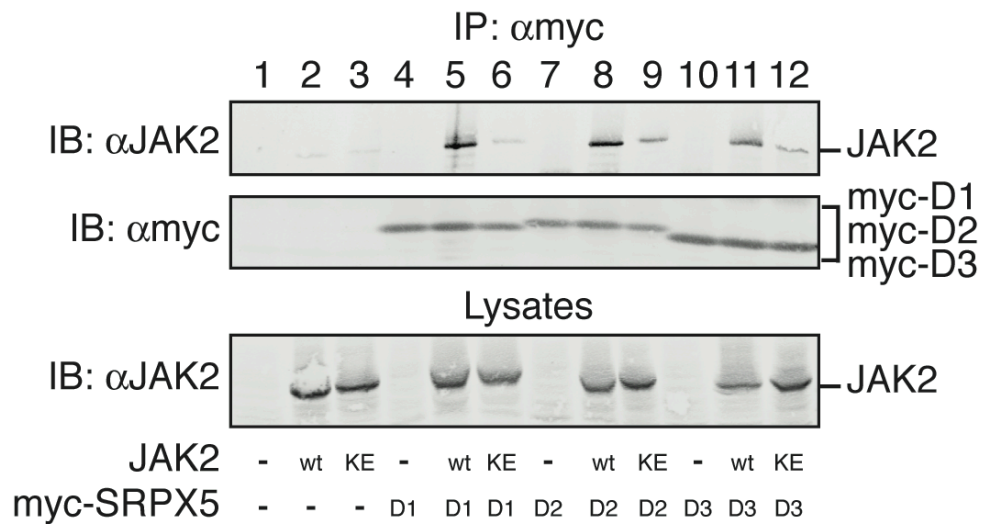


Figure 2.5 All three SRPX5 domains of SSG1 interact with kinase-active JAK2.

293T cells were transfected with 1.5 μ g of vector or cDNA encoding the designated myc-SRPX5 domains of SSG1 (D1, D2, or D3) and 1 μ g vector or of cDNA encoding either wild-type JAK2 (wt) or JAK2 (K882E) (KE). The myc-SRPX5 domains of SSG1 were immunoprecipitated (IP) with α myc and immunoprecipitated proteins immunoblotted (IB) with α JAK2 (top panel). The blot was reprobed with α myc (middle panel). Lysates were immunoblotted with α JAK2 (bottom panel). The migration of JAK2 and of the myc-SRPX5 domains is indicated. n=3

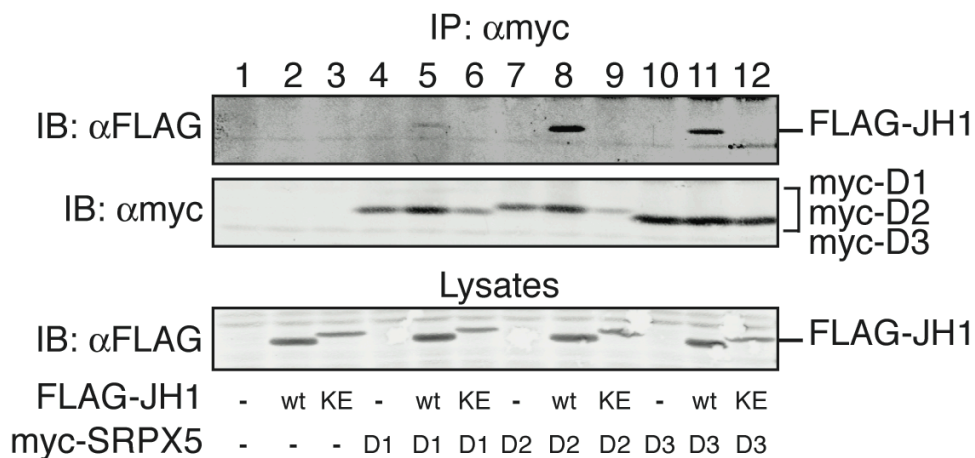


Figure 2.6 The SRPX5 domains of SSG1 interact with the kinase-active kinase domain of JAK2.

293T cells were transfected with 1.5 μ g of vector or cDNA encoding one of the myc-SRPX5 domains of SSG1 (D1, D2, and D3) and either 1 μ g of vector or cDNA encoding wild-type FLAG-tagged JAK2 (797-1132) [FLAG-JH1 wt; designated wt] or 3 μ g FLAG-tagged JAK2 (797-1132, K882E) [FLAG-JH1 (K882E), designated KE]. The myc-SRPX5 domains of SSG1 were immunoprecipitated (IP) with α myc and precipitated proteins immunoblotted (IB) with α FLAG (top panel). The blot was reprobed with α myc (middle panel). Lysates were immunoblotted with α FLAG (bottom panel). The migration of FLAG-JH1 and of the myc-SRPX5 domains is indicated. n=4

Full-length SSG1 interacts with full-length, kinase-active JAK2.

We have shown that truncated versions of the yeast two-hybrid clone of SSG1 (Fig. 4) as well as all three individual SRPX5 domains within SSG1 bind preferentially to kinase-active JAK2 (Fig. 5). To confirm that full-length SSG1 and JAK2 interact in mammalian cells, an HA tag was inserted after the signal peptide at the thirtieth amino acid in full-length SSG1 and a myc tag fused at the C-terminus (denoted SSG1-HA-myc). This cDNA expresses a full-length protein of the appropriate size (~110kDa) for full-length SSG1. SSG1-HA-myc was co-expressed in 293T cells with either wild-type FLAG-tagged JAK2 or JAK2 (K822E). JAK2 co-immunoprecipitated SSG1-HA-myc to a much greater extent than JAK2 (K882E) (Fig. 7, top panel). These findings show that like SSG1 (477-949) and the SRPX5 domains of SSG1, full-length SSG1 interacts preferentially with tyrosyl phosphorylated, kinase-active JAK2. Similar results were obtained using full-length SSG1-myc (data not shown).

SSG1 interacts with full-length JAK2 better than JH1.

All three of the SRPX5 domains bind kinase-active JAK2 (Fig. 5), however D1 appears to bind full-length JAK2 to a greater extent than to the JH1 domain alone (Figs. 5 and 6). Therefore, we hypothesized that full-length SSG1 might bind full-length JAK2 at more than one site. To compare the binding of full-length SSG1 to both full-length JAK2 and to the JH1 domain of JAK2, SSG1-HA-myc was co-expressed in 293T cells with FLAG-JAK2 or FLAG-JH1. FLAG-JAK2 and FLAG-JH1 were immunoprecipitated using α FLAG and the immunoprecipitated proteins were blotted with α HA (Fig. 8). Full-length JAK2 co-immunoprecipitated SSG1-HA-myc (Fig. 8, lane 3) to a much greater extent than JH1 (Fig. 8, lane 4). This finding suggests that the three SRPX5 domains in SSG1 interact with multiple sites in JAK2 and that at least some of these binding sites are outside of the JH1 domain.

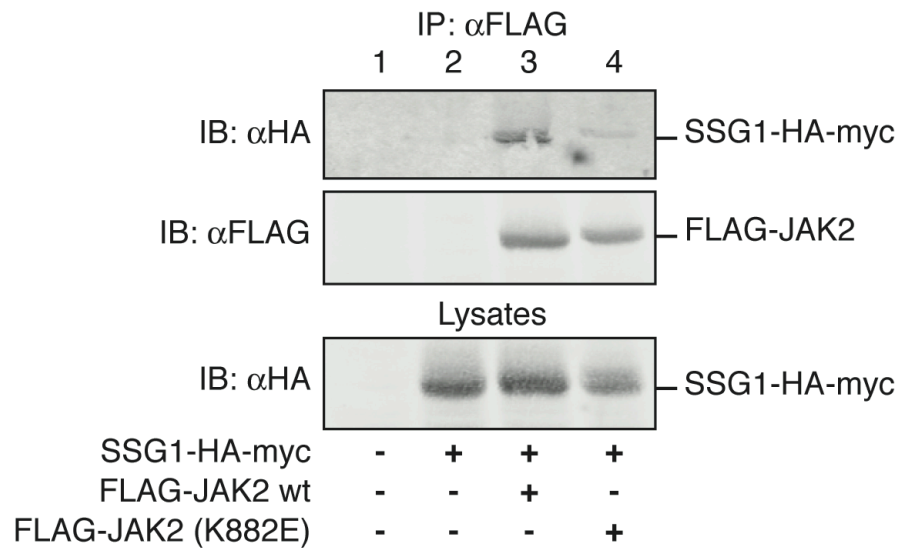


Figure 2.7 Full-length SSG1 interacts with full-length, kinase-active JAK2.

293T cells were transfected with 3 μ g empty vector (lane 1) or cDNA encoding SSG1-HA-myc (lanes 2-4), in combination with 2 μ g of empty vector (lanes 1 and 2) or cDNA encoding wild-type FLAG-tagged JAK2 (lane 3) or FLAG-tagged JAK2 (K882E) (lane 4). FLAG-JAK2 was immunoprecipitated (IP) using α FLAG agarose beads and precipitated proteins immunoblotted (IB) with α HA (top panel). The blot was reprobed with α FLAG to show relative levels of FLAG-JAK2 and FLAG-JAK2 (K882E) (second panel). Cell lysates were immunoblotted with α HA (lower panel). The migration of the FLAG-tagged JAK2 constructs and SSG1-HA-myc is indicated. n=5

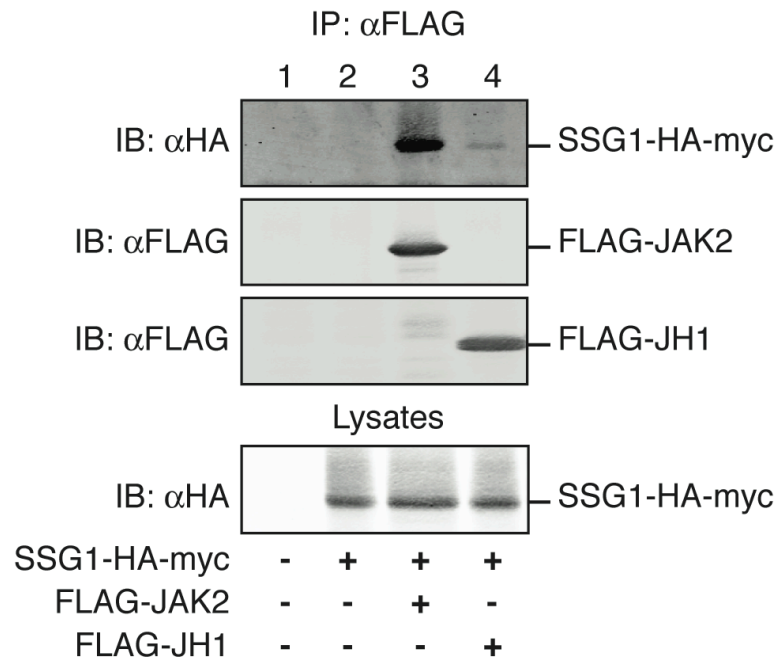


Figure 2.8 SSG1 interacts with full-length JAK2 better than JH1.

293T cells were transfected with 3 μ g of cDNA encoding full-length SSG1-HA-myc or empty vector and co-transfected with 2 μ g cDNA encoding either wild-type FLAG-JAK2 or FLAG-JH1 or vector. JAK2 and the kinase domain were immunoprecipitated (IP) using α FLAG and the precipitated proteins were immunoblotted (IB) with α HA (top panel). The blot was reprobbed with α FLAG and the regions of the blot containing FLAG-JAK2 (second panel) and FLAG-JH1 (third panel) are shown. Lysates were immunoblotted with α HA. The migration of SSG1-HA-myc, FLAG-JAK2, and FLAG-JH1 is indicated. n=2

SRPX2, another SRPX5 domain-containing protein, binds kinase-active JAK2.

We hypothesized that the SRPX5 domains in SSG1 may represent a novel JAK2 binding domain. To test whether other SRPX5 domain-containing proteins would bind preferentially to kinase-active JAK2, SRPX2-myc was co-expressed in 293T cells with FLAG-tagged JAK2 or JAK2 (K882E). As observed for SSG1, SRPX2 co-immunoprecipitated preferentially with wild-type JAK2 (Fig. 9).

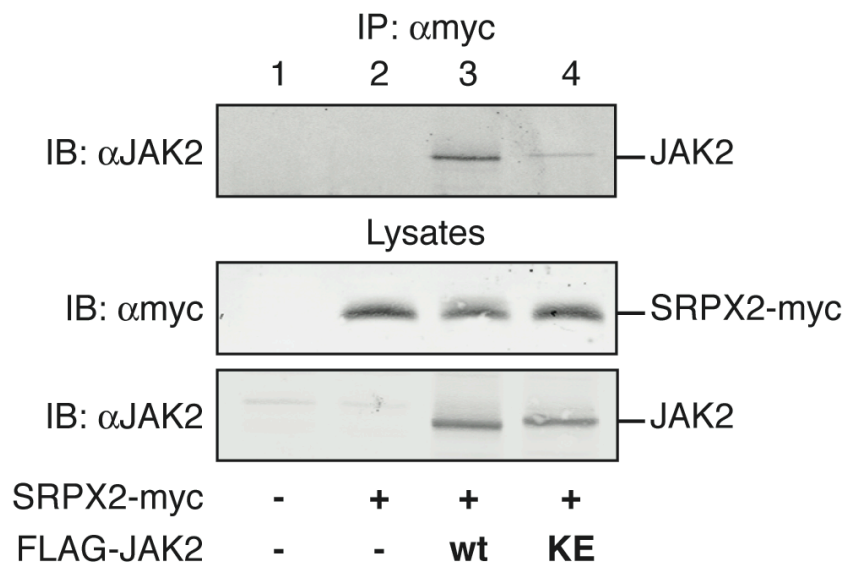


Figure 2.9 SRPX2, another SRPX5 domain-containing protein, binds kinase-active JAK2.

293T cells were transfected with 3 μ g of cDNA encoding SRPX2-myc or vector and 2 μ g of cDNA encoding either FLAG-tagged wild-type JAK2 (wt), JAK2 (K882E) (KE), or vector. SRPX2-myc was immunoprecipitated (IP) with α myc and immunoprecipitated proteins immunoblotted (IB) with α JAK2 (top panel). Lysates were immunoblotted with α myc (middle panel) and reprobbed with α JAK2 (bottom panel). The migration of FLAG-JAK2 and of SRPX2-myc are indicated. n=3

Discussion

In this study, we identified a new JAK2 binding protein, SSG1, by screening a rat adipose cDNA library with JAK2 using the yeast two-hybrid system. Although SSG1 had been previously cloned (22), its size and sequence were reported incorrectly and nothing was known about its function at the cellular level. Here, we provide evidence that rat SSG1 is a protein of 949 amino acids (~110 kDa) that is identical to rat DRO1 (24) and rat CL2 (23), binds to activated JAK2 and is phosphorylated by JAK2. We also provide evidence that the SRPX5 domain within SSG1 is a novel protein-protein interaction domain that is necessary and sufficient for binding to the kinase domain of kinase-active, tyrosyl phosphorylated JAK2.

SSG1 is a protein of unknown function that is expressed in a number of tissues in both the embryo and adult. It has been shown to be downregulated in thyroid, ovarian, pancreatic, and colon cancer cell lines and tumors (23, 24). Overexpression of SSG1 in colorectal and pancreatic cancer cell lines inhibits malignant growth and suppressed anchorage independent growth (24), suggesting that SSG1 may be a tumor suppressor. It is intriguing to speculate whether the interaction between SSG1 and JAK2 affects the apparent tumor suppressing functions of SSG1. Murine SSG1 (Urb) has also been recently shown to be highly expressed in adipose tissue (28), the tissue used to prepare the library used in our yeast two-hybrid screen. SSG1 mRNA expression was found to be downregulated in obese mouse models, including ob/ob, KKAY and diet-induced obese mice. In 3T3-L1 adipocytes, insulin, TNF- α , H₂O₂ and hypoxia decrease SSG1 mRNA levels, suggesting that these factors may contribute to the down-regulation of SSG1 seen with obesity (28). The presence of an N-terminal signal peptide suggests that SSG1 is a secreted protein. However, secretion of SSG1 has only been detected in a few studies (26-28), and whether it is secreted remains somewhat controversial (24). If it is a secreted protein, it is intriguing to speculate that association with JAK2 prior to secretion may affect the rate of secretion of SSG1. Alternatively, if a portion of SSG1 remains in an intracellular compartment, it seems possible that association with SSG1 could affect the trafficking of JAK2, or initiate SSG1-dependent cellular signaling pathways. The demonstration that SSG1 (472-788) appears to be phosphorylated on tyrosines when co-

expressed with kinase-active JAK2 but not kinase-inactive forms of JAK2 suggests that SSG1 could recruit and bind downstream signaling molecules that contain SH2 or PTB or other phosphotyrosine binding domains.

Having identified SSG1 as a JAK2 interacting protein, we focused in this manuscript on the molecular basis for that interaction. Defining the regions of interaction was of great interest because SSG1 clearly bound preferentially to the kinase-active, tyrosyl phosphorylated form of JAK2. Thus, in the yeast two-hybrid assay which is generally considered to assess direct interactions, SSG1 (477-949) preferentially interacted with kinase-active JAK2 and not kinase-inactive JAK2 (K882E). Both full-length SSG1 and the yeast two-hybrid fragment of SSG1, SSG1 (477-949), co-immunoprecipitate from mammalian cells with wild-type, tyrosyl phosphorylated JAK2 but not with kinase-inactive JAK2 (K882E) or JAK2 (Y1007F). This suggested that SSG1 might be binding to phosphorylated tyrosines within JAK2. However, SSG1 lacks SH2, PTB, or C2 domains, domains known to bind to phosphorylated tyrosines (52-58). Thus, it seems possible that SSG1 might be binding to JAK2 via a novel phosphotyrosine binding domain.

Experiments using both N- and C-terminally truncated forms of SSG1 identified a region of SSG1, which binds to tyrosyl phosphorylated JAK2. We have called this domain the SRPX5 domain because it was first described as an uncharacterized, C-terminal fifth domain in the protein SRPX (29). This domain was subsequently identified in SRPX2/SRPUL (33) and then in SSG1 which has three SRPX5 domains (25) which share roughly 30% amino acid identity with the single SRPX5 domain in SRPX and SRPX2. SRPX and SRPX2 belong to the selectin gene family which is currently comprised of five genes: SRPX, SRPX2, selectin P precursor (SELP), selectin E precursor (SELE), and selectin-like protein (SVEP1) (59). Selectins are receptors at the cell surface that are important in cell adhesion and the binding of carbohydrates to facilitate cell rolling (60, 61). The SRPX and SRPX2 genes encode for proteins with similar domain structures which include a signal peptide, three sushi domains, and a hyalin repeat (59). SELP, SELE, and SVEP1 also contain sushi domains (62). It has been suggested that the sushi domains and the hyalin repeat play a role in cell adhesion

(62, 63). Of the selectin family of proteins, only SRPX and SRPX2 have the SRPX5 domain.

Results using truncated forms of JAK2 implicated the kinase domain of JAK2 (JH1 domain) in SSG1 binding. This finding adds SSG1 to the growing list of proteins (e.g. SOCS-1 (11), SOCS-3 (12), PTP-1B (13), and Sts-1 (14)) that appear to bind to the JH1 domain of JAK2. Although, all three of the SRPX5 domains in SSG1 bound to the JH1 domain of JAK2, the second (D2) and third (D3) domains were much more effective than the first (D1) domain. In contrast, full-length JAK2 did not show differential binding to the different SRPX5 domains of SSG1 (i.e. binding of D1, D2 and D3 were roughly equivalent). In addition, full-length SSG1 bound to full-length JAK2 much better than it did to the JH1 domain of JAK2. Furthermore, when we attempted to determine which tyrosines in JAK2 bound to SSG1, we were unable to disrupt binding of either full-length SSG1 or isolated SRPX5 domains to JAK2 by mutating single tyrosines in JAK2, except for those tyrosines whose mutation inhibited JAK2 activity and thus phosphorylation of all the tyrosines within JAK2 (data not shown). This raised the possibility that the individual SRPX5 domains in SSG1 bind multiple phosphorylated tyrosines in JAK2 and that maximal binding of SSG1 to JAK2 may involve at least two sites of interaction, with at least one in the JH1 domain and one lying outside the JH1 domain.

If the SRPX5 domain is a novel JAK2 binding domain and possibly a phosphotyrosine binding domain, we reasoned that other proteins which contain the SRPX5 domain should also bind JAK2. In agreement with this hypothesis, SRPX2 (SRPUL) was also found to bind kinase-active JAK2 but not kinase-dead JAK2 (K882E). Thus, we have identified SRPX2 as another JAK2 interacting protein. In addition, SRPX is a likely candidate to bind JAK2. Little is known about SRPX2 and what is known sheds little light on its cellular function. SRPX2 mRNA is expressed in the heart, ovary, and placenta (33) and protein was visualized in the brain (64). Inhibition of expression of E2A-HLF oncoprotein using a dominant negative form of E2A-HLF suppressed the gene expression of SRPX2 (33). Mutations in SRPX2 may play a role in Rolandic/Sylvian epileptic seizures and perisylvian polymicrogyria (59, 64). A human mutation, N372S, results in partial gain of N-glycosylation resulting in increased SRPX2 protein secretion

as well as protein misfolding (64). Human variation, R75K, in the hypervariable loop in the first sushi domain of SRPX2 (59) has been hypothesized to have functional importance in protein-protein interactions and play a role in two disorders of the Rolandic/Sylvian speech area in the brain (59). The determination that JAK2 binds SRPX2 (SRPUL) suggest that investigations of ligands that activate JAK2 in the affected tissues could be fruitful for better understanding and potentially managing these medical conditions.

