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**Characterization of Nucleo-cytoplasmic Transport of the
Transcription Factor STAT5a**

A Dissertation Presented

by

Janaki Kannan Iyer

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ABSTRACT OF THE DISSERTATION

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Signal Transducers and Activators of Transcription (STATs) are a family of latent DNA binding proteins that play critical roles in cytokine signaling. As the name suggests, these proteins have the dual role of transducing signals from the cell membrane into the nucleus where they can perform their role as transcription factors. STATs are normally present in the cell in an inactive state and are activated by phosphorylation on a conserved tyrosine. Tyrosine phosphorylation confers a conformational change and the ability to bind specific target DNA. Thus STATs must

have a cytoplasmic presence to transduce signals from the cell membrane and have the ability to enter the nucleus to fulfill their role as transcription factors.

STAT5a plays an important role in a number of physiological processes like hematopoiesis and mammary gland development. Accurate cellular localization of STAT5a is necessary to execute its function as a signaling molecule and transcription factor. STAT5a responds to cytokines such as growth hormone, prolactin, and interleukin-2, and undergoes tyrosine phosphorylation to form dimers with the ability to bind DNA. This study explores the nuclear trafficking of STAT5a both prior to and following tyrosine phosphorylation. STAT5a shows a constitutive nuclear presence in the absence of tyrosine phosphorylation. With the use of live cell imaging we demonstrate the continuous shuttling of STAT5a in and out of the nucleus. Evaluation of a series of mutations and deletions identifies a region within the coiled coil domain of STAT5a that is critical for nuclear import of both unphosphorylated and tyrosine phosphorylated forms. The mechanism that regulates transport of STAT5a through nuclear pore complexes into the nucleus is therefore independent of tyrosine phosphorylation. However, following tyrosine phosphorylation STAT5a accumulates in the nucleus due to its retention by DNA binding.

STAT5a is also continually exported from the nucleus. A region in the DNA binding domain shows the presence of an NES that can be recognized by CRM1. The positioning of the NES might suggest that it is accessible when STAT5a is not bound to DNA. These findings should lay a foundation for further studies that involve targeting the activity of STAT5a in pathological conditions.

Dedication

To My Family

With Lots of Love

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Abbreviations

aa	Amino acid
Ab	Antibody
Arm	Armadillo
AML	Acute Myelogenous Leukemia
BSA	Bovine Serum Albumin
CAS	Cellular Apoptosis Susceptibility
cDNA	Complementary Deoxyribonucleic Acid
CML	Chronic Myelogenous Leukemia
CNTF	Ciliary Neurotrophic Factor
CRM1	Chromosome Region Maintenance 1
CT-1	Cardiotrophin-1
Cys	Cysteine
DIC	Differential Interference Contrast
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
ds	Double strand
DTT	Dithiothreitol
EDTA	Ethylene Diaminetetra-acetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol-tetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay

EPO	Erythropoietin
FBS	Fetal Bovine Serum
FERM	Band 4.1, Ezrin, Radixin and Moesin
GAP	GTPase Activating Protein
GAS	Interferon- γ activated site
G-CSF	Granulocyte Colony Stimulating Factor
GDP	Guanosine 5' diphosphate
GEF	Guanine-nucleotide Exchange Factor
GFP	Green fluorescent protein
GH	Growth Hormone
GHBP	Growth hormone Binding Protein
GHR	Growth Hormone Receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GTP	Guanosine 5' triphosphate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HTLV	Human T-cell Lymphotropic Virus
I β B	Importin- β 1 Binding
IFN	Interferon
IL	Interleukin
IRF	Interferon Regulatory Factor
ISGF3	Interferon Stimulated Gene Factor-3
ISRE	Interferon Stimulated Response Element
JAK	Janus Kinase

JH	JAK Homology
KCl	Potassium chloride
kDa	kilodalton
LIF	Leukemia Inhibitory Factor
LMB	Leptomycin B
LMP2	Low molecular mass polypeptide 2
MEF	Mouse Embryonic Fibroblasts
μg	microgram
μL	microliter
mg	milligram
ml	milliliter
mm	millimeter
mM	milli molar
NaCl	Sodium chloride
NaF	Sodium fluoride
NES	Nuclear Export Signal
NK	Natural Killer
NLS	Nuclear Localization Signal
NTF2	Nuclear Transport Factor 2
NPC	Nuclear Pore Complex
OSM	Oncostatin M
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PL	Placental Lactogen
PMSF	Phenylmethylsulphonyl Fluoride
Prl	Prolactin
pY	Phosphotyrosine
RT	Room temperature
PTK	Protein tyrosine kinase
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecylsulfate
SH2	Src Homology 2
STAT	Signal Transducer and Activator of Transcription
SV40	Simian Virus 40
TBS	Tris Buffered Saline
TNF	Tumor Necrosis Factor
TPO	Thrombopoietin
Tris	tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene-sorbitan-monolaurate
U	Unit
VEGF	Vascular Endothelial Growth Factor
WT	wild type

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Chapter 1

Introduction

All living organisms, unicellular or multicellular, have evolved mechanisms to sense and appropriately respond to changes in their environment. They have evolved proteins that can recognize extracellular changes and signal these changes to the nucleus which results in specific changes in gene expression. In higher organisms, these signaling pathways are tightly regulated by soluble factors like cytokines and growth factors. These molecules play critical roles in physiological processes like embryogenesis, growth, immune responses, etc. A number of cytokines and growth factors signal through a family of transcription factors called Signal Transducers and Activators of Transcription (STAT). In this dissertation I have focused on a member of this family designated as STAT5a.

STAT5a plays critical roles in physiological processes including mammary gland development and hematopoiesis. In addition to this, it has been linked to cell survival, proliferation, and cancer. STAT5a is normally present in cells in an inactive state. The process of activation of STAT5a is initiated when a cytokine binds to its specific cell surface receptor. This binding results in the activation of one or more members of a family of tyrosine kinases called Janus kinases (JAKs) that are usually associated with the cytokine receptor. The activated JAKs can then activate STAT5a by phosphorylating it on a conserved tyrosine. Tyrosine phosphorylation confers a conformational change and the ability to bind specific target DNA and activate transcription. Thus, STAT5a can act as a direct link between cell surface receptors and gene expression. This entire signal transduction pathway begins once a cytokine binds to its cognate receptor.

Cytokines and Cytokine receptors

Cytokines play critical roles in the regulation of various immunological and non-immunological processes. They are low molecular weight proteins or

glycoproteins that are secreted by various cells of the body in response to stimuli. There are over 100 cytokines that have been characterized and they can be classified in various ways based on their similarity in structure, the receptors that they bind, or the common cellular sources that secrete them. On the basis of structure, cytokines can be classified into four main families as follows (Goldsby, 2000):

1. Hematopoietin family or Class 1 family
2. Interferon family or Class 2 family
3. Tumor necrosis factor (TNF) family
4. Chemokine family

Cytokines are able to regulate physiological processes in an interactive and co-ordinated manner due to their ability to exhibit the properties of pleiotropy, redundancy, synergism, antagonism and cascade induction (Cohen and Cohen, 1996). They bind to specific target cell surface receptors with high affinities, thereby enabling them to function at very low concentrations. Cytokine receptors are membrane bound proteins. Based on structure most cytokine receptors fall under one of the following families (Figure 1):

1. Immunoglobulin receptor superfamily
2. Class 1 cytokine receptor family
3. Class 2 cytokine receptor family
4. TNF receptor family
5. Chemokine receptor family

Most cytokines bind to receptors belonging to class 1 or class 2 families. Class 1 cytokine receptors include the gp130 family, the common γ chain (γc) family, the common β chain (βc) family and the single chain family of receptors. The Class 2 cytokine receptor family include receptors for interferons, IL-10 and IL-10-related cytokines. Both of these families share conserved features. They both have conserved cysteines and most of them are composed of more than one polypeptide chain which includes a cytokine specific recognition subunit and a signal transducing subunit (Bazan, 1990; Langer et al., 2004). Class 1 cytokine receptors possess a conserved

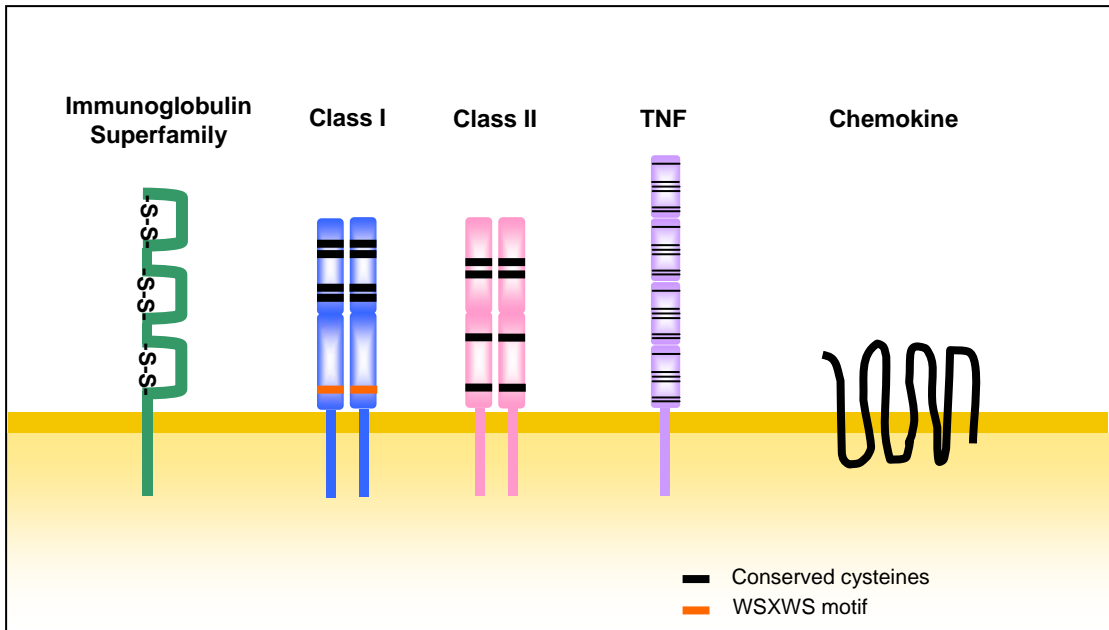


Figure 1: Cytokine receptor families (Goldsby, 2000)
Representation of various cytokine receptor families

WSXWS (Trp-Ser-any amino acid-Trp-Ser) motif that is absent in Class 2 cytokine receptors.

Various cytokines belonging to all four families can induce the tyrosine phosphorylation of STAT5a. Since cytokine receptors do not possess intrinsic tyrosine kinase activity they themselves are unable to phosphorylate STAT5a. One family of cytoplasmic tyrosine kinases, the Janus kinases, is known to associate with cytokine receptors and mediate the phosphorylation of STAT5a.

Janus kinases (JAKs)

Between the late 1980s and early 1990s, a search for protein kinases using PCR based or low stringency hybridization techniques led to the discovery of a novel family of non-receptor tyrosine kinases (Firmbach-Kraft et al., 1990; Wilks, 1989). Analysis of the primary structure of these kinases revealed the presence of two kinase domains. Thus from “Just Another Kinase” these kinases were named after the Roman mythological God Janus and the family of Janus kinases was born (Wilks et al., 1991).

In vertebrates, the family comprises of four members called JAK1, JAK2, JAK3 and TYK2. They all share seven regions of homology named JH1 to JH7 from the carboxyl to the amino terminus (Figure 2). JH1 is the kinase domain while JH2 corresponds to a kinase-like or pseudokinase domain. Although this domain lacks kinase activity, it has been shown to play an important role in regulating the activity of JAKs (Chen et al., 2000; Saharinen and Silvennoinen, 2002; Saharinen et al., 2000). JH3 and JH4 form an SH2-like domain while JH4-JH7 correspond to the FERM (Band 4.1, ezrin, radixin and moesin) homology domain (Al-Lazikani et al., 2001; Girault et al., 1999).

JAKs play critical roles in transducing signals from the cell surface to within the cell. The importance of JAKs in cytokine signaling was shown in mutant cell lines that were defective in the interferon response (Muller et al., 1993; Velazquez et al., 1992; Watling et al., 1993). Complementation of these mutant cell lines with JAKs

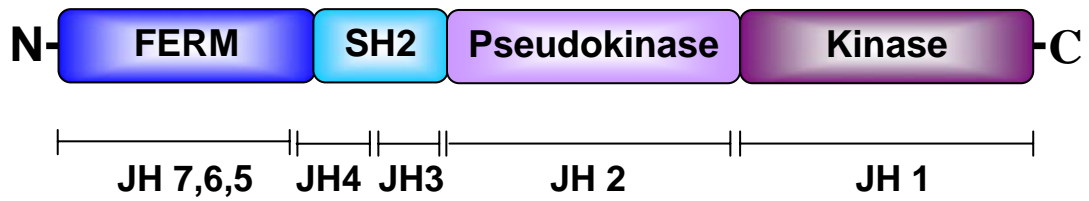


Figure 2: Domain organization of JAKs

JAKs possess an amino terminal FERM domain, an SH2 domain, a pseudokinase domain and a carboxyl kinase domain. JAKs also possess seven regions of homology named JH1-JH7 starting from the carboxyl end to the amino terminal end.

restored their responsiveness to interferons. Now it is known that many cytokines and growth factors can activate the JAKs (Table 1).

Table 1: Activation of different JAKs by cytokines (Yamaoka et al., 2004)

Cytokine receptor	JAK Kinase
γ c containing receptors or similar: IL-2, IL-4, IL-7, IL-9, IL-15, IL-13 etc	JAK1, JAK3
β c containing receptors: IL-3, IL-5, GM-CSF	JAK 2
gp130 containing receptors or similar: IL-6, IL-11, OSM, CNTF, LIF, CT-1, IL-12, G-CSF etc	JAK1, JAK2, TYK2
Homodimeric receptors: GH, prolactin, EPO, TPO	JAK2
IFN receptor: IFN α/β , IFN γ , IL-10	JAK1, JAK2, TYK2

Studies with knock-out mice have reinforced the importance of JAKs in cytokine signaling and immunity (Table 2). JAK1 knock-out mice die perinatally, probably, due to neurological defects that prevent them from suckling. They also display defective lymphoid development and function (Rodig et al., 1998). Mice lacking JAK2 show embryonic lethality due to a defect in erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998). In humans, JAK3 deficiency has been associated with severe combined immunodeficiency (SCID) (Cacalano et al., 1999; Macchi et al., 1995; Russell et al., 1995). JAK3 knock-out mice also display a SCID phenotype but do not have non-immune defects (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995). TYK2 knock-out mice show defective immune responses (Karaghiosoff et al., 2000; Shimoda et al., 2000).

Table 2: Phenotype of mice deficient in JAKs

JAK kinase	KO mice phenotype
JAK1	Perinatal death, neurological defects, block in lymphocyte development, reduced response to IL-6 and LIF, no response to IL-2-4,7,9,13,15, no response to IFNs and IL-10.
JAK2	Embryonic death around day 12, lack of definitive erythropoiesis, no response to IFN-gamma, reduced response to EPO, TPO, IL-3, IL-5.
JAK3	Viable and fertile, SCID phenotype, hypoplastic thymus, peripheral T cells present as activated but non-functional, developmental pro-B to pre-B maturation block, lack of NK cells, Peyer patches, fewer lymph nodes.
TYK2	Viable and fertile, resistant to LPS, susceptible to parasite infections.

JAKs are constitutively associated with the membrane proximal regions of cytokine receptors (Argetsinger et al., 1993; Silvennoinen et al., 1993; Witthuhn et al., 1993). The binding of a cytokine to its cognate receptor results in a conformational change in the receptor which promotes the activation of JAKs through reciprocal interaction between two juxtapositioned JAKs. This enables the JAKs to phosphorylate each other on their activation loops and thereby become fully activated. These activated JAKs can now phosphorylate their substrates on tyrosine residues (Yamaoka et al., 2004; Yeh and Pellegrini, 1999). One of the well characterized substrates of JAKs is a family of transcription factors called Signal Transducers and Activators of Transcription (Darnell et al., 1994).

Signal Transducers and Activators of Transcription (STAT)

In the 1980s, various experiments were performed to gain more insight into the mechanism that led to the induction of various genes following IFN α or IFN γ treatment. Studies showed that the DNA element responsible for the IFN α response

was a 12-15 bp highly conserved region and was termed Interferon Stimulated Response Element (ISRE) (Cohen et al., 1988; Dale et al., 1989b; Kessler et al., 1988; Levy et al., 1988; Reich et al., 1987; Rutherford et al., 1988; Shirayoshi et al., 1988). It was then shown that a factor with the ability to bind DNA was formed minutes after cells were treated with IFN α . This factor was named Interferon Stimulated Gene Factor-3 (ISGF3) (Dale et al., 1989a; Levy et al., 1989; Levy et al., 1988; Porter et al., 1988). Further analysis of this factor revealed the presence of several polypeptides of different masses: 113 kDa, 91 kDa, 84 kDa and 48 kDa (Fu et al., 1990).

The 48 kDa protein was shown to belong to a family of transcription factors called Interferon Regulatory Factors (IRFs) and was named IRF9. IRF9 is also known as p48 and ISGF3 γ . IRFs are a family of transcription factors that play important roles in innate and adaptive immune responses. The family comprises of nine members that are designated as IRF1-9 (Taniguchi et al., 2001). IRF9 is essential for an effective antiviral response by IFN α/β and IFN γ (Bluyssen et al., 1996).

Sequence analysis of the 91 kDa and 84 kDa proteins revealed that they were identical except for the last 38 amino acids which were absent in the 84 kDa protein. The 113 kDa protein was ~ 40% similar to the 91 kDa protein indicating that the two proteins belonged to the same family of proteins. The amino acid sequence of these proteins did not match any previously known proteins and thus led to the discovery of a new family of proteins called Signal Transducer and Activator of Transcription (STAT). The first members of this family were the 91 kDa and the 113 kDa protein and were named STAT1 and STAT2 respectively. Further studies have shown that in addition to STAT1 and STAT2, there are five more members of this family in mammals and they have been named STAT3, STAT4, STAT5a, STAT5b and STAT6 (Azam et al., 1995; Hou et al., 1994; Mui et al., 1995; Wakao et al., 1995; Yamamoto et al., 1994; Zhong et al., 1994).

It was soon demonstrated that STATs are phosphorylated on a single conserved tyrosine residue following IFN α treatment (Schindler et al., 1992). Mutation of this conserved tyrosine residue renders the proteins unable to function as transcription factors. The kinases responsible for the phosphorylation of STATs

belong to the family of JAKs (Darnell et al., 1994). Cytokine binding promotes the activation of JAKs that in turn can phosphorylate the STATs. This signal transduction pathway is commonly referred to as the JAK-STAT signaling pathway.

A large number of cytokines have been shown to activate the JAK-STAT pathway and the mechanism of activation is similar in most cases. Some cytokines can also function as hormones. Hormones are molecules that are usually synthesized by specialized cells of an endocrine gland. The hormone is then secreted into the blood stream where it can mediate its effects on distant parts of the body in an endocrine manner. Cytokines on the other hand can be synthesized by a wide variety of cells and usually exert their effects in an autocrine or paracrine manner. One of the cytokines that also falls in the category of a hormone is growth hormone (GH).

GH is a member of the hematopoietin family of cytokines and the receptor it binds to belongs to the Class 1 cytokine receptor family. The GH signaling pathway can activate the JAK-STAT pathway resulting in the phosphorylation of several STATs that include STAT1, STAT3, STAT5a and STAT5b (Herrington et al., 2000). STAT5a and STAT5b are known to play critical roles in GH signaling and hence GH has been used extensively in my studies to evaluate the localization of phosphorylated STAT5a. The section below describes how GH activates the JAK-STAT signal transduction pathway which results in the tyrosine phosphorylation of STAT5a.

JAK-STAT signaling pathway: Signaling through the growth hormone receptor

Growth hormone is a member of an evolutionary related family of cytokines that include prolactin (Prl) and placental lactogen (PL) (Horseman and Yu-Lee, 1994). It is expressed primarily in the anterior pituitary gland (Li CH, 1945) and is synthesized as a precursor protein with a signal peptide. This peptide is then cleaved to give a 191 aa protein which is 22 kDa. Cleavage of the signal occurs as the hormone is transferred across the rough endoplasmic reticulum into storage granules (Kopchick and Andry, 2000; Strobl and Thomas, 1994). A 20 kDa variant of GH is also synthesized through alternative splicing which generates a deletion of aa residues 32-46 (Baumann, 1991; Lewis et al., 1980). In addition to the pituitary gland, GH is

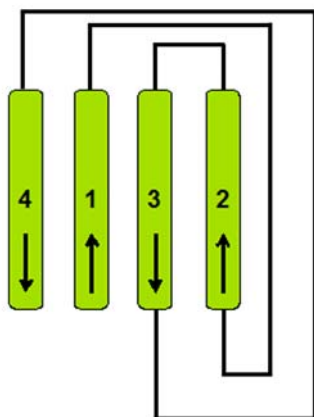
also expressed in human lymphoid cells where it can modulate immune function (Smal et al., 1985).

Since GH is a member of the hematopoietin family of cytokines it shares a structural framework that is commonly observed in this family. All members share a four alpha helix bundle structure (Figure 3). In GH, the 4 alpha helices (21-30 aa) are arranged in a left handed bundle orientation in a unique up-up-down-down topology. There are two disulfide bridges (C35-165 and C182-189 in human GH) which are conserved between GH, Prl and PL (Nicoll et al., 1986). The central core of GH is made up of ~20 hydrophobic aa which aid in keeping the 4-helix bundle stable (Abdel-Meguid et al., 1987; de Vos et al., 1992). Binding of the GH to its receptor is the first event in the signaling pathway.

The GH receptor (GHR) is a member of the Class 1 cytokine receptor family (Bazan, 1990). It is 620 amino acids long and is composed of an extracellular domain (246 aa), a transmembrane region (24 aa) and a cytoplasmic domain (350 aa) (Figure 4). A soluble form of the receptor which is identical to the extracellular domain of the full length receptor is also present and is found circulating in the blood stream. This circulating form of the receptor is called GH Binding Protein (GHBP). GHBP helps in increasing the half life of circulating GH by binding to it and preventing its removal from the blood (Dastot et al., 1996; Ross et al., 1997).

The GHR shares certain features that are characteristic of the Class 1 cytokine receptor superfamily. It possesses a single membrane spanning domain and shows the presence of two fibronectin III modules in the extracellular domain. There are four conserved cysteines (cys) and a conserved tryptophan that is adjacent to the second conserved cys in the N-terminal fibronectin domain. The C-terminal fibronectin domain shows the presence of the WSXWS or equivalent motif that is conserved in all members of the superfamily. In GHR this motif has conservative substitutions and the sequence of the motif in human GHR is YGEFS. This motif has been shown to play important roles in ligand binding. The cytoplasmic domain shows the presence of two conserved motifs named Box 1 and Box 2.

a.



b.

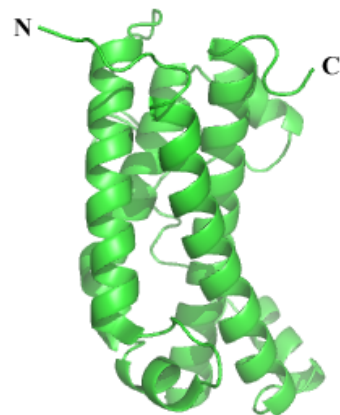


Figure 3: Structure of GH

GH is composed of four α -helices that show a unique up-up-down-down topology (a) and are arranged in a left handed bundled orientation (b) (Abdel-Meguid et al., 1987; de Vos et al., 1992).