In summary, we identified SSG1/DRO1/CL2/Urb as a novel JAK2 binding protein of Mr~110,000. The interaction with JAK2 occurred via the SRPX5 domains of SSG1. Because the three SRPX5 domains of SSG1 only interact with kinase-active, tyrosyl phosphorylated JAK2, the SRPX5 domain is quite possibly a novel phosphotyrosine binding domain with at least some specificity for JAK2. Consistent with the SRPX5 domain serving as a JAK2 binding site, we demonstrated that a second SRPX5 domain-containing protein, SRPX2 (SRPUL), could also bind activated, tyrosyl phosphorylated JAK2. The fact that SSG1 is expressed in a large number of tissues both in the embryo and adult, that its level of expression appears to be highly regulated, and that it has been implicated in such a wide variety of functions, including tumor suppression, suggests that SSG1 may play an important and fundamental role in cell function. The fact that it appears to be secreted from adipocytes and downregulated in obesity raises the possibility SSG1 plays a role in fat and carbohydrate metabolism. We also observed that SSG1 is tyrosyl phosphorylated in the presence of activated JAK2. Therefore, JAK2 has the potential to influence SSG1 at multiple levels ranging from localization of SSG1 to specific cellular regions to regulation of SSG1 function.

¹The abbreviations used are: C2, conserved domain 2 for PKC δ ; DRO1, down-regulated by oncogene1; drs, downregulated by Src; ER, endoplasmic reticulum; GH, growth hormone; JAK2, Janus kinase 2; PAGE, polyacrylamide gel electrophoresis; PTB, phosphotyrosine binding domain; PY, phosphotyrosine; SH2, Src-homology 2; SOCS, suppressor of cytokine signaling; SRPUL, sushi repeat protein upregulated in leukemia; SRPX, sushi repeat containing protein, X-linked; SSG1, steroid-sensitive gene-1; Stat, signal transducer and activator of transcription; Urb, upregulated in brown adipose tissue of bombesin-receptor-subtype-3-deficient mice.

Note: This chapter will be submitted for publication, in modified form, to Biochemistry under the title: “Identification of steroid-sensitive gene-1 as a novel JAK2 binding protein and the SRPX5 domain as a novel phosphotyrosine binding domain” by Erin E. O’Leary, Anna M. Mazurkiewicz-Muñoz, Lawrence S. Argetsinger, Hung T. Huynh, and Christin Carter-Su. Dr. Anna Mazurkiewicz-Muñoz provided part of Fig. 2.1 and Figs. 2.2-2.4 while I provided part of Fig. 2.1 and Figs. 2.5-2.9.

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CHAPTER III

PHYSIOLOGICAL CONSEQUENCES OF THE INTERACTION BETWEEN JAK2 AND SSG1

Abstract

Growth hormone (GH) is an important regulator of body growth and metabolism. GH binding to its receptor activates the receptor associated tyrosine kinase, JAK2, which in turn, initiates multiple signaling cascades, including activation of Stats 1, 3, 5a, and 5b. Previously, we identified a novel JAK2 binding protein, steroid-sensitive gene-1 (SSG1), as a binding partner for activated JAK2. This study aims to identify the physiological consequences of the interaction between SSG1 and JAK2. In this study, we show that SSG1 does not alter JAK2 kinase activity and only modestly increases the phosphorylation of Stat5b on Tyr694. In contrast, SSG1 enhances phosphorylation of Stat3 on Tyr705 in the presence and absence of GH. Antibody to phospho-Ser727 of Stat3 revealed that SSG1 does not affect phosphorylation of Ser 727. However, phosphatase experiments suggest that SSG1 may enhance GH-independent phosphorylation of a different residue in Stat3. We also show that SSG1 negatively regulates the GH-responsive gene, *c-fos*. By fluorescent microscopy, we show that SSG1 co-localizes with markers for both the ER and the Golgi. By confocal microscopy, we saw that portion of JAK2 co-localizes with SSG1 in this subcellular compartment. We also demonstrate that SSG1 contains a functional signal peptide and is a secreted protein, which is blocked by BFA. We also show that JAK2 enhances both the secretion and cleavage of SSG1. Interestingly, we also detect extracellular staining of SSG1, which is enhanced by the presence of wild-type JAK2.

Introduction

The cytokine/hematopoietin family of receptors includes receptors for growth hormone (GH), prolactin, erythropoietin, leptin, and multiple interleukins and interferons. The Janus family of tyrosine kinases includes JAK1, JAK2, JAK3, and Tyk2 (1-5). Cytokine receptors signal through the associated JAK family kinases with nearly two-thirds of the cytokine receptor family activating JAK2 (6, 7). Activation of JAK family kinases promotes growth, proliferation, and differentiation in the cell types in which they are expressed and activated. It is not surprising, then, that activating mutations in JAK kinases and dysregulation of JAK signal transduction have deleterious effects, causing various cancers and immune disorders (reviewed in (8)).

GH is an important regulator of body growth and metabolism, yet the mechanism by which GH elicits these responses is not completely understood. In GH signal transduction, GH binding to the GH receptor activates the associated tyrosine kinase, JAK2, resulting in tyrosyl phosphorylation of both JAK2 and the GH receptor (9-19). These phosphorylated tyrosine residues in JAK2 and the GH receptor serve as docking sites for downstream signaling molecules and recruit a number of signaling molecules to the GH receptor-JAK2 complex, some of which also become phosphorylated by JAK2. Proteins recruited to the GH receptor-JAK2 complex include SH2B1 (SH2B) and SH2B2 (APS), adaptor proteins which bind to phosphotyrosine 813 in JAK2 and enhance kinase activity of JAK2 and cell motility (12, 20, 21); Shc which activates the Ras-MAP kinase pathway (7, 22, 23); insulin receptor substrates 1/2 which leads to the activation of the phosphoinositide 3-kinase pathway (reviewed in (23)); proteins involved in the regulation of the cytoskeleton (reviewed in(23)); and signal transducers and activators of transcription (Stat) proteins (reviewed in (23-25)).

Stat proteins are latent cytoplasmic transcription factors that are activated by phosphorylation by JAK kinases with Stats 1, 3, 5a, and 5b playing roles in mediating responses to GH (23-26). Phosphorylation by JAK2 allows for the Stat proteins to homo- (27) or heterodimerize (28-30) via reciprocal phosphorylated tyrosine-SH2 domain interactions. Dimerization allows for translocation to the nucleus where the Stat proteins bind to DNA and act as transcription factors for target genes (31, 32) including the GH-

regulated target genes *c-fos*, *c-jun*, *IGF-1*, and *serine protease inhibitor (Spi) 2.1* (33-37). Stat proteins differ in their DNA binding specificities as well as in their tissue distribution, resulting in a large number of genes being regulated by Stat transcription factors.

Previously, we identified steroid-sensitive gene-1 (SSG1) in a yeast two-hybrid screen as a novel protein, which binds to kinase-active JAK2, but not inactive JAK2 (O'Leary et al., *manuscript in preparation*). We also showed that SSG1 is phosphorylated by JAK2 and identified a novel JAK2 binding domain in SSG1 (O'Leary et al., *manuscript in preparation*). SSG1 was initially identified as a gene that was downregulated in response to estrogen using differential display of uterine tissues from ovariectomized versus ovariectomized/estrogen-treated rats (38). SSG1 was subsequently independently identified as DRO1, CL2, Urb, and Equarin in different contexts. Rat SSG1/DRO1 (downregulated by oncogenes-1) was identified using subtractive suppression hybridization as a protein whose gene expression was decreased in cells neoplastically transformed by β -catenin (39). Rat SSG1/CL2 was also identified as a protein induced by the adenovirus E1A gene but downregulated in adenomas and carcinomas (40). These results led both Bommer et al. (39) and Visconti et al. (40) to propose that SSG1/DRO1/CL2 may function as a tumor suppressor. The gene encoding murine SSG1, also known as Urb, was identified by differential display as a gene upregulated in brown adipose tissue with a proposed function in energy metabolism (41). Liu et al. (42) identified SSG1 through a differential display as a gene that is downregulated during osteoblast differentiation, suggesting a role for SSG1/Urb in bone development. A chicken ortholog of SSG1, Equarin, was identified using a signal sequence trap screen and implicated in eye development (43). However, the role SSG1 has at the cellular level, as well as how the cell regulates SSG1 remains unclear.

This study aimed to identify the physiological consequences of the previously described interaction between SSG1 and JAK2. We show that SSG1 does not enhance JAK2 kinase activity and only modestly increases phosphorylation of Stat5b on Tyr694, which is required for activation of Stat5b. In contrast, SSG1 enhances phosphorylation of Stat3 on Tyr705 and may promote the phosphorylation of another residue in Stat3. SSG1 negatively regulates basal and GH-dependent *c-fos* gene transcription. We show

that SSG1 co-localizes with both ER and Golgi markers and that JAK2 partially co-localizes with SSG1 in COS-7 cells. Finally, we show that SSG1 has a functional signal peptide resulting in SSG1 secretion. SSG1 secretion and cleavage are enhanced in the presence of kinase-active JAK2 but not kinase-inactive JAK2 (K882E). Because JAK2 has also been shown not only to bind SSG1 but also to phosphorylate SSG1 on tyrosines, these results raise the possibility that the interaction of SSG1 with JAK2 enhances SSG1 secretion.

Materials and Methods

Cells and reagents.

293T and COS-7 cells were from American Type Culture Collection. Chinese hamster ovary cells expressing amino acids 1-454 of rat GH receptor (CHO454) were provided by Dr. G. Norstedt (Karolinska Institute, Stockholm, Sweden) (44). The stock of 3T3-F442A preadipocyte cells were kindly provided by H. Green (Harvard University). Dulbecco's Modified Eagle Medium (DMEM), antibiotic-antimycotic, trypsin-EDTA, and Magic Mark molecular weight standards were from Invitrogen. Calf serum and fetal bovine serum were from Atlanta Biologicals. Bovine serum albumin was from Intergen (CRG-7) and Proliant. Recombinant 22,000-Da Growth Hormone was a kind gift from Eli Lilly & Co. (Indianapolis, IN) and was used at a concentration of 500 ng/ml unless otherwise noted. Aprotinin, leupeptin, Triton X-100, and alkaline phosphatase were from Roche. Lambda protein phosphatase (λ ppase) was from New England Biolabs. Recombinant protein A-agarose was from Repligen. Hybond-C Extra nitrocellulose paper was from Amersham Biosciences. The QuickChange Site-Directed Mutagenesis Kit was from Stratagene. Trichloroacetic acid was from J. T. Baker. Paraformaldehyde was from Electron Microscopy Sciences. Brefeldin A dissolved in ethanol was from Sigma.

Antibodies.

Polyclonal antibody to JAK2 (α JAK2, 1:7500), antibody recognizing a peptide containing phosphorylated tyrosines 1007 and 1008 of JAK2 (α pY1007/1008 JAK2, 1:2000), monoclonal anti-phosphotyrosine antibody (α PY; 4G10, 1:7500), and monoclonal pY705Stat3 (α pY705Stat3, 1:1000) were from Millipore, Inc. and were used for immunoblotting. Monoclonal JAK2 (691) antibody (Invitrogen) was used for immunoblotting at 1:2000. Monoclonal antibody to influenza virus hemagglutinin (HA) tag (α HA; HA.11) was from Covance and used at a dilution of 1:100 for immunoprecipitation and 1:1000 for immunoblotting. Rat monoclonal HA antibody (α HA; 3F10) used at a dilution of 1:2000 was from Roche. Monoclonal antibody against myc-tag (α myc; 9E10) was from Santa Cruz Biotechnology, Inc. and used at a dilution of

1:100 for immunoprecipitation and 1:10,000 for immunoblotting. Polyclonal antibody against the myc-tag (α myc; A14) was from Santa Cruz Biotechnology, Inc. and used at a dilution of 1:1000 for immunoblotting and 1:200 for immunocytochemistry. Polyclonal Stat3 antibody (α Stat3, K-15, 1:1000), and monoclonal Stat5b antibody (α Stat5b, G-2, 1:5000) were from Santa Cruz Biotechnology, Inc. Monoclonal pS727Stat3 antibody (α pS727Stat3, 1:000) was from Cell Signaling. Polyclonal antibody against pY694Stat5 (α pY694Stat5, 1:7500) was from Zymed. Monoclonal anti-FLAG M2 antibody (α FLAG, 1:1000) was from Sigma. Anti-FLAG M2 antibody conjugated to agarose beads was from Sigma. Affinity purified anti-mouse IgG, anti-rabbit IgG, anti-rat IgG, and anti-GFP antibodies conjugated to IRDyes 680 and 800 were from Rockland, Molecular Probes, and Licor were used at a dilution of 1:20,000 for western blotting. Polyclonal antibody against giantin (α Giantin, 1:500) was from Covance. Polyclonal antibody against calnexin (α Calnexin, 1:500) was from Stressgen. AlexaFluor 488 conjugated anti-mouse IgG (1:500, Molecular Probes), AlexaFluor 594-conjugated anti-rabbit IgG (1:500, Molecular Probes), and Oregon Green anti-rabbit IgG (1:1000, Molecular Probes) were used to visualize the proteins by immunocytochemistry.

Plasmids.

The *prk5* expression vector encoding wild-type murine JAK2 (45) or murine JAK2 K882E in which the critical lysine in the ATP-binding domain is mutated to a glutamate (46) were provided by Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). The mammalian expression vector encoding murine wild-type JAK2 with an N-terminal FLAG tag, FLAG-JAK2, was described previously (47). FLAG-JAK2 K882E was prepared using primers 5' GGTGGTCGCTGTGgAgAAACTCCA GCACAGCAC 3' and 5' GTGCTGTGCTGGAGTTTcTcCACAGCGACCACC 3' using the QuikChange mutagenesis kit. The mammalian expression vector encoding FLAG-Stat3 was kindly provided by R. Jove (City of Hope National Medical Center, CA). The GFP-Stat5b expression vector was previously described (48). The mammalian expression vector pLM108 encoding rat GHR was provided by G. Norstedt (Karolinska Institute, Sweden). Mammalian expression vectors encoding SSG1 with a C-terminus myc tag (SSG1-myc) and SSG1 with an HA tag after amino acid 30 and a C-terminal

myc tag (SSG1-HA-myc) were described previously (O'Leary et al., *manuscript in preparation*). *c-fos*/Luc reporter plasmid, containing the 379 base pairs of mouse *c-fos* promoter immediately 5' of the transcription start site, cloned upstream of a luciferase gene (49) was provided by Dr. J. Schwartz (University of Michigan). The RSV β -galactosidase plasmid was provided by Dr. M. Uhler (University of Michigan). GFP-JAK2 was created by first inserting a NotI site (underlined) in pEGFP-C1 vector with primers 5' TCGAGGTTCGAGGGATCCGATGCGGCCGCACGT 3' and 5'CTAGACGTGCGGCCGCATCCGATCCGTCGACC 3'. Wild-type JAK2 was subcloned from prk5-JAK2 into the pEGFP-C1 NotI vector using the Sall and NotI restriction sites to create an N-terminal fusion protein, GFP-JAK2. The same primers listed above were used to produce the K882E mutation in GFP-JAK2, GFP-JAK2 K882E. All constructs and mutations were verified by sequencing.

Cell culture and transfection.

293T and 3T3-F442A preadipocyte cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin 0.25 μ g/ml amphotericin B, and 8% calf serum. COS-7 cells were grown in the same medium except containing 8% fetal bovine serum instead of calf serum. 293T and COS-7 cells were transiently transfected by calcium phosphate precipitation (50) and assayed between 24 and 48 h after transfection. 3T3-F442A cells were electroporated using the Nucleofactor system (Amaxa). For GH treated cells, cells were washed twice in DMEM, and medium was replaced with DMEM supplemented with 1% BSA for 18 h prior to GH addition for the indicated time. For Brefeldin A (BFA) treated cells, cells were washed once in DMEM and medium was replaced with DMEM supplemented with 1% calf serum with the indicated concentration of BFA for 16 h.

CHO454 cells were cultured in F12 medium supplemented with 1 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B, and 8% fetal bovine serum. CHO454 cells grown in 35 mm wells were transiently transfected by calcium phosphate precipitation with 0.5 μ g *c-fos*/Luc, 0.1 μ g RSV β -galactosidase plasmid DNA and increasing amounts of cDNA encoding SSG1. 24 h after

transfection, cells were incubated overnight in serum-free medium containing 1% BSA. Cells were then treated for 4 h with 500 ng/ml of GH at 37°C and rinsed three times with PBS (10 mM sodium phosphate, 137 mM NaCl [pH 7.4]). In a separate series of experiments, CHO454 were transiently transfected with 0.5 µg *c-fos*/Luc, 0.1 µg RSV β-galactosidase, and varying amounts of cDNA encoding SSG1 and either JAK2 or JAK2 (K882E) and assayed after 24 h incubation in medium containing 1% BSA. All experiments were carried out at 37°C. The amount (µg) of cDNA/well in all experiments was kept constant using empty vector.

Immunoprecipitation and immunoblotting.

Cells were washed three times with chilled PBSV (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na₃VO₄, pH 7.4) and solubilized in lysis buffer (50mM Tris [pH 7.5], 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na₃VO₄) containing 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Lysates were centrifuged at 16,750 xg for 10 min at 4°C. The cell lysate was incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose during an 1 h incubation at 4°C. In the experiments in which proteins were immunoprecipitated with αFLAG conjugated agarose beads, lysate was incubated with the beads for 2 h at 4°C. Both sets of beads were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS-PAGE sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue). The solubilized proteins were separated on a 5-12% SDS-PAGE gradient gel. Proteins in the gel were transferred to a nitrocellulose membrane and detected by immunoblotting with the indicated antibody using the Odyssey Infrared Imaging System (LI-COR Biosciences). The intensity of the bands in immunoblots was quantified using LI-COR Odyssey 2.1 software. Values for phosphorylated proteins were normalized to total level of unphosphorylated protein with background subtracted values. Experiments were performed at least 2 times, usually 3 or more, with similar results. Means ± SEM are included.

Whole cell lysate.

Cells were washed three times with chilled PBSV (10 mM sodium phosphate, 137 mM NaCl, 1 mM Na₃VO₄, pH 7.4) followed by the addition of boiling SDS-PAGE sample buffer to the cells. Samples were then boiled and vortexed several times. Samples were diluted 1:4 with lysis buffer before proteins were separated on a 5-12% SDS-PAGE gradient gel, transferred to nitrocellulose membrane, and detected by immunoblotting with the indicated antibody using the Odyssey Infrared Imaging System (LI-COR Biosciences). In some cases, cells were scraped in PBSV, briefly centrifuged and then resuspended in PBSV. Equal volumes were separated into two tubes: one for solubilizing protein cell lysates and one to create whole cell lysates.

Phosphatase assay.

Proteins were dephosphorylated as previously described (20). Briefly, cells were stimulated with 500 ng/ml of GH for the indicated times. FLAG-Stat3 was immunoprecipitated with α FLAG conjugated agarose beads. The immunoprecipitates were washed in 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5 and then incubated in 100 μ l of the same buffer containing 40U of alkaline phosphatase with or without 10 mM Na₃VO₄ for one hour at 37°C. For λ phosphatase, immunoprecipitates were washed in 50 mM Tris-HCl, 100 mM NaCl, 2mM MnCl₂, 0.1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, pH 7.5, then incubated with 50 μ l of the same buffer containing 200U λ phosphatase with or without 10 mM Na₃VO₄ at 30°C for 30 min. The dephosphorylation reactions were terminated by adding SDS-PAGE sample buffer and boiling. As controls, immunoprecipitates were treated identically, but no phosphatase was added. The resultant proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

TCA precipitation.

10 h post-transfection, medium was changed to 1% calf serum. Medium was collected 26 h post-transfection and briefly centrifuged at 2500 rpm for 2 min to remove dead cells. Proteins in the medium were precipitated by adding TCA (15% final concentration) to 1mL of medium and incubating on ice for 5 min. Precipitated proteins

were pelleted by centrifugation at 16,750 xg for 5 min. The pellet was resuspended in 1% SDS at 37°C for 5-10min. To neutralize the acid, 1-3 μ l of 10N NaOH was added to each sample.

Immunocytochemistry.

COS-7 cells on coverslips were transfected using calcium phosphate precipitation. 24 h later, cells were fixed by adding 16% paraformaldehyde (PFA) directly to the growth medium for a final concentration of 4% PFA and incubated for 15 min at 37°C. The 4% PFA-containing medium was removed and the cells were permeabilized with 0.1% TritonX-100 in PBS containing 4% PFA for 7 min at room temperature. The cells were then washed twice with PBS and incubated in 5% fat free milk in PBS for 30 min at room temperature. The cells were incubated with primary antibody in 5% milk in PBS at the indicated dilutions for 30 min. The cells were then rinsed twice with PBS before the addition of the secondary antibody in 5% milk in PBS and incubation for 30 min. The chromosomal/nuclear stain DAPI (Molecular Probes) was added to double distilled H₂O (ddH₂O) at a concentration of 2 ng/ml and incubated for 10 min at room temperature. The cells were then rinsed in ddH₂O to wash away residual salts and excess DAPI. Prolong Gold antifade reagent (Molecular Probes) was used to mount the coverslip on the microscope slide.