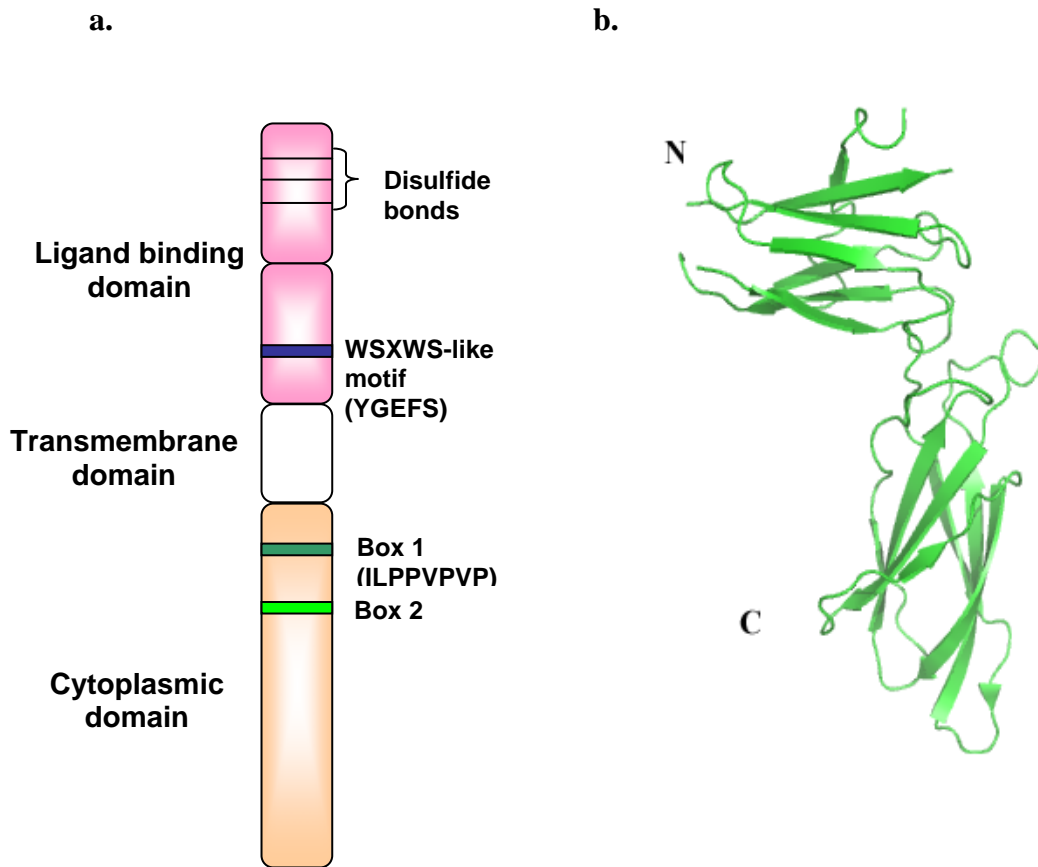


Figure 4: Structure of GH receptor

a. The GHR is composed of a ligand binding domain that has conserved disulfide bonds and a WSXWS-like motif, a transmembrane domain and a cytoplasmic domain that shows the presence of two conserved motifs called Box 1 and Box 2.

b. The structure of the extracellular domain of GHR has been resolved and shows a structure similar to fibronectin repeats (de Vos et al., 1992).

The consensus sequence for Box 1 is Ψ -X-X-X-Al-P-X-P or Al- Ψ -P-X-Al-P-X-P where Ψ stands for any hydrophobic amino acid, X is any amino acid, and Al is any aliphatic amino acid (Kopchick and Andry, 2000). In GHR the sequence of this motif is ILPPVPVP. Box 2 begins with a cluster of hydrophobic amino acids and ends with 1 or 2 positively charged amino acids. In GHR, Box 2 is located ~30 amino acids carboxy terminal to Box 1 and spans about 15 aa. Mutation or deletion of Box 1 and/or Box 2 in GHR results in defective receptor-mediated signal transduction (Argetsinger and Carter-Su, 1996; Carter-Su et al., 1996; Kopchick and Andry, 2000).

Based on crystallography, size exclusion chromatography, calorimetry, and fluorescence quenching experiments using solubilized extracellular domain of GHR, it is known that one GH molecule is complexed with two GHR molecules (Figure 5) (de Vos et al., 1992). Studies have shown that GH possesses two receptor-binding sites. Site 1 is a high affinity binding site that is comprised of residues on helix 1, short segments in the loop before helix 2 and residues on helix 4 (24 residues) of GH. On the other hand, site 2 is a lower affinity binding site which in turn is composed of residues in the N-terminal region, the beginning of helix 1 and residues on helix 3 (~13 amino acids) (Wells and de Vos, 1993). These sites sequentially interact with binding pockets in two discrete receptor molecules. Thus GH binds to one GHR molecule through the high affinity site 1 and can then bind to a second GHR molecule via site 2. This promotes receptor dimerization and subsequent signal transduction. Experiments have determined that GH can still bind to the receptor even if it lacks site 2 but binding is completely abolished if site 1 is rendered non-functional (Cunningham et al., 1991; Fuh et al., 1992).

It is now known that receptor dimerization is a common method of signal transduction in cytokine signaling (Imada and Leonard, 2000; Kishimoto et al., 1994). In case of the GHR, dimerization of the receptors causes the JAKs to be spatially positioned such that it results in the transphosphorylation and catalytic activation of the kinase domain (Figure 6). JAK2 is the primary Janus kinase that is associated with GHR and binds to the Box 1 region of the receptor (Argetsinger et al., 1993). Mutation or deletion of this region results in the abrogation of JAK activation and



Figure 5: Structure of GH complexed with GHR

One GH molecule (magenta) can bind to two GH receptor molecules (green)
(de Vos et al., 1992).

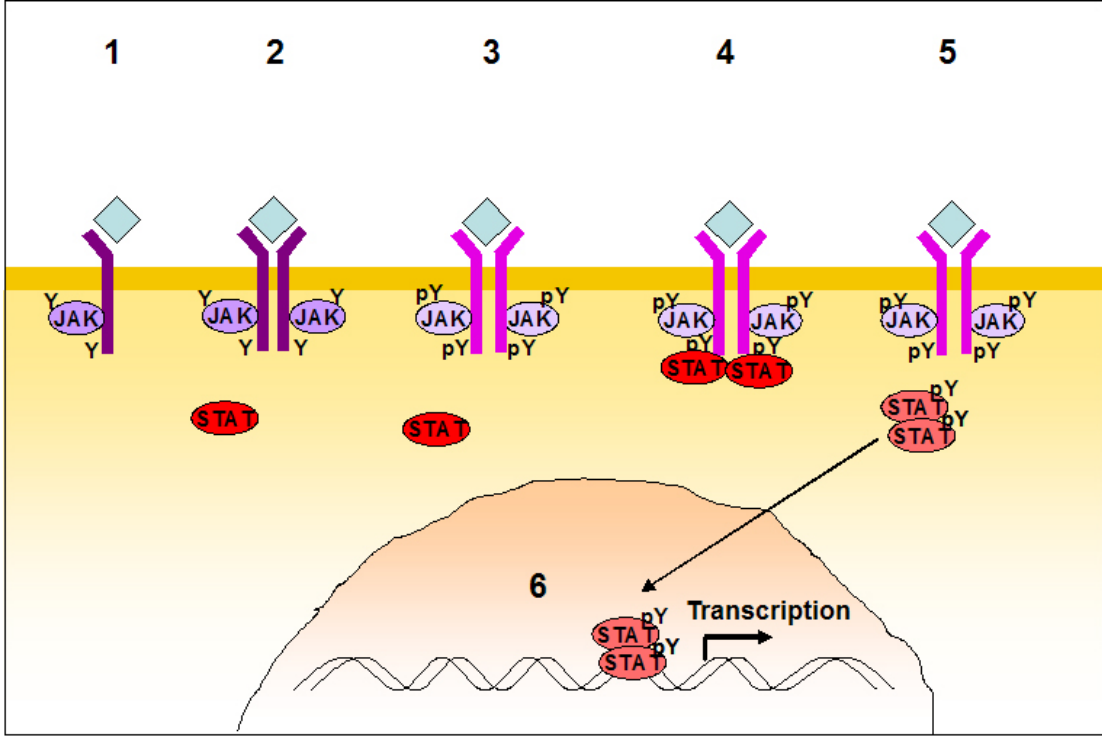


Figure 6: JAK-STAT signaling pathway via GH

During GH signaling, one GH molecule binds to the extracellular domain of one GH receptor molecule that interacts with JAKs through its cytoplasmic domain (1). Binding of GH to the receptor promotes receptor dimerization (2) that results in the activation of JAKs. The activated JAKs phosphorylate the receptor on tyrosine residues (3) which form docking sites for STATs (4). The STATs bind to the phosphotyrosine residues through their SH2 domains and are phosphorylated by JAKs (5). The phosphorylated STATs translocate into the nucleus where they can bind to DNA and drive the transcription of target genes (6).

downstream signaling (Dinerstein et al., 1995; Tanner et al., 1995). The activated JAKs phosphorylate the receptor on tyrosine residues which creates docking sites for molecules that possess an SH2 domain. STATs are one such class of molecules. By binding to phosphorylated tyrosines on the receptor or JAKs through their SH2 domains, the STATs are brought to closer proximity of the JAKs, enabling the JAKs to phosphorylate STATs. The GH signaling pathway results in the phosphorylation of several STATs that include STAT1, STAT3, STAT5a and STAT5b (Herrington et al., 2000).

STATs: Evolution and structure

Even though STATs were first discovered in mammalian systems, they have also been found in lower organisms. The slime mold *Dictyostelium discoideum* which is a facultative organism that exists as a single-cell amoeba but forms a multi-cellular organism upon starvation has three STATs (Dd-STATa, Dd-STATb and Dd-STATc) which play important roles in growth, development and stress responses (Araki et al., 2003; Kawata et al., 1997; Zhukovskaya et al., 2004). STATs have also been found in insects like the fruit fly (Hou et al., 1996) and mosquitoes (Barillas-Mury et al., 1999; Lin et al., 2004), and in the nematode *C. elegans* (Liu et al., 1999; Wang and Levy, 2006a; Wang and Levy, 2006b). D-STAT or the STAT found in *Drosophila melanogaster* plays important roles in various physiological processes like embryonic segmentation, larval hematopoiesis, tracheal development etc., while the mosquito STATs play a role in the immune responses. The STAT found in *C. elegans* regulates larval development.

Since STATs are found in organisms across the animal kingdom, it is hypothesized that STATs have evolved from an ancient gene that underwent various duplication events in the course of evolution to finally give rise to seven STATs in mammals (Barillas-Mury et al., 1999; Copeland et al., 1995). It is believed that the primordial STAT gene was first duplicated by tandem gene duplication. This was then followed by two additional whole genome duplication events. The final duplication event was probably a tandem duplication event that gave rise to the STAT

members STAT5a and STAT5b. Based on this model and by performing sequence alignments, the mammalian STATs can be distributed into two groups. STATs 1, 2, 3 and 4 fall in one group while STATs 5a, 5b and 6 belong to the second group. This model is supported by the fact that the genes encoding mammalian STATs are found clustered in the genome. In humans, STAT1 and STAT4 are located on chromosome 2, STAT2 and STAT6 are found on chromosome 12, and STAT3, STAT5a and STAT5b are present on chromosome 17. A similar feature of clustering is observed in mice as well (Barillas-Mury et al., 1999; Copeland et al., 1995).

All STATs, whether in vertebrates or invertebrates, are activated by phosphorylation on a conserved tyrosine residue and share a common domain organization (Figure 7). They possess an N-terminal region followed by a coiled coil domain that mediates protein-protein interactions (Xu et al., 1996). A DNA binding domain follows the coiled coil domain and this domain enables the STATs to bind specific DNA elements in the promoters of target genes. They all possess a Src Homology 2 (SH2) domain that can interact with phosphotyrosine residues present on JAKs, cell surface receptors or other STAT molecules. In the latter case, the interaction results in the formation of a STAT dimer that has the ability to bind DNA. At the carboxyl terminal end, STATs possess a transactivation domain that can interact with transcriptional coactivators like CBP/p300 and thereby increase the efficiency of transcription of target genes. This domain organization is conserved in all mammalian STATs while some invertebrate STATs lack the N-terminal region.

It was initially believed that inactive unphosphorylated STATs exist as monomers and following activation the tyrosine phosphorylated STATs form dimers with the ability to bind DNA. However evidence from techniques like size exclusion chromatography, sedimentation equilibrium analysis and X-ray crystallography show that STATs can exist as dimers even in the absence of phosphorylation (Braunstein et al., 2003; Mao et al., 2005; Neculai et al., 2005; Stancato et al., 1996). The crystal structures of the core fragment (includes the coiled coil domain, DNA binding domain, SH2 domain and conserved tyrosine) of unphosphorylated STAT1 and



Figure 7: Domain organization of STATs

STATs share a common domain organization. They possess an amino terminal domain (N-term), followed by a coiled coil domain (CC), a DNA binding domain (DBD), an SH2 domain (SH2), a linker bearing the conserved tyrosine that is phosphorylated (Y) and a transcriptional activation domain (TAD).

STAT5a has been resolved and in both instances the STATs are dimeric and have a similar structure (Figure 8).

Following tyrosine phosphorylation, there are no major changes in the structure of STATs but there are extensive changes in the arrangement of the monomers relative to each other (Figure 9) (Becker et al., 1998; Chen et al., 1998; Soler-Lopez et al., 2004). The structures of phosphorylated STAT molecules have been solved in the absence and presence of DNA. Surprisingly, the arrangement of the monomers of phosphorylated STAT unbound to DNA was different from the STAT bound to DNA. The crystal structure of phosphorylated *Dictyostelium* STAT unbound to DNA revealed that the monomers are present in an open extended conformation which is different from phosphorylated STAT1 and STAT3 bound to DNA (Soler-Lopez et al., 2004). The monomers in phosphorylated STAT1 and STAT3 are arranged in a closed conformation in a manner that appears as if they are embracing the DNA (Becker et al., 1998; Chen et al., 1998). Thus, the STAT dimers undergo significant conformational changes depending on their phosphorylation status.

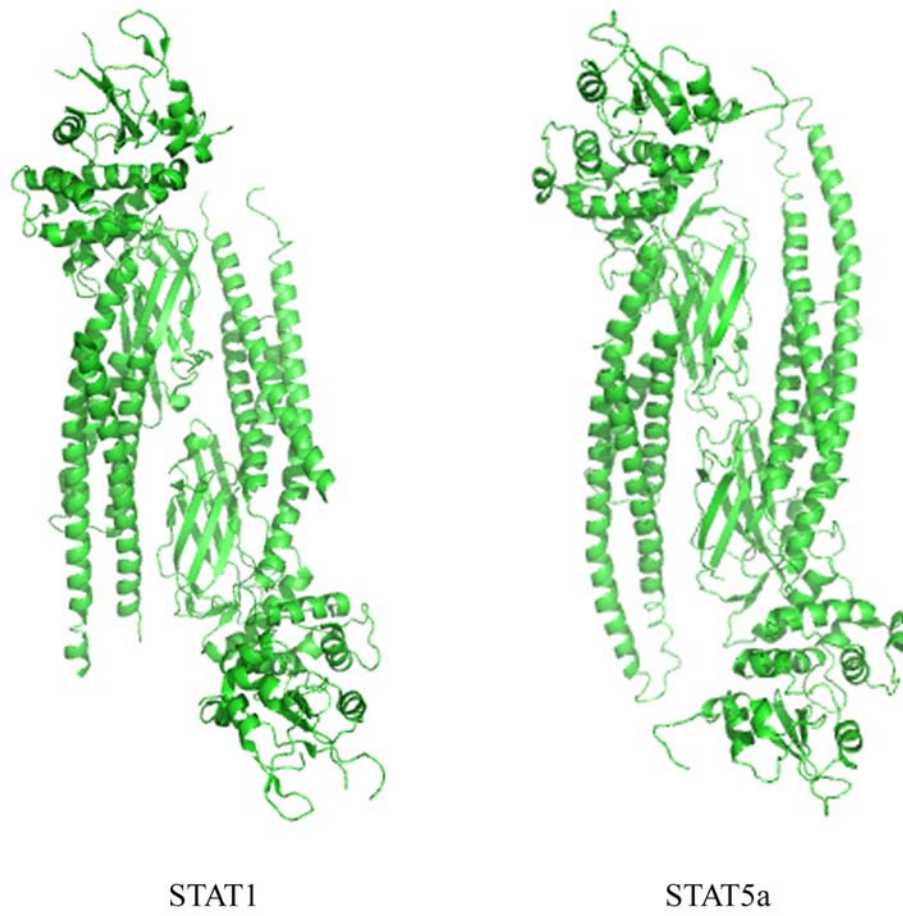


Figure 8: Structure of unphosphorylated STAT dimers

Structure of unphosphorylated STAT1 (left) and STAT5a (right) (Mao et al., 2005; Neculai et al., 2005).

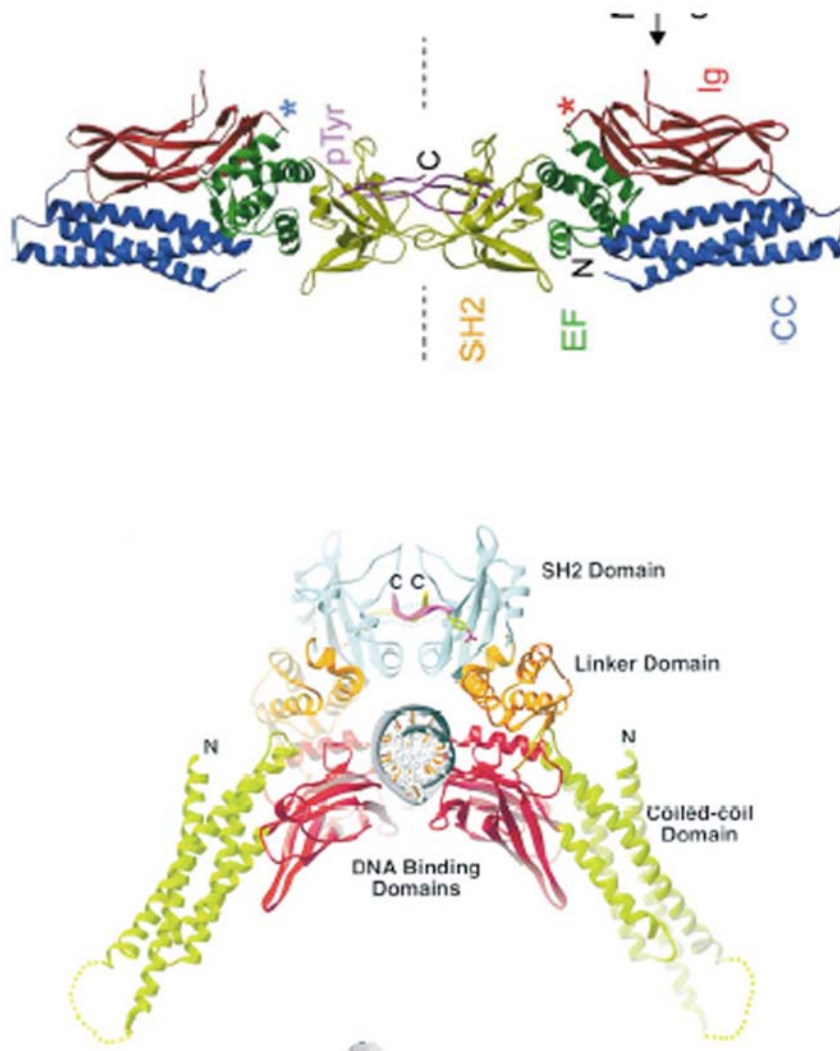


Figure 9: Structure of phosphorylated STATs

Structure of phosphorylated STAT dimers (Chen et al., 1998; Soler-Lopez et al., 2004). The top structure represents phosphorylated *Dictyostelium* STAT unbound to DNA while the bottom structure represents phosphorylated STAT1 bound to DNA.

Biological functions of STATs in mammals

The seven mammalian STATs have similar domain structure and similar modes of activation. This might suggest that they have similar biological functions. However, studies using knock out mice have revealed that individual STATs can mediate distinct physiological processes (Akira, 1999; Ihle, 2001) (Table 3).

Table 3: Phenotype of mice deficient in individual STATs (Paukku and Silvennoinen, 2004)

Targeted STAT	Phenotype of KO mice
STAT1	Susceptible to viral infections and tumors, defective interferon response
STAT2	Defective interferon response
STAT3	Embryonic lethal
STAT4	Abrogation of IL-12 signaling and impaired T _H 1 cell differentiation
STAT5a	Defective mammary gland development during pregnancy, no lactation in females
STAT5b	Defect in growth hormone signaling
STAT5a and STAT5b	Perinatal death of mice, severe anemia in survivors, reduction in growth, defective T-cell proliferation
STAT6	Impaired IL-4 and IL-13 signaling, defective T _H 2 cell differentiation

STAT1 is activated by a number of cytokines like IFNs, GH, thrombopoietin, IL-6 and IL-10 while STAT2 is predominantly activated by IFN α (Table 4). Deletion of STAT1 or STAT2 results in mice that develop normally and are viable; however they show severe defects in IFN-dependent immune responses against viruses and pathogenic micro-organisms (Durbin et al., 1996; Meraz et al., 1996; Park et al.,

2000). This phenotype reinforces the important roles played by these STATs in IFN signaling pathways.

STAT3 is activated by a range of cytokines and hormones (Table 4). Deletion of STAT3 in mice is embryonic lethal and the embryos die prior to gastrulation. It is believed that this embryonic lethality is due to the inability of the extraembryonic cells to initiate implantation or provide the necessary nutritional support required for viability (Takeda et al., 1997). Tissue specific deletion of STAT3 has revealed critical roles played by STAT3 in IL-2, IL-6 and IL-10 signaling pathways (Levy and Lee, 2002).

STAT4 is primarily activated in response to IL-12 and hence it comes as no surprise that the phenotype of STAT4 deficient mice is similar to mice lacking IL-12. IL-12 is a macrophage derived cytokine that mediates the differentiation of naïve T-cells to T helper cells 1 (T_H1). The T-cells of STAT4 null mice are unresponsive to IL-12 and show severely impaired T_H1 cell development (Kaplan et al., 1996; Thierfelder et al., 1996).

The principal cytokines that activate STAT6 are IL-4 and IL-13. Thus it is expected that STAT6 knock out mice would lack many of the physiological functions that have been associated with IL-4, which indeed is the case. The mice show impairment in T-cell development toward T_H2 cells and B-cells have a defect in class switching resulting in the inability to produce IgE (Shimoda et al., 1996; Takeda et al., 1996).

STAT5a and STAT5b are activated by numerous cytokines and hormones (Table 4). Both proteins share ~94% identity and differ most significantly at the carboxyl terminus. Due to the similarity between these two proteins we would expect mice lacking either of the proteins to display a similar phenotype. Mice deficient in STAT5a or STAT5b or both have been generated and interestingly they all display different phenotypes. STAT5a knock out mice develop normally and are fertile. However due to impaired mammary gland development during pregnancy, the mice are unable to nurse their offsprings. Macrophages derived from the bone marrow of these mice exhibit defective GM-CSF induced gene expression and proliferation

while T-cells showed a defect in IL-2 induced proliferation. The mice also exhibited defective natural killer (NK) cell development (Feldman et al., 1997; Liu et al., 1997; Nakajima et al., 1997). STAT5b knock out mice also developed normally but displayed defective growth hormone signaling that resulted in the loss of sexually dimorphic growth. The mice also exhibited a defect in IL-2 induced proliferation and NK cell development (Imada et al., 1998; Udy et al., 1997).

Table 4: Activation of STATs by cytokines, growth factors or G-proteins; adapted from (Imada and Leonard, 2000; Paukku and Silvennoinen, 2004)

Ligand	Activated STATs
Cytokines	
β c family: IL-3, IL-5, GM-CSF	1, 3, 5, 6
gp130 family: CNTF, CT-1, G-CSF, IL-6, IL-11, IL-12, IL-23, OSM etc.	1, 3, 4, 5
γ c family: IL-2, IL-4, IL-7, IL-9, IL-15, GH, Epo, Prl, Tpo	1, 3, 5, 6
IFN family: IFN α / β , IFN γ , IL-10 etc.	1, 3, 5
	1, 2, 3, 4, 5, 6
Growth factors	
EGF, PDGF, Insulin, VEGF etc	1, 3, 5, 6
G proteins	
Angiotensin II, Follicle stimulating hormone, Serotonin	1, 2, 3, 5

Mice deficient in both STAT5a and STAT5b were generated and the phenotype observed was more severe than the individual knock outs (Cui et al., 2004; Moriggl et al., 1999; Teglund et al., 1998). Most of the mice died perinatally due to severe anemia and other physiological defects that are not known. The mice that did manage to survive were weak and had only one-third the body weight of wild type counterparts. In another study STAT5a/b knock out mice were generated in such a way that the mice produced defective STAT5 proteins (Teglund et al., 1998). These mice were viable but females were sterile. The growth of the mice was reduced and they showed defects in T-cell proliferation. The embryos of the double knock out mice showed increased numbers of apoptotic erythroid lineage cells in the fetal liver and were anemic. By comparing the phenotypes of the double knock out mice with

their single knock out counterparts, it is evident that STAT5a and STAT5b proteins are able to functionally complement each other to a certain extent.

The two fraternal twins: STAT5a and STAT5b

STAT5 was first discovered in ovine mammary gland tissues as a prolactin-stimulated transcription factor and was initially named mammary gland factor (Wakao et al., 1995). Later studies using PCR techniques revealed the presence of two closely related proteins that displayed ~94% identity at the amino acid level. These two proteins were then designated STAT5a and STAT5b (Azam et al., 1995; Mui et al., 1995). The genes encoding these proteins are localized in the same chromosome (chromosome 17 in humans) and it is believed that there was a duplication of the STAT5 progenitor gene that gave rise to these two genes.

Like all STAT members, STAT5a and STAT5b are activated by tyrosine phosphorylation on a conserved tyrosine residue (Y694 in STAT5a and Y699 in STAT5b). Following tyrosine phosphorylation, the proteins gain the ability to bind DNA and transcribe target genes. Studies have revealed that STAT5 can recognize and bind to canonical interferon- γ activated sites (GAS). GAS sites are distinct DNA regulatory sequences that were first identified in the promoters of IFN γ inducible genes and hence were given the name interferon- γ activated site (Lew et al., 1989). They are characterized by the inverted repeat of GAA sequence. STAT5a and STAT5b bind to GAS sites that have the general sequence TTCNNGAA (Grimley et al., 1999).

STAT5a and STAT5b have been shown to regulate the expression of many genes like β -casein, α 2-macroglobulin, cyclin D1, Bcl-x, oncostatin M, etc (Grimley et al., 1999). They can bind similar as well as distinct enhancer elements in the promoters of response genes. For example, both STAT5a and STAT5b can bind a DNA element present in the β -casein gene promoter, but STAT5b can bind to a DNA target in β -macroglobulin gene more efficiently than STAT5a (Boucheron et al., 1998). Thus this difference in DNA binding enables STAT5a and STAT5b to regulate the expression of distinct target genes.

Unlike some STATs, STAT5a and STAT5b are activated by many stimuli (Figure 10) (Grimley et al., 1999). Cytokines belonging to Class 1 and Class 2 families can phosphorylate STAT5a and STAT5b. As mentioned before, these cytokine receptors do not possess intrinsic kinase activity and hence the STATs are phosphorylated by JAKs that are associated with the cytokine receptors. Cytokines like TNF, angiotensin II and follicle stimulating hormone can also phosphorylate these STATs through the JAKs (Guo et al., 1998; McWhinney et al., 1998). The receptors for angiotensin II and follicle stimulating hormone are seven-pass transmembrane receptors that are G-protein coupled (Ali et al., 1997). STAT5a and STAT5b can also be phosphorylated by growth factors that bind to receptors that possess intrinsic tyrosine kinase activity. These include epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Chen et al., 1997; David et al., 1996; Valgeirsdottir et al., 1998). These receptor tyrosine kinases can phosphorylate the STATs independent of JAKs. There have been reports that some non-receptor tyrosine kinases like the Src or Tec family of tyrosine kinases, and oncogenic tyrosine kinases like TEL-JAK, BCR-Abl etc., can also activate STAT5a and STAT5b (Chai et al., 1997; de Groot et al., 1999; Welte et al., 1999; Yu et al., 1997). Thus STAT5a can be activated by a variety of cytokines and kinases.

STAT5a and Cancer

Cell division is a tightly regulated process. Cells can progress through the cell cycle only when conditions are conducive for cell division. Usually, environmental stimuli trigger the expression of mitogenic factors that signal cells to progress through the cell cycle. In some instances cells can behave aberrantly in which case they are programmed to undergo cell death. Cancer results due to a breakdown in the regulation of the cell cycle which leads to uncontrolled and unregulated cell division. Cancer cells gain the ability to proliferate in the absence of mitogenic signals and are able to circumvent cell death.

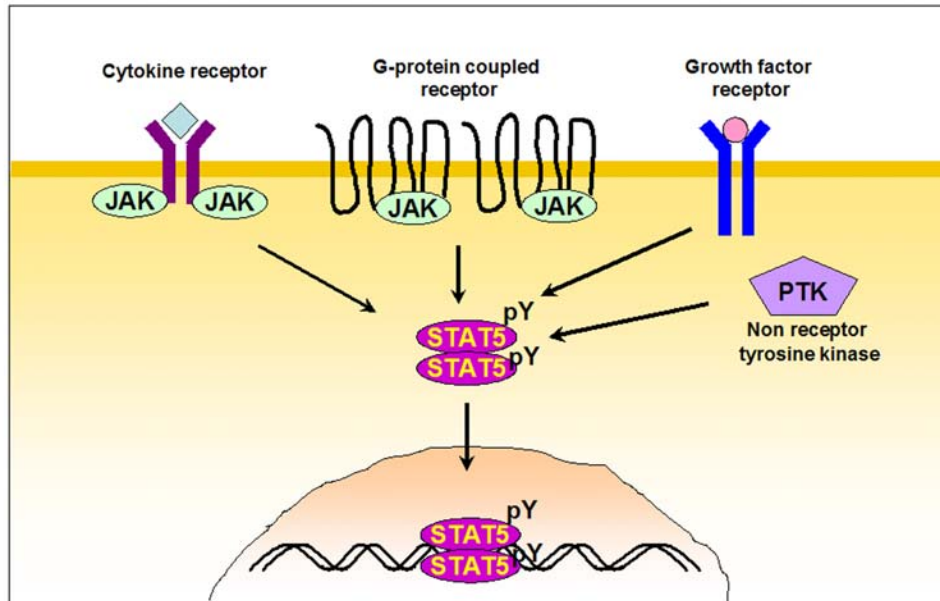


Figure 10: Mechanisms of STAT5 activation

STAT5 can be phosphorylated in many ways. Cytokine receptors can phosphorylate STAT5 through JAKs that are constitutively associated with the receptor. Binding of cytokine to its receptor activates JAKs which in turn phosphorylates STAT5. STAT5 can also be phosphorylated by JAKs that are associated with G-protein coupled receptors. Growth factor receptors that possess intrinsic kinase activity phosphorylate STAT5 following ligand binding. Some non-receptor tyrosine kinases can also phosphorylate STAT5.

One of the main classes of protein that play critical roles in cell survival, proliferation and differentiation are tyrosine kinases. The kinase activity of tyrosine kinases is regulated in cells but in many cancers, tyrosine kinases are frequently found to be constitutively active. Since some STATs like STAT1, STAT3, STAT5a and STAT5b are activated by a variety of kinases, they are also found to be constitutively active in many cancers (Table 5). Among the STATs, STAT3 is most commonly associated with tumorigenesis while STAT5 takes second place (Yu and Jove, 2004).

Numerous studies have associated STAT5a with breast cancer. STAT5a was shown to be phosphorylated and display a nuclear localization in many human breast cancer tissues (Cotarla et al., 2004). Another report involving rat mammary gland adenocarcinomas revealed that the carcinomas showing increased STAT5a nuclear accumulation were more likely to be highly proliferative (Shan et al., 2004). Mouse models have been developed that show that reduction in levels of STAT5a delayed mammary cancer progression (Ren et al., 2002).

The transcriptional activity of STAT5a can be inhibited by dominant negative mutants of STAT5a (Moriggl et al., 1996; Wang et al., 1996). One such mutant was generated by deleting a portion of the transactivation domain of STAT5a. This dominant negative mutant can be tyrosine-phosphorylated, bind to DNA and dimerize with wild type STAT5a. However such a dimer cannot mediate the transcription of target genes. Thus it can intervene with the transcriptional activity of endogenous STAT5. Inhibition of STAT5 activity by using such dominant negative mutants of STAT5a induced apoptosis of T47D (human ductal breast epithelial tumor) cells and T47D-induced tumors in nude mice (Yamashita et al., 2003; Yamashita et al., 2004).

STAT5a has also been implicated in leukemias like chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML). Studies have shown that STAT5a is necessary for the efficient induction and maintenance of CML in mice (Ye et al., 2006) and disruption of STAT5 activity resulted in decreased proliferative potential and enhanced apoptosis of blast cells obtained from patients suffering from CML and AML (Baskiewicz-Masiuk and Machalinski, 2004). STAT5a is also an

essential mediator of proliferation in the T cell acute lymphoblastic leukemia caused by the TEL-JAK2 chromosomal translocation (Lacronique et al., 2000; Schwaller et al., 2000).

Table 5: STATs that are found activated in tumors; adapted from (Yu and Jove, 2004)

Liquid tumors	Activated STAT
Acute lymphoblastic leukemia	1, 3, 5
Acute myelogenous leukemia (AML)	1, 3, 5
Anaplastic large cell lymphoma	3
Burkitt's lymphoma	3
Chronic myelogenous leukemia (CML)	5
Cutaneous T-cell lymphoma	3
Erythroleukemia	1, 5
Hodgkins lymphoma	3
HTLV-1 dependent leukemia	3, 5
Non-Hodgkins lymphoma	3

Solid tumors	Activated STATs
Brain tumors	1,3
Breast cancer	1,3,5
Head and neck cancer	1,3,5
Hepatocellular carcinoma	3
Lung cancer	1,3
Ovarian cancer	3
Pancreatic cancer	3
Prostate cancer	3

In order for STAT5a to transcribe its target genes it must be transported into the nucleus. Nuclear transport of macromolecules like proteins and RNA is a complex process that involves many players. Depending on the size of the macromolecule, the mechanism of transport can be by passive diffusion or energy-dependent active transport. The following section describes the basic aspects of nuclear transport of proteins.

Nuclear transport

The nucleus is separated from the cytoplasm by the nuclear envelope but maintains communication with the cytosol via the nuclear pore complexes (NPCs). Hence the transport of molecules across the nuclear membrane occurs through the NPC. The NPC is a massive macromolecular assembly with eight fold symmetry and can be divided into three basic elements: the cytoplasmic ring with filaments extending from it, a central core and a nuclear ring that culminates into a basket (Figure 11). The cytoplasmic and nuclear rings are connected to the central core by vertical spoke elements. Eight filaments of 2-3 nm in diameter and ~ 50 nm in length emanate from the cytoplasmic ring into the cytoplasm. Similarly, eight filaments extend from the nuclear ring and join at a smaller ring structure that forms the nuclear basket (Adam, 2001; Fahrenkrog and Aebi, 2003; Stoffler et al., 2003).

The NPC is made up of ~30 proteins that have been named nucleoporins (Nups) which are present in multiple copies to yield ~500-1000 molecules per NPC (Figure 10). Some of the nucleoporins play a role in maintaining the structural integrity of the NPC while others play a role in the transport of molecules across the NPC. The Nups that mediate nuclear transport show the presence of multiple phenylalanine-glycine (FG) dipeptidyl repeats. These FG repeats play important roles in mediating the transport of molecules across the NPC (Fahrenkrog, 2006; Lim and Fahrenkrog, 2006; Stoffler et al., 2006).

Any molecule smaller than 9 nm or ~50 kDa can freely pass through the NPC by passive diffusion but larger molecules have to be actively transported into the nucleus. This process is mediated by nuclear shuttling receptors belonging to 3 main classes:

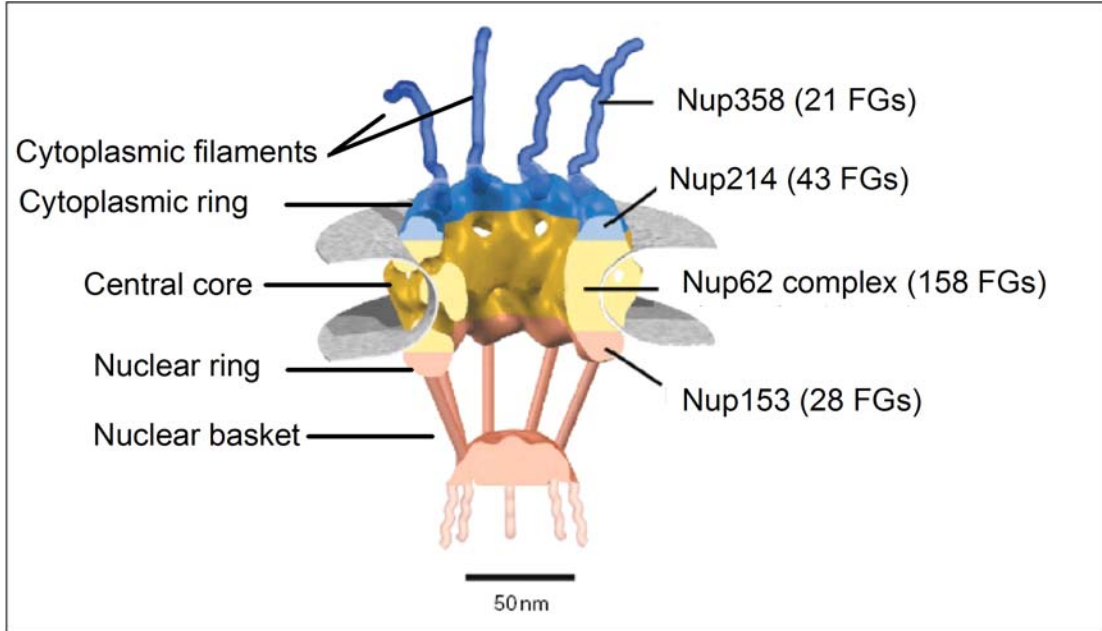


Figure 11: Composition of nuclear pore complex

The NPC is composed of a cytoplasmic ring with filaments extending from it, a central core and a nuclear ring that culminates into a basket (Fahrenkrog and Aebi, 2003). The NPC is made of nucleoporins (Nups) that play important roles in nuclear transport (Fahrenkrog et al., 2004).

the karyopherin- β family that mediate the nuclear import and export of many proteins, small homodimeric nuclear transport factor 2 (NTF2) which mediates the import of the small GTPase Ras-like protein Ran into the nucleus, and Tap/Mex67 family that mediates RNA transport (Fried and Kutay, 2003; Weis, 2002).

The mechanism of nuclear transport of proteins mediated by the karyopherin- β family is best studied. The members of the karyopherin- β family are also referred to as importins and exportins and this nomenclature will be used in this dissertation. As the name suggests, importins mediate the nuclear import of macromolecules while exportins mediate the export of macromolecules. The direction of transport is regulated by the small Ras-related protein, Ran. There is an asymmetrical distribution of the Ran-GTP and Ran-GDP bound forms across the nuclear membrane. This is due to the presence of the Ran guanine nucleotide exchange factor (RanGEF) in the nucleus and Ran GTPase-activating protein (RanGAP) in the cytoplasm which results in a high concentration of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm. This gradient of Ran is critical for the import and export of proteins and other molecules across the nuclear membrane.

a. Nuclear import:

Proteins that have to be imported into the nucleus need to possess a signal that can be recognized by importins or other transport molecules. This signal is usually a sequence of amino acids and is designated as a nuclear localization signal (NLS). The classical NLSs contain either a single or monopartite stretch of basic amino acids (e.g., SV40 NLS-PKKKRRV) or a bipartite sequence of basic amino acids (e.g., Nucleoplasmin NLS-KRPAATKKAGQAKKKK) (Gorlich and Mattaj, 1996; Lange et al., 2007). The basic residues in these NLSs play critical roles in nuclear import as mutating them inhibits nuclear import.

The NLS in proteins are usually recognized by members of the importin- α family of shuttling receptors. The importin- α proteins share similar domain organization (Figure 12). The amino terminal region is comprised of a domain termed as imporin- β 1 binding domain (I β B). This domain, as the name suggests, interacts with another shuttling receptor, importin- β 1. The central region comprises of ten

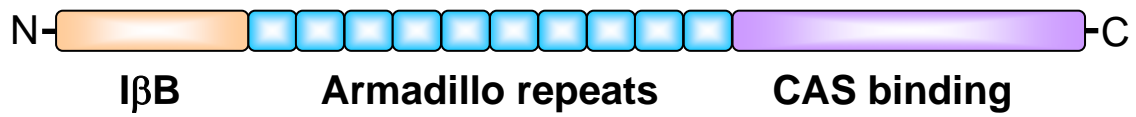


Figure 12: Domain organization and structure of importin- α

Above: Importin- α s are composed of an importin- β binding domain (I β B), Armadillo (Arm) repeats and a CAS binding domain.

Below: The structure of importin- α (shown in green) is mainly α -helical. The SV40 nuclear localization signal (shown in red) binds to the Arm repeats of importin- α .

repeat motifs called Armadillo (Arm) repeats. Classical NLSs interact with this region of importin- α . Structural studies have revealed that each armadillo repeat is composed of three α -helices that form a cylindrical superhelix. The basic amino acids on classical NLSs can bind to the grooves formed by Arm2-4 and Arm7-8. The carboxyl terminal region of importin- α interacts with the protein CAS (Cellular Apoptosis Susceptibility) that mediates the nuclear export of importin- α s (Chook and Blobel, 2001). The nuclear export of importin- α s is necessary so that they can carry out successive rounds of nuclear import.

In the classical nuclear import pathway, basic NLSs present on cargos (proteins) are recognized directly by the importin- α molecules (Figure 13). Importin- α s bind to the importin- β 1 and this complex along with the cargo is competent to translocate into the nucleus. Importin- β 1 interacts with the FG repeats of the nucleoporins in the NPC resulting in the import of the whole complex into the nucleus. Inside the nucleus, Ran-GTP binds to importin- β 1 causing the dissociation of the importins from the cargo. The importins are then exported into the cytoplasm where they can begin another cycle of nuclear import.

b. Nuclear export:

Nuclear export of proteins is mediated by exportins. Similar to nuclear import, proteins that need to be exported must possess a nuclear export signal (NES) which can be recognized by the shuttling receptors, exportins. The most well characterized exportin is the protein CRM1 (Chromosome Region Maintenance 1). NESs recognized by CRM1 contain a stretch of hydrophobic residues, especially leucines. The consensus NES can be denoted as LxxxLxxLxL, where L stands for leucine and x for any amino acid (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997).

Figure 13 depicts the classic nuclear export pathway which involves CRM1, Ran-GTP and the protein that will be exported (Askjaer et al., 1998). CRM1 binds to NES sequences of the cargo and this complex along with Ran-GTP can interact with the nucleoporins and mediate the export of the complex out of the nucleus. In the cytoplasm, Ran-GTP is rapidly converted to Ran-GDP due to the presence of

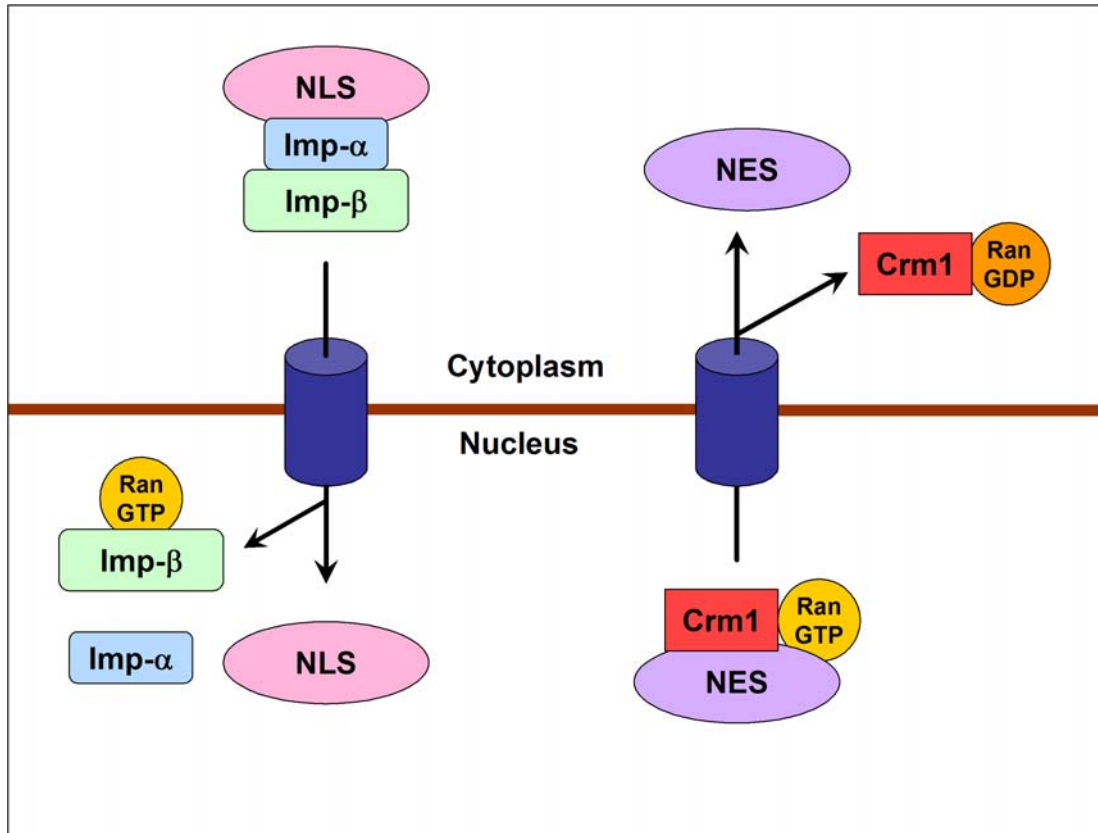


Figure 13: Nuclear trafficking of proteins across the NPC

Left: Proteins that possess an NLS are targeted for nuclear import. The NLS is recognized by importin- α that interacts with importin- β . Importin- β can directly interact with nucleoporins of the NPC and mediate the import of the complex into the nucleus. Once in the nucleus, importin- β interacts with the small GTPase protein Ran bound to GTP which results in the dissociation of the complex and release of cargo.