Microscopy.

The cells were visualized by fluorescence microscopy (Nikon Eclipse TE200) with a 60X oil objective. Images were captured using a CoolSnap HQ digital camera (Roper Scientific) and viewed using MetaVue imaging software (Molecular Devices, Sunnyvale, Ca). Pictures were captured using equal exposure times to assess differences in signal intensity. SSG1 images showing extracellular staining were taken in a different focal plane than the JAK2 images; SSG1 images were focused on the coverslip; JAK2 images were focused on the cell. The intensity of the extracellular staining due to secreted SSG1-myc was assessed by a blinded observer. Briefly, the observer randomly selected 8-10 isolated SSG1-myc positive cells per experiment per condition. Using MetaVue's "trace region" function, the observer manually selected a region of

extracellular staining that surrounded the cell and used the “region statistics” function to quantify the average signal intensity of the region selected. The trace was then dragged outside of the area of SSG1-myc secretion and a second average intensity measurement was taken and used to calculate the background signal. Background subtracted values were plotted. The experiment was performed 3 times. Means \pm SEM are shown.

Confocal microscopy.

Analysis of SSG1-myc and GFP-JAK2 subcellular distribution was performed with an Olympus FluoView 500 laser scanning confocal microscope utilizing a 60X oil-immersion objective and FluoView version 5.0 software. AlexaFluor 594 fluorescence was excited with a Green Helium Neon (HeNe) laser at 543 nm and emission was measured through a 560 nm long pass filter (560 and above). GFP and/or Oregon Green conjugated secondary fluorescence was excited with an Argon (Ar) laser at 488 nm and emission was measured through a 505-525 nm filter. Images depicting co-localization of SSG1 and JAK2 were captured using sequential scanning to avoid fluorescence cross talk.

Luciferase assay.

Cells were solubilized in 100 mM potassium phosphate, 0.2% Triton X-100, 1 mM dithiothreitol, pH 7.8. Luciferase or β -galactosidase activity was measured using an Opticomp Luminometer (MGM Instruments, Inc., Sherman, CT). The luciferase values were normalized to β -galactosidase activity.

Results

The effect of SSG1 on JAK2 kinase activity.

We have previously shown that SSG1 binds JAK2 in a kinase-dependent manner (O'Leary et al., *manuscript in preparation*). To determine the physiological importance of the SSG1-JAK2 interaction, we first examined whether SSG1 affects JAK2 kinase activity. 293T cells were co-transfected with cDNA encoding vector alone or SSG1-HA-myc, which has an internal HA tag after amino acid 30 and a C-terminal myc tag, in the presence or absence of cDNA encoding FLAG-tagged JAK2. FLAG-JAK2 was immunoprecipitated with α FLAG-M2 agarose beads and the immunoprecipitated proteins were immunoblotted with α pY1007/pY1008. Tyrosine 1007 is the activating tyrosine in JAK2, thus, the amount of pY1007 generally reflects the amount of kinase-active JAK2 (14). SSG1 was found not to affect the kinase activity of JAK2 when normalized to the amount of JAK2 (Fig. 1, compare lanes 3 and 4). Similar results were obtained using SSG1-myc which has only the C-terminal myc tag (data not shown). In four experiments, the mean \pm SEM for pY1007/pY1008 JAK2 normalized to the amount of JAK2 in the presence of SSG1 versus the absence of SSG1 was 0.99 ± 0.10 .

The effect of SSG1 on phosphorylation of Stat5b and Stat3.

We next examined whether overexpression of SSG1 affects the ability of GH to stimulate the phosphorylation of Stat5b and Stat3 on Tyr694 and Tyr705, respectively. Phosphorylation on these tyrosines by JAK2 is a necessary first step in the activation by GH of Stats 5b and 3, respectively (23-26). 293T cells were co-transfected with cDNA encoding GFP-Stat5b or FLAG-Stat3, rat GH receptor, and SSG1-myc. Cells were incubated in serum-free medium for 18 h, and then stimulated with or without 500ng/ml GH for 15 min. Figure 2 reveals a very modest increase ($28 \pm 11\%$, $p < 0.05$, $n = 5$) in the levels of GH-dependent phosphorylation of Tyr694 in Stat5b SSG1-expressing cells compared to control cells.

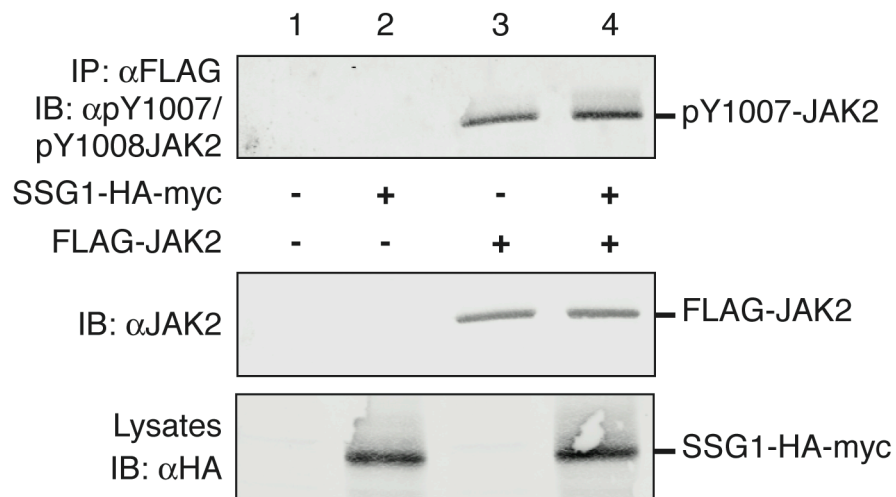


Figure 3.1 The effect of SSG1 on JAK2 kinase activity.

293T cells were transfected with 3 μ g empty vector or cDNA encoding SSG1-HA-myc in combination with 2 μ g of empty vector or cDNA encoding FLAG-JAK2. FLAG-JAK2 was immunoprecipitated (IP) with α FLAG agarose beads and immunoprecipitated proteins were immunoblotted (IB) with α pY1007/pY1008 (top panel). This blot was reprobbed with α JAK2 to show relative levels of immunoprecipitated JAK2 (second panel). Lysates from the transfected cells were immunoblotted with α HA to reveal levels of expression of SSG1-HA-myc. The migration of FLAG-JAK2 and SSG1-HA-myc is indicated. n=4

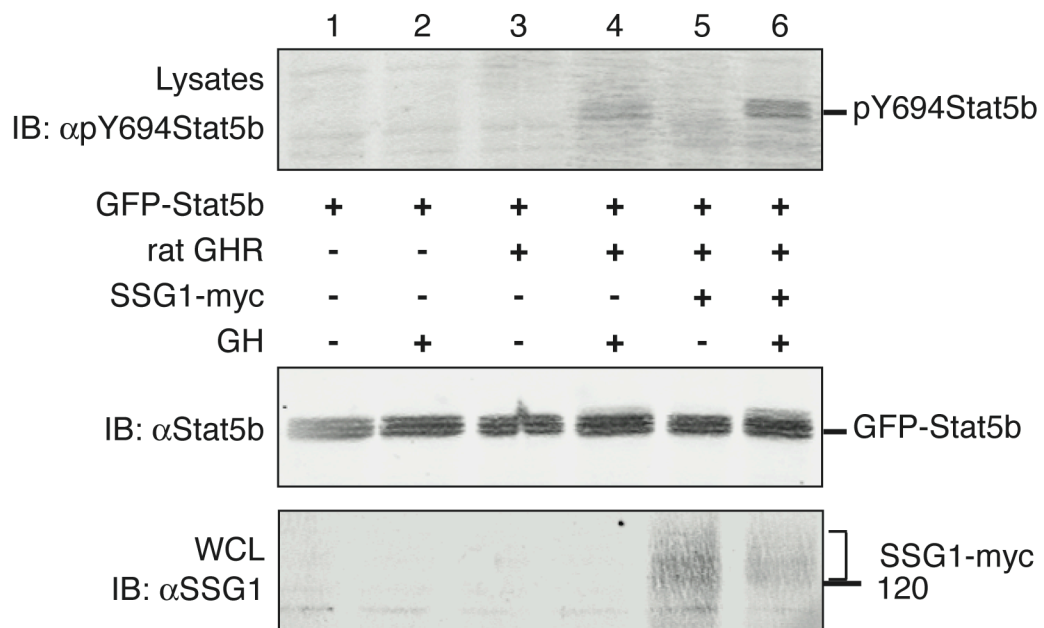


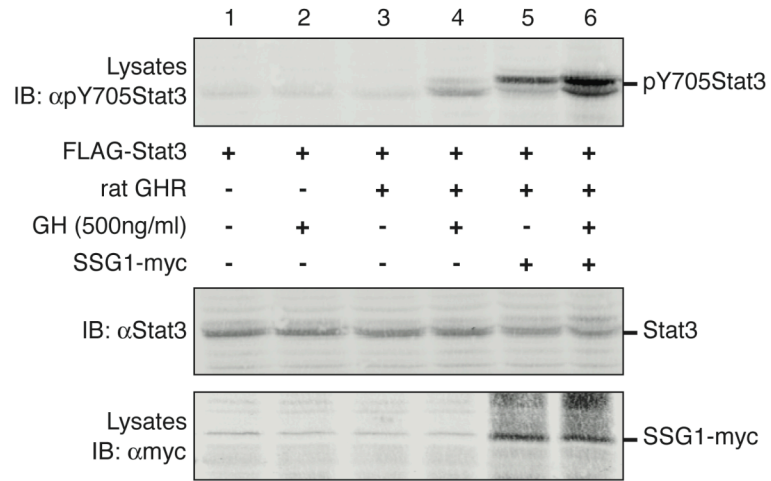
Figure 3.2 The effect of SSG1 on tyrosyl phosphorylation of Stat5b.

293T cells were co-transfected with cDNA encoding GFP-Stat5b (500ng), rat GHR (250 ng), and SSG1-myc (1 μ g) as indicated. 35 h post-transfection, cells were washed and medium was replaced with 1% BSA/DMEM for 17 h. Cells were stimulated with 500 ng/ml GH for 15 min, and lysates were prepared. Lysates were immunoblotted (IB) with α pY694-Stat5 (top panel) and reprobred with α Stat5b (middle panel). Whole cell lysates were immunoblotted with α SSG1 (bottom panel). The migration of GFP-Stat5b and SSG1-myc is indicated. n=5

In contrast, SSG1 had several effects on phosphorylation in Stat3. SSG1 dramatically promoted both basal (Fig. 3A, compare lanes 3 and 5) and GH-dependent phosphorylation of Tyr705 (Fig. 3A, compare lanes 4 and 6). SSG1 increased basal levels of pTyr705 to levels similar to (Fig. 3B) or even above (Fig. 3A) GH-stimulated levels in control cells. Levels of Tyr705 phosphorylation in GH-treated cells were on average 4.33 ± 0.622 times higher in cells expressing SSG1 than in control cells. The two pTyr705-Stat3 bands observed in the SSG1-expressing cells in both the absence and presence of GH migrated slightly higher than the corresponding bands observed for the control, GH-treated cells. Overexpression of SSG1 also significantly changed the distribution between the two pTyr705-Stat3 bands so that in SSG1 overexpressing cells, the upper band dominated.

An upward shift in migration of a protein is consistent with the presence of one or more posttranslational modifications. Because both Stat3 bands are detected with α pY705, the upward shift must be due to some other post-translational modification. Stat3 has also been shown to be phosphorylated on Ser727. We therefore examined whether SSG1 affected phosphorylation of Stat3 on Ser727. Cells were prepared as for Fig. 3A. Confirming the results shown in Fig. 3A, lysates immunoblotted with α pY705-Stat3 showed that SSG1 promotes the phosphorylation of Tyr705 in Stat3 both in the absence and presence of GH (Fig. 3B, second panel). In contrast, Stat3 was phosphorylated on Ser727 in the absence of GH, and when normalized to levels of Stat3, phosphorylation of Ser727 was not reproducibly affected by either GH stimulation or expression of SSG1 (Fig. 3B, top panel). Furthermore, both the upper and lower Stat3 bands were phosphorylated on Ser727, indicating that the difference between the upper and lower Stat3 bands is not phosphorylation of Stat3 on Ser727. Taken together, these results suggest that SSG1 enhances the phosphorylation of Tyr705 on Stat3 both in the presence and absence of GH. It also most likely promotes one or more additional post-translational modifications that results in decreased migration of Stat3 in SDS-PAGE gels.

A



B

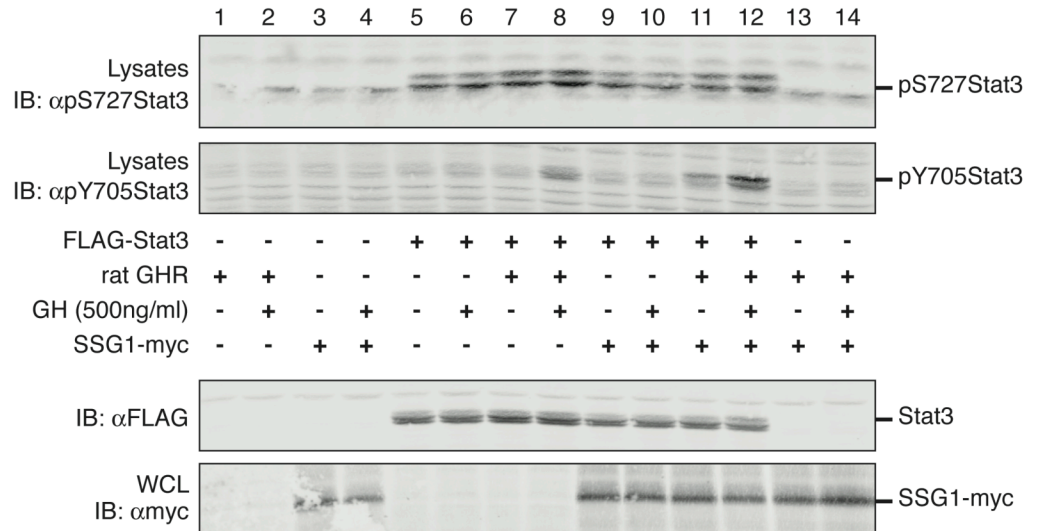
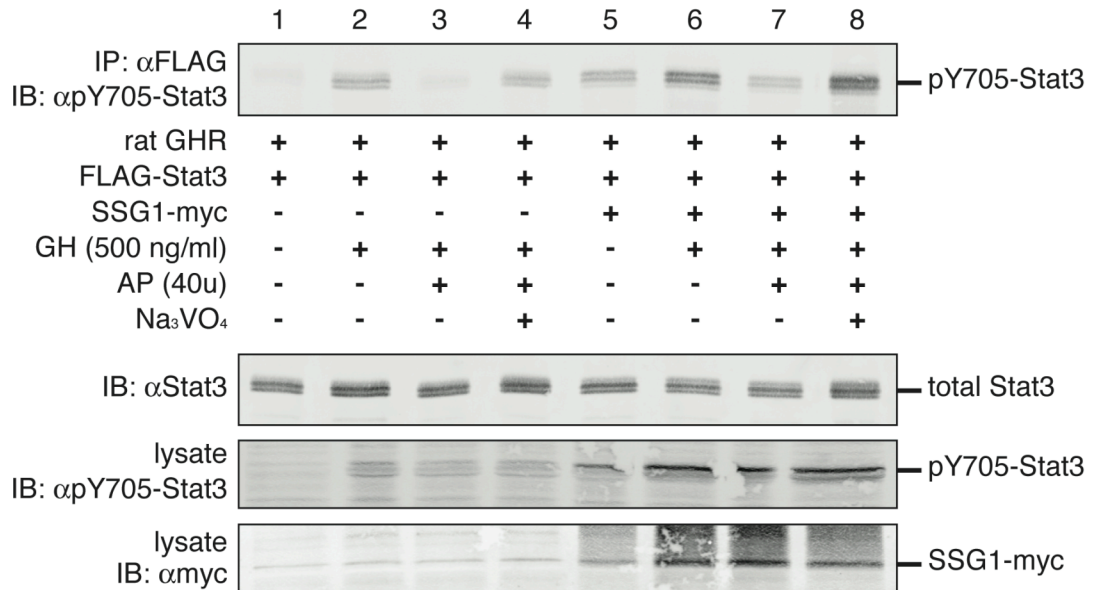
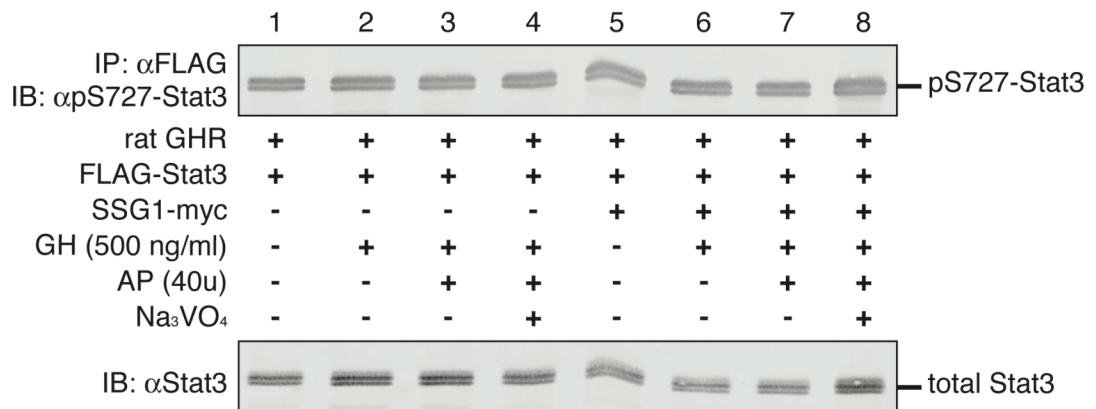


Figure 3.3 SSG1 stimulates the tyrosyl phosphorylation of Stat3.

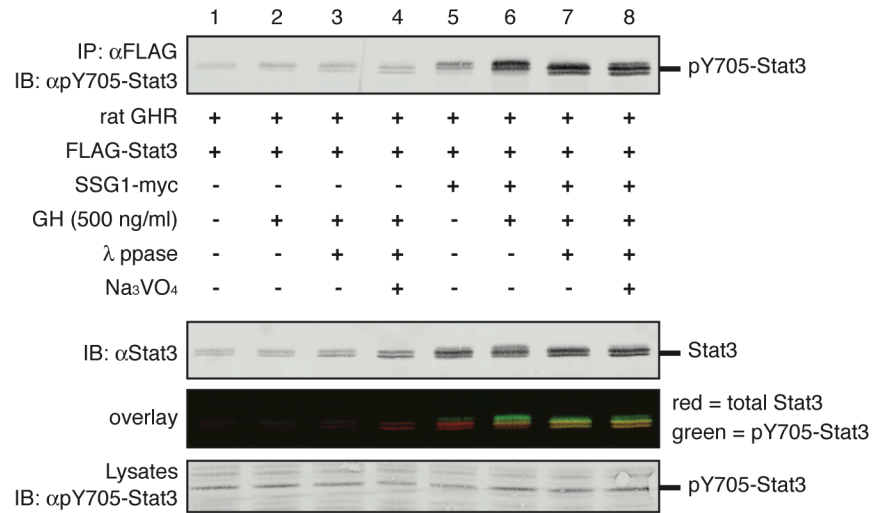
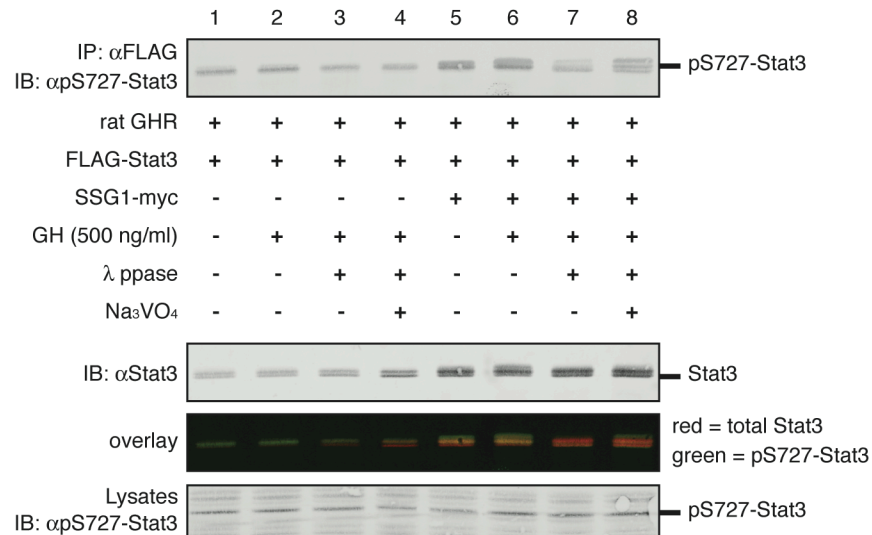
293T cells were transfected with cDNA encoding FLAG-Stat3 (500 ng), rat GHR (250 ng), and SSG1-myc (1 μ g). 35 h post-transfection, cells were washed and medium was replaced with 1% BSA/DMEM for 18 h. Cells were stimulated with 500 ng/ml GH for 15 min, and lysates were prepared. (A) Lysates were immunoblotted (IB) with α pY705-Stat3 (top panel) and reprobed with α Stat3 (middle panel). A second set of lysates was probed α myc (bottom panel). (B) Lysates were blotted with α pS727-Stat3 (top panel), α pY705-Stat3 (second panel), or α FLAG (third panel). Whole cell lysates were probed with α myc (bottom panel). The migration of FLAG-Stat3 and SSG1-myc is indicated. n=4-6

To gain insight into whether the SSG1-induced upward shift in migration of Stat3 is due to phosphorylation, we performed a phosphatase assay using alkaline phosphatase, a non-specific protein phosphatase that dephosphorylates phosphotyrosines, phosphoserines, and phosphothreonines. FLAG-Stat3 and rat GH receptor were co-expressed with or without SSG1-myc in 293T cells. The following day, cells were incubated in serum-free medium for 18 h, and then stimulated with or without 500ng/ml GH for 15 min. FLAG-Stat3 was immunoprecipitated with α FLAG-M2 agarose beads, washed and then incubated in the presence or absence of alkaline phosphatase in the presence or absence of sodium orthovanadate, an inhibitor of alkaline phosphatase. Immunoblotting the immunoprecipitates with α pY705-Stat3 revealed that alkaline phosphatase dephosphorylated Tyr705 of Stat3 in both control (- SSG1) (Fig 4A, lane 3) and SSG1-expressing cells (Fig 4A, lane 7) and that sodium orthovanadate blocked dephosphorylation (Fig 4A, compare lanes 3 and 4; lanes 7 and 8). Immunoblotting the cell lysates with α pY705-Stat3 show that Stat3 was phosphorylated on Tyr705 before phosphatase treatment. In contrast, alkaline phosphatase did not dephosphorylate Ser727 in Stat3 in control (-SSG1) or SSG1-expressing cells (Fig. 4B, top panel), suggesting that pSer727 was protected from dephosphorylation. Further, alkaline phosphatase did not change the ratio of the different Stat bands seen using α Stat3, α pSer727-Stat3, or α pY705-Stat3, indicating that either the upper band contains a phosphorylated amino acid, that like pSer727, is not susceptible to desphosphorylation by alkaline phosphatase or contains a different post-translational modification (e.g. acetylation, methylation, sumoylation) than the bottom band.