Right: Proteins bearing an NES can be exported out of the nucleus. The NES is recognized by Crm1 which along with Ran-GTP forms a trimeric complex. This complex is exported out of the nucleus through the NPC. In the cytoplasm, Ran-GTP is converted to Ran-GDP which triggers the dissociation of the complex and release of cargo.

RanGAP in the cytoplasm. This nucleotide conversion of Ran leads to the dissociation of the complex and thus the completion of nuclear export.

The export activity of CRM1 can be inhibited by an anti-fungal antibiotic synthesized by the bacteria *Streptomyces species*. This drug, designated as Leptomycin B (LMB), can interact with CRM1 and covalently modify a critical cysteine residue thereby preventing CRM1 from functioning as an exportin (Kudo et al., 1999; Kudo et al., 1998; Nishi et al., 1994). The effect of LMB on CRM1 is irreversible and has aided in the better understanding of the process of CRM1-mediated nuclear export. The process of nuclear import and export is summarized in Figure 13.

In order for STATs to fulfill their role as transcription factors they must translocate from the cytoplasm to the nucleus where they can regulate gene expression. The molecular mass of STATs varies from 90 kDa to 130 kDa. Thus they are above the threshold of passive diffusion which would suggest that their transport into the nucleus would be energy dependent. The cellular trafficking of some of the STAT members has been explored. Due to their similarity in domain arrangement and structure, it would be expected that they all show similar nuclear trafficking patterns. However, various studies on different STATs have demonstrated otherwise (Reich and Liu, 2006).

STAT1 cellular trafficking

STAT1 was the first STAT whose intracellular trafficking was studied (McBride and Reich, 2003; Reich, 2007). Latent STAT1 was found to reside in the cytoplasm of cells (Figure 14). This cytoplasmic localization might be due to the inability of latent STAT1 to be imported into the nucleus or due to a faster rate of nuclear export. On treating cells with the CRM1 exportin inhibitor LMB, STAT1 remained cytoplasmic suggesting that its exclusion from the nucleus was probably due to a lack of nuclear import. Following cytokine stimulation, STAT1 rapidly accumulated in the nucleus (McBride et al., 2002; Sekimoto et al., 1996). Cytokine stimulation results in the phosphorylation of STAT1 that confers a conformational

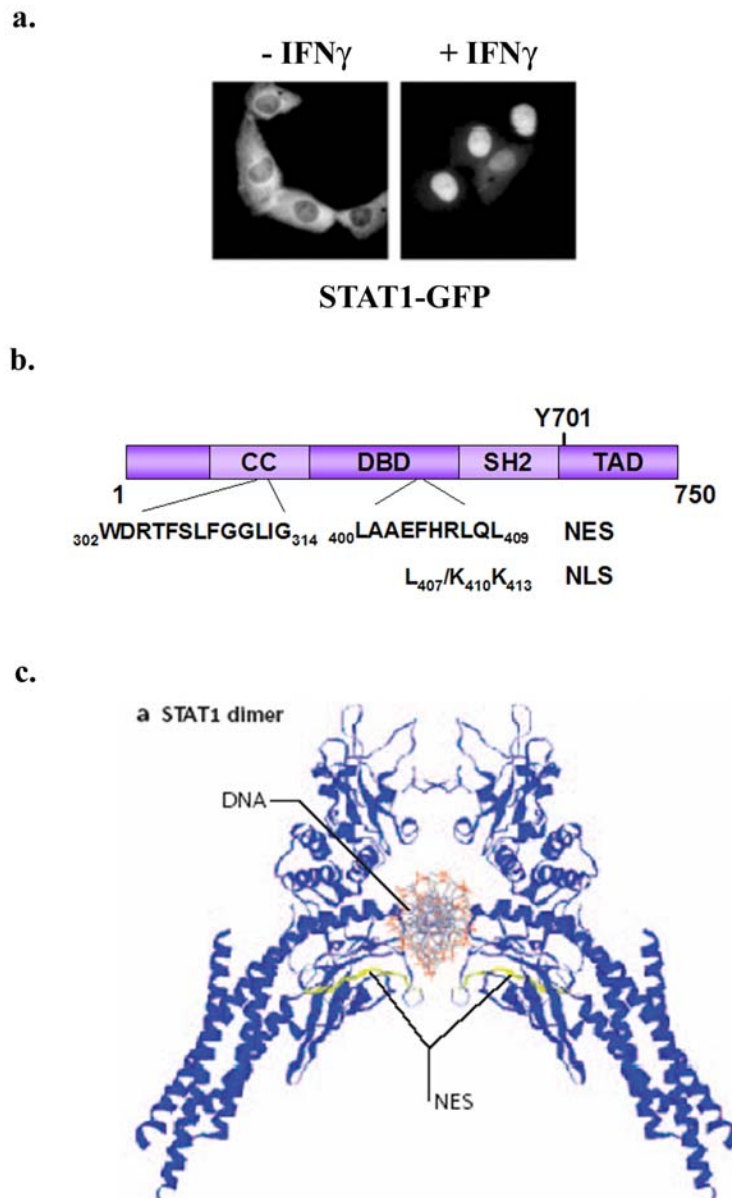


Figure 14: STAT1 nuclear trafficking (McBride and Reich, 2003)

- a. Unphosphorylated STAT1-GFP is primarily cytoplasmic in the absence of any stimulation but accumulates in the nucleus following cytokine stimulation (IFN γ).
- b. Depiction of NESs and motifs critical for nuclear import of STAT1.
- c. Position of motifs that play a role in nuclear transport of STAT1 in the structure phosphorylated STAT1 dimer bound to DNA (Chen et al., 1998).

change enabling STAT1 to bind to DNA. It is now known that in addition to DNA binding, this conformational change exposes a conformational NLS that is recognized by importin- α 5 which then mediates the nuclear import of STAT1. Usually classical NLSs interact with the Arm repeats of importins but in the case of STAT1, the NLS interacts with the carboxyl domain of importin- α 5 (Sekimoto et al., 1997).

Phosphorylated STAT1 is dephosphorylated in the nucleus by nuclear phosphatases (ten Hoeve et al., 2002). The dephosphorylation results in the dissociation of STAT1 from DNA which exposes a leucine-rich NES in the DNA binding domain. This classical NES is recognized by the exportin CRM1 which can then mediate the nuclear export of STAT1 (McBride et al., 2000). One study has shown the presence of an additional NES in the coiled coil domain of STAT1 (Begitt et al., 2000). Thus tyrosine phosphorylation acts as a regulatory switch in the nuclear trafficking of STAT1. Interestingly, The NES and NLS of STAT1 reside in the DNA binding domain. This overlap in the NES and NLS of STAT1 regulates its cellular localization based on its DNA binding ability (Figure 14).

STAT2 cellular trafficking

STAT2 also shows a cytoplasmic localization in the unphosphorylated state (Figure 15) (Banninger and Reich, 2004; Frahm et al., 2006). Treatment of cells expressing unphosphorylated STAT2 with LMB results in the nuclear presence of STAT2 indicating that STAT2 can enter in the nucleus even in the unphosphorylated state but is rapidly exported out of the nucleus by CRM1. Studies have revealed that the nuclear import of unphosphorylated STAT2 is mediated by the transcription factor IRF9 which constitutively associates with STAT2. In cells lacking IRF9, STAT2 is cytoplasmic and remains cytoplasmic even after LMB treatment. The NES of STAT2 has been identified and it resides in the carboxyl terminus of the protein (Banninger and Reich, 2004).

Following cytokine stimulation, STAT2 accumulates in the nucleus in a manner similar to STAT1 and this nuclear accumulation is dependent on the presence of STAT1. In the absence of STAT1, STAT2 fails to accumulate in the nucleus

following cytokine stimulation. Some studies have shown that a motif rich in arginines and lysines found in the DNA binding domain of STAT1 and STAT2 plays a role in the nuclear import of these STATs following IFN stimulation (Fagerlund et al., 2002).

STAT3 cellular trafficking

Unlike STAT1 and STAT2, STAT3 shows a nuclear presence even in the absence of phosphorylation (Figure 16) (Liu et al., 2005). This nuclear presence is independent of tyrosine phosphorylation and it was further demonstrated that STAT3 shuttles between the nucleus and cytoplasm in an unphosphorylated state (Liu et al., 2005; Prana da et al., 2004). Further studies have shown that the amino acids 150-162 in the coiled coil domain of STAT3 played a critical role in its nuclear import. The basic residues in the motif were not required for nuclear import which makes this region an unconventional NLS.

The nuclear import of unphosphorylated STAT3 is mediated by importin- α 3. Following tyrosine phosphorylation STAT3 remains in the nucleus and the nuclear import can be mediated by importin- α 5 and importin- α 3 (Ma and Cao, 2006). STAT3 can be exported out of the nucleus by CRM1. It has been demonstrated that STAT3 has multiple NESs that can be recognized by CRM1: one is present in the coiled coil domain of while two reside in the DNA binding domain (Figure 15) (Bhattacharya and Schindler, 2003).

Thus each STAT differs in its nucleocytoplasmic trafficking. The unphosphorylated STATs show distinct cellular localization patterns, use distinct domains for transport and utilize different transporter proteins for their nuclear import. This dissertation explores the trafficking of another member of the family, STAT5a. Experiments have been performed to better understand the differences between the nuclear import and export of unphosphorylated and phosphorylated STAT5a. By understanding the nucleocytoplasmic transport of STAT5a, the information obtained should aid in the development of specific methods to inhibit the activity of STAT5a in various pathological conditions without affecting the activity of other STATs.

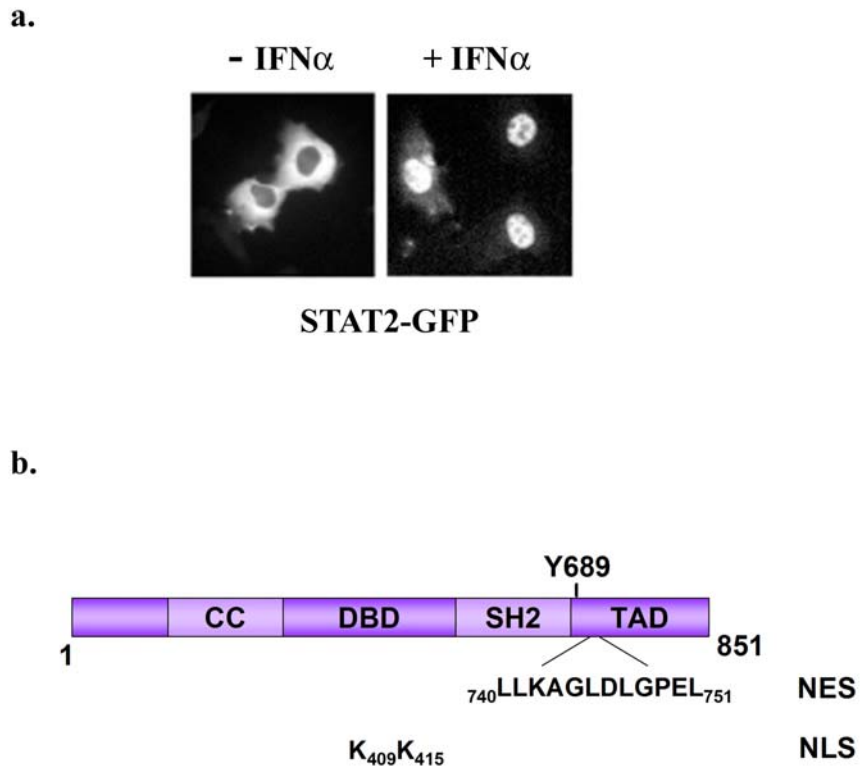
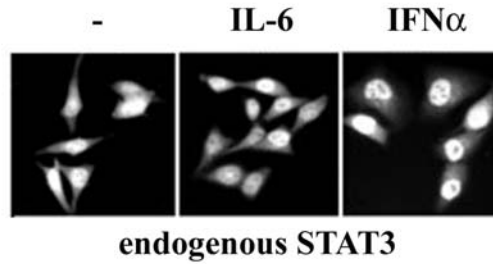


Figure 15: STAT2 nuclear transport

a. STAT2-GFP is cytoplasmic prior to cytokine stimulation and is nuclear following cytokine stimulation (IFN α).

b. Depiction of NES and NLS in STAT2 (Banninger and Reich, 2004; Fagerlund et al., 2002).

a.



b.

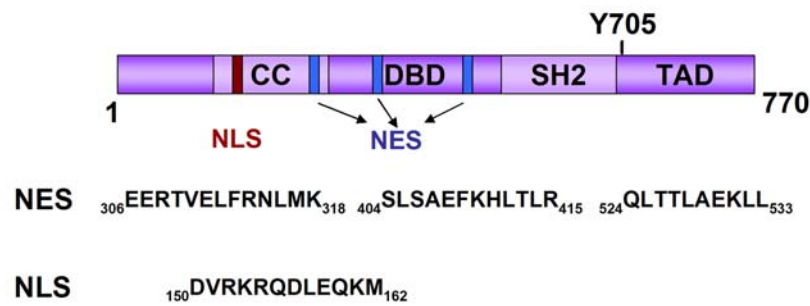


Figure 16: STAT3 nuclear trafficking

a. Endogenous STAT3 shows a nuclear presence both before and following ligand stimulation (Liu et al., 2005).

b. Position of NLS and NES motifs in STAT3 (Bhattacharya and Schindler, 2003; Liu et al., 2005).

Chapter 2

Materials and methods

Cell culture and Reagents

HeLa, COS1, HT1080, U3A (gift from Dr. George R. Stark, Cleveland Clinic Foundation Research Institute, Cleveland, OH, USA) and HEC1B cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum. MCF7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Recombinant human growth hormone (PBL Biomedical Laboratories, New Brunswick, NJ, USA) was used at 1 ng/mL. Recombinant human epidermal growth factor (Sigma-Aldrich Corp., St. Louis, MO, USA) was used at 100 ng/mL. Recombinant human IFN α (Hoffman-LaRoche, Nutley, NJ, USA) was used at 1000 U/mL. Leptomycin B (gifted from Barbara Wolff-Winiski, Novartis Research Institute, Vienna, Austria) was used at 10 nM. DNA transfections were performed using Fugene 6 (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's instructions. Antiphosphotyrosine STAT5 (Cell Signaling Technology, Beverly, MA, USA) and anti-STAT5a (L-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a 1:1000 dilution for Western blotting. Anti-green fluorescent protein (GFP) conjugated to IRDye 88 (Rockland Immunochemicals, Gilbertsville, PA, USA) was used at a 1:5000 dilution for Western blots. Anti-V5 (Invitrogen, Grand Island NY, USA) was used at a dilution of 1:5000 for Western blotting and 1:200 for immunofluorescence. Anti-GFP (Roche Diagnostics Corp., Indianapolis, IN, USA), murine anti-STAT1 (Martinez-Moczygemba et al., 1997), anti-STAT3 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-STAT5a (L-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-STAT5 (C-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-V5 (Invitrogen, Grand Island NY, USA) or control (MOPC-141; Sigma-Aldrich Corp., St. Louis, MO, USA) were used at 1 μ g for electrophoretic mobility shift assays.

Plasmid constructs

The plasmid encoding rat growth hormone receptor was a kind gift from Dr. Christin Carter-Su (University of Michigan, Ann Arbor, MI, USA). The plasmid vectors pEGFP-N1 and pEF1/V5-His were purchased from Clontech (Mountain View, CA, USA) and Invitrogen (Grand Island NY, USA) respectively.

Site directed mutagenesis

Various point mutants of STAT5a were created by using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). This process utilizes plasmid DNA purified from bacteria and two complementary synthetic oligonucleotides containing the desired mutation at the central region of the primers. In a 50 μ L reaction volume, 50 ng of plasmid DNA, 125 ng of each primer, 0.1 mM dNTP, *Pfu Turbo* (Stratagene, La Jolla, CA, USA) buffer and 1 unit of *Pfu Turbo* were included. *Pfu Turbo* (Stratagene, La Jolla, CA, USA) is a high fidelity DNA polymerase with proof-reading activity and was used to minimize random mutations. A thermal cycle reaction of 95° C for 30 seconds, 55° C for 1 minute and 68° C for 14 minutes was repeated 20 times. The reaction was then digested with 10 units of *Dpn I* restriction endonuclease for 5 hours at 37° C. *Dpn I* is known to recognize and cleave methylated DNA. This would result in the digestion of only the original template DNA. Chemically competent bacteria were transformed with this reaction and plated on appropriate selection media. Plasmids were purified from single colonies and screened for the desired mutations by DNA sequencing.

Plasmid constructs: Generation of STAT5a mammalian expression constructs

Full-length human STAT5a cDNA without stop codon was amplified by polymerase chain reaction with primers containing *Bgl* II and *Hind* III restriction sites and cloned into the vector pEGFP-N1 (Clontech, Mountain View, CA, USA) to generate enhanced green fluorescent protein (EGFP)-tagged STAT5a. Similarly, STAT5a cDNA without stop codon was amplified by polymerase chain reaction with

primers containing *Spe* I and *Not* I restriction sites and cloned into the vector pEF1/V5-His (Invitrogen, Grand Island NY, USA) to generate V5 epitope-tagged STAT5a. cDNAs of various deletion mutants of STAT5a were also cloned into pEGFP-N1 or pEGFP2-N1 vector between the *Bgl* II and *Hind* III sites to create GFP fusion proteins. The STAT5a(Δ 142-149)-GFP internal deletion mutant was generated by inserting STAT5a(1-141) between *Bgl* II and *Hind* III sites of the pEGFP-N1 vector to create STAT5a(1-141)-GFP. STAT5a(150-794) was then inserted between the *Hind* III and *Kpn* I sites of STAT5a(1-141)-GFP. The *Hind* III site was then removed by site-directed mutagenesis resulting in STAT5a(Δ 142-149)-GFP. The STAT5a(Δ 341-365)-GFP internal deletion mutant was generated in a similar manner. Briefly, STAT5a(1-340) was inserted between the *Bgl* II and *Hind* III sites of pEGFP-N1 vector to generate STAT5a(1-340)-GFP. STAT5a(366-794) was then inserted between the *Hind* III and *Kpn* I sites of STAT5a(1-340)-GFP. The *Hind* III site was then removed by site-directed mutagenesis resulting in STAT5a(Δ 341-365)-GFP.

Plasmid constructs: Generation of GFP-tagged STAT5a peptides

Phosphorylated complementary oligodeoxynucleotide primers encoding the amino acids 409-422 of STAT5a were mixed in equal amounts and subjected to 95° C for 30 seconds followed by 65° C for 5 minutes. The reaction was then allowed to cool gradually to room temperature. This resulted in the annealing of the oligodeoxynucleotides. The annealed primers were then inserted between the *Bgl* II and *Hind* III sites of the vector pEGFP-N1 to generate STAT5a (409-422)-GFP. The construct, STAT5a(529-540), was generated in a similar manner as described above.

DNA transfections into mammalian cells

Transfections were performed with Fugene 6 (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were plated so that they would be ~50 % confluent at the time of transfection. The desired amount of Fugene was mixed into 50 μ L (for cells in one well of a 12-well plate) or

100 μ L (for cells plated in one well of a 6-well plate, one 35-mm dish, one 60-mm dish or one 100-mm dish) of serum free DMEM and incubated at room temperature (RT) for 5 minutes. The desired amount of DNA was then added in and incubated at RT for 15 minutes. After the time of incubation, the Fugene:DNA complex was then added dropwise to the cells. The amount of DNA and Fugene used for transfection was:

Type of tissue culture plate	DNA (μg)	Fugene (μL)
1 well of 12-well plate	0.2	0.4
1 well of 6-well plate	0.5	1.0
35-mm dish	0.5	1.0
60-mm dish	1.0	2.0
100-mm dish	2.5	5.0

Western blot analysis

Cells were lysed in 50 mM Tris (pH 8.0), 5 mM EDTA, 0.5% Nonidet P-40, 280 mM NaCl, 1mM PMSF, 1X protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA), 1mM NaF, and 1mM sodium vanadate at 4° C for 30 min. The lysates were centrifuged at 12,000 g for 10 min, and the supernatants obtained were boiled in sodium dodecyl sulfate sample buffer. The cell lysates were separated on 8% denaturing polyacrylamide gels and transferred to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL, USA). The membranes were incubated in blocking solution (3% non-fat dry milk in TBS-Tween: 20 mM Tris pH 7.5, 137 mM NaCl, 0.05% Tween 20) at 4° C for 2 hours with gentle agitation. The membranes were then incubated with primary antibodies that were diluted in blocking solution (STAT5 phosphotyrosine antibody was diluted in a solution containing 3% BSA in TBS-Tween) at 4° C overnight. After overnight incubation, the membranes were washed thrice with TBS-Tween for 15 minutes each time followed by incubation with appropriate secondary antibodies. The membranes were again washed and the specific proteins were detected using the Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA).

Electrophoretic Mobility Shift Assays (EMSA)

a. Preparation of extracts

Cells were scraped from plates and incubated with a hypotonic buffer (1.5 mM HEPES pH 7.9, 0.02 mM spermine, 0.05 mM spermidine, 0.2 mM EDTA, 8 mM KCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1X protease inhibitor cocktail, 1mM NaF, and 1mM sodium vanadate) for 20 minutes at 4° C. The cells were then passed 15 times through a 25 gauge syringe. 0.1 volume of restoration buffer (150 mM HEPES pH 7.9, 2 mM spermine, 5 mM spermidine, 20 mM EDTA, 800 mM KCl, 5% glycerol) was added followed by centrifugation at 2,000 rpm for 10 minutes at 4° C. The pellet was suspended in a hypertonic buffer composed of 20 mM HEPES pH 7.9, 0.2 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 400 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1X protease inhibitor cocktail, 1mM NaF, and 1mM sodium vanadate for 20 minutes at 4° C followed by centrifugation at 10,000 rpm for 10 minutes at 4° C. The supernatant obtained was diluted with 1 volume of a buffer containing 20 mM HEPES pH 7.9, 0.2 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 400 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF. Protein concentration was determined by the BioRad assay and 10 µg of the lysates were used for the EMSA.

b. DNA binding assay

10 µg of cell lysate was incubated in 15 µL of reaction buffer containing 12 mM HEPES pH 7.9, 10% glycerol, 5 mM MgCl₂, 0.12 mM EDTA, 0.6 mM EGTA, 0.5 mM DTT, 2 µg poly (dI-dC), 0.5 µg of non-specific plasmid DNA. The reaction was incubated with ³²P radiolabeled double-stranded (ds) DNA oligonucleotide representing the prolactin response element of the β-casein gene promoter (5'-AGATTTCTAGGAATTCAA-3') for 30 min at RT (33, 34). In some instances, the reaction was incubated with specific antibodies (1 µg) or 100-fold excess non-radiolabeled dsDNA oligonucleotide for 30 minutes at RT before the addition of

radiolabeled DNA. Reactions were separated on non-denaturing polyacrylamide gels, exposed to X-ray films at -70°C and finally subjected to autoradiography.

Fluorescence microscopy

Cells were seeded on glass coverslips, transfected, serum starved, and observed after 48 hours of transfection. Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were observed with a Zeiss LSM 5 laser scanning microscope using a 40X oil objective [Plan-Neofluar, numerical aperture 1.3, differential interference contrast microscopy objective (DIC)]. GFP was excited at 488 nm using an argon laser, and emission was collected using a 505 nm long pass filter. Images were captured using the Zeiss LSM 5 Pascal imaging software and processed and presented using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Images shown are representative of ~90% of the cell population.

Immunofluorescence

Cells were seeded on glass coverslips, transfected with STAT5a-V5, serum-starved and evaluated after 48 h of transfection. Cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 for 5 min and then blocked with 3% BSA in PBS for 60 min at room temperature. STAT5a was detected with anti-V5 antibody and a rhodamine-conjugated secondary antibody. Immunofluorescence was observed under a Zeiss LSM 5 laser scanning microscope using a 40X oil objective (Plan-Neofluar, numerical aperture 1.3, DIC objective). Images were captured using Zeiss LSM 5 Pascal imaging software and processed and presented using Adobe Photoshop. Images shown are representative of ~90% of the cell population.

Live cell imaging

HeLa cells were plated on glass bottom tissue culture dishes (Mattek Corporation, Ashland, MA, USA) and transfected with STAT5a-GFP or GFP. After 18 hours of transfection, the cells were serum-starved for 18 hours. The cell culture

plates were mounted on a Zeiss LSM 510 META NLO two-photon laser scanning microscope system (Zeiss). The cells were maintained at 37°C and 5% CO₂ for the entire length of the experiment using the Zeiss Tempcontrol 37-2 Digital and CTI Controller 3700. All analyses were performed using a 40X oil objective (Plan-Neofluar, numerical aperture 1.3, DIC objective). GFP was excited with an argon laser at 488 nm, and emission was collected using a 505 nm long pass filter.

For performing fluorescence recovery after photobleaching (FRAP) in the nucleus, a region of interest in the nucleus was bleached at 100% power of an argon laser at 488 nm for 70 seconds. For fluorescence recovery after photobleaching (FLIP) analysis, a region of interest in the cell was bleached every 12 seconds at maximum laser intensity for 10–75 minutes, depending on the protein expressed in cells. Images were acquired using LSM 510 Meta version 3.2 imaging software. The images were processed and presented using Adobe Photoshop. The fluorescence intensity of the bleached area and other regions on the test cell and a neighboring control cell was quantified automatically using the mean region of interest (ROI) function of the LSM Imaging software and graphically depicted using Microsoft Excel.

Chapter 3

Characterization of Nuclear Import of the STAT5a Transcription Factor

Abstract

The STAT5a transcription factor has been shown to play critical roles in various physiological processes including mammary gland development and hematopoiesis. There have also been numerous findings that have linked the transcriptional activity of STAT5a to cell survival, proliferation, and cancer. To execute its function as a signaling molecule and transcription factor, accurate cellular localization of STAT5a is essential.

This chapter explores the nuclear import of STAT5a both before tyrosine phosphorylation and after tyrosine phosphorylation. Studies involving live cell imaging demonstrate that unphosphorylated STAT5a is constitutively imported into the nucleus. Evaluation of a series of mutations and deletions identifies a region within the coiled coil domain of STAT5a that is critical for nuclear import of both unphosphorylated and tyrosine-phosphorylated forms. The mechanism that regulates transport of STAT5a through nuclear pore complexes into the nucleus is therefore independent of tyrosine phosphorylation.

Following, tyrosine phosphorylation, STAT5a accumulates in the nucleus. Studies performed in living cells indicate that DNA binding is critical for this nuclear accumulation. Thus tyrosine phosphorylated STAT5a accumulates into the nucleus due to its retention by DNA binding. These findings should provide a foundation for further studies that involve targeting the activity of STAT5a.

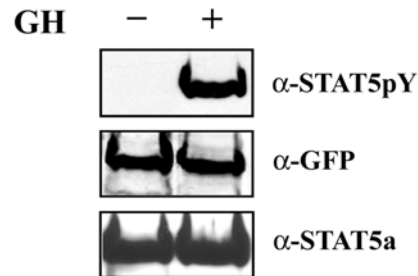
Results

STAT5a nuclear import in independent of tyrosine phosphorylation:

In 1962 a remarkable protein extracted from the jelly fish *Aequorea victoria* was characterized that had the ability to fluoresce green when excited by blue light (Shimomura et al., 1962). This 27 kDa green fluorescent protein (GFP) did not require any exogenous factors other than oxygen to fluoresce. Even though the protein was discovered in the 1960s, its practical application was recognized only in the 1990s after its successful cloning and heterologous expression (Chalfie et al., 1994; Prasher et al., 1992). Presently various mutants of GFP have been created that can cover most of the visible spectra. These fluorescent proteins can emit their fluorescence in live or fixed specimens and have proven to be remarkable tools in studying protein expression, protein movement, cell motility, etc. By labeling proteins of interest with these fluorescent proteins, their cellular localization and trafficking can be extensively studied (Chudakov et al., 2005).

To evaluate the cellular localization of STAT5a, a fusion protein was generated by linking STAT5a to enhanced green fluorescence protein (GFP) to create STAT5a-GFP. In order to ensure that the STAT5a-GFP retained the functional characteristics of STAT5a, tyrosine phosphorylation and DNA binding ability after cytokine stimulation were evaluated (Figure 17). STAT5a can be activated by various cytokines and growth factors including growth hormone (GH). I evaluated the phosphorylation of STAT5a-GFP by performing Western blotting with lysates from cells treated with GH for 30 minutes and antibodies that detect specific phosphorylation of STAT5a on tyrosine 694 (Figure 17a). Phosphorylation was clearly detected only in response to GH treatment. The DNA binding ability of phosphorylated STAT5a-GFP was then determined by performing an electrophoretic mobility shift assay (EMSA) using the β -casein gene response element as a probe (Smit et al., 1997). In response to GH, DNA-binding complexes were observed (Figure 17b). Inclusion of specific antibodies to GFP in the reaction abrogated the appearance of the complex, thus confirming the presence of STAT5a-GFP in the

a.



b.

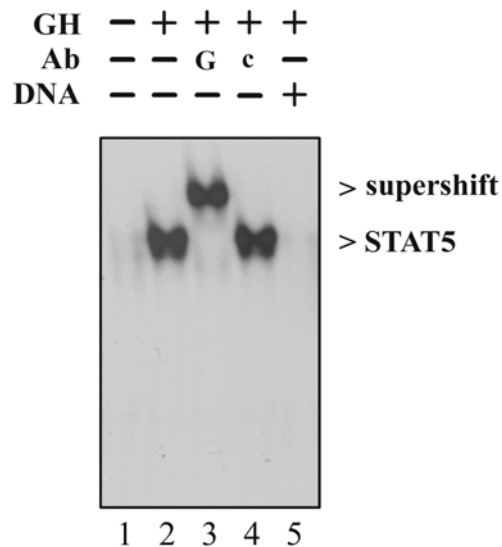


Figure 17: Characterization of STAT5a-GFP

HeLa cells expressing GH receptor (GHR) and STAT5a-GFP were serum deprived and untreated or treated with GH for 30 min (Iyer and Reich, 2007).

a. Western blots of protein lysates from cells untreated (-) or treated (+) with GH were performed with antibodies to phosphorylated STAT5a (α -STAT5pY), GFP (α -GFP), or STAT5a (α -STAT5a).

b. An EMSA was performed with lysates from cells untreated (lane 1) or treated with GH (lanes 2-5) and a radiolabeled DNA fragment corresponding to the β -casein gene response element. Additions to the binding reactions include control (c) antibody (Ab) (lane 4), antibody to GFP (G) (lane 3), or 100-fold excess unlabeled specific DNA (lane 5).

complex. These results demonstrated that STAT5a-GFP was able to function in a manner similar to STAT5a.

The localization of this protein was then evaluated by fluorescence microscopy (Figure 18). HeLa cells expressing STAT5a-GFP and GH receptor (GHR) were serum starved to reduce the basal level of phosphorylation, fixed and observed prior to and following GH stimulation using a confocal microscope. STAT5a-GFP was present in both nuclear and cytoplasmic compartments of the cell in the absence of GH stimulation. However, following GH stimulation STAT5a-GFP was predominantly nuclear. Thus STAT5a was present in the nucleus both before and after tyrosine phosphorylation.

To ensure that the localization pattern of STAT5a was not influenced by GFP, the localization of STAT5a tagged to the epitope V5 was evaluated (Figure 19). The V5 epitope is a small peptide composed of 14 amino acids which makes it considerably smaller than GFP. The STAT5a-V5 protein also retained all the functional characteristics of STAT5a as demonstrated by tyrosine phosphorylation and DNA binding following GH stimulation (Figure 19a and 19b). The localization pattern of STAT5a-V5 in cells was analyzed by indirect immunofluorescence using antibodies against the V5 epitope. As shown in figure 19c, STAT5a-V5 was present in the cytoplasm and nucleus in the absence of GH stimulation and accumulated into the nucleus following GH treatment. Thus STAT5a-GFP and STAT5a-V5 show similar localization patterns in cells.

From these results it is evident that unlike latent STAT1 and STAT2, unphosphorylated STAT5a shows a constitutive nuclear presence but this nuclear presence is not as prominent as latent STAT3. Thus the localization of unphosphorylated STAT5a differs from other STAT members. There is no known function of unphosphorylated STAT5a in the nucleus so it is not clear whether the nuclear presence of STAT5a is critical for its function. Following tyrosine phosphorylation the nuclear presence of STAT5a is required so that it can function as a transcription factor and regulate the expression of target genes.

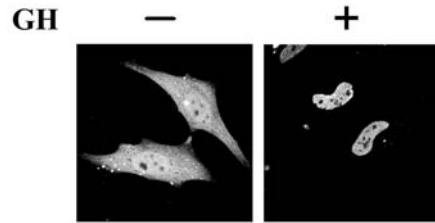


Figure 18: Localization of STAT5a-GFP before and after GH treatment

HeLa cells expressing GH receptor and STAT5a-GFP were serum deprived and left untreated (-) or treated with GH (+) for 30 min. The localization of STAT5a-GFP was analyzed by confocal microscopy (Iyer and Reich, 2007).

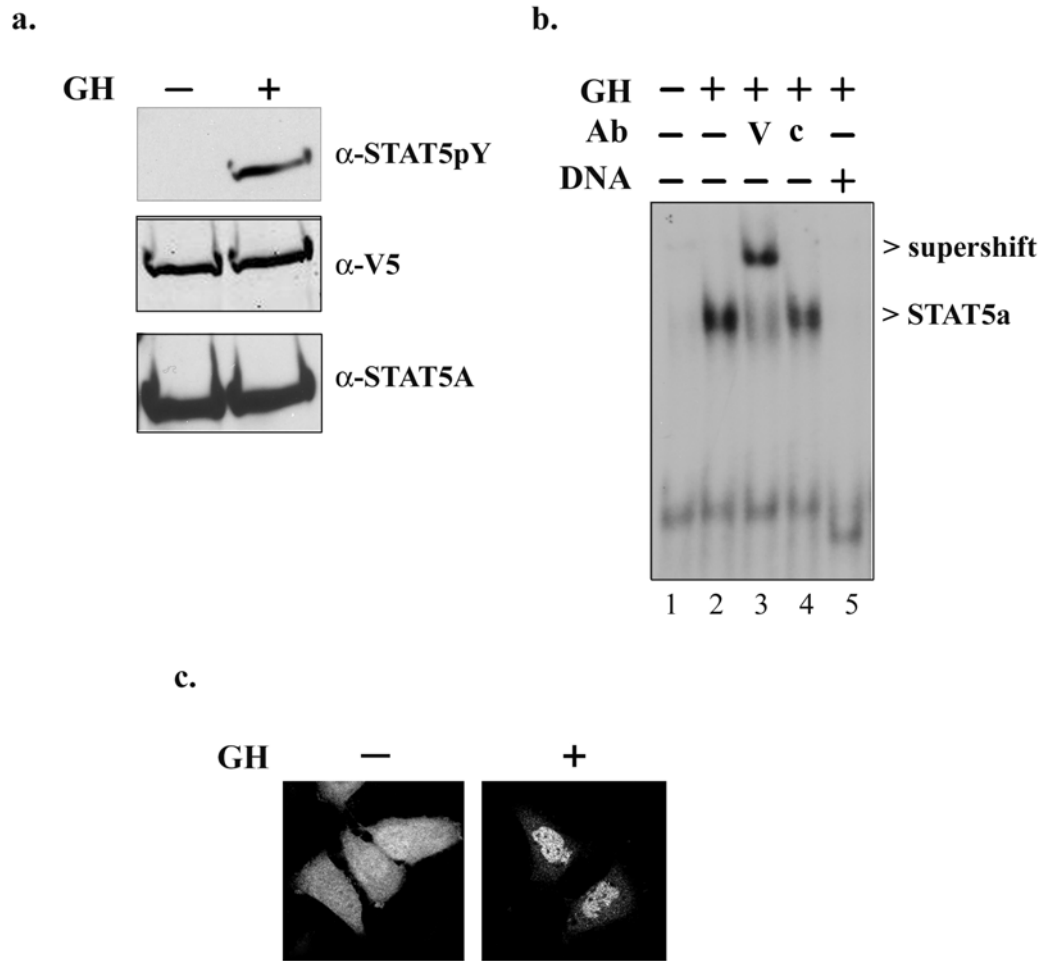


Figure 19: Characterization of STAT5a-V5

HeLa cells expressing GH receptor and STAT5a-V5 were serum deprived and untreated or treated with GH for 30 min (Iyer and Reich, 2007).

a. Western blots of lysates from cells untreated (-) or treated (+) with GH were performed with antibodies to phosphorylated STAT5a (α -STAT5pY), V5 (α -V5), or STAT5a (α -STAT5a).

b. EMSA was performed with lysates from cells untreated (lane 1) or treated with GH (lanes 2-5) and a radiolabeled DNA fragment corresponding to the β -casein gene response element. Additions to the binding reactions include control (c) antibody (Ab) (lane 4), antibody to V5 (V) (lane 3), or 100-fold excess unlabeled specific DNA (lane 5).

c. Confocal microscopy of latent unphosphorylated STAT5a in serum-deprived cells expressing STAT5a-V5 (-) or in cells stimulated with GH (+).

All the experiments mentioned so far were performed in HeLa cells, a human cervix epitheloid carcinoma cell line. To ensure that the localization of unphosphorylated STAT5a that was observed was not limited to HeLa cells, the cellular localization of STAT5a-GFP was analyzed in other cell lines (Figure 20). Various cell lines like the Green monkey kidney cell line COS-1, human breast carcinoma cell line MCF-7, human fibrosarcoma cell line HT1080, human STAT1 deficient cell line U3A and the human endometrial carcinoma cell line HEC 1B were transfected with STAT5a-GFP. In all cases unphosphorylated STAT5a-GFP showed similar localization patterns and was present in the nucleus in addition to the cytoplasm.

Since STAT5a can be activated by a variety of ligands, there is a possibility that a certain population of STAT5a is tyrosine phosphorylated in the cell even in the absence of added exogenous ligand. This population of phosphorylated STAT5a might contribute to its nuclear presence in unstimulated cells. To rule out this possibility two mutations were introduced in STAT5a. The critical tyrosine residue that is phosphorylated was mutated to phenylalanine (Y694F) and the critical arginine residue in the SH2 domain that mediates phosphotyrosine-SH2 domain interactions was mutated to alanine (R618A). This created the STAT5a-RY mutant that cannot associate with other STATs *via* reciprocal phosphotyrosine and SH2 domains. The cellular localization of STAT5a-RY-GFP in multiple cell lines like HeLa and U3A cells prior to tyrosine phosphorylation was similar to STAT5a-GFP and the protein showed a constitutive nuclear presence (Figure 21). Thus the nuclear presence of STAT5a is independent of tyrosine phosphorylation.

Unphosphorylated STAT5a has a high mobility in the nucleus and cytoplasm

The localization of proteins is usually studied after fixing cells with agents like paraformaldehyde or methanol. However this method cannot be employed to study the mobility or spatio-temporal dynamics of proteins in cells. The development of photobleaching techniques like fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) has enabled us to study the mobility of proteins in living cells. Both these techniques require that the protein of interest be

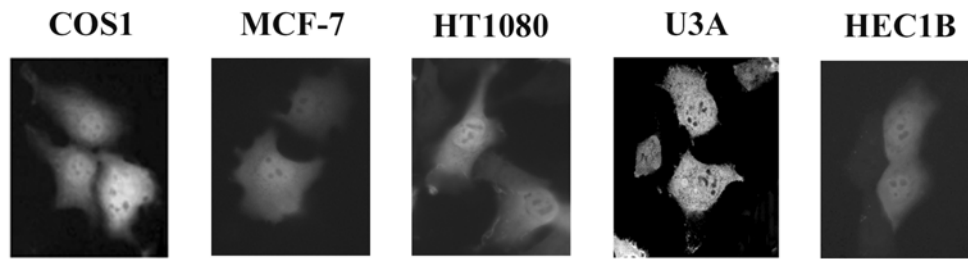


Figure 20: Cellular localization of STAT5a-GFP in various cell lines

The cellular localization of STAT5a-GFP was observed by fluorescence microscopy in various cell lines that were serum deprived

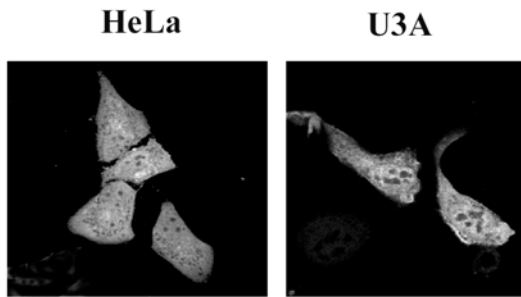


Figure 21: Cellular localization of STAT5a-RY-GFP

HeLa and U3A cells were transfected with STAT5a-RY-GFP and the localization pattern was evaluated by confocal microscopy following serum deprivation (Iyer and Reich, 2007).

fluorescently labeled. This can easily be achieved *in vivo* by labeling the protein of interest with a fluorescent protein like GFP. The FRAP photobleaching technique was used to study the mobility of STAT5a in living cells. In the case of FRAP, a region of interest (ROI) in the cell expressing the protein of interest is bleached using a high intensity laser (Figure 22). This results in the loss of fluorescence in that region. The recovery of the fluorescence in that bleached region over time will depend on the mobility of the proteins in the cell. Highly mobile proteins will occupy the region faster than proteins that move relatively slowly.

The FRAP technique was used to assess the movement of STAT5a in the cytoplasm. A small region in the cytoplasm was subjected to a high intensity laser resulting in the loss of fluorescence in that region (Figure 23). The recovery of fluorescence intensity was then monitored with time. As shown in figure 23, the region of interest (ROI) regained its fluorescence by one minute. This indicates that unphosphorylated STAT5a rapidly moves in the cytoplasm.

A similar analysis was performed to study the mobility of STAT5a-GFP within the nucleus of cells. FRAP was performed in a small region in the nucleus which resulted in the decrease of fluorescent intensity in that region. This was apparent when the intensity of fluorescence in the ROI was quantitatively measured over a period of time. As shown in figure 24b, the recovery in fluorescence was complete within a minute. Thus unphosphorylated STAT5a moves rapidly in the nucleus as well.

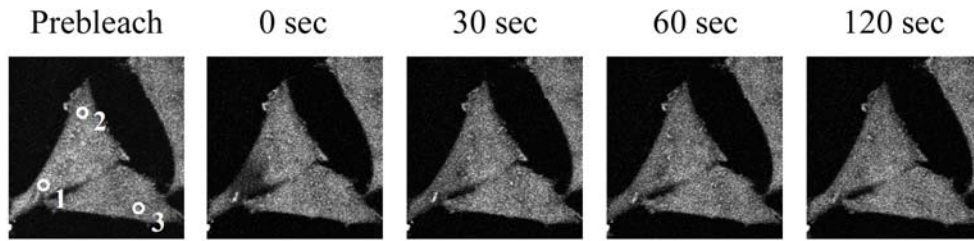
Fluorescence Recovery After Photobleaching (FRAP)



Figure 22: Principle behind the FRAP photobleaching technique

In the FRAP technique, a region in the cell (white circle) expressing a fluorescently tagged protein is subjected to the beam of a high intensity laser resulting in the bleaching of fluorescence in that region. The rate of recovery of fluorescence in the bleached area will depend on the mobility of the fluorescent protein.

a.



b.

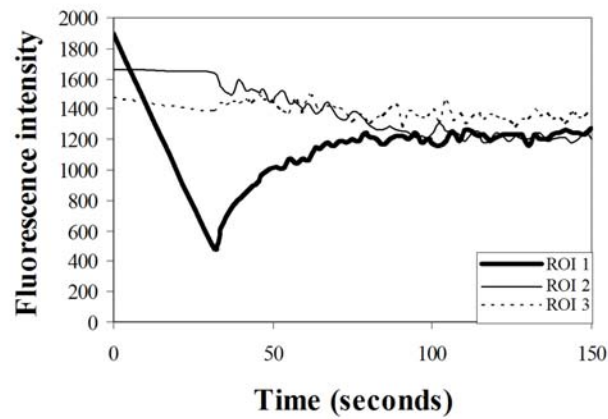
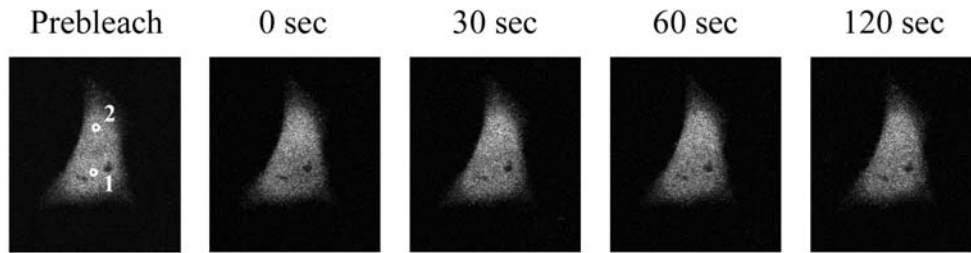


Figure 23: Mobility of STAT5a in the cytoplasm as observed by FRAP

a. A HeLa cell expressing STAT5a-GFP was subjected to cytoplasmic FRAP within ROI 1. Subsequent recovery of fluorescence is shown with time.

b. Quantitative measurement of relative fluorescence intensity in the bleached region (ROI 1) relative to a site in the cytoplasm of the photobleached cell (ROI 2) or an adjacent cell (ROI 3) shown in (a).

a.



b.

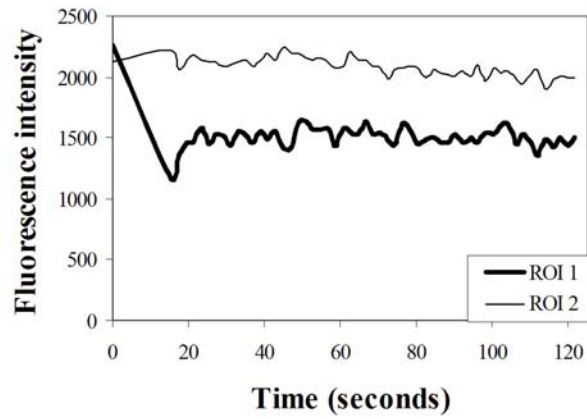


Figure 24: Mobility of STAT5a in the nucleus as observed by FRAP

a. A HeLa cell expressing STAT5a-GFP was subjected to nuclear FRAP within ROI 1. Subsequent recovery of fluorescence is shown with time.

b. Quantitative measurement of relative fluorescence intensity in the bleached region (ROI 1) relative to a site in the cytoplasm of the photobleached cell (ROI 2).