Because alkaline phosphatase did not dephosphorylate pSer727, we repeated the experiment using λ phosphatase, another phosphatase with activity towards phosphorylated serine, threonine, and tyrosine residues. Immunoblots using α pY705-Stat3 revealed that in contrast to alkaline phosphatase, λ phosphatase did not dephosphorylate pTyr705-Stat3 in control (- SSG1) or SSG1-expressing cells (Fig. 5A, top panel). However, the Stat3 bands containing pY705-Stat3 migrated as lower molecular weight proteins after λ phosphatase treatment in SSG1-expressing cells but not control cells (- SSG1) (Fig. 5A, compare lanes 6 and 7), suggesting that an amino acid in

A**B****Figure 3.4 The effect of alkaline phosphatase on Stat3.**

293T cells were co-transfected with 500 ng of cDNA encoding FLAG-Stat3, 250 ng rat GHR, and 1 μ g SSG1-myc. 10 h post-transfection, cells were washed and media was replaced 1% BSA/DMEM for 16 h. Cells were stimulated with 500ng/ml GH for 15 min. FLAG-Stat3 was immunoprecipitated (IP) with α FLAG agarose beads. Immunoprecipitated proteins were incubated in the presence or absence of 40U of alkaline phosphatase (AP) in the presence or absence of 10 mM sodium orthovanadate (Na₃VO₄) for 1 h and immunoblotted (IB) with α pY705-Stat3 (A, top panel) or α pS727-Stat3 (B, top panel). Immunoprecipitated proteins were reprobbed with α Stat3. Lysates were immunoblotted (IB) with α pY705-Stat3 (A, third panel) and α myc (A, bottom panel). The migration of pY705-Stat3, pS727-Stat3, FLAG-Stat3, and SSG1-myc is indicated. n=2

A**B****Figure 3.5 The effect of λ phosphatase on Stat3.**

500 ng of cDNA encoding FLAG-Stat3, 250 ng rat GHR, and 1 μ g SSG1-myc were co-expressed in 293T cells. 30 h post-transfection, cells were washed and media was replaced with 1% BSA/DMEM for 18 h. Cells were stimulated with 500ng/ml GH for 15 min. FLAG-Stat3 was immunoprecipitated (IP) with α FLAG agarose beads. Immunoprecipitated proteins were incubated in the presence or absence of 200U of λ phosphatase (λ ppase) in the presence or absence of 10 mM sodium orthovanadate (Na_3VO_4) for 30 min and immunoblotted (IB) with α pY705-Stat3 (A, top panel) or α pS727-Stat3 (B, top panel). Immunoprecipitated proteins were reprobed with α Stat3. Lysates were immunoblotted (IB) with α pY705-Stat3 (A, bottom panel) or α pS727-Stat3 (B, bottom panel). The migration of pY705-Stat3, pS727-Stat3, and FLAG-Stat3 is indicated. n=2

Stat3 other than Tyr705 is being dephosphorylated by λ phosphatase. In fact, in the presence of λ phosphatase, the migration of the two Stat3 bands in SSG1-expressing cells became similar to the migration of the two Stat3 bands from control cells (- SSG1). Treating with sodium orthovanadate partially prevented the dephosphorylation, revealing three distinct Stat3 bands, all of which are phosphorylated on Tyr705. λ phosphatase was able to dephosphorylate Ser727 of Stat3 in control and SSG1-expressing cells (Fig. 5B). The addition of orthovanadate partially restored pSer727. It also revealed the presence of three bands all of which are phosphorylated on Ser727. These findings suggest that: Stat3 migrates as two distinct bands in control cells; SSG1 increases the apparent molecular weight of Stat3; phosphatase treatment returns the apparent molecular weight of Stat3 in SSG1-expressing cells back to the apparent molecular weight in control cells without altering phosphorylation of Tyr705; and partial inhibition of the phosphatases reveal that Stat3 undergoes at least two post-translational modifications in addition to phosphorylation on Tyr705 and Ser727. One is present even in the absence of SSG1 accounting for the detection of two bands by α Stat3. The second modification appears to be a phosphorylation that is enhanced by SSG1.

SSG1 negatively regulates the GH-responsive gene *c-fos*.

Because SSG1 enhances Stat3 phosphorylation, we hypothesized that SSG1 might affect Stat3-mediated gene expression. The finding that SSG1 promotes the phosphorylation of Tyr705 suggested that SSG1 might increase basal and GH-stimulated expression of Stat3 regulated genes. However, the ability of SSG1 to enhance phosphorylation of an additional, as yet, unidentified site leaves open the possibility that SSG1 might actually inhibit expression of Stat3 regulated genes. GH rapidly (15-30 min) induces the expression of *c-fos* (34) which encodes a transcription factor implicated in cell growth and differentiation (51). The regulatory region of *c-fos* contains multiple regulatory sequences, including the sis-inducible element (SIE) which binds Stat3 and Stat1 homo- and heterodimers. To determine whether SSG1 regulates *c-fos* promoter expression, SSG1-myc was expressed in GH-responsive CHO454 cells in combination with a luciferase reporter driven by wild type *c-fos* promoter (from -379 to +1). CHO454 cells stably express a truncated form of the GH receptor (GHR 1-454) that is capable of

stimulating expression of *c-fos* (52, 53). These cells were chosen because they express higher levels of GH receptor than CHO cells expressing full-length GH receptor. GH treatment doubled *c-fos* promoter expression (Fig. 6A) as reported previously (54). Overexpression of SSG1 inhibited both basal and GH-stimulated expression of the *c-fos* reporter.

Because SSG1 binds to JAK2, we tested whether SSG1 blunts the stimulatory effect of JAK2 on *c-fos* promoter activation. SSG1 was co-expressed with either JAK2 or JAK2 (K882E), along with the *c-fos* driven luciferase reporter, in CHO454 cells (Fig. 6B). JAK2 (K882E) lacks a critical lysine in the ATP binding loop and as a consequence, is completely inactive as a kinase (46). Overexpressed JAK2 stimulated *c-fos* promoter activity to ~300% of control values. At the lowest level (25 ng cDNA) of SSG1 expression tested, stimulation of *c-fos* reporter activity by JAK2 was not significantly different from that seen in the absence of SSG1; however, 100 ng of SSG1 cDNA decreased basal *c-fos* reporter activity by ~50% and JAK2-stimulated *c-fos* reporter activity by ~70%. As expected, JAK2 (K882E) did not stimulate *c-fos* promoter activity in the presence (Fig. 6B) or absence (data not shown) of SSG1. These findings suggest that SSG1 blunts the stimulatory effect of kinase-active JAK2 on *c-fos* gene transcription.

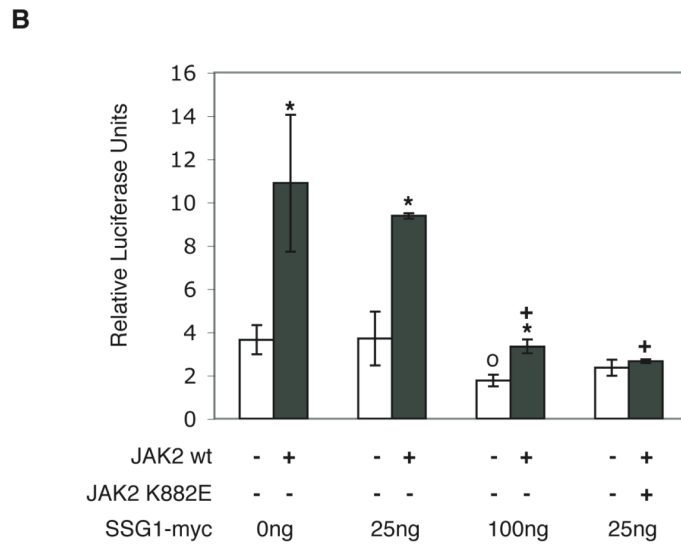
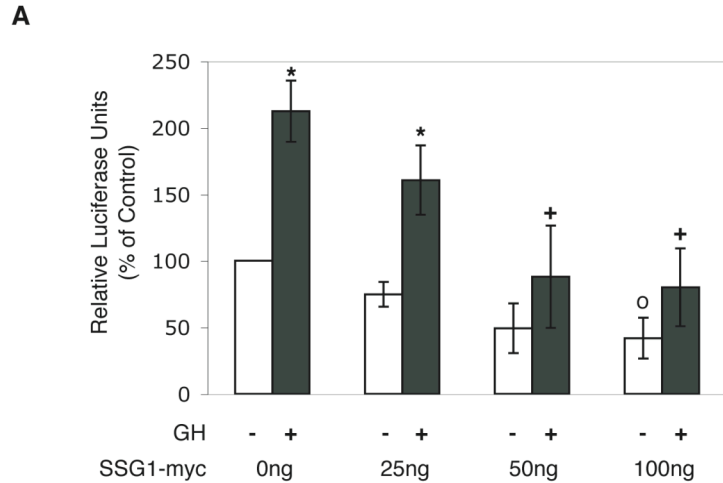


Figure 3.6 SSG1 negatively regulates the GH-responsive gene *c-fos*.

(A) CHO454 cells were transfected with cDNA encoding SSG1-myc (0, 25, 50, or 100 ng cDNA as indicated) in combination with a luciferase reporter driven by wild type *c-fos* promoter (from -379 to +1). After 48 h, cells were treated with vehicle (open bars) or GH (black bars) for 4 h and analyzed for luciferase activity. The data are expressed as the mean \pm SEM of three independent experiments each assayed in triplicate. The one-tailed, paired Student's *t* test was used to assess statistical differences. Significant differences ($p < 0.05$) are denoted with a circle (o) when controls with SSG1 are significantly different than controls without SSG1; a plus (+) when GH-treated with SSG1 are significantly different than GH-treated without SSG1; and an asterisk (*) when GH-treated are significantly different than control samples for a given concentration of SSG1. (B) CHO454 cells were transfected with cDNA encoding SSG1-myc (0, 25, 100 μ g cDNA as indicated) in combination with cDNA encoding either JAK2 or JAK2 (K882E) as indicated and luciferase reporter driven by wild type *c-fos* promoter (from -379 to +1). After 48 h, cells were analyzed for luciferase activity. The data are expressed as the mean \pm SEM of nine (0 ng SSG1 cDNA) or three (25 and 100 ng SSG1 cDNA) replicates in a representative experiment. The one-tailed, paired Student's *t* test was used to assess statistical differences. Significant differences ($p < 0.05$) are denoted with a circle (o) when controls (0 ng JAK2) with SSG1 are different than controls without SSG1; a plus (+) when JAK2 expressing cells with SSG1 are different than JAK2 expressing cells without SSG1; and an asterisk (*) when control cells with SSG1 are different than JAK2 expressing cells with SSG1.

Localization of SSG1.

SSG1 contains several putative nuclear localization sequences, suggesting that SSG1 might have a nuclear function that might contribute to its inhibitory effect on *c-fos* gene transcription. To examine the subcellular localization of SSG1, full-length SSG1-HA-myc, which has a tag at both the C-terminus and 30 amino acids after the N-terminus, was overexpressed in COS-7 cells (Figs. 7A-C). The cells were fixed and immunostained using α HA (Figs. 7A and C) and α myc (Fig 7B) to visualize SSG1. α myc and α HA staining was seen in the perinuclear region in COS-7 cells (Fig. 7) in a pattern suggesting the endoplasmic reticulum and/or Golgi apparatus. The fact that the staining with α myc and α HA was indistinguishable argues against SSG1 being cleaved into N- and C-terminal cleavage products capable of localizing to different parts of the cell.

To test whether SSG1 co-localizes in the endoplasmic reticulum or Golgi apparatus, SSG1-myc was overexpressed in COS-7 cells. SSG1-myc localized in a manner indistinguishable from the subcellular localization observed with SSG1-HA-myc (Fig. 7D and G). These cells were also immunostained with a marker for the Golgi (Giantin), an endoplasmic reticulum marker (calnexin), or a nuclear marker (DAPI). SSG1-myc co-localized with both the Golgi marker, Giantin, (Fig. 7E-F) and the ER marker, calnexin, (Fig. 7H-I) in COS-7 cells. Only rarely was SSG1 seen in the nucleus (data not shown). This localization pattern is not consistent with a nuclear function for SSG1. Rather, it is more consistent with the localization pattern of proteins in the secretory pathway. These data suggest that SSG1 is unlikely to act directly in the nucleus to regulate gene expression.

Localization of SSG1 in the presence of wild-type JAK2.

Because of our finding that SSG1 immunoprecipitates with wild-type JAK2, but not kinase-inactive JAK2 (Fig. 7P), we next examined the subcellular localization of SSG1 and JAK2. Full-length SSG1-myc was co-expressed with GFP or GFP-JAK2 in COS-7 cells. The cells were fixed and immunostained with α myc and visualized by confocal microscopy. As in Figs. 7A and D, SSG1-myc was present in the perinuclear region and in a pattern consistent with it being in the ER and Golgi (Fig 7, panels J-O).

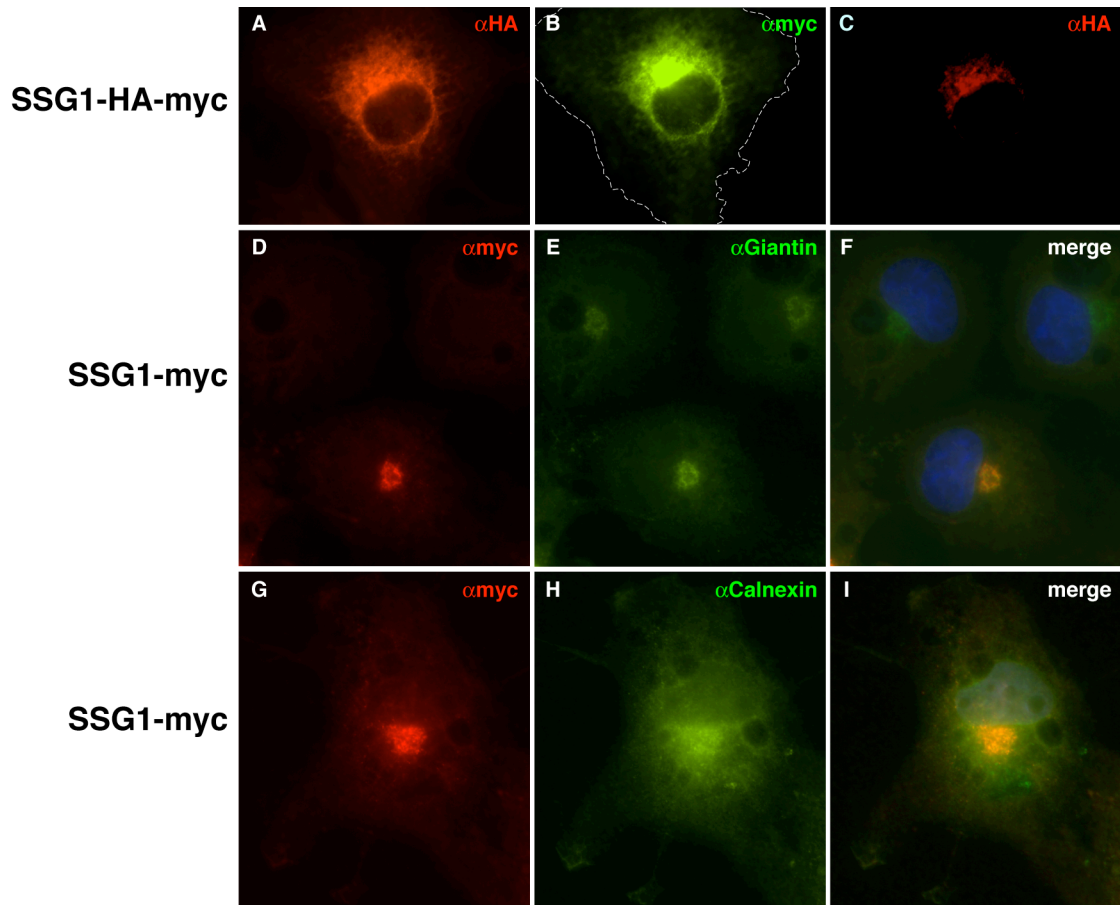


Figure 3.7A-I Localization of SSG1.

COS-7 cells were transfected with 500 ng of cDNA encoding SSG1-HA-myc (panels A-C) or with 500ng of cDNA encoding SSG1-myc (panels D-I) alone. Cells were fixed with 4% paraformaldehyde and immunostained using α myc or α HA (as indicated) followed by staining with AlexaFluor594 (panels A, C, D, F, G, and I) or AlexaFluor488 (panel B). Cells were stained with the Golgi marker, Giantin, (panels E and F) or the ER marker, calnexin, (panels H and I) followed by staining with Oregon Green. Nuclei were visualized with DAPI (panels F, and I). Panels A-I are fluorescent images. Exposure times were selected to avoid saturation where possible. Panel C is a shorter exposure of the cell in panel A. The dotted line represents the cell boundaries.

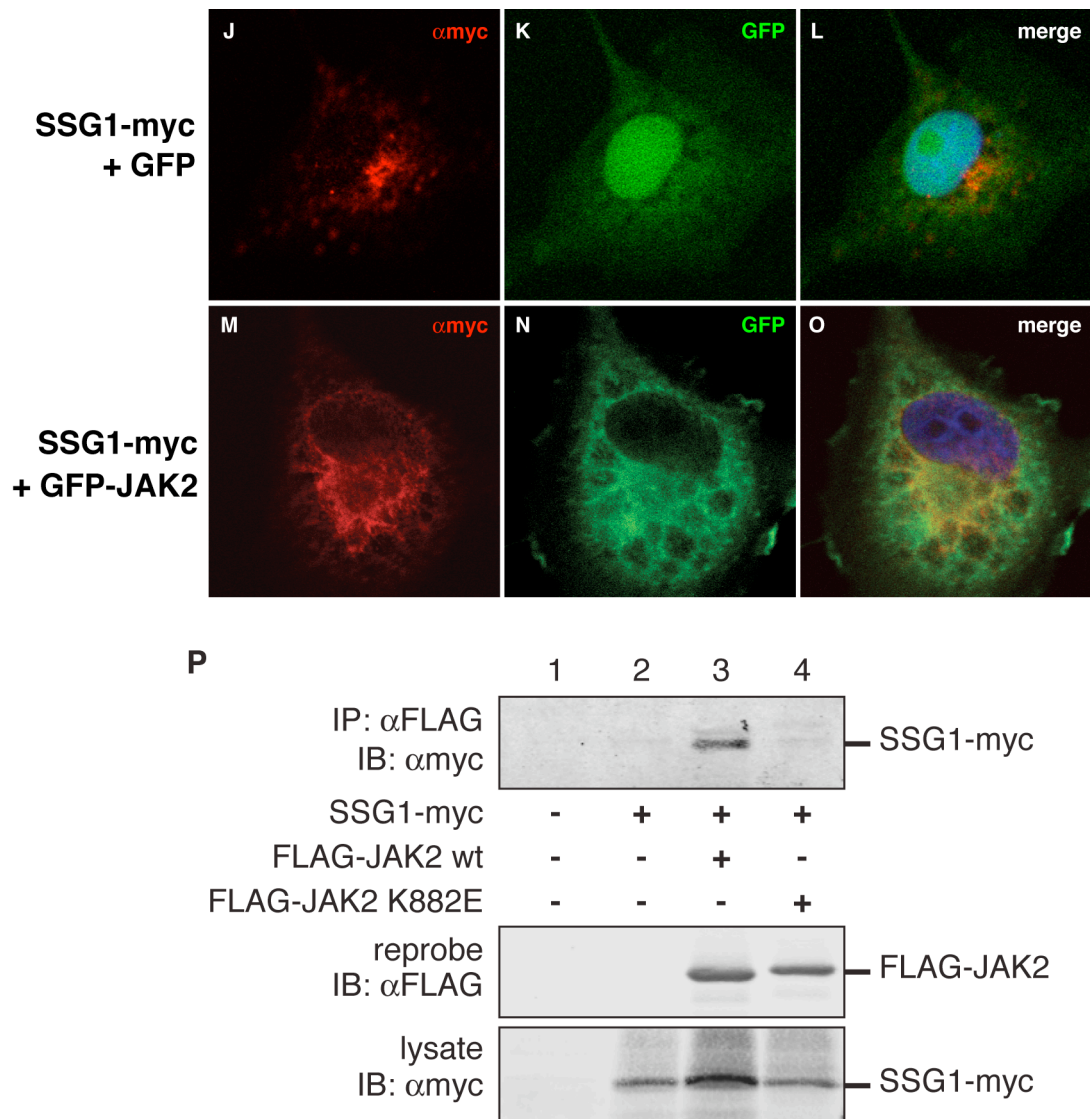


Figure 3.7J-P Localization of SSG1.