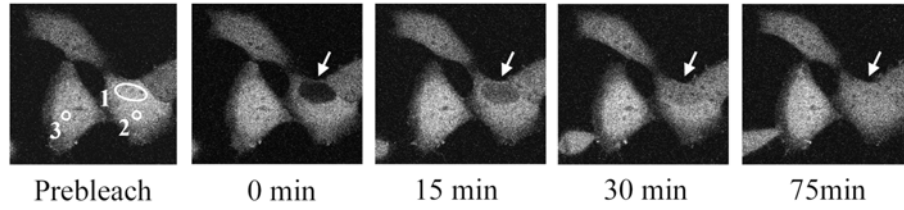
Unphosphorylated STAT5a is actively imported into the nucleus of cells

The experiments described above were able to provide information on the mobility of STAT5a within the cytoplasm and nucleus of living cells. They however cannot give information on the mobility of STAT5a between the cytoplasmic and nuclear compartments of the cell. I have already showed that unphosphorylated STAT5a shows a constitutive nuclear presence which is independent of phosphorylation. To evaluate the actual import of unphosphorylated STAT5a from the cytoplasm into the nucleus of cells, the FRAP technique was employed. The entire nucleus of a cell expressing STAT5a-GFP was subjected to a high intensity laser resulting in the effective bleaching of fluorescence in the entire nucleus (Figure 25). Thus any recovery of fluorescence in the nucleus would be due to the import of unbleached STAT5a-GFP from the cytoplasm into the nucleus. Recovery of fluorescence in the nucleus was observed within 15 minutes and by 75 minutes the recovery was complete. Thus STAT5a-GFP can be constitutively imported into the nucleus.

STAT5a is a 94 kDa protein and thus it cannot passively diffuse through the NPC. However, a protein like GFP is small enough to diffuse through the NPC and thus it would be predicted that its nuclear import would be considerably faster than STAT5a-GFP. To test this hypothesis, I performed FRAP analysis in the nucleus of cells expressing GFP. The fluorescence of the entire nucleus was bleached by passing a high intensity laser through the nucleus. As depicted in figure 26, the recovery of fluorescence in the nucleus was complete by 3 minutes which is definitely faster than STAT5a-GFP (Figure 25).

To determine whether tyrosine phosphorylation was required for the constitutive nuclear import of STAT5a, I determined the nuclear import of a mutant of STAT5a, STAT5a-RY-GFP. This mutant of STAT5a cannot be phosphorylated and cannot associate with other STATs *via* reciprocal phosphotyrosine and SH2 domain interactions. The entire nucleus of a cell expressing STAT5a-RY-GFP was subjected to a high intensity laser resulting in the effective bleaching of fluorescence in the entire nucleus. As shown in figure 27, the recovery of fluorescence in the

a.



b.

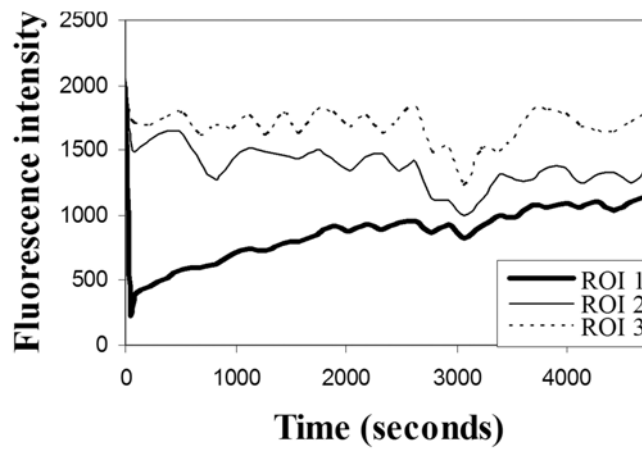
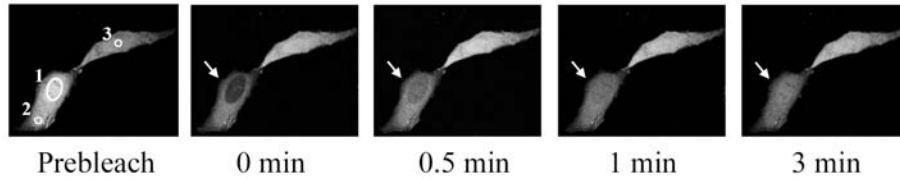


Figure 25: Nuclear FRAP in cells expressing STAT5a-GFP (Iyer and Reich, 2007)

a. A HeLa cell expressing STAT5a-GFP was subjected to nuclear photobleaching within ROI 1. Subsequent recovery of nuclear fluorescence is shown with time.

b. Quantitative measurement of relative fluorescence intensity in the nucleus that was bleached (ROI 1) relative to a site in the cytoplasm of the photobleached cell (ROI 2) or nucleus of an adjacent cell (ROI 3) shown in (a).

a.



b.

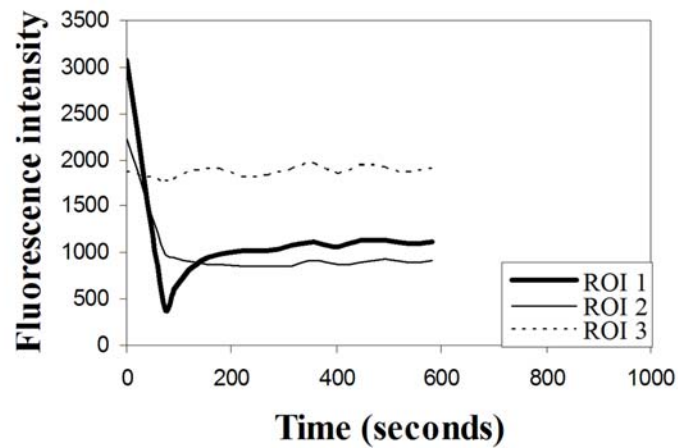


Figure 26: Nuclear FRAP in cells expressing GFP (Iyer and Reich, 2007)

a. A HeLa cell expressing GFP was subjected to nuclear photobleaching within ROI 1. Subsequent recovery of nuclear fluorescence is shown with time.

b. Quantitative measurement of relative fluorescence intensity in the nucleus that was bleached (ROI 1) relative to a site in the cytoplasm of the photobleached cell (ROI 2) or an adjacent cell (ROI 3) shown in (a).

nucleus was complete by 60 minutes. This time of recovery was very similar to the recovery that was observed wild type STAT5a-GFP (Figure 25). Thus from all the results of these studies it can be concluded that unphosphorylated STAT5a-GFP is constitutively imported into the nucleus and this import is independent of tyrosine phosphorylation.

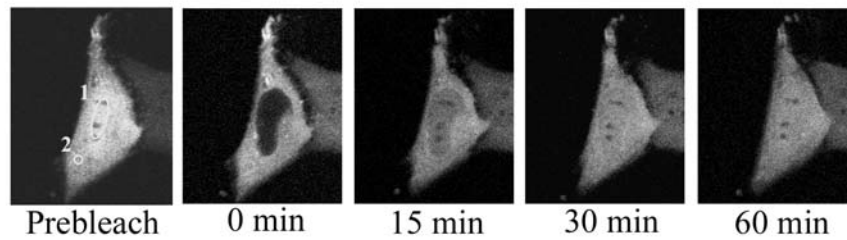
Recognition of a motif of STAT5a that is critical for nuclear import

Since STAT5a is imported into the nucleus it is likely to possess a motif that functions as a nuclear localization signal (NLS). Classical NLSs usually contain one or two stretches of basic residues and these basic residues play critical roles in nuclear import (Gorlich and Mattaj, 1996; Lange et al., 2007). After scanning the primary amino acid sequence of STAT5a for putative classical NLSs, I was able to identify two candidate sequences. One was present in the coiled coil domain while the other was in the DNA binding domain.

The NLS in the coiled coil domain resides between the amino acids 241-258 and the primary amino acid sequence is as follows: RKQQTII LDDEL IQWKRR (basic residues are underlined). All the basic residues in this motif were mutated to alanines and the cellular localization of this mutant, STAT5a-RK-GFP, was evaluated (Figure 28). The mutated protein was present in the nucleus and cytoplasm of cells indicating that it had the ability to be imported into the nucleus. Thus this motif did not function as an NLS and was not required for nuclear import.

The motif present in the DNA binding domain is between the amino acids 422 to 430: KRIKRADRR (basic residues are underlined). As depicted, this motif is rich in basic amino acids which is a hallmark of classical NLSs. The basic residues in this motif were also mutated to alanines to generate the mutant STAT5a-KR-GFP. When this protein was expressed in HeLa cells, it also showed a nuclear presence indicative of the fact that it could be imported into the nucleus (Figure 28). Hence this motif also did not function as an NLS.

a.



b.

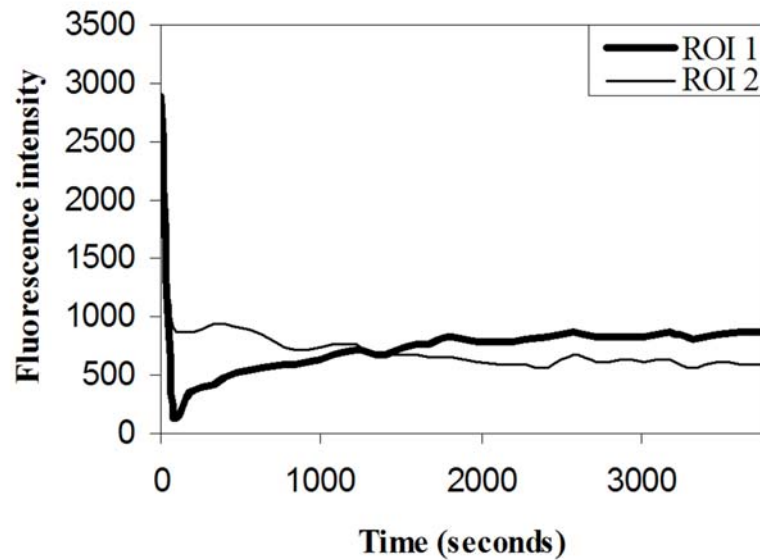


Figure 27: Nuclear FRAP of STAT5a-RY-GFP with live cell imaging (Iyer and Reich, 2007)

a. A HeLa cell expressing STAT5a-RY-GFP was subjected to nuclear photobleaching within ROI 1. Subsequent recovery of nuclear fluorescence is shown with time.

b. Quantitative measurement of relative fluorescence intensity in the nucleus that was bleached (ROI 1) relative to a site in the cytoplasm of the photobleached cell (ROI 2) or an adjacent cell (ROI 3) shown in (a).

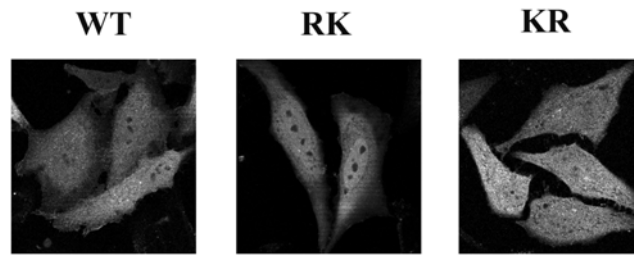


Figure 28: Cellular localization of STAT5a-RK-GFP and STAT5a-KR-GFP

HeLa cells were transfected with various STAT5a mutants and their cellular localization was evaluated by confocal microscopy.

A recent publication had reported the requirement of the amino acids 341-365 of STAT5a for nuclear import (Onishi et al., 1998). These residues reside in the DNA binding domain of STAT5a and have the sequence: VLKTQTKFAATVRLLVGGKLVHNMN. Since this sequence was not rich in basic residues and did not resemble any classical NLS, I tested the localization of STAT5a with an internal deletion of the amino acids 341-365 (Figure 29). This protein showed a cytoplasmic localization in cells but following treatment of cells with an inhibitor of the export transporter CRM1, leptomycin B, the protein showed a nuclear presence in multiple cell lines. This result indicates that the 341-365 a.a. deletion mutant is imported to the nucleus and is exported effectively. Hence this region is not required for the nuclear import of STAT5a.

A logical approach was then used to evaluate the region of STAT5a that was critical for its nuclear import. I created a series of STAT5a deletion mutants and tagged them with GFP. The smaller deletion fragments were tagged with two tandem copies of GFP to prevent nuclear import by passive diffusion. The cellular localization of these deletion proteins was evaluated by fluorescence microscopy. As shown in figure 30, an amino terminal fragment comprised of the N-terminal region and the coiled coil domain (amino acids 1-330) tagged to two GFP molecules showed a greater nuclear presence than wild type STAT5a-GFP. On the other hand, a deletion mutant comprised of the DNA binding domain, SH2 domain, and transcriptional activation domain (aa 331-794) showed a cytoplasmic localization. This suggested that the region required for nuclear import was present within the amino acids 1-330.

Further analysis of additional deletion constructs revealed that STAT5a containing amino acids 150-794 showed a cytoplasmic localization but a slightly larger deletion mutant comprised of the amino acids 145-794 showed a nuclear presence. The cytoplasmic localization of STAT5a150-794 would suggest that it lacked an NLS while the constitutive nuclear presence of STAT5a145-794 would indicate that it did possess an NLS that enabled its nuclear import. Thus the amino acids in the vicinity of 145-150 were required for nuclear import.

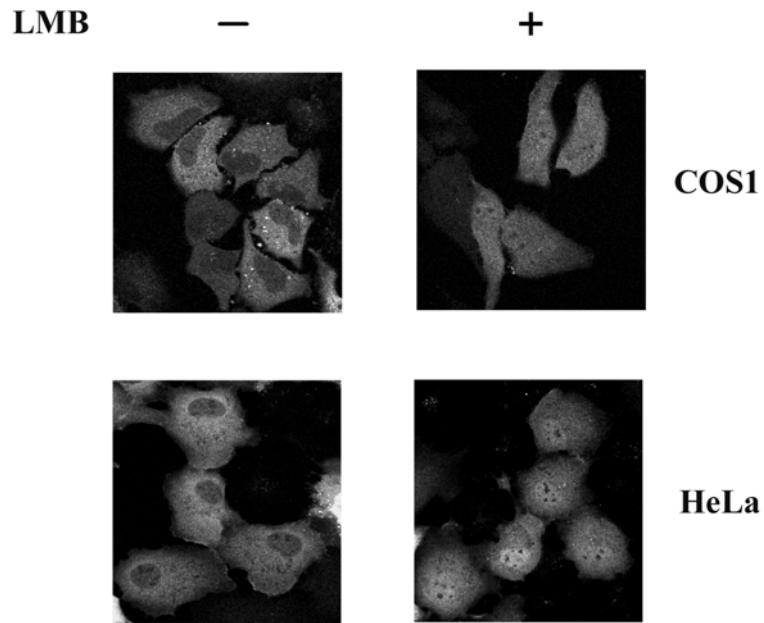


Figure 29: Nuclear accumulation of STAT5a(Δ 341-365)-GFP in the presence of the CRM1 inhibitor, Leptomycin B (LMB)

COS1 cells or HeLa cells expressing with STAT5a(Δ 341-365)-GFP were untreated (-) or treated (+) with 10 nM LMB for 2 hours. Localization was determined by confocal microscopy.

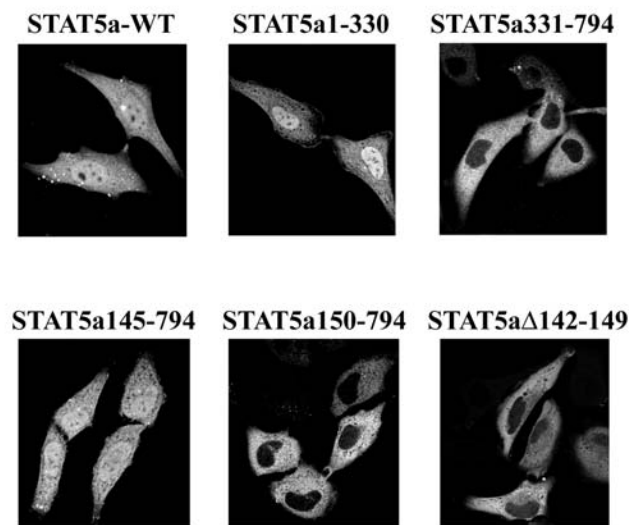
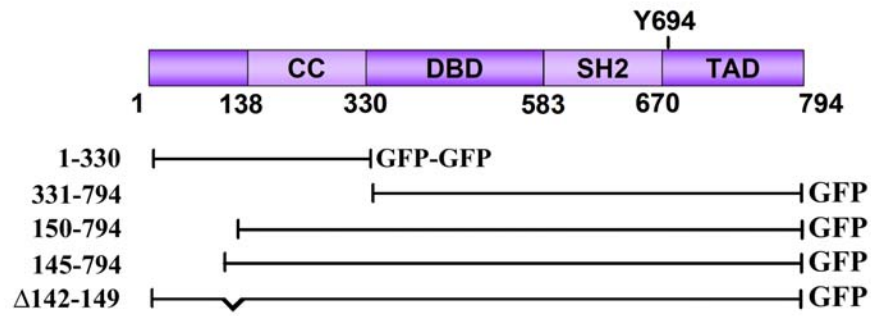


Figure 30: Identification of a region of STAT5a required for nuclear import (Iyer and Reich, 2007)

a. Diagram of STAT5a functional motifs including the coiled coil (CC), DNA binding domain (DBD), SH2 domain, transcriptional activation domain (TAD), and specific phosphorylated tyrosine (Y694). Linear depiction of deletion mutations with encoding amino acid residues or amino acid residues removed (Δ) linked to GFP or two copies of GFP in tandem (GFP-GFP).

b. Confocal microscopy of STAT5a deletion constructs.

Due to the difference in the cellular localization of the deletion mutants STAT5a145-794 and STAT5a150-794, I generated a mutant of STAT5a with an internal deletion of the amino acids 142-149. Evaluation of the cellular localization of this internal deletion mutant, STAT5a (Δ 142-149), revealed that it was restricted to the cytoplasm (Figure 30). This cytoplasmic localization could be due to the inability of the mutant to be imported into the nucleus or due to a faster rate of nuclear export. I evaluated the localization of this protein in the presence of the CRM1 inhibitor LMB to determine whether nuclear export contributed to the cytoplasmic localization of STAT5a (Δ 142-149) (Figure 31). There was no change in its cellular localization following treatment with LMB indicating that CRM1 did not mediate the nuclear export of this mutant. Thus the amino acids 142-149 were necessary for the nuclear import of unphosphorylated STAT5a.

To ensure that this mutant retains the functional characteristics of STAT5a, I analyzed the ability of this mutant to be tyrosine phosphorylated and bind DNA following cytokine stimulation. Tyrosine phosphorylation was evaluated by Western blotting with lysates of cells treated with cytokines and antibodies that detect specific phosphorylation of STAT5a on tyrosine 694. Interestingly, tyrosine phosphorylation could not be detected in response to GH or IFN α treatment but was detected following EGF treatment (Figure 32a). It is possible that the region 142-149 was required for interaction with the cytokine receptor or JAKs but further studies need to be performed to verify this possibility. Thus, these results indicated that the mutant had the ability to be tyrosine phosphorylated in response to EGF.

I then checked the DNA binding ability of this mutant by performing an EMSA using COS1 cell lysates transfected with STAT5a(Δ 142-149)-GFP (Figure 32b). The β -casein gene response element was used as a probe for the EMSA. In response to EGF, DNA-binding complexes were observed that were not seen in the absence of EGF. Inclusion of specific antibodies to GFP, STAT5a or STAT5 in the reaction abrogated the appearance of the complex, thus confirming the presence of STAT5a(Δ 142-149)-GFP in the complex. These results demonstrate that

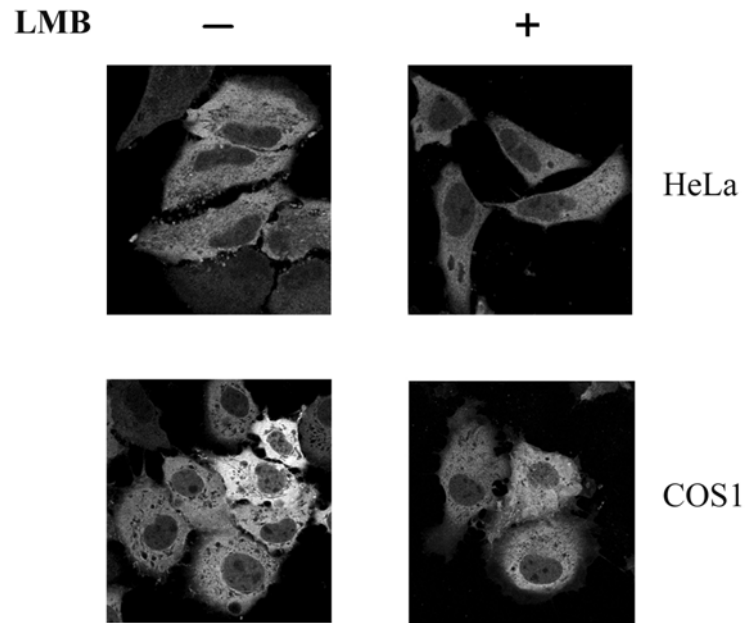


Figure 31: Cellular localization of STAT5a(Δ 142-149)-GFP in the presence of the CRM1 inhibitor, Leptomycin B (LMB)

COS1 cells or HeLa cells expressing with STAT5a(Δ 142-149)-GFP were untreated (-) or treated (+) with 10 nM LMB for 2 hours. Localization was determined by confocal microscopy.

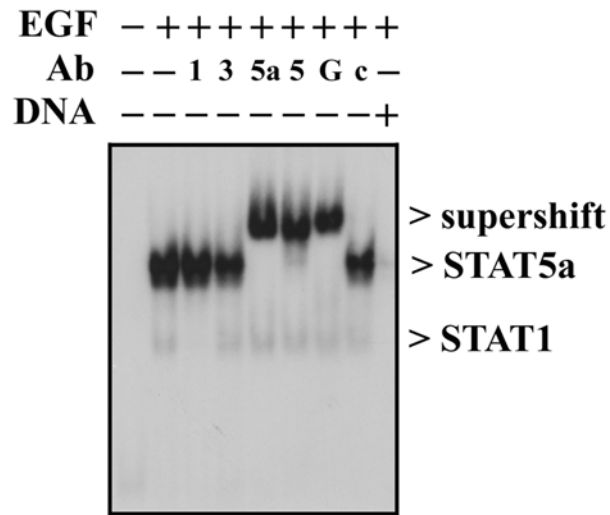
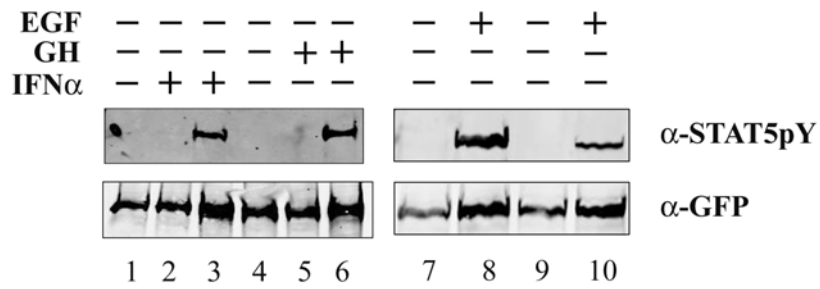


Figure 32: Characterization of STAT5a(Δ 142-149)-GFP

a. Western blot analysis of protein lysates from COS1 cells expressing STAT5a(Δ 142-149)-GFP (lanes 1, 2, 4, 5, 9, 10) or STAT5a-GFP (lanes 3, 6, 7, 8) treated with EGF, GH or IFN α was performed with antibodies to phosphorylated STAT5a (α -STAT5pY) or GFP (α -GFP).

b. EMSA with COS1 lysates containing STAT5a (Δ 142–149)-GFP from untreated cells (-) or cells treated with EGF (+). Specific antibody (Ab) to STAT1 (1), STAT3 (3), STAT5a (5a), STAT5 (5), GFP (G), or a control antibody (c) was added to the DNA-binding reactions. As a specific competitor 100-fold excess unlabeled DNA was added (DNA). The mobility of STAT5a-DNA complexes and the antibody-mediated supershift is noted. The mobility of endogenous STAT1 is also indicated (Iyer and Reich, 2007).

STAT5a(Δ 142-149)-GFP retained some of the functional characteristics of wild type STAT5a.

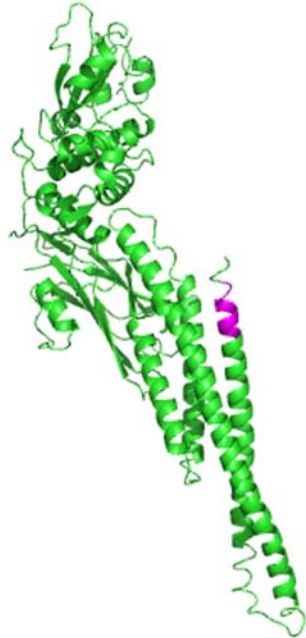
The crystal structure of unphosphorylated STAT5a dimer has been solved. The location of the amino acids 142-149 was determined in the structure and its position is depicted in figure 33. This sequence is part of an α -helix in the coiled coil domain and from its position in the structure it is evident that this region is not buried inside the molecule. Thus this region is on the outer surface of STAT5a and hence is exposed to the solvent. This can enable it to interact with transporter molecules or nucleoporins of the NPC that can in turn mediate the nuclear import of STAT5a.

Redistribution of STAT5a following tyrosine phosphorylation

As described above unphosphorylated STAT5a shows a cytoplasmic and nuclear presence. However, following GH treatment, there is a dramatic accumulation of STAT5a into the nucleus (Figure 34). This feature was observed in HeLa cells as well as the STAT1 deficient cell line, U3A (Figure 34a). A similar nuclear accumulation was also observed following IFN α treatment in HeLa cells and EGF treatment in COS1 cells (Figure 34b). Since all these cytokines stimulate the tyrosine phosphorylation of STAT5a, it can be concluded that tyrosine phosphorylation of STAT5a leads to its nuclear accumulation in cells.

To evaluate the kinetics of phosphorylation, I treated cells expressing STAT5a-GFP with GH for different periods of time. Phosphorylation was detected by Western blotting using antibodies specific to phosphorylated STAT5a at tyrosine 694 (Figure 35). By 10 minutes of ligand addition, phosphorylation of STAT5a could be detected. The phosphorylation could be detected for as long as 24 hours in the presence of GH. I also analyzed the cellular localization of STAT5a at different times of GH treatment (Figure 36). Even though tyrosine phosphorylation could be detected within 10 minutes of cytokine stimulation, complete nuclear accumulation of STAT5a was detected only by 30 minutes. This amount of time might be required for active nuclear import and DNA binding ability of a major population of phosphorylated STAT5a. The protein remained nuclear for as long as 24 hours in the

a.



b.



Figure 33: Location of amino acids 142-149 in STAT5a

The amino acids 142-149 are depicted in magenta in the structure of unphosphorylated STAT5a (Iyer and Reich, 2007). The location of the amino acids is shown in a STAT5a monomer (a) and unphosphorylated dimer (b).

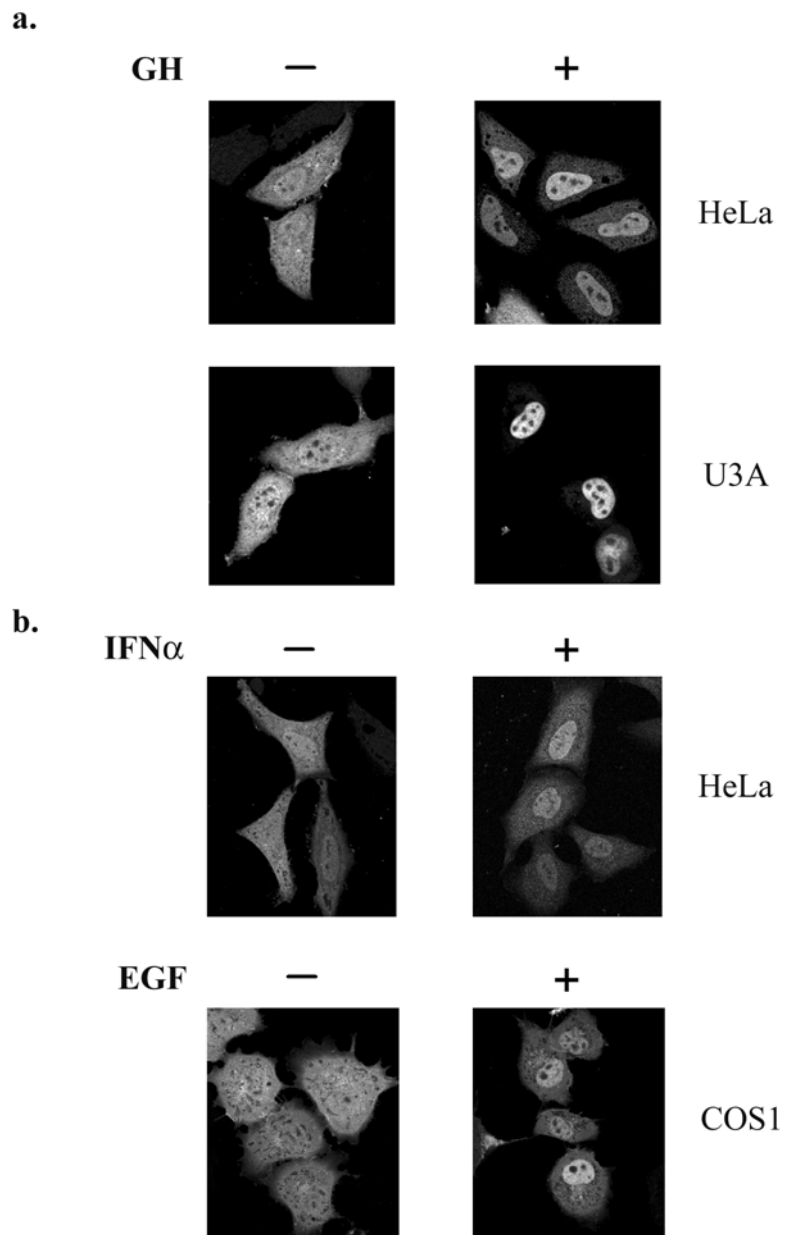


Figure 34: Cellular localization of STAT5a following cytokine stimulation

a. HeLa cells or U3A cells expressing GHR and STAT5a-GFP were untreated (-) or treated (+) with GH.

b. HeLa (top panel) cells or COS1 (bottom panel) cells expressing STAT5a-GFP were untreated (-) or treated (+) with IFN α (top panel) or EGF (bottom panel). Localization patterns were determined by confocal microscopy

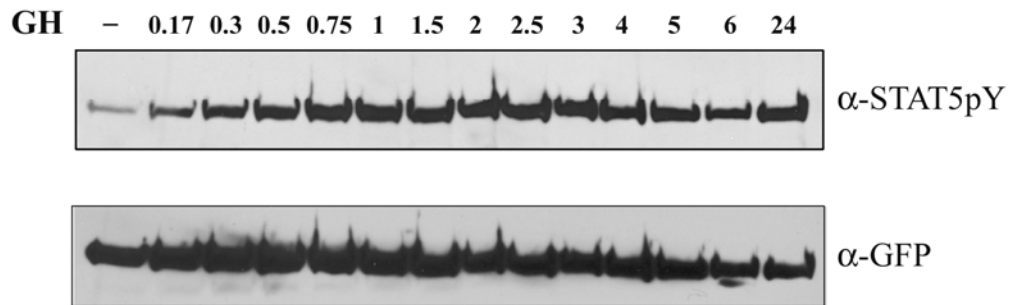


Figure 35: Phosphorylation kinetics of STAT5a-GFP

HeLa cells expressing the GHR and STAT5a-GFP were treated with GH for various periods of time. Cell lysates were prepared and Western blot analysis was performed with antibodies to phosphorylated STAT5a (α -STAT5pY) and GFP (α -GFP).

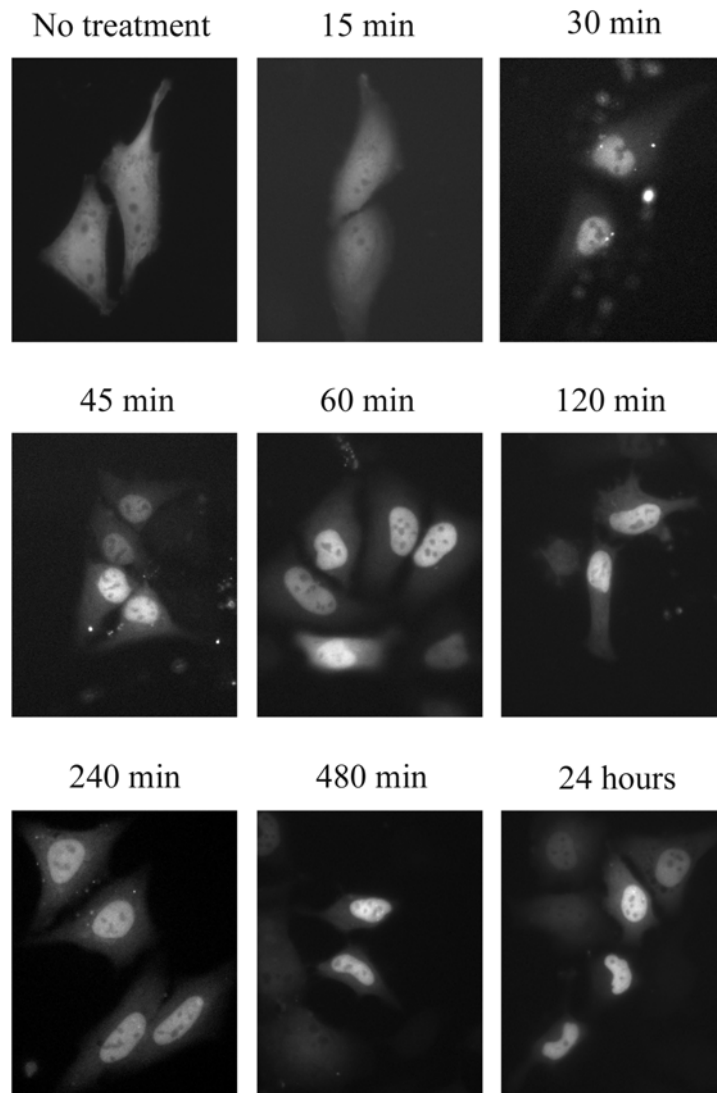


Figure 36: Localization kinetics of STAT5a following treatment with GH

HeLa cells expressing the GHR and STAT5a-GFP were treated with GH for various periods of time. The cellular localization was evaluated at indicated time points by fluorescence microscopy.

presence of GH. Thus STAT5a is rapidly phosphorylated following ligand stimulation and accumulates in the nucleus by 30 minutes of ligand treatment. It remains tyrosine phosphorylated and nuclear in the presence of GH for as long as 24 hours.

It is now evident that the amino acids 142-149 are necessary for the nuclear import of unphosphorylated STAT5a (Figure 30). To determine whether these amino acids were critical for the nuclear import of tyrosine phosphorylated STAT5a, I analyzed the localization of STAT5a(Δ 142-149) following EGF treatment in COS1 cells. There was no change in the cellular localization of this mutant following EGF treatment and the mutant remained cytoplasmic (Figure 37). As shown in figure 32, this mutant was phosphorylated following EGF treatment and had the ability to bind DNA in the phosphorylated state. The absence of nuclear accumulation of this mutant in response to EGF might be due to the fact that it could not be imported into the nucleus. Thus the amino acids 142-149 are critical for the nuclear import of unphosphorylated and phosphorylated STAT5a.

From figures 21 and 27, it is evident that tyrosine phosphorylation was not necessary for the nuclear import of unphosphorylated STAT5a. I tested whether phosphorylation was required for the nuclear accumulation of STAT5a following cytokine stimulation. For these experiments, I used a mutant of STAT5a in which the tyrosine residue that is phosphorylated was mutated to phenylalanine (Y694F) and the arginine residue in the SH2 domain that mediates phosphotyrosine-SH2 domain interactions was mutated to alanine (R618A). As mentioned before, this STAT5a-RY mutant does not have the ability to associate with other STATs *via* reciprocal phosphotyrosine and SH2 domain interactions. The inability of this mutant to be phosphorylated was evaluated by Western blotting using cell lysates stimulated with cytokines and using antibodies that detect specific phosphorylation of STAT5a on tyrosine 694 (Figure 38, top panel). Phosphorylation could not be detected in the presence or absence of GH treatment. The mutant also failed to bind DNA as revealed by electrophoretic mobility shift assays (Figure 38, bottom panel). Thus STAT5a-RY-GFP cannot be tyrosine phosphorylated and does not have the ability to bind DNA.

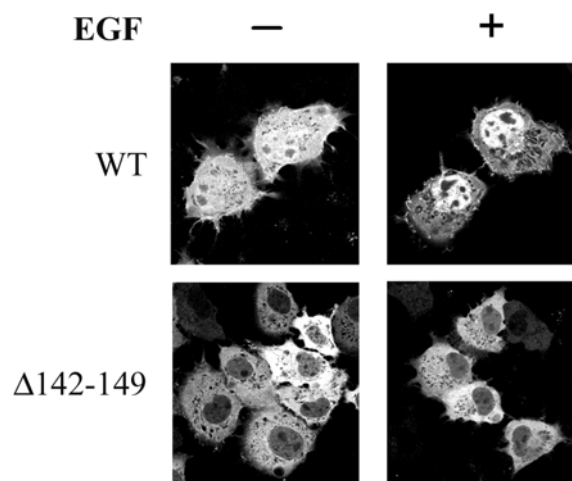


Figure 37: Cellular localization of STAT5a($\Delta 142-149$)-GFP in the presence of EGF (Iyer and Reich, 2007)

COS1 cells expressing STAT5a-GFP (top panel) or STAT5a($\Delta 142-149$)-GFP (bottom panel) were untreated (-) or treated (+) with EGF. The localization pattern was analyzed by confocal microscopy.

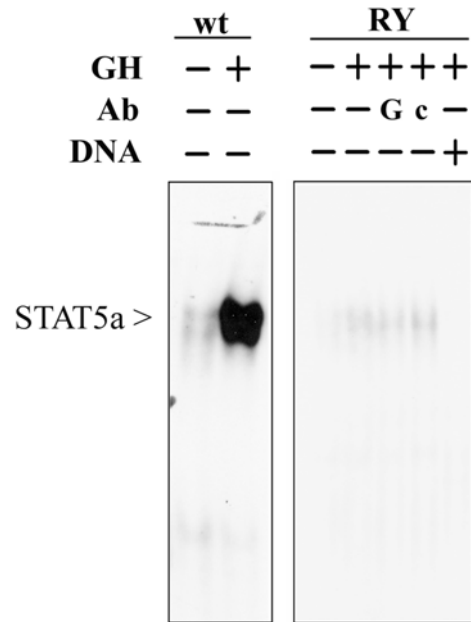
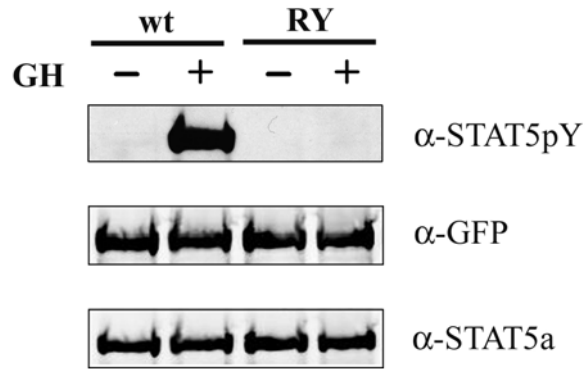


Figure 38: Characterization of STAT5a-RY-GFP (Iyer and Reich, 2007)

a. Western blot analysis of lysates from HeLa cells expressing STAT5a-GFP (wt) or STAT5a-RY-GFP (RY) treated GH was performed with antibodies to phosphorylated STAT5a (α-STAT5pY), GFP (α-GFP) or STAT5a (α-STAT5a).

b. EMSA with HeLa lysates expressing STAT5a-GFP (wt) or STAT5a-RY-GFP from untreated cells (-) or cells treated with GH (+). Specific antibody (Ab) to GFP (G), or a control antibody (c) was added to the DNA-binding reactions. As a specific competitor, 100-fold excess unlabeled DNA was added (DNA) to the reaction.

I then evaluated the cellular localization of this mutant following GH treatment (Figure 39). HeLa cells were co-transfected with STAT5a-RY-GFP and GHR and the cellular localization was observed by confocal microscopy. The mutant was present in the nucleus and cytoplasm in the absence of GH treatment but failed to accumulate into the nucleus in response to GH stimulation. This would suggest that tyrosine phosphorylation plays an important role in nuclear accumulation. Since this mutant also has the inability to bind DNA it is also possible that the property of DNA binding is required for the nuclear accumulation of STAT5a following tyrosine phosphorylation.

DNA binding is required for the nuclear accumulation of phosphorylated STAT5a

To determine if DNA binding was critical for the nuclear accumulation of phosphorylated STAT5a, I evaluated the localization patterns of mutants of STAT5a that were unable to bind DNA. There have been a few studies that have identified several residues in the DNA binding domain of other STATs that are necessary for DNA binding. In the case of STAT1, the residues E428 and E429 are critical for DNA binding (Horvath et al., 1995). These glutamic acids are conserved among all STAT members including STAT5a.

I analyzed the contribution of these two amino acids to the DNA binding ability of STAT5a. These two residues were mutated to alanines to generate the protein STAT5a-EE-GFP. I evaluated the tyrosine phosphorylation and DNA binding ability of this mutant by Western blotting and EMSA respectively (Figure 40). Western blotting was performed on lysates from cells expressing STAT5a-EE-GFP that were either untreated or treated with GH (Figure 40a). Tyrosine phosphorylation was detected only in response to GH and EMSA analysis revealed that the mutant was able to efficiently bind DNA (Figure 40b). DNA binding complexes were observed following GH stimulation and addition of specific antibodies to GFP led to a supershift of the complex. This result confirmed the ability of the protein STAT5a-EE-GFP to bind DNA.

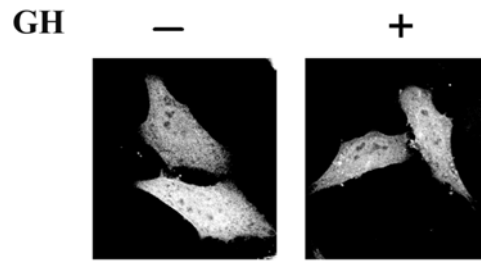


Figure 39: Cellular localization of STAT5a-RY-GFP in the presence of GH (Iyer and Reich, 2007)

HeLa cells expressing GHR and STAT5a-RY-GFP were untreated (-) or treated (+) with GH for 30 minutes. The cellular localization was evaluated by confocal microscopy.

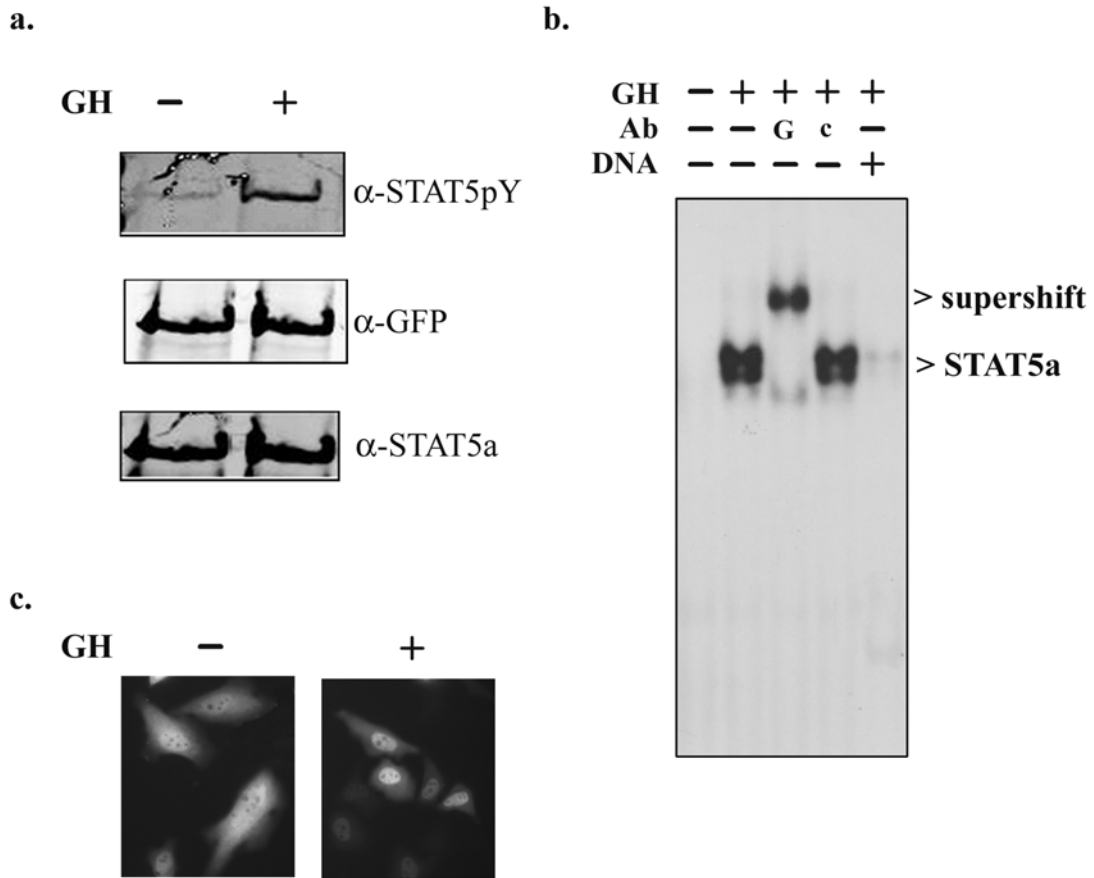


Figure 40: Characterization of STAT5a-EE-GFP

a. Western blot analysis of protein lysates from HeLa cells expressing STAT5a-EE-GFP treated with GH was performed with antibodies to phosphorylated STAT5a (α-STAT5pY), GFP (α-GFP) or STAT5a (α-STAT5a).

b. EMSA with HeLa lysates expressing STAT5a-EE-GFP from untreated cells (-) or cells treated with GH (+). Specific antibody (Ab) to GFP (G), or a control antibody (c) was added to the DNA-binding reactions. As a specific competitor, 100-fold excess unlabeled DNA was added (DNA) to the reaction.

c. HeLa cells expressing GHR and STAT5a-EE-GFP were untreated (-) or treated (+) with GH for 30 minutes. The cellular localization was evaluated by confocal microscopy.

The cellular localization of this protein was also evaluated in HeLa cells both prior to and following tyrosine phosphorylation (Figure 40c). Before treatment with GH, the protein was present in the nucleus and cytoplasm of cells and following stimulation with GH, the protein accumulated in the nucleus. Thus even though these residues are necessary for DNA binding in STAT1, they are not required for the DNA binding ability of STAT5a.