COS-7 cells were transfected with 500 ng of cDNA encoding SSG1-myc in combination with 500ng GFP (panels J-L) or in combination with 500 ng of GFP-JAK2 (panels M-O). Cells were fixed with 4% paraformaldehyde and immunostained using α myc followed by staining with AlexaFluor594 (panels J and M). Nuclei were visualized with DAPI (panels L, and O). Panels J-O are confocal images. Exposure times were selected to avoid saturation where possible. (P) 3 μ g of cDNA encoding SSG1-myc was expressed alone or with 2 μ g of cDNA encoding either wild-type FLAG-JAK2 or FLAG-JAK2 (K882E) in 293T cells. FLAG-JAK2 was immunoprecipitated (IP) with α FLAG agarose beads and immunoprecipitated proteins were immunoblotted (IB) with α myc (top panel). This blot was reprobated with α FLAG to show relative levels of immunoprecipitated JAK2 (second panel). Lysates from the transfected cells were immunoblotted with α myc to reveal levels of expression of SSG1-myc. The migration of FLAG-JAK2 and SSG1-myc is indicated.

GFP-JAK2 overlapped with SSG1-myc staining, although JAK2 localization was more widespread (Fig 7, panels M-O). This raises the possibility that JAK2 is likely to be interacting with SSG1 in the ER and Golgi.

SSG1 is a secreted protein.

The finding that SSG1 co-localizes with both an ER marker, calnexin, and a Golgi marker, Giantin, (Fig. 7 D-I) and also contains a putative signal peptide suggested that SSG1 was likely to be a secreted protein. Previous reports in the literature had conflicting evidence about whether SSG1 was secreted. Several groups suggest that SSG1 is not a secreted protein (39, 55). Others have suggested that SSG1 is secreted (42, 56). Mu et al. (43) were unable to detect C-terminally tagged Equarin-L in the culture medium; however, they reported detecting Equarin-S (which contains the N-terminal 601 of 958 amino acids) by western blot analysis. To examine whether the signal peptide at the N-terminus of SSG1 serves as a functional secretion signal, 293T cells were transfected with cDNA C-terminally tagged SSG1-myc, or SSG1-HA-myc which has an HA tag just following the putative signal peptide. Ten hours after transfection, the medium was changed to 1% calf serum. After 16 h, medium was collected, proteins were precipitated using trichloroacetic acid (TCA), and TCA precipitated proteins were immunoblotted with α myc. Cell lysates were also prepared and SSG1 in the cell lysate were blotted with α myc. Fig. 8A (lanes 1-3) shows that SSG1 migrates as a tight band of ~120kDa and a diffuse band of ~125-175kDa suggesting that SSG1 undergoes post-translational modification. Blotting TCA precipitated proteins (lanes 4-6) with α myc revealed a diffuse band migrating ~125-175kDa only in SSG1-expressing cells, consistent with the presence of only the glycosylated forms of SSG1 in the medium.

To confirm that SSG1 is secreted from cells, we added 1 μ g/ml Brefeldin A (BFA) to cells expressing SSG1-myc during the 16 h incubation with 1% serum. BFA blocks secretion. Only minimal cell death was detected. TCA precipitated proteins were immunoblotted with α myc. As shown in lane 8 of Fig. 8A, SSG1-myc was detected in the medium from untreated (no BFA) cells. However, BFA completely blocked secretion

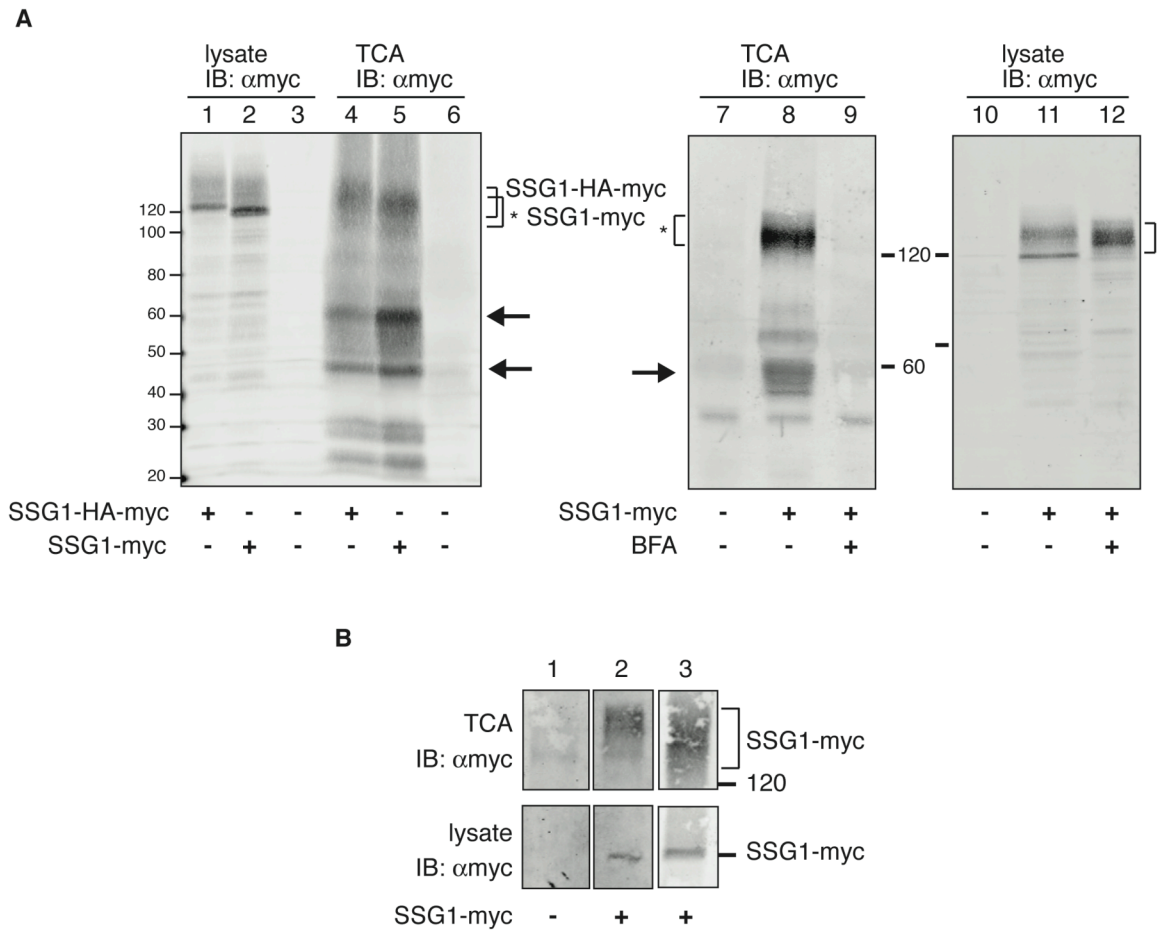


Figure 3.8 SSG1 is a secreted protein.

(A) 293T cells were transfected with 3 μ g empty vector or cDNA encoding SSG1-myc or SSG1-HA-myc as indicated. Cells in lanes 7-12 were treated with or without the 1 μ g/ml of BFA for 16 h as indicated. Cell lysates were prepared (lanes 1-3, 10-12), and proteins in the medium were precipitated using TCA (lanes 4-6, 7-9). Proteins were resolved by SDS-PAGE and immunoblotted (IB) with α myc. The migration of the molecular weight standards, SSG1-HA-myc, and SSG1-myc is indicated. (B) cDNA encoding SSG1-myc or empty vector were introduced into 3T3-F442A preadipocyte cells by electroporation. TCA precipitates and cell lysates were prepared. For reference, SSG1-myc overexpressed in 293T cells was included as a positive control (lane 3, light exposure). The migration of SSG1-myc is indicated.

of SSG1-myc (Fig. 8A, lane 9). Taken together, these results suggest that SSG1 is a highly glycosylated protein secreted by the constitutive secretory pathway.

To test whether SSG1 is secreted from a GH-responsive cell line that contains endogenous SSG1, SSG1-myc was expressed in 3T3-F442A preadipocytes. Medium was collected and proteins were precipitated using TCA. A band corresponding to SSG1-myc from 3T3-F442A was present in the TCA precipitates only in lanes where SSG1-myc had been overexpressed, suggesting that SSG1-myc is also secreted from 3T3-F442A cells (Fig. 8B, top panel). Again, SSG1-myc from 3T3-F442A cells migrated at a higher molecular weight than SSG1-myc found in the cell lysate, suggesting that SSG1-myc undergoes post-translational modification before being secreted. 3T3-F442A cells appeared to secrete the majority of protein made as there was very little SSG1-myc present in the cell lysate. Lysates prepared from both transfected cell lines show that SSG1-HA-myc or SSG1-myc is expressed in the cell before it is secreted. Further, when the lysate is blotted for SSG1, there is a smear above SSG1 consistent with SSG1 undergoing post-translational modification/N-glycosylation prior to being secreted from the cell.

SSG1 secretion is enhanced by kinase-active JAK2.

Previously, we have shown that kinase-active JAK2, not kinase-inactive JAK2, interacts with SSG1 (O'Leary et al., *manuscript in preparation*, Fig. 7P of this manuscript). Having just demonstrated that at least a portion of SSG1 is secreted (Fig. 8), we hypothesized that JAK2 kinase activity may affect the secretion of SSG1. To test this hypothesis, SSG1-HA-myc was co-expressed with FLAG-tagged JAK2 or JAK2 (K882E) in 293T cells. Medium was collected after 16 h, and proteins were precipitated using TCA. The precipitated proteins were resolved by SDS-PAGE and immunoblotted with α myc or α HA. As shown in Fig. 8, a band corresponding to SSG1 was present in the medium (Fig. 9, lane 1). An additional band assumed to be a C-terminal cleavage product due to the presence of the C-terminal myc tag migrated as a ~60 kDa protein. The amount of this 60kDa protein, as well as total SSG1, was significantly greater when JAK2 was overexpressed than in control cells when JAK2 was not expressed or when JAK2 (K882E) was co-expressed. Consistent with the presence of secreted SSG1, a band

of ~125-175,000 was also present in the medium when proteins were blotted with α HA. As expected from the presence of C-terminal cleavage products detected with α myc, N-terminal cleavage products detected with α HA migrating with molecular weights ~80,000 and ~40,000 when wild-type JAK2 was co-expressed (Fig. 9, lane 5) than in control or JAK2 (K882E) expressing cells. These results suggest that both secretion and post-translation processing (i.e. proteolytic cleavage) of SSG1 is enhanced in the presence of wild-type JAK2. This increased expression due to the presence of JAK2 appears to be at least partially specific, since JAK2 did not appear to increase the secretion of SRPX2, a JAK2 interacting protein belonging to the selectin family (data not shown).

Localization of SSG1 in the presence of wild-type JAK2.

Because it seems that JAK2 enhances SSG1 secretion, we wanted to determine if extracellular SSG1 could be visualized by microscopy. To confirm our results in Fig. 9, we overexpressed SSG1-myc in COS-7 cells, fixed the cells in the presence of medium, and immunostained using α myc. Visualizing SSG1-myc overexpressing cells by fluorescence microscopy revealed low, but detectable α myc staining in a halo-like pattern surrounding the cell which we believe is SSG1 secreted into the extracellular matrix (Fig. 10). This halo was only present in SSG1 transfected cells. When GFP-JAK2 was co-expressed with SSG1-myc, the halo-like effect was enhanced (Fig. 10C, compare to 10A). This result was specific to kinase-active JAK2. When kinase-dead GFP-JAK2 (K882E) was co-expressed with SSG1-myc, the halo pattern (Fig. 10E) was similar to that seen when SSG1-myc was expressed alone (Fig. 10A). Quantification of the intensity of the “halo” reveals that wild-type JAK2 enhances the presence of extracellular SSG1 by more than three-fold (Fig. 10M). Taken together, these data suggest that a portion of SSG1-myc co-localizes with GFP-JAK2 (Fig. 7O) and that co-expression with wild-type JAK2 enhances the secretion of SSG1-myc.

BFA inhibits SSG1 extracellular staining.

To confirm that the staining seen in the extracellular matrix surrounding SSG1-expressing cells was secreted SSG1, we overexpressed SSG1-myc in COS-7 cells. Cells

were treated with or without 10 μ g Brefeldin A (BFA) overnight. Cells were fixed and stained as above. Cells which were not treated with BFA had a similar staining pattern as before, with SSG1 localizing to the ER, Golgi, and in the extracellular matrix (Fig. 10, panels G, I, and J). When the cells were treated with BFA, SSG1 localization was completely intracellular (dark extracellular background) with SSG1 staining in the ER (Fig. 10, panels H, K, and L). There was no extracellular staining in of SSG1 overexpressing cells treated with BFA. From this, we conclude that SSG1 is a secreted protein which can be seen in the extracellular matrix and perhaps binds to a component of the extracellular matrix.

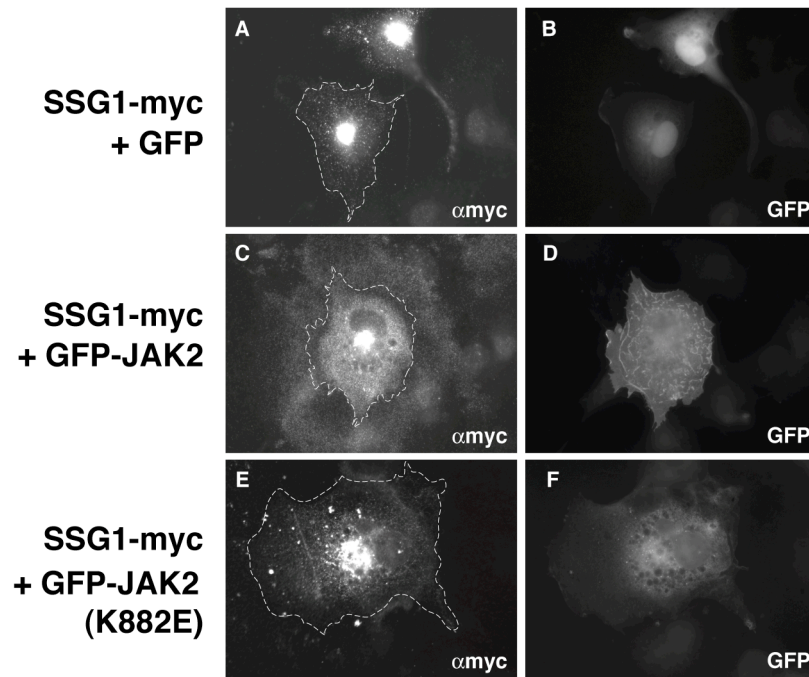


Figure 3.10A-F Localization of SSG1 in the presence of wild-type JAK2.

COS-7 cells were transfected with 500 ng of cDNA encoding SSG1-myc alone (panels A-B) or in combination with cDNA encoding 500 ng of GFP-JAK2 (panels C-D) or GFP-JAK2 (K882E) (panels E-F). 24 h after transfection, cells were fixed with 4% PFA and immunostained with α myc followed by AlexaFluor594. Cells were visualized using a Nikon Eclipse TE200 fluorescent microscope. The dotted line represents the cell boundaries.

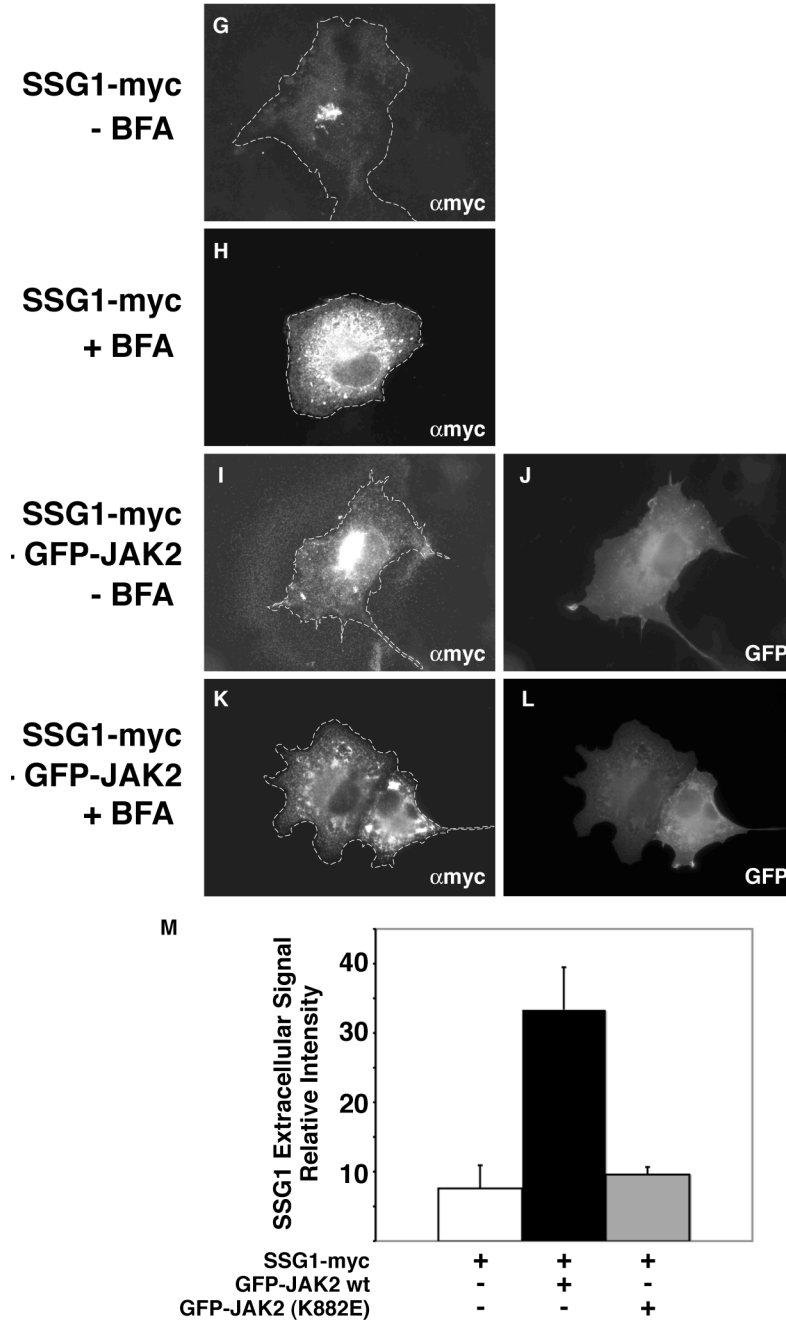


Figure 3.10G-M Localization of SSG1 in the presence of wild-type JAK2.

COS-7 cells were transfected with 500 ng of cDNA encoding SSG1-myc alone (panels G-H) or in combination with cDNA encoding 500 ng of GFP-JAK2 (panels I-L). Cells were treated with (panels H, K, and L) or without (G, I, and J) 10 μ g/ml BFA for 16 h prior to fixation. 24 h after transfection, cells were fixed with 4% PFA and immunostained with α myc followed by AlexaFluor594. Cells were visualized using a Nikon Eclipse TE200 fluorescent microscope. (M) The intensity of extracellular SSG1 was calculated as described in Materials & Methods. The data are expressed as the mean \pm SEM of three independent experiments in which 8-10 cells were assessed for each condition. The dotted line represents the cell boundaries.

Discussion

Previously, we identified SSG1 as a binding partner for kinase-active JAK2 from a yeast two-hybrid screen of a rat adipocyte cDNA library (O'Leary et al., *manuscript in preparation*). We confirmed in a mammalian system that SSG1 interacts with kinase-active wild-type JAK2, but not kinase-dead JAK2 (K882E) and identified the interacting regions within SSG1 to be the SRPX5 domains, named for their similarity to an uncharacterized fifth domain within SRPX (O'Leary et al., *manuscript in preparation*). Based on this interaction, we hypothesized that SSG1 might affect signaling molecules in the GH signaling pathway. We also hypothesized that JAK2 might affect the function of SSG1.

In this paper, we have shown that SSG1 does not affect JAK2 kinase activity, and only modestly affects phosphorylation of Stat5 on Tyr694, which is required for GH-activation of Stat5. However, SSG1 was shown to enhance the phosphorylation of Stat3 on Tyr705 and likely promote the phosphorylation of another amino acid in Stat3 in both the presence and the absence of GH stimulation. Because phosphorylation of Tyr705 is required for activation of Stat3, we predicted that this increase in Stat3 Tyr705 phosphorylation would enhance Stat3-mediated gene transcription. However, the expression of a *c-fos* promoter construct containing a Stat3 responsive sis-inducible element was found to be negatively regulated by the presence of SSG1. One can envision several ways in which SSG1-induced phosphorylation of this as yet unidentified amino acid could negatively regulate *c-fos* gene expression. It could interfere with the ability of Stat3 to form dimers, bind to importin- α 3 and be transported into the nucleus, bind to DNA, or interact with co-factors. Phosphorylation could directly affect these events or indirectly affect them by affecting the ability of Stat3 to undergo other post-translational modifications, such as acetylation. Acetylation of Stat3 has been reported to be important for Stat3 to form stable dimers and induce gene transcription (57). Until we map the site of phosphorylation, we also cannot rule out the possibility that the SSG1-induced changes in Stat3 phosphorylation and inhibition of GH- and JAK2-induced *c-fos* promoter activity are independent events. The inhibition of *c-fos* promoter activity could be mediated by a response element in the *c-fos* promoter other than the SIE that binds

Stat3 since this promoter construct also contains a serum response element (SRE), CRE (CCAAT/enhancer-binding protein beta (C/EBP β) response element), and AP1 response element which bind other transcription factors important in regulating *c-fos* gene expression (49).

There are clearly multiple candidates for the proposed SSG1-promoted site of phosphorylation in Stat3. A number of amino acids in Stat3 in addition to Ser727 and Tyr705 have been shown to be phosphorylated by mass spectroscopy. These sites include: Thr268 (58), Ser269 (58), Ser273 (58), Tyr539 (59, 60), Tyr686 (60, 61), and Ser691 (62). It is not known whether phosphorylation of these sites in Stat3 alters the function of Stat3 nor is it known what the kinase is for these sites. It will be interesting to determine if the SSG1-promoted phosphorylation in Stat3 coincides with any of these predicted sites or is a previously unidentified site, and what kinase is responsible for that phosphorylation.

Overexpression of SSG1 in colorectal and pancreatic cancer cell lines led to the inhibition of malignant growth and suppression of anchorage independent growth (39), suggesting that SSG1 may function as a tumor suppressor. SSG1 has also been reported to be downregulated in a number of cancer cell lines and tumors including, thyroid, ovarian carcinoma, pancreatic, and colon cancer (39, 40). Our observation that SSG1 is a negative regulator of GH- and JAK2-dependent *c-fos* gene transcription is consistent with SSG1 being a tumor suppressor. Constitutive activation of JAK2 and/or Stat proteins has been associated with various cancers including thyroid, ovarian, pancreatic, and colon cancers (63-74).

Fluorescent and confocal microscopy revealed SSG1 to be present in the perinuclear region of the cell as well as in a distinct punctate pattern throughout the cytoplasm suggestive of it being in the ER and Golgi. In fact, SSG1 co-localized with both ER and Golgi markers. Bommer et al. (39) also reported partial overlap of human SSG1 with Golgi and ER markers, while others reported perinuclear staining of SSG1 (40-42). SSG1 has several putative nuclear localization sequences (NLS). However, only rarely did we see SSG1 in the nucleus. Bommer et al. (39) and Lui et al. (42) also reported an absence of SSG1 in the nucleus. However, Visconti et al. (40) reported seeing some nuclear-nucleolar localization of SSG1-GFP in 5-10% of cells. The basis for

this apparent discrepancy in results is unknown. However, the Visconti report of nuclear localization leaves open the possibility that SSG1, or an SSG1 cleavage product, can accumulate in the nucleus but only under certain conditions not met in our experiments. Visualizing SSG1-HA-myc, which has an HA tag just downstream of the putative signal sequence and a C-terminal myc tag, with α myc and α HA revealed the same subcellular localization as each other and as SSG1-myc suggesting that SSG1 does not get cleaved, with one of the cleavage products going to the nucleus. It also reveals that the HA tag does not interfere with protein folding and/or trafficking.

Although we observed co-precipitation of SSG1 with JAK2 and decreased expression of a *c-fos* promoter construct, suggesting a cellular function for SSG1, we were intrigued by the presence of a putative signal peptide in SSG1. Literature reports were contradictory about whether this was a functional signal peptide resulting in the secretion of SSG1. Bommer et al. (39) did not find SSG1 in conditioned medium of COS and 293 cells overexpressing human SSG1 assessed by western blot analysis. Cha et al. (55) also failed to detect endogenous human SSG1 in conditioned medium of dermal papilla cells. However, Liu et al. (42) reported detecting by western blot analysis the presence of overexpressed C-terminally tagged murine SSG1 in conditioned medium. Mu et al. (43) failed to detect C-terminally tagged Equarin-L in the medium but were able to detect Equarin-S. Because of this controversy, we examined whether we could detect any secreted SSG1. Using both western blot analysis of proteins that had been TCA precipitated from the medium of SSG1-HA-myc or SSG1-myc overexpressing cells and immunofluorescence of those cells fixed in the presence of medium, we readily detected extracellular SSG1. The fact that Brefeldin A blocked the appearance of extracellular SSG1 assessed by either assay provides strong support for at least a portion of SSG1 being secreted. While this manuscript was being written, Okada et al. (56) also reported that they were able to detect SSG1 in the medium from cells stably expressing SSG1 as well as endogenous SSG1 secreted from cultured human adipocytes derived from adipose derived stromal cells.

We observed that SSG1 in cell lysates migrates primarily as a tight band of Mr ~120,000 whereas the soluble secreted SSG1 detected by immunoblotting migrates as a broad band of Mr ~125,000-175,000. This increased apparent molecular weight of

extracellular SSG1 along with its diffuseness suggests that secreted SSG1 is highly glycosylated. Lui et al. (42) also saw this dual migration pattern of a tight band and a more diffuse, slower migrating band for murine SSG1. SSG1 contains several predicted N-glycosylation sites. Consistent with SSG1 being subject to N-linked glycosylation, Lui et al. (42) report that treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, reduced the amount of the diffuse SSG1 band but not of the faster migrating, tight SSG1 band. Bommer et al. (39) also reported that treating cells with tunicamycin reduces the apparent molecular weight of cellular SSG1.

The fact that we visualized extracellular SSG1 by fluorescent microscopy suggests that at least a portion of secreted SSG1 may bind to the extracellular matrix. Two other groups have reported results consistent with secreted SSG1 being in the extracellular matrix (42, 43). This raises the possibility that SSG1 might be working locally as an autocrine or paracrine factor, along the lines of members of the FGF family, which are also reported to bind to extracellular matrix components (reviewed in (75)).

By expressing the double tagged SSG1-HA-myc with an HA tag just downstream of the signal sequence and an N-terminal myc tag, we were able to show that cells secrete full-length SSG1. In addition, we observed both a C-terminal fragment of SSG1 in cell medium that is recognized by α myc and several N-terminal fragments recognized by α HA. The N-terminal fragments migrated primarily as ~80 kDa and 40 kDa proteins, with presumably the 40 kDa fragment being a further proteolytic product of the 80 kDa fragment since both contain the HA tag. The C-terminal fragment migrated primarily as a ~60 kDa fragment and sometimes as a ~40 kDa fragment. SSG1 cleavage products have also been reported by others (39, 43, 56), although significant differences exist in the sizes of the fragments reported for both cell lysates and cell medium. In our hands, SSG1 fragments were more reproducibly detected in the TCA precipitate from the medium than in cell lysates, suggesting that the cleavage occurs after SSG1 is secreted. There is precedence for secreted proteins being proteolyzed. For example, secreted members of the FGF family of growth factors have been shown to be proteolyzed by proteases in the extracellular matrix (reviewed in (75)). Of potential relevance is the finding that there are two alternatively spliced gene products of Equarin, one that encodes a long form (Equarin-L, 958 amino acids) and one that encodes a short form (Equarin-S)

that corresponds to the first 601 amino acids of Equarin-L. Two human SSG1 transcripts have been identified; however, they are predicted to encode the same open reading frame; only one transcript has been reported for rat and mouse (38-42). Thus, while it seems unlikely that there is a splice variant of rodent or human SSG1 equivalent to Equarin-S, it is possible that a short form equivalent to Equarin-S is produced by proteolytic cleavage. GH receptor provides precedence of a protein that exists in both a long and a short form with the short form being either the proteolytic cleavage product of the long form (e.g. human, rabbit) or the product of a separate transcript (e.g. mouse, rat) (76, 77). Identification of any protease(s) responsible cleaving SSG1 and the cleavage site(s) along with the fate of the cleavage product(s) would provide insight into whether secreted SSG1 functions as a full-length protein or one or more proteolytic cleavage products.

An intriguing question that arises from our work is how SSG1 can be secreted and also bind JAK2. One possibility is that there are two separate pools of SSG1. One is secreted and one is not. In support of this, preliminary studies not shown using *in vitro* transcription and translation of SSG1 transcript in the presence and absence of microsomes showed that most SSG1 was not protected against proteolysis by trypsin, as would be expected if all the translated SSG1 were inside the microsomes as predicted for a secreted protein. In contrast, secreted alkaline phosphatase was not degraded. Consistent with the presence of two pools of SSG1, there are reports in the literature that suggest that proteins containing a signal peptide can localize to the cytosol and Golgi as well as be secreted ((78-80) and reviewed in (81)). Since SSG1 at least partially appeared to co-localize with JAK2 in the ER, our data raise the possibility that some SSG1 interacts with JAK2 in the ER without being fully taken up into Golgi vesicles and subsequently secreted. Preliminary data not shown also suggest that there may be a small pool of SSG1 that is cytoplasmic or even present at the plasma membrane that could interact with cytoplasmic JAK2. The interaction of one of these pools of SSG1 with JAK2 may be important for the enhanced GH-dependent phosphorylation of Stat3 and/or decreased GH- and JAK2-dependent expression of the *c-fos* promoter construct. Lavoie et al. (82) demonstrated that JAK2 has kinase activity in an *in vitro* microsome system replicating transitional ER membranes. There are also reports in the literature demonstrating that JAK2 interacts with cytokine receptors in the ER and is important for

the processing and cell surface localization of cytokine receptors (83, 84). Therefore, it is possible that SSG1 interacts with JAK2 in this cellular compartment.

Intriguingly, our data reveal that SSG1 secretion is enhanced when SSG1 is co-expressed with wild-type kinase-active JAK2 but not kinase-dead JAK2 (K882E) (Figs. 9 and 10). Because we have found that SSG1 forms a complex with kinase-active JAK2 but not kinase-inactive JAK2 (K882E), it is tempting to speculate that this interaction is critical for the stimulatory effect of JAK2 on SSG1 secretion. One can envision phosphorylation of SSG1 by JAK2 somehow enhancing SSG1 secretion from the cell, since JAK2 appears capable of phosphorylating SSG1 on tyrosines (O'Leary et al., *manuscript in preparation*). There is a great deal of precedence for secreted proteins being phosphorylated, and that phosphorylation regulates the function of the secreted protein (85-87). Our observation that the presence of wild-type but not kinase-dead JAK2 substantially increased the amount of cleaved SSG1 found in the medium raises the possibility that phosphorylation of secreted SSG1 increases its susceptibility to cleavage. If instead there are two pools of SSG1, the ability of JAK2 to bind SSG1 and to stimulate its secretion may be independent events. This would allow for the possibility that JAK2 promotes secretion of SSG1 indirectly by phosphorylating a protein involved in the trafficking or secretion of SSG1. The fact that overexpression of JAK2 does not appear to enhance the secretion of another JAK2 binding protein, SRPX2 (data not shown), argues against the stimulatory effect of JAK2 on SSG1 secretion being a non-specific effect of JAK2 on secretion.

JAK2 is activated by nearly two-thirds of the cytokine receptors including the leptin receptor. SSG1 has a wide tissue distribution (38-40) with high expression in adipose tissue (56). It seems likely that SSG1 plays a role in signaling not just by GH, but also by other ligands that bind to members of the cytokine family of receptors. To support this hypothesis, Aoki et al. (41) reported that patterns of gene expression are similar for murine SSG1 and leptin, another cytokine that activates JAK2. They demonstrated that SSG1 is expressed white and brown adipose tissue as well as in the chroid plexus in the brain, regions where the long form of the leptin receptor are also expressed (41). They proposed that SSG1 plays a role in energy metabolism based on their finding that expression of SSG1 is upregulated in brown adipose tissue from

bombesin-receptor-subtype (BRS)-3-deficient mice which display mild obesity (41, 88). An inhibitory role of SSG1 on leptin-dependent gene expression would be consistent with this finding because BRS-3-deficient mice exhibit characteristics associated with reduced leptin signaling (obesity, hyperphagy and reduced metabolic rate) (88). Further support that SSG1 may play a role in energy metabolism came from a study reporting that mouse models of obesity demonstrate a downregulation of SSG1 mRNA levels (56). Okada et al. (56) proposes that this is not a function of SSG1 acting intracellularly. Rather, they propose that SSG1 is a novel adipocytokine. It is intriguing to speculate that secreted SSG1 may play a role in carbohydrate metabolism similar to other secreted proteins important in leptin signaling (i.e. adiponectin).

It is intriguing to think that SSG1 may have dual functions: tumor suppressor and regulator of energy metabolism. Indeed, SSG1 has been identified in multiple different screens assessing different functions in different cells and tissues. In this study, we show that SSG1 does not affect JAK2 kinase activity or alter the phosphorylation of Stat5b. Rather, SSG1 enhances the tyrosyl phosphorylation of Stat3 and most likely a novel, unidentified site within Stat3. SSG1 also negatively regulates *c-fos* gene expression, consistent with SSG1 functioning as a tumor suppressor. SSG1 localizes to the ER and Golgi, with a portion co-localizing with wild-type JAK2. We also report that SSG1 contains a functional signal peptide resulting in SSG1 secretion, which is blocked with BFA. When co-expressed with JAK2, SSG1 secretion is enhanced, as is proteolysis of the secreted SSG1. Because SSG1 is highly expressed in adipose tissue and downregulated in obesity, secreted SSG1 may play a role in maintaining energy balance.

The abbreviations used are: BSA, bovine serum albumin; BFA, Brefeldin A; DMEM, Dulbecco's modified Eagle's medium; DRO1, down-regulated by oncogene1; drs, downregulated by Src; ER, endoplasmic reticulum; GH, growth hormone; JAK2, Janus kinase 2; PFA, paraformaldehyde; PAGE, polyacrylamide gel electrophoresis; PY, phosphotyrosine; SDS, sodium dodecyl sulfate; SOCS, suppressor of cytokine signaling; SSG1, steroid-sensitive gene-1; Stat, signal transducer and activator of transcription; TCA, trichloroacetic acid; Urb, upregulated in brown adipose tissue of bombesin-receptor-subtype-3-deficient mice.

Note: This chapter will be submitted for publication, in modified form, to *Molecular Endocrinology* under the title: "Physiological Consequences of the interaction between JAK2 and SSG1" by Erin E. O'Leary, Anna M. Mazurkiewicz-Muñoz, Travis Maures, Lawrence S. Argetsinger, and Christin Carter-Su. Dr. Anna Mazurkiewicz-Muñoz provided of Fig. 3.6 (which I also repeated). Dr. Travis Maures performed the staining and microscopy on Figs. 3.7 and 3.10 while I provided Figs. 3.1-3.5, 3.8-3.9, and parts of Figs 3.7 and 3.10.

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CHAPTER IV

CONCLUSIONS & FUTURE DIRECTIONS

Summary

JAK2 is an important signaling protein in GH signal transduction, yet few proteins have been identified as JAK2-interacting proteins. Steroid-sensitive gene-1 (SSG1) was identified in our lab as a novel binding protein of kinase-active JAK2 from a yeast two-hybrid screen of a rat adipocyte cDNA library. SSG1 has also previously been identified by others using numerous different screens thus suggesting a potential role for SSG1 in a variety of functions including tumor suppression and regulation of energy metabolism. However, these studies revealed very little about SSG1 at the cellular level. My hypothesis was that SSG1 is a multi-functional protein having a synergistic relationship with JAK2. SSG1 regulates GH signaling by interacting with JAK2 thus affecting downstream GH-stimulated signaling molecules and gene expression. Conversely, JAK2 regulates the function of and cellular trafficking of SSG1.

SSG1 as a JAK2 interacting protein

In Chapter II of this thesis, I describe the molecular basis for the interaction between JAK2 and SSG1. This was of particular interest because SSG1 interacts preferentially with kinase-active JAK2; however SSG1 contains no known phosphotyrosine binding motifs. Therefore, I characterized the regions of interaction within both SSG1 and JAK2. In SSG1, I demonstrated that the three consensus regions, the SRPX5 domains, interact with kinase-active JAK2 but not kinase-inactive JAK2. The SRPX5 domain was found to be necessary and sufficient for binding to kinase-active JAK2. These results suggested that this domain may be a novel protein-protein

interaction domain. To further support the hypothesis that the SRPX5 domain is a novel protein-protein interaction domain, I showed that SRPX2, another SRPX5 domain-containing protein, interacts with kinase-active JAK2. All three SRPX5 domains (D1, D2, and D3) of SSG1 interact with the JH1 kinase domain of JAK2. The interaction of JH1 with D2 was the strongest, while the interaction with D1 was the weakest. The finding that the SRPX5 domains interacted preferentially with the kinase-active form of JH1 compared to the kinase-inactive JH1 (K882E) and the kinase-active form of JAK2 but not the kinase-inactive JAK2 (K882E) suggests that the SRPX5 domain may define a novel phosphotyrosine-binding domain.

I also demonstrated that full-length SSG1 preferentially interacts with kinase-active JAK2 compared to kinase-inactive JAK2. When I tested the ability of SSG1 to bind to JH1 compared to full-length JAK2, I observed that SSG1 interacts with full-length JAK2 better than JH1. This result was not entirely surprising since all three of the SRPX5 domains of SSG1 bind JAK2. This result suggested that there are at least two regions of interaction between SSG1 and JAK2, with one site of interaction within the JH1 domain and one site of interaction outside of the JH1 domain of JAK2. Further, in Chapter III of this thesis, I was able to demonstrate that a portion of JAK2 co-localizes with SSG1 in the Golgi/ER compartment of the cell. It has been reported that JAK2 has kinase activity in the ER (1), and therefore, it is possible that SSG1 complexes with JAK2 in the ER, resulting in JAK2 phosphorylating SSG1. Previous data from our lab demonstrated that SSG1 is phosphorylated by JAK2 (2). From the data presented in Chapter II of this thesis, I concluded that full-length SSG1 is a binding partner for full-length, kinase-active JAK2 (Fig. 4.1).

SSG1 as a regulator of Stat3 signaling

In Chapter II of this thesis, I showed that SSG1 binds JAK2 in a kinase-dependent manner. To determine the physiological importance of the SSG1-JAK2 interaction, I examined whether SSG1 affects GH-stimulated signaling molecules in the JAK-Stat pathway, and regulates GH-induced gene expression. I first looked to see if SSG1 affects JAK2 kinase activity. In Chapter III of this thesis, I show that overexpression of SSG1

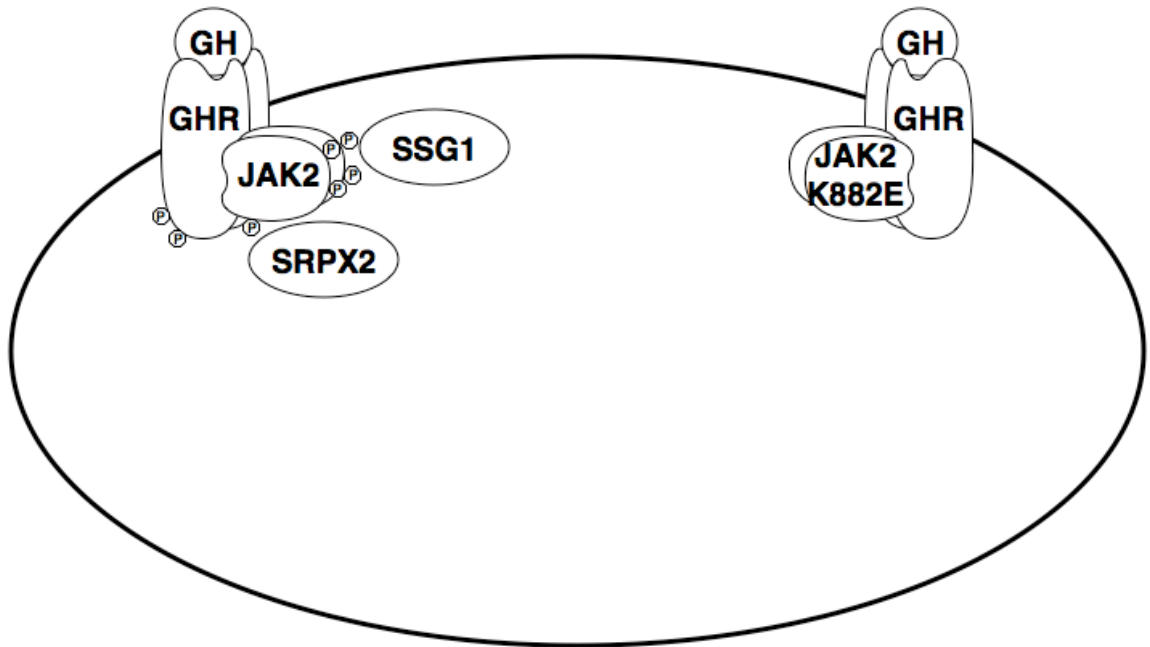


Figure 4.1 Schematic representation of SSG1 as a JAK2 interacting protein.

Full-length SSG1 is a binding partner for full-length kinase-active JAK2. SSG1 interacts with kinase-active JAK2 but not kinase-inactive JAK2 (K882E) via the SRPX5 domains within SSG1. SRPX2, another SRPX5 domain-containing protein, also interacts with kinase-active JAK2.

does not alter JAK2 kinase activity. I next looked at the downstream GH signaling molecules Stat3 and Stat5b. GH-dependent phosphorylation of Tyr694, required for Stat5b transcriptional activation, was only modestly increased in the presence of SSG1. Interestingly, SSG1 substantially promoted the phosphorylation of tyrosine 705 of Stat3 required for transcriptional activation, both in the absence and presence of GH stimulation. Perhaps the most intriguing finding of this thesis project was that SSG1 also appears to promote the phosphorylation of a novel site in Stat3 as seen with the upward mobility shift of the Stat3 band in cells overexpressing SSG1 in the absence of GH stimulation. Stat3 migrates as two distinct bands in control cells, and in the presence of SSG1 the apparent molecular weight of Stat3 increased. λ phosphatase treatment reduced the migration of the two Stat3 in SSG1-expressing cells to that seen in control cells without altering phosphorylation of Tyr705. Experiments using alkaline and λ phosphatases also reveal that Stat3 undergoes at least two post-translational modifications in addition to phosphorylation on Tyr705 and Ser727: one is present even in the absence of SSG1, accounting for the two bands detected by α Stat3; the second is a phosphorylation that is enhanced by SSG1. Expression of a *c-fos* promoter reporter construct, which contains a Stat1/Stat3 binding site, was negatively regulated in cells overexpressing SSG1. This finding suggests that SSG1 may blunt Stat3 activity perhaps as a consequence of this novel phosphorylation. The inhibition of Stat3 would be consistent with SSG1 functioning as a tumor suppressor. Constitutive activation of the Stat3 pathway has been linked with numerous cancers ((3-7) reviewed in (8, 9)). SSG1's proposed role as a tumor suppressor is based on several findings. Bommer et al. (10) demonstrated that overexpression of SSG1 in colorectal and pancreatic cancer cell lines leads to the inhibition of malignant growth and suppression of anchorage independent growth. SSG1 has been reported to be downregulated a number of cancer cell lines and tumors including thyroid, ovarian carcinoma, pancreatic and colon cancer (10, 11). Taken together, these findings support my hypothesis that SSG1 regulates GH signaling by interacting with JAK2 and affecting downstream GH-stimulated signaling molecules and gene expression (Fig. 4.2).

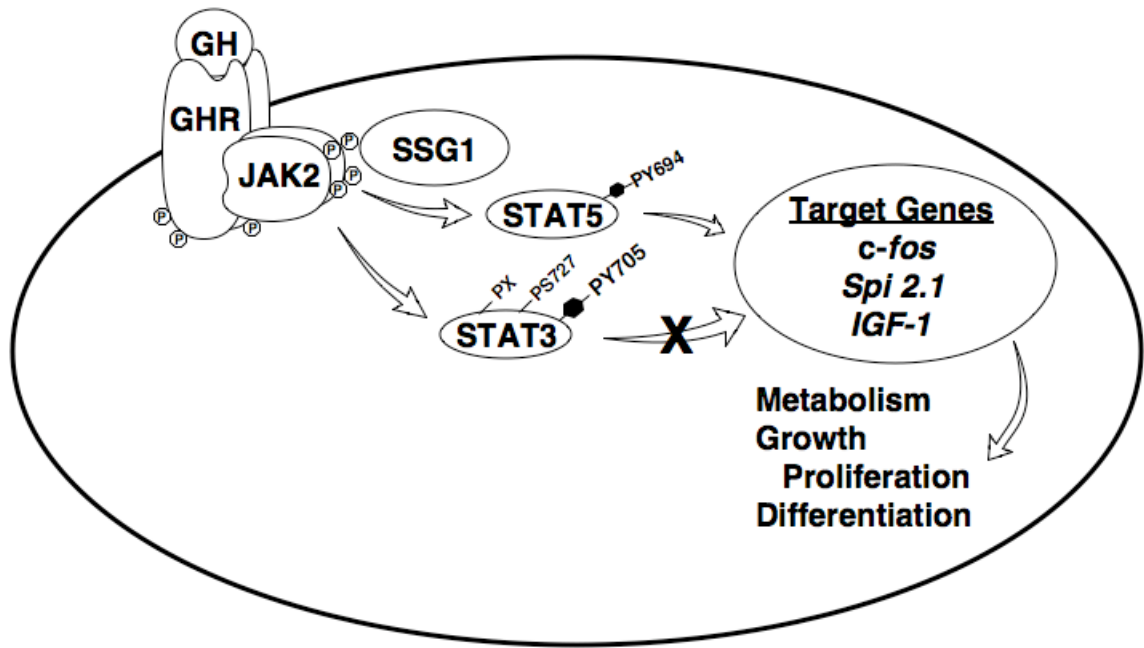


Figure 4.2 Schematic representation of SSG1 as a regulator of Stat signaling.

SSG1 binds JAK2 in a kinase-dependent manner, but does not affect JAK2 kinase activity. SSG1 only modestly increases phosphorylation of Tyr694 of Stat5b. SSG1 promotes the phosphorylation of Tyr705 in Stat3 in the absence and presence of GH stimulation. SSG1 does not affect the phosphorylation of Ser727 of Stat3, but SSG1 appears to promote the phosphorylation of a novel site in Stat3. SSG1 negatively regulates *c-fos* gene expression.

SSG1 as a secreted protein

In Chapter III of this thesis, I also show that JAK2 may regulate the secretion of SSG1. SSG1 contains a functional signal peptide. I demonstrated that SSG1 is a secreted protein from cells overexpressing epitope-tagged SSG1 as SSG1 is found in the medium. Secreted SSG1 migrated as a diffuse band corresponding to a molecular weight of ~125,000-175,000 compared to a tighter, faster migrating band of ~120,000 observed in the cell lysate. This suggests that SSG1 undergoes post-translational modifications before being secreted and is consistent with the presence of predicted N-glycosylation sites within SSG1. I was able to block the appearance of SSG1 in the medium completely by treating the cells with Brefeldin A, an inhibitor of the constitutive secretory pathway. When SSG1 was co-expressed with wild-type JAK2, more SSG1 was detected in the medium of JAK2 overexpressing cells compared to control cells. This SSG1 came in the form of full-length SSG1 as well as cleaved SSG1, with the combined amount of these bands being greater when the cells co-expressed JAK2 than when SSG1 was expressed alone or with kinase-dead JAK2 (K882E). These results suggest that JAK2 enhances the secretion and possibly the cleavage of SSG1. SSG1 cleavage products were most commonly seen when looking at secreted SSG1. While SSG1 has protease cleavage sites, our data suggest that most of the proteolytic cleavage of SSG1 occurs once SSG1 has been secreted from the cell. There are reports in the literature that trafficking of cytokine receptors to the membrane requires interaction with JAK2 in the ER/Golgi, with JAK2 being important for the processing and cell surface localization of cytokine receptors (12, 13). It has also been reported that JAK2 has kinase activity in the ER (1). Our findings that SSG1 co-immunoprecipitates with kinase-active JAK2 and that JAK2 partially co-localizes with SSG1 in what appears to be the ER/Golgi complex suggest that SSG1 and JAK2 may functionally interact in the ER/Golgi. Previous data from our lab demonstrated that SSG1 is phosphorylated by JAK2 (2). It is possible that JAK2 phosphorylation of SSG1 enhances the activity of secreted SSG1 or its susceptibility to proteases. Therefore, it is possible that SSG1 is phosphorylated by JAK2 in the ER and that this phosphorylation aids in the enhanced processing/secretion of SSG1. Taken

together, these findings support my hypothesis that JAK2 regulates the secretion and processing of SSG1 (Fig. 4.3).

Future Directions

This thesis project leaves many questions unanswered. The potential that SSG1 has both an intracellular function as well as extracellular function is interesting. The following future directions probe both the interaction between SSG1 and JAK1 as well as the potential that secreted SSG1 may have a physiological role.

SSG1 interaction with JAK2

Does SSG1 interact with JAK2 in vivo?

In Chapter II of this thesis, I demonstrated that full-length SSG1 and JAK2 interact in an overexpression system. Because both JAK2 and SSG1 are expressed in adipose tissue and SSG1 was identified from a yeast two-hybrid screen of a rat adipocyte cDNA library, we would predict that endogenous SSG1 and JAK2 would interact *in vivo* in adipocytes. To test this hypothesis, 3T3-F4442A preadipocytes, which express endogenous SSG1 (preliminary data, not shown), would be treated with GH to activate JAK2. JAK2 would then be immunoprecipitated and immunoprecipitates would be immunoblotted with α SSG1 to detect GH-dependent JAK2-SSG1 interaction *in vivo*. If we are unable to detect this interaction in preadipocytes, preadipocytes will be differentiated into adipocytes, which have a higher level of expression of SSG1 than preadipocytes (preliminary data, not shown). Our data suggest that SSG1 is mostly secreted from these cells, and as a result, we can detect a minimal amount of SSG1 in the cell lysate, which might make detecting the interaction of SSG1 with JAK2 difficult. Thus, it may be necessary to use multiple plates of cells to detect the interaction of endogenous SSG1 with endogenous JAK2. It may also be possible to increase intracellular levels of SSG1 using Brefeldin, A which causes SSG1 to accumulate in the cell. This may or may not cause SSG1 to accumulate in the right pool to interact with

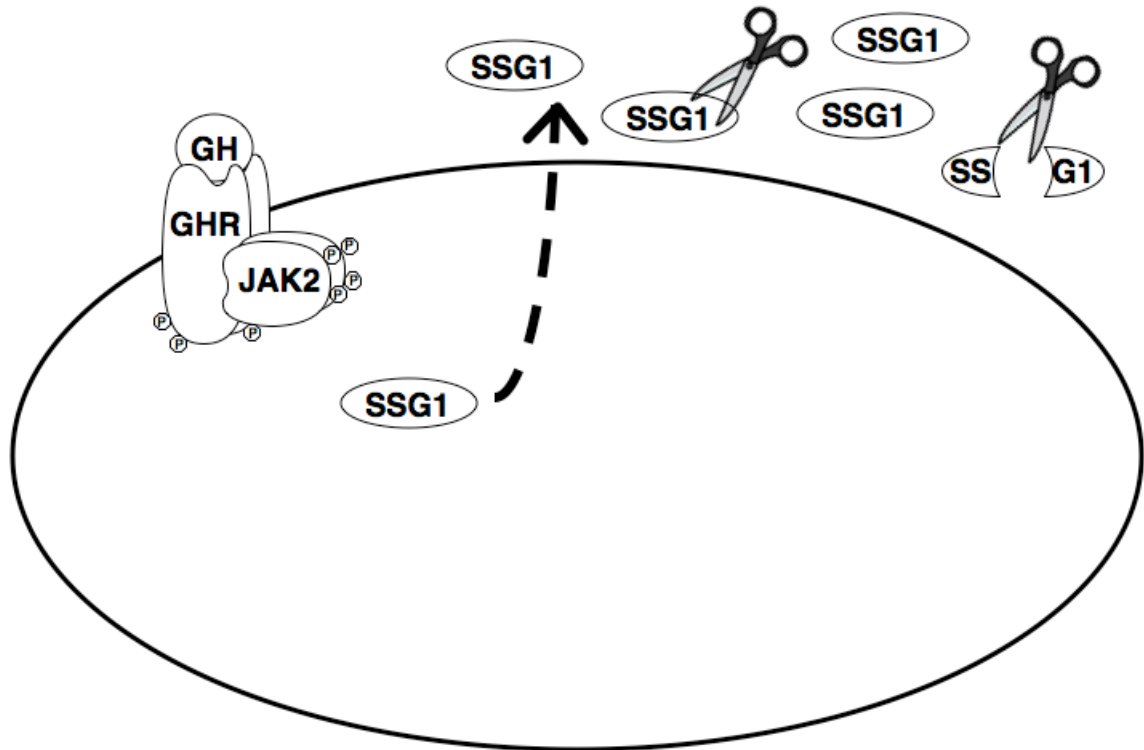


Figure 4.3 Schematic representation of SSG1 as a secreted protein.

SSG1 contains a functional signal peptide resulting in SSG1 secretion. SSG1 secretion and proteolytic processing, resulting in cleavage fragments of SSG1, is enhanced in the presence of wild-type JAK2. Therefore, JAK2 regulates the secretion and processing of SSG1. Finally, SSG1 secretion is blocked by Brefeldin A.

JAK2. We have a limited amount of the α SSG1 antibody; therefore, we may need to make a new, high affinity antibody to SSG1 for these experiments to be successful.

Does the SRPX5 domain represent a novel phosphotyrosine-binding domain?

In Chapter II of this thesis, I demonstrated that all three of the SRPX5 domains in SSG1 interacts with the activated form of JAK2 as well as the activated JH1 kinase domain of JAK2. From these results, we predict that the SRPPX5 domain represents a novel phosphotyrosine-binding domain. Definitive proof for the hypothesis of the SRPX5 domain being a phosphotyrosine-binding domain will come from solving the crystal structure of the SRPX5 domain in complex with a phosphopeptide. Additional support for this hypothesis would be obtained by showing that the isolated SRPX5 domain of SRPX2 and the SRPX5 domain of SRPX also bind to the kinase-active JAK2 and not to the kinase-inactive JAK2. Identification of the tyrosine (or tyrosines) within JAK2 that bind the SRPX5 domain would also provide additional support.

The role SSG1 plays in Stat3 phosphorylation

Identify the novel site of phosphorylation on Stat3 and determine its function.

In Chapter III of this thesis, I demonstrate that SSG1 promotes the phosphorylation of Stat3 on Tyr705 as well as the phosphorylation of another residue within Stat3. To determine the novel phosphorylation site in Stat3, cDNA encoding Stat3 will be overexpressed in 293T cells in the presence or absence of SSG1-myc and rat GH receptor. Cells will be incubated in serum-free medium overnight and stimulated with GH for 15 min. Phospho-Stat3 will be purified from cell lysates and analyzed by mass spectroscopy to identify the novel site of phosphorylation seen in the presence of SSG1. Several candidates exist as sites of this novel phosphorylation, though SSG1 may promote the phosphorylation of a completely novel, uncharacterized residue. Potential sites of Stat3 phosphorylation identified by mass spectroscopy include: Thr268 (14), Ser269 (14), Ser273 (14), Tyr539 (15, 16), Tyr686 (16, 17), Ser691 (18). If mass spectroscopy fails to identify this novel phosphorylation site, two dimensional

phosphopeptide mapping combined with site-directed mutagenesis can be done to see if any of the above sites is the site of SSG1-regulated phosphorylation in Stat3. Identification of this site will allow us to create non-phosphorylatable mutants by site-directed mutagenesis as well as mutants that mimic phosphorylation if this site is a Ser or Thr. These mutants will be useful tools to determine the function of this novel phosphorylation site in Stat3. The characterized post-translational phosphorylation sites within Stat3 (i.e. Ser727 and Tyr705) have been shown to play a role in protein-protein interaction, nuclear translocation, DNA binding, and transcriptional activation resulting in regulation of apoptosis, the cell cycle, and cell growth. Therefore, these mutant constructs will allow us to determine if this novel site also plays a role in any of these functions using the appropriate assay to test the specific function.

Does the novel phosphorylation site on Stat3 impair Stat3 dimer formation?

Once this novel phosphorylation site has been identified, it will be important to determine if it plays a role in any of the downstream events of activated Stats: dimerization, translocation, DNA binding, transcriptional activation. To determine if this novel phosphorylation site on Stat3 interferes with Stat3 dimer formation, Stat3 mutant constructs will be made in which the phosphorylation site has been mutated to the appropriate non-phosphorylated amino acid analog. Additionally, if this site is a Ser or Thr, mutants that mimic phosphorylation will be created. Both mutant and wild-type Stat3 proteins will be tagged with FLAG or with myc. Combinations of mutant and wild-type differentially tagged Stat3 constructs will be overexpressed in 293T cells in the absence or presence of SSG1 and rat GH receptor. GH will be added, FLAG-Stat3 will be immunoprecipitated with α FLAG, and immunoprecipitates will be immunoblotted with α myc. Co-immunoprecipitation of the differentially tagged wild-type constructs will serve as positive controls for dimer formation. A finding that the differentially tagged wild-type Stat3 proteins do not co-immunoprecipitate in the presence of SSG1 would suggest that SSG1 prevents the association between the two Stat3 molecules. An additional finding that the mutated Stat3 proteins lacking the proposed site of SSG1-induced phosphorylation can dimerize in response to GH would provide evidence that the

SSG1-dependent phosphorylation site is responsible for lack of dimerization of wild-type Stat3 in SSG1 overexpressing cells. If dimers are unable to form in the presence of SSG1 as a result of this novel phosphorylation, activated Stat3 might not be able to translocate to the nucleus to initiate gene transcription. This would support the results in Chapter III of this thesis where I demonstrate that SSG1 negatively regulates *c-fos* gene transcription.

Does the novel phosphorylation inhibit Stat3 translocation to the nucleus?

If SSG1 does not affect Stat3 dimer formation, I would next test whether SSG1 prevents Stat3 dimers from translocating to the nucleus. cDNA encoding wild-type or mutant GFP-tagged Stat3 will be transfected into COS-7 cells along with cDNA encoding rat GH receptor in the presence or absence of cDNA encoding SSG1. Cells will be grown on coverslips and incubated in serum-free medium overnight and then stimulated with GH for 15 min. GH rapidly promotes the phosphorylation of Stat3 and its accumulation in the nucleus. Nuclear accumulation of GFP-Stat3 in living cells can be visualized by fluorescent microscopy. Cells can also be fixed, stained for pTyr705-Stat3 (in red) and visualized to determine nuclear accumulation of the pTyr705 Stat3 dimers. A finding that wild-type Stat3 fails to accumulate in the nucleus in the presence of SSG1 would suggest that this novel phosphorylation promoted by SSG1 interferes with the nuclear accumulation of Stat3. Further support would come from experiments where the mutant GFP-Stat3 lacking the proposed site of phosphorylation is able to translocate to the nucleus in the presence of SSG1 suggesting that SSG1-dependent phosphorylation is important for the nuclear translocation of Stat3. Taken together, these results would be consistent with the novel phosphorylation site in Stat3, promoted by the presence of SSG1, interfering with the nuclear accumulation of Stat3. However, if wild-type GFP-Stat3 still accumulates in the nucleus in the presence of SSG1, this would suggest that this novel phosphorylation site is not important for the nuclear translocation of Stat3.

If the immunocytochemistry results are ambiguous, 293T cells will be co-transfected with cDNA encoding wild-type and mutant FLAG-Stat3 and rat GH receptor in the presence or absence of SSG1. Cells will be incubated in serum-free medium overnight and stimulated with GH for 15 min. Nuclear and cytoplasmic fractions will be

harvested and samples will be immunoblotted with α pTyr705-Stat3 to determine if SSG1 prevents nuclear accumulation of pTyr705-Stat3 and whether this can be attributed to the novel phosphorylation site in Stat3. A finding of less wild-type pTyr705-Stat3 in the nuclear fraction of SSG1-expressing cells compared to control cells would be consistent with the novel phosphorylation site in Stat3 promoted by SSG1 interfering with the nuclear accumulation of Stat3. Further support that SSG1-dependent phosphorylation of Stat3 is important for the nuclear translocation of Stat3 would be if mutant GFP-Stat3 is detected in the nuclear fraction in the presence of SSG1.

In this series of experiments, a finding that wild-type Stat3 is not seen in the nucleus upon GH stimulation when SSG1 is co-expressed would suggest SSG1 interferes with nuclear translocation of Stat3. If wild-type Stat3 is still able to translocate to the nucleus in the nucleus, this would suggest that function of the novel phosphorylation site in Stat3 is not related to nuclear translocation.

Does SSG1 alter Stat3 binding to the promoter of target genes?

Another important function of Stat3 is the ability of Stat3 dimers to bind to DNA to regulate gene transcription. If SSG1 does not alter the ability of Stat3 to dimerize or translocate to the nucleus, it is possible SSG1 alters the ability of the Stat3 dimer to bind to DNA. To test this hypothesis, I performed electrophoretic mobility shift (EMSA) assays using the m67 region of the SIE as a probe for Stat3 binding. I overexpressed cDNA encoding FLAG-Stat3 and rat GH receptor in the presence and absence of cDNA encoding SSG1 in 293T cells. I prepared nuclear extracts and performed a radioactive EMSA to look at the ability of Stat3 to bind DNA in the presence and absence of SSG1. Unfortunately, I was unable to conclude if the presence of SSG1 had any effect on the ability of Stat3 to bind DNA. The amounts of Stat3 I had to overexpress to detect Stat3 binding to the probe no longer demonstrated the SSG1-dependent phosphorylation detected by immunoblotting, presumably due to saturation of a rate-limiting endogenous protein.

As an alternative, chromatin immunoprecipitation (Chip) assays, which also test the ability of proteins to bind DNA, may be able to be performed. Again, the levels of

proteins needed to detect this interaction may be an important limitation. Therefore, it may be necessary to perform the Chip assay in 3T3-F442A cells where endogenous SSG1 has been knocked down and compare the ability of endogenous Stat3 to bind *c-fos* in the presence and absence of endogenous SSG1 using an antibody to total Stat3. In this experiment, if SSG1 is affecting the ability of Stat3 to bind *c-fos*, I would predict that less Stat3 would be detected in the presence of endogenous SSG1 compared to when SSG1 is knocked down. This would be consistent with SSG1 interfering with Stat3's ability to bind DNA due to the phosphorylation promoted by SSG1. Because SSG1 is expressed in the liver (19), GH-responsive liver cell lines lacking endogenous Stat3 can be obtained. Wild-type or mutant Stat3 could be introduced into these cells, and then stimulated with GH. A Chip assay using total Stat3 antibody will be performed comparing the ability of wild-type and mutant Stat3 to bind *c-fos* in the presence of endogenous SSG1. In these experiments, a finding that wild-type Stat3 fails to bind to DNA upon GH stimulation when SSG1 is co-expressed would suggest SSG1 interferes with the ability of Stat3 to bind DNA. If mutant Stat3 is able to bind DNA, this would support the hypothesis that SSG1-dependent phosphorylation is important in regulating the ability of Stat3 to bind DNA. If wild-type Stat3 in the presence of SSG1 is still able to bind DNA, it would suggest that SSG1 may be interfering with other transcriptional machinery to negatively regulate gene transcription.

Does Stat3 enhance or inhibit other GH-regulated genes in the presence of SSG1?

To determine the effect of SSG1 on Stat3-regulated gene expression, I would compare gene expression profiles of Stat3-mediated genes from 3T3-F442A cells expressing endogenous SSG1 to cells with SSG1 knocked down. Cells would be incubated in serum-free medium overnight and stimulated with GH to activate JAK2, which, in turn, would activate Stat3. RNA would be harvested from these cells and expression of established Stat3-regulated genes would be analyzed by QT-PCR. If SSG1 negatively affects the Stat3 signaling pathway, I would predict that a subset of Stat3-mediated genes would have reduced expression when SSG1 is expressed compared to cells in which SSG1 has been knocked down.

SSG1 and signaling

Does SSG1 influence other signaling pathways activated by GH?

In Chapter III of this thesis, I demonstrate that SSG1 affects phosphorylation of Stat3. SSG1 also modestly affects the phosphorylation of Stat5b on Tyr694, another important transcription factor activated in response to GH stimulation. GH stimulation also leads to the activation of the Ras-MAPK and PI-3K/Akt pathways. To determine if SSG1 affects other signaling pathways activated by GH, cDNA encoding SSG1-myc will be stably overexpressed in 3T3-F442A cells. Cells will be incubated in serum-free medium overnight and stimulated with GH for different lengths of time. Cell lysates will be prepared and immunoblotted with α pERK1/2 and α pAkt. The presence of SSG1 may or may not affect the activation of these pathways.

Additionally, these pathways activate transcription factors important in regulating *c-fos* gene transcription. For instance, the transcription factor C/EBP β is phosphorylated on Thr188 by MAPK in response to GH (20), and mutation of this site results in inhibition of *c-fos* gene transcription suggesting that phosphorylation of this site in C/EBP β is necessary for maximal transcriptional activation (20). Therefore, it will be interesting to determine if SSG1 plays a role in other signaling pathways activated by GH, which may shed some light on the results presented in Chapter III in which SSG1 negatively regulates *c-fos* gene transcription.

Does knock down of SSG1 alter phosphorylation and signaling of Stat3? Other signaling pathways activated by GH?

I have demonstrated that overexpressed SSG1 alters the GH-induced Stat3 signaling pathway. It will be interesting to determine what happens to signaling pathways when SSG1 is knocked down. To test this hypothesis, SSG1 RNAi constructs will be engineered and stably introduced into 3T3-F442A cells to knock down endogenous levels of SSG1. Control cell lines will also be created for comparison. Cells will be incubated in serum-free medium overnight and then stimulated with GH. Lysates

will be immunoblotted with antibodies for: pTyr705-Stat3, pTyr694-Stat5b, pERK1/2 and pAkt to determine if knockdown of SSG1 has any effect on these GH signal transduction pathways. If endogenous SSG1 is knocked down, then I would predict that phosphorylation of Tyr705 of Stat3 after GH stimulation would not be different compared to phosphorylation of Tyr705 in the presence of endogenous SSG1, nor would Stat3 be phosphorylated on this novel residue. However, it is possible that this novel phosphorylation in the presence of SSG1 primes Stat3 allowing for the enhanced phosphorylation of Tyr705. If this is the case, I would predict that knocking down SSG1 may reduce the levels of phosphorylation of Tyr705 of Stat3. Additionally, if SSG1 affects the MAPK and/or PI-3K/Akt pathways (tested above), I would expect to see changes in these pathways when SSG1 is knocked down. To determine the effect of SSG1 on gene expression mediated by activation of these pathways, I would compare gene expression profiles from 3T3-F442A cells expressing endogenous SSG1 to cells with SSG1 stably knocked down. Cells would be incubated in serum-free medium overnight and stimulated with GH. RNA would be harvested from these cells and gene expression would be analyzed by QT-PCR. If SSG1 negatively affects these signaling pathways, I would predict that a subset of genes would have reduced expression when SSG1 is expressed compared to cells in which SSG1 has been knocked down.

SSG1 has been shown to be upregulated in adipose tissue (21, 22). Therefore, it will be interesting to determine if SSG1 knock down cells can be differentiated into adipocytes. Control cell lines and SSG1 knockdown cell lines will be differentiated into adipocytes and stained with Oil Red-O to determine if knockdown of SSG1 has any effect on the ability of preadipocytes to differentiate into adipocytes. QT-PCR analysis on other adipocyte specific genes can also be performed to see if relative amounts of gene expression are altered when SSG1 is knocked down.

Does SSG1 influence cell migration or cell motility?

SSG1/CL2/DRO1 was found to be downregulated in human carcinoma cell lines and tumors suggesting that SSG1 may function as a tumor suppressor (10, 11). Bommer et al. (10) demonstrated that ectopic expression of SSG1/DRO1 in cancer cell lines

reduced colony formation, growth in soft agar, and inhibited anchorage-independent cell growth. Therefore, it would be interesting to see if SSG1 affects the ability of cells to migrate in the presence or absence of growth hormone. GH has been shown to promote cell motility (23, 24). To test this hypothesis, I would perform a migration assay comparing control cells and SSG1-expressing cells or control cells and SSG1 knockdown cells. Cells would be transfected with control vector or cDNA encoding full-length SSG1. Alternatively, SSG1 knockdown cells can also be used as a control for cells expressing endogenous levels of SSG1. Cells would be grown to confluency, wounded, incubated in serum-free medium overnight, and then stimulated with or without GH. The ability of cells to migrate into the wound would be assessed and compared for control and SSG1-expressing cells and/or SSG1 knockdown cells. A decrease in migration of SSG1-expressing cells would be consistent with SSG1 functioning as a tumor suppressor.

Is SSG1 involved in other cytokine signaling pathways?

JAK2 is activated by nearly two-thirds of the cytokine receptors including the leptin receptor, and it has been reported that SSG1 has a wide tissue distribution (10, 11, 19) with high expression in adipose tissue (22). Therefore, it seems likely that SSG1 plays a role in signaling not just by GH, but also by other ligands that bind to members of the cytokine family of receptors. To support this hypothesis, Aoki et al. (21) reported that patterns of gene expression are similar for murine SSG1 and leptin, another cytokine that activates JAK2. They demonstrated that SSG1 is expressed in white and brown adipose tissue as well as in the chroid plexus in the brain, regions where the long form of the leptin receptor is also expressed (21). They proposed that SSG1 plays a role in energy balance based on their finding that expression of SSG1 is upregulated in brown adipose tissue from bombesin receptor subtype (BRS)-3-deficient mice which display mild obesity (21, 25). An inhibitory role of SSG1 on leptin-dependent gene expression would be consistent with this finding because BRS-3-deficient mice exhibit characteristics associated with reduced leptin signaling (obesity, hyperphagy and reduced metabolic rate) (25).

To examine whether SSG1 is involved in signaling by leptin, we can take advantage of the leptin (L. Rui) or EPO/leptin (M. Myers) systems developed by our collaborators at the University of Michigan. These stable cell lines expressing either the leptin receptor or the EPO-leptin chimeric receptor (EPO ligand binding and transmembrane domains fused to the intracellular leptin receptor) are useful tools in studying leptin signaling. Leptin activates Stat3 (26), and these tools will help to determine if SSG1 can also influence Stat3-mediated gene expression by another cytokine other than GH. To test the hypothesis that SSG1 regulates leptin-stimulated gene expression, I would overexpress SSG1-myc or vector in either of these cell lines. Cells would be incubated in serum-free medium overnight and stimulated with the appropriate ligand to activate the associated JAK2 molecules, which in turn, would activate Stat3. RNA would be harvested from these cells and expression of Stat3-regulated genes would be analyzed by QT-PCR. If SSG1 also negatively affects the Stat3 signaling pathway in the leptin system, I would predict that a subset of Stat3-mediated genes would have reduced expression when SSG1 is overexpressed. I would also test the effect SSG1 has on leptin signaling and gene expression using the siRNA constructs created above. I would predict that leptin-stimulated Stat3-mediated gene transcription would be unchanged or possibly elevated when SSG1 has been knocked down.

Does SSG1 interact with JAK family members or other receptor tyrosine kinases?

In Chapter II of this thesis, I demonstrated that full-length SSG1 interacts preferentially with activated JAK2 but not kinase-dead JAK2. It would be interesting to determine if SSG1 is a binding partner for the other JAK family members or activated receptor tyrosine kinases. The experimental design would be the same as that used for JAK2. Briefly, SSG1-myc would be overexpressed in 293T cells with or without the JAK family member. JAK would be immunoprecipitated with JAK specific antibody and immunoprecipitates would be immunoblotted with α myc for SSG1. If SSG1 interacted with any of the JAK family members, preferential binding to the kinase-active form would be assessed by overexpressing SSG1 alone or in the presence of activated JAK or

kinase-dead JAK2. Similar experimental design would be used to determine if SSG1 is a binding partner for receptor tyrosine kinases. In these experiments, ligand-activated or constitutively activated forms of the receptors would be used. In these sets of experiments, I would predict that SSG1 would interact with activated forms of the JAK family members and potentially interact with the activated forms of the receptor tyrosine kinases.

Because SSG1 interacts with kinase-active JAK2 and I hypothesize that the SRPX5 domain is a novel phosphotyrosine binding domain, it will be useful to determine which phosphotyrosines in JAK2 are responsible for binding SSG1. To identify these phosphorylated tyrosine(s) in JAK2 is responsible for binding to SSG1, immobilized peptides containing non-phosphorylated or phosphorylated tyrosines from JAK2 will be used. Lysates from cells overexpressing SSG1 will be incubated with these JAK2 peptides. We predict that SSG1 will interact with some of the phosphorylated peptides but not the non-phosphorylated peptides of JAK2.

Secreted SSG1

Is secreted SSG1 phosphorylated on tyrosines?

Previous data from our lab demonstrated that SSG1 is phosphorylated by JAK2 (2). In Chapter III of this thesis, I demonstrated that SSG1 is a secreted protein. Because it has been reported that JAK2 has kinase activity in the ER (1), it is possible that SSG1 is phosphorylated by JAK2 in this subcellular compartment prior to secretion. It will be interesting to determine if secreted SSG1 is phosphorylated on tyrosines. To test this hypothesis, SSG1-myc will be co-expressed with FLAG-tagged JAK2 or JAK2 (K882E) in 293T cells. Medium will be changed to 1% serum ten hours post-transfection and collected after 16 h of incubation with the cells. Phosphatase inhibitors will be added to the medium and proteins will be precipitated using TCA. The precipitated proteins will be resolved by SDS-PAGE and immunoblotted with α pY to determine if secreted SSG1 is phosphorylated. If my hypothesis is correct, I would expect to see phosphorylated SSG1 secreted when co-expressed with wild-type JAK2 but not when co-expressed with

JAK2 (K882E). If I cannot definitively say if the band detected is indeed, phosphorylated SSG1, I can purify secreted SSG1 and blot with α pY.

Does GH stimulate the secretion of SSG1?

As shown in Chapter III of this thesis, SSG1 secretion from 293T cells was enhanced when JAK2 was co-expressed but not when JAK2 (K882E) was expressed. Secretion was also blocked when cells were treated with Brefeldin A. It will be interesting to see if GH promotes secretion of SSG1. Initially, I would co-transfect 293T cells with cDNA encoding rat GH receptor in the presence or absence of SSG1. Cells would be incubated in serum-free medium overnight and then stimulated with GH. Medium from transfected cells would be collected, proteins in the medium would be precipitated with TCA, and samples would be immunoblotted with α myc to see SSG1 secreted into the medium. The caveat to this experiment is that large amounts of SSG1 seem to be secreted in the basal state; therefore, an increase may not be detected when cells are stimulated with GH. Because I do not know how long cells must be stimulated with GH, I would do a GH timecourse and look to see if secretion of SSG1 is altered with varying times of GH treatment. This experiment can also be performed in GH-treated 3T3-F442A preadipocytes, which express endogenous SSG1, using an antibody to detect SSG1. The SSG1 knock down stable cell line could also serve as a negative control since no SSG1 is expressed, there will be no SSG1 secretion.

Is secreted SSG1 able to enter cells? Are there SSG1 receptors? What is the function of this intracellular SSG1?

Because SSG1 is a secreted protein, it is intriguing to speculate that SSG1 interacts with a cell surface receptor and/or is internalized and therefore has an intracellular function. Eventually, I would like to determine if there are cell surface receptors for SSG1 and/or if SSG1 or an SSG1 cleavage product gets internalized into cells resulting in an intracellular function for SSG1 in these cells.

Cleaved SSG1

What are the proteases that cleave SSG1?

In Chapter III of this thesis, I demonstrate that secreted SSG1 is seen both as a full-length protein as well as a series of N- or C-terminal truncated proteins. Because secreted SSG1 was seen as both a full-length protein as well as these various cleavage products, I hypothesize that the majority of proteolytic cleavage occurs once SSG1 has been secreted. SSG1 cleavage products were most often seen when visualizing secreted SSG1; however, SSG1 truncation products were also seen in the cell lysate, suggesting that some intracellular proteases are also able to recognize and cleave SSG1 inside the cell. It will be interesting to determine whether cleavage is physiological or adventitious and if physiological, what proteases cleave full-length SSG1 into these N- and C-terminal fragments and whether cleavage products have distinct functions.

Physiological function of SSG1

What is the physiological role for SSG1 in vivo?

One way to determine the physiological role for SSG1 would be to generate SSG1 knockout mice. Because SSG1 has a wide tissue distribution and seems to have several possible functions, including a role in embryogenesis (27), conditional knockout mice with SSG1 deleted in particular tissues will help to determine the tissue-specific function of SSG1 in adult mice. For instance, a mouse with SSG1 conditionally deleted in adipose tissue would help determine the role SSG1 plays in energy metabolism. Likewise, a conditional knockout in bone will help determine if SSG1 plays a role in bone formation. These mice would be valuable tools to examine the physiological effects of SSG1 *in vivo*.

What role, if any, does secreted SSG1 play in adipogenesis and osteoblastogenesis?

SSG1 has high expression in adipose tissue (22) and is down regulated in bone (27). These tissues are derived from a common progenitor cell. Therefore, it is not surprising that a subset of genes is upregulated in one tissue that is downregulated in the other. Wnt10b is a secreted protein that inhibits adipogenesis and stimulates

osteoblastogenesis (reviewed in (28)). To test whether SSG1 stimulates adipogenesis and inhibits osteoblastogenesis, adipose and bone tissue from the SSG1 conditional, whole-body knockout mice will be analyzed for difference in fat depots as well as bone density compared to wild-type controls. To determine if SSG1 expression plays a role in fate determination of these cells, SSG1 knockdown cells can be differentiated into adipocytes or osteoblasts and the rate of differentiation compared to control cells. I would predict that cells expressing SSG1 would differentiate into adipocytes, but that these cells may not differentiate into osteoblasts as well as control cells. In contrast, I would predict that cells in which SSG1 has been knocked down would differentiate into osteoblasts more robustly than control (+ SSG1) cells. Similarly, it would be interesting to determine if cells treated with purified SSG1 would differentiate into adipocytes, and conversely, if cells treated with purified SSG1 would inhibit differentiation into osteoblasts.

Because SSG1 is upregulated in adipose tissue (22), is expressed in regions of the brain similar as the leptin receptor (21), and is a secreted protein, it is tempting to speculate that secreted SSG1 may function in maintaining energy balance. Further support that SSG1 may play a role in energy metabolism came from a study reporting that mouse models of obesity demonstrate a downregulation of SSG1 mRNA levels in adipose tissue (22). Okada et al. (22) propose that SSG1 is a novel adipocytokine. It is intriguing to speculate that secreted SSG1 plays a role in carbohydrate metabolism similar to other secreted proteins such as leptin and adiponectin.

Conclusion

In this thesis, I have examined the cellular function of SSG1 (Fig. 4.4). I first demonstrated that SSG1 interacts with activated JAK2. SSG1 promotes the GH-dependent phosphorylation of Tyr705. It also appears to stimulate phosphorylation of a novel residue on Stat3. SSG1 appears to negatively regulate *c-fos* gene expression. I also showed that SSG1 localizes to the ER and Golgi, and that a portion of JAK2 co-localizes with SSG1. SSG1 contains a functional signal peptide resulting in SSG1 secretion, which is blocked with Brefeldin A. When co-expressed with JAK2, SSG1

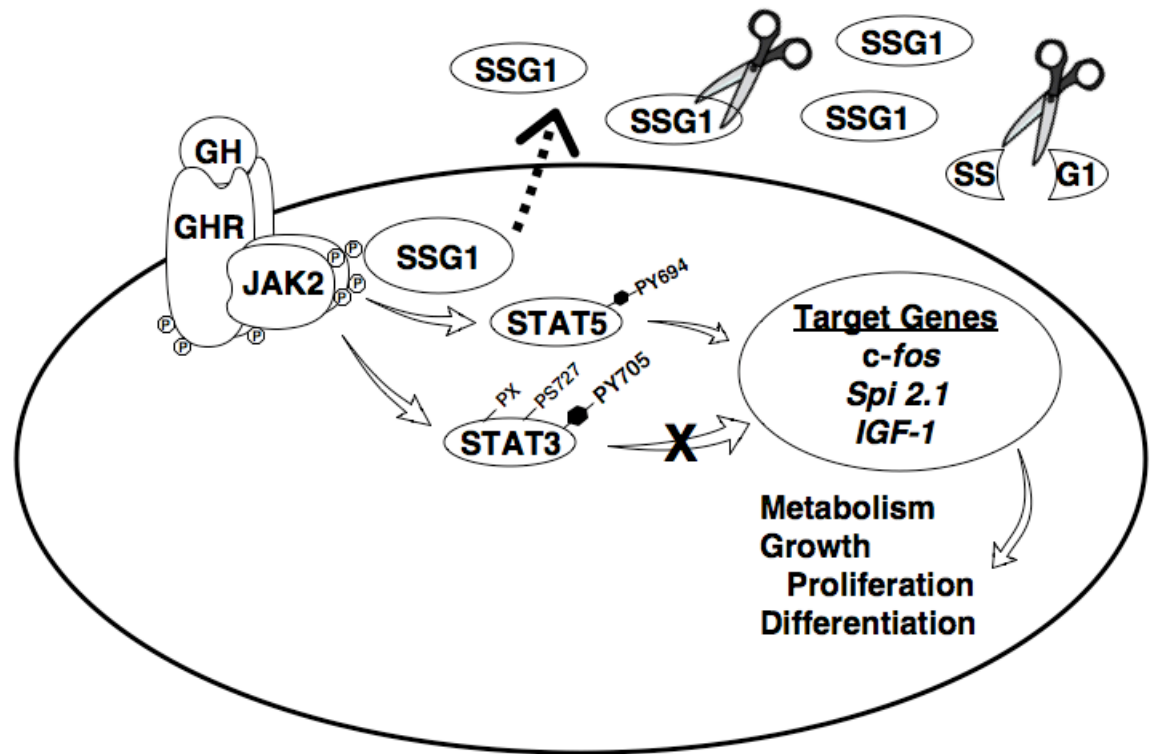


Figure 4.4 Model of SSG1 in GH Signal Transduction.

SSG1 interacts with kinase-active JAK2, but does not alter the kinase activity of JAK2. SSG1 promotes phosphorylation of Tyr705 in Stat3 in the absence and presence of GH stimulation. SSG1 also appears to stimulate the phosphorylation of a novel residue on Stat3. SSG1 contains a functional signal peptide resulting in SSG1 secretion. JAK2 enhances the secretion and proteolytic processing of SSG1.

secretion is enhanced as is the detection of proteolytic cleavage products. SSG1 does not appear to be cleaved into products that function in different parts of the cell. Because SSG1 is highly expressed in adipose tissue and downregulated in obesity, secreted SSG1 may play a role in maintaining energy balance. Taken together, with published results, these results support the hypothesis that SSG1 is a multi-functional protein. SSG1 interacts with JAK2. Conversely, JAK2 regulates the function of and cellular trafficking of SSG1. It will be interesting to determine if the SRPX5 domain is a novel phosphotyrosine binding domain and the physiological function of SSG1; and if, in fact, SSG1 has dual functions in the cell.

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