I then analyzed the DNA binding ability of another mutant of STAT5a that was originally created to evaluate nuclear import. In this mutant, a motif in the DNA binding domain that was rich in basic amino acids (422-KRIKRADRR-430) was mutated such that all the basic residues were replaced by alanines to generate STAT5a-KR-GFP. This mutant was efficiently phosphorylated in response to GH as demonstrated by Western blotting (Figure 41a). Tyrosine phosphorylation was detected only after GH stimulation by using antibodies specific to phosphorylated STAT5a. I also analyzed the DNA binding ability of this mutant by performing EMSAs on cell lysates expressing STAT5a-KR-GFP (Figure 41b). The results of the EMSA analysis revealed that this mutant was unable to bind DNA even though it was efficiently tyrosine phosphorylated following GH treatment.

To assess the effect of DNA binding on the nuclear accumulation of phosphorylated STAT5a, the cellular localization of STAT5a-KR-GFP was evaluated prior to and following GH stimulation (Figure 41c). The cellular localization of HeLa cells expressing STAT5a-KR-GFP revealed that the protein was present in the nucleus and cytoplasm in the absence of any cytokine stimulation. However, following treatment with GH, STAT5a-KR-GFP failed to show the nuclear accumulation that is observed with phosphorylated wild type STAT5a. Thus the ability to bind DNA is necessary for the nuclear accumulation of tyrosine phosphorylated STAT5a.

Some of the basic residues in this motif of STAT5a that are conserved in STAT1 and STAT2 have been shown to play critical roles in nuclear import in addition to DNA binding (Fagerlund et al., 2002; Melen et al., 2001). In the case of STAT5a, the basic residues do not seem to affect the import of STAT5a.

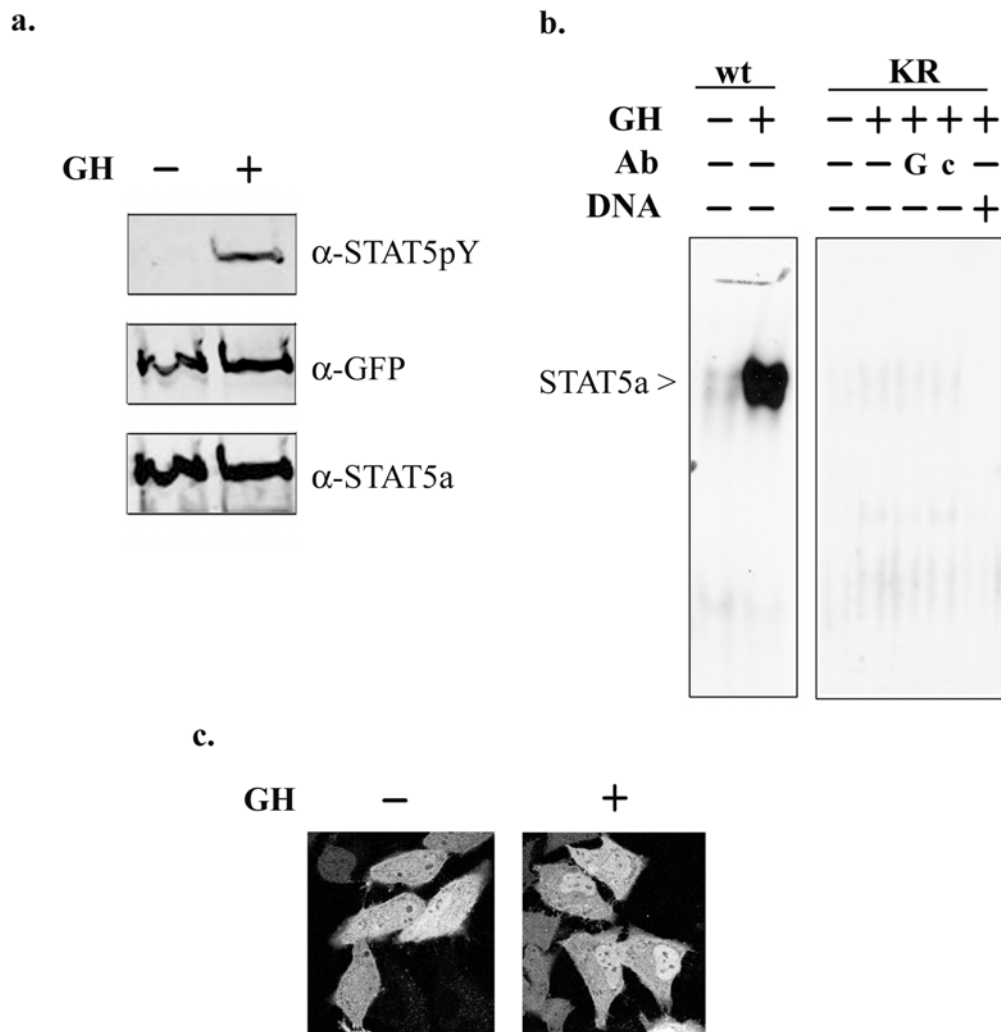


Figure 41: Characterization of STAT5a-KR-GFP (Iyer and Reich, 2007)

a. Western blot analysis of protein lysates from HeLa cells expressing STAT5a-KR-GFP treated with GH was performed with antibodies to phosphorylated STAT5a (α-STAT5pY), GFP (α-GFP) or STAT5a (α-STAT5a).

b. EMSA with HeLa lysates expressing STAT5a-KR-GFP from untreated cells (-) or cells treated with GH (+). Specific antibody (Ab) to GFP (G), or a control antibody (c) was added to the DNA-binding reactions. As a specific competitor, 100-fold excess unlabeled DNA was added (DNA) in the reaction.

c. HeLa cells expressing GHR and STAT5a-KR-GFP were untreated (-) or treated (+) with GH for 30 minutes. The cellular localization was evaluated by confocal microscopy.

Mobility of phosphorylated STAT5a is reduced in the nucleus

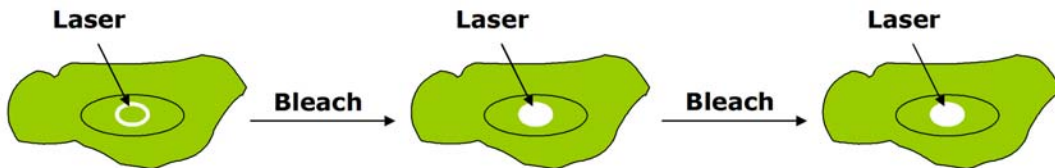
Tyrosine phosphorylated STAT5a has the ability to bind DNA and it is possible that this would result in the reduced mobility of STAT5a in the nucleus. To determine the mobility of STAT5a in the nucleus, the FLIP photobleaching technique was employed in the nucleus of living cells expressing STAT5a-GFP before and following GH stimulation. The FLIP technique involves focusing a high intensity laser continuously in a ROI of a living cell (Figure 42). Any fluorescent molecules passing through the ROI will be bleached resulting in a loss of fluorescence intensity in that region. If the proteins are not mobile then the loss in fluorescence intensity will be limited to the ROI in the cell (Figure 42a). On the other hand, if the fluorescent proteins are mobile, the loss in fluorescence intensity will not be restricted to the ROI and will spread throughout the cell (Figure 42b).

The mobility of unphosphorylated STAT5a-GFP in the nucleus was first evaluated in HeLa cells expressing STAT5a-GFP by performing a FLIP in the nucleus (Figure 43a and 43b, top panel). A small region of interest (ROI) in the nucleus was selected and a high intensity laser was continuously directed to this ROI. Since the action of the laser was continuous, any fluorescent molecules passing through this region would be bleached. The FLIP analysis revealed that the fluorescence of unphosphorylated STAT5a was lost throughout the nucleus in ~2 min. This result demonstrates the rapid movement of unphosphorylated STAT5a in the nucleus.

A similar analysis was performed in cells treated with GH (Figure 43a and 43b, bottom panel). GH treatment resulted in the tyrosine phosphorylation and nuclear accumulation of STAT5a. When a FLIP was performed in a small ROI in the nucleus, the pattern of fluorescence loss in the nucleus was significantly different from that observed with unphosphorylated STAT5a. The region of the nucleus that was exposed to the laser rapidly lost its fluorescence intensity, whereas the other areas of the nucleus retained fluorescence for a significant period of time. This result suggests that tyrosine phosphorylated STAT5a has slower mobility in the nucleus.

Fluorescence Loss In Photobleaching (FLIP)

a. Low mobility proteins



b. High mobility proteins

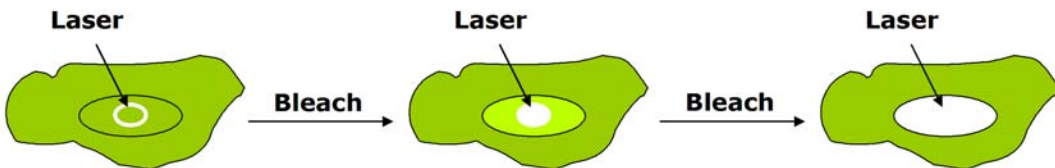


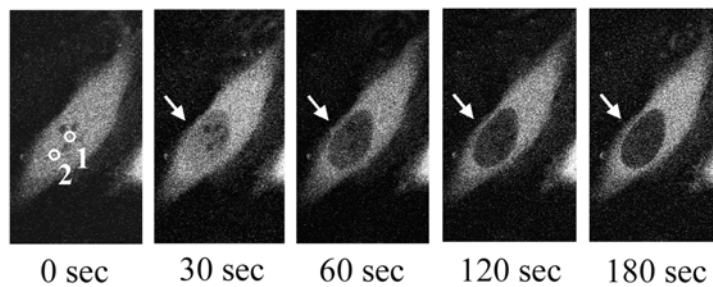
Figure 42: Principle behind the FLIP photobleaching technique

In the FLIP technique, a region in the cell (white circle) expressing a fluorescently tagged protein is subjected to a continuous high intensity laser beam resulting in the bleaching of fluorescence in that region.

a. If the fluorescent protein has low mobility, the loss in fluorescence will be restricted to the ROI (white circle)

b. If the fluorescent protein has high mobility, the loss in fluorescence will not be limited to the ROI (white circle) and spread beyond the ROI.

Unphosphorylated STAT5a-GFP



Phosphorylated STAT5a-GFP

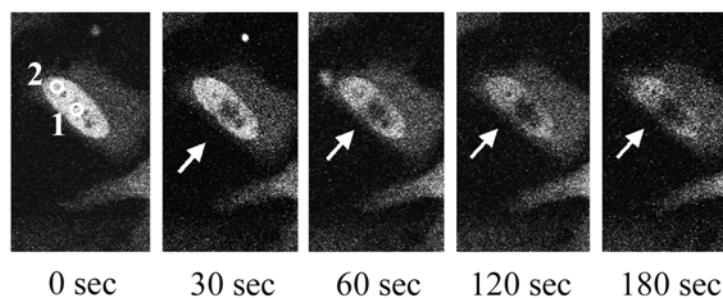


Figure 43a: Live cell imaging with nuclear FLIP reveals reduced movement of phosphorylated STAT5a-GFP (Iyer and Reich, 2007)

Nuclear FLIP in a cell expressing unphosphorylated STAT5a-GFP (top panel) or tyrosine-phosphorylated STAT5a-GFP (bottom panel). The laser was directed to ROI 1 in both cases.

b.

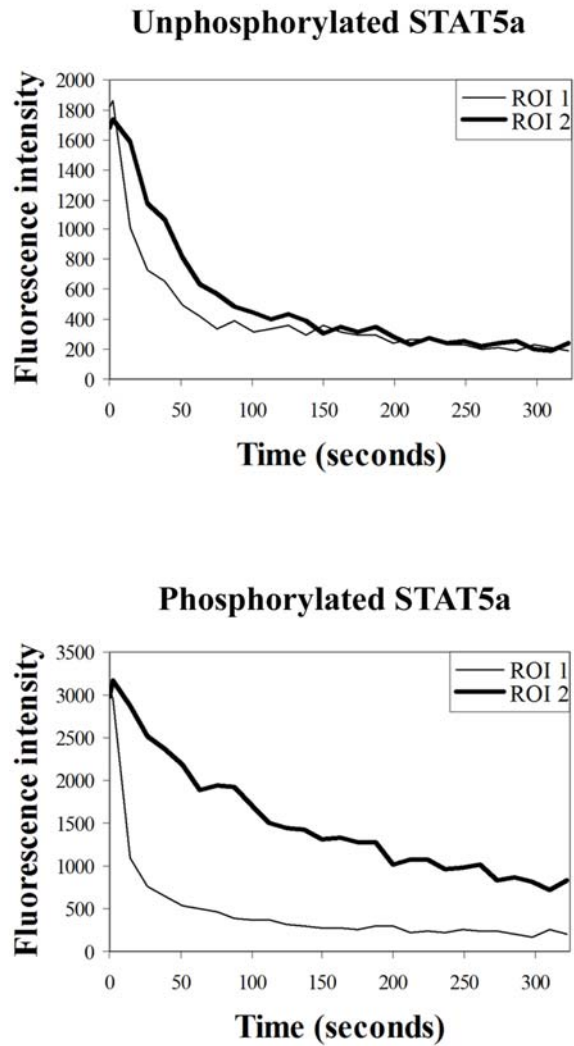


Figure 43b: Live cell imaging with nuclear FLIP reveals reduced movement of phosphorylated STAT5a-GFP (Iyer and Reich, 2007)

Quantitative measure of fluorescence intensity at a focal point of the laser (ROI 1) compared with a distant site in nucleus (ROI 2) for images presented in (a).

To further evaluate whether the reduction in the mobility of tyrosine-phosphorylated STAT5a in the nucleus was due to DNA binding, I tested the behavior of a mutant of STAT5a that was efficiently tyrosine phosphorylated but was unable to bind DNA. HeLa cells were transfected with a DNA binding mutant of STAT5a, STAT5a-KR-GFP, and the mobility of this mutant in the nucleus was first analyzed in the absence of GH stimulation. By performing a FLIP in a small region in the nucleus, I observed that the pattern of fluorescence loss in the nucleus was very similar to unphosphorylated wild type STAT5a-GFP (Figure 44). However, when the FLIP analysis was performed in the nucleus of cells expressing STAT5a-KR-GFP after GH treatment, the pattern of fluorescence loss in the nucleus was significantly different from that observed with phosphorylated wild type STAT5a. Phosphorylated STAT5a-KR-GFP showed a higher mobility than phosphorylated STAT5a-GFP as evidenced by the rapid bleaching of fluorescence in the entire nucleus (Figure 44). Thus DNA binding is the cause of nuclear retention of STAT5a following tyrosine phosphorylation.

Summary

The current study explores the nuclear import of STAT5a both before phosphorylation and after tyrosine phosphorylation. Unphosphorylated STAT5a is present in the cytoplasm and nucleus of cells and can be constitutively imported into the nucleus of living cells. The mechanism that regulates transport of STAT5a through the nuclear pore complexes into the nucleus is independent of tyrosine phosphorylation. A region within the coiled coil domain of STAT5a is necessary for the nuclear import of both unphosphorylated and phosphorylated STAT5a. Following tyrosine phosphorylation, STAT5a accumulates in the nucleus. Studies performed in living cells indicate that the property of DNA binding is critical for this nuclear accumulation. Thus tyrosine phosphorylated STAT5a accumulates into the nucleus due to its retention by DNA binding.

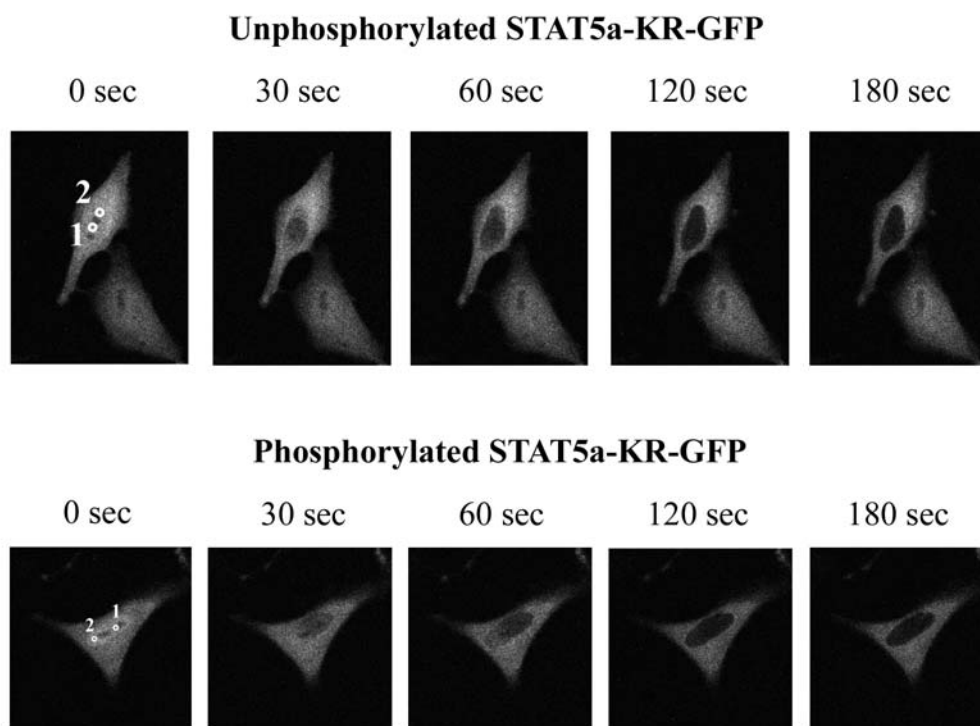


Figure 44a: Live cell imaging with nuclear FLIP in cells expressing STAT5a-KR-GFP

Nuclear FLIP in a cell expressing unphosphorylated STAT5a-KR-GFP (top panel) or tyrosine-phosphorylated STAT5a-KR-GFP (bottom panel) (Iyer and Reich, 2007). The laser was directed to ROI 1 in both cases.

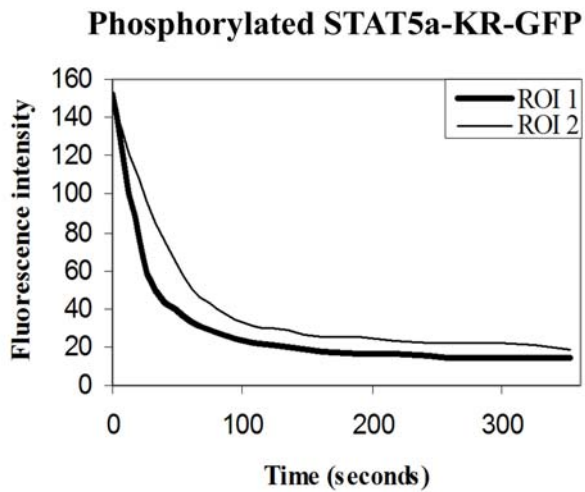
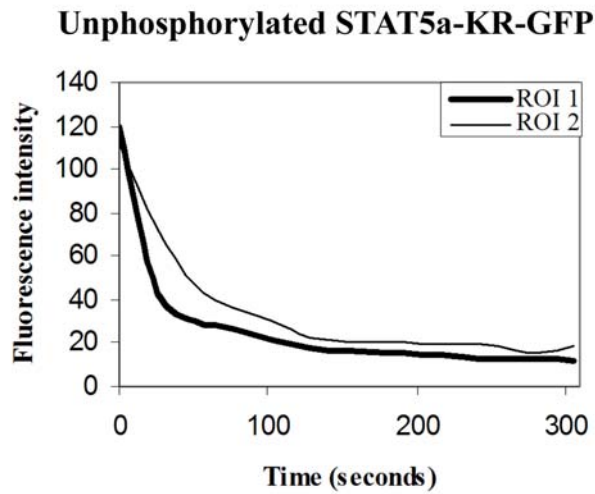


Figure 44b: Live cell imaging with nuclear FLIP in cells expressing STAT5a-KR-GFP

Quantitative measurement of fluorescence intensity at a focal point of the laser (ROI 1) compared with a distant site in nucleus (ROI 2) for images presented in (a) (Iyer and Reich, 2007).

Chapter 4

Characterization of Nuclear Export of the STAT5a Transcription Factor

Abstract

Latent STAT5a is present in the nucleus and cytoplasm of cells and accumulates in the nucleus following activation by tyrosine phosphorylation. This chapter explores the nuclear export of STAT5a both before and after tyrosine phosphorylation. Studies performed using living cells demonstrated that unphosphorylated STAT5a is constitutively exported out of the nucleus. This nuclear export of unphosphorylated STAT5a is independent of tyrosine phosphorylation. Further studies revealed that the exportin CRM1 did not play a significant role in the nuclear export of unphosphorylated STAT5a.

Cytokine stimulation results in the tyrosine phosphorylation and nuclear accumulation of STAT5a. The nuclear export of tyrosine phosphorylated STAT5a is slower than unphosphorylated STAT5a as demonstrated by studies performed in living cells. On removal of the cytokine, STAT5a starts appearing back to the cytoplasm presumably due to dephosphorylation and nuclear export. This nuclear export of STAT5a can be delayed without affecting its dephosphorylation kinetics in the presence of the CRM1 inhibitor; Leptomycin B. Studies with a DNA binding mutant of STAT5a also revealed that CRM1 mediates its nuclear export only after cytokine stimulation.

The NES activity of two candidate NES sequences in the DNA binding domain of STAT5a was studied. One of these sequences is recognized by CRM1 while the other one is not. The positioning of the NESs might suggest that it is accessible to proteins that mediate nuclear export when STAT5a is not bound to DNA. These findings should aid in the development of specific methods that involve targeting the activity of STAT5a.

Results

Nuclear export of unphosphorylated STAT5a

Unphosphorylated STAT5a is present in the nucleus and cytoplasm of cells. To determine whether it was being actively exported from the nucleus into the cytoplasm, the photobleaching technique Fluorescence Loss In Photobleaching (FLIP) was employed (Figure 45). In this technique, a small region of interest (ROI) in the cytoplasm was subjected to a high intensity laser which would bleach any fluorescent molecules passing through the ROI. If the molecules are highly mobile in the cytoplasm, the fluorescence intensity of entire cytoplasm is expected to decrease with time. Similarly, if the protein is exported out of the nucleus into the cytoplasm, the fluorescence intensity of the nucleus would also decrease with time even though the activity of the laser was restricted to a region in the cytoplasm.

The nuclear export of a small protein like GFP was evaluated before studying the nuclear export of STAT5a. GFP is a 27kDa protein that can freely diffuse through the nuclear pore complex (NPC). Hence its nuclear export should be fairly rapid. I analyzed the nuclear export of GFP by performing a FLIP in a small region in the cytoplasm of HeLa cells expressing GFP. As shown in figure 46, by 15 minutes the fluorescence of the entire cell including the nucleus was lost. Thus, based on the FLIP technique, it took ~15 minutes for the fluorescence of a small molecule like GFP to be lost from the entire cell.

STAT5a (92 kDa) is significantly larger than GFP in molecular mass and this prevents it from exiting the nucleus by diffusion. Thus its rate of nuclear export should be slower than GFP. To determine whether this was indeed the case, FLIP was performed in a small region in the cytoplasm of HeLa cells expressing unphosphorylated STAT5a-GFP. As shown in figure 47, the fluorescence intensity in the entire cytoplasm was decreased by 10 minutes, indicating the rapid movement of STAT5a in the cytoplasm. However, it took 75 minutes for the nuclear fluorescence intensity to be lost suggesting that STAT5a was exported out of the nucleus at a rate slower than GFP. To rule out the possibility, that the decrease in nuclear fluorescence

Fluorescence Loss In Photobleaching (FLIP)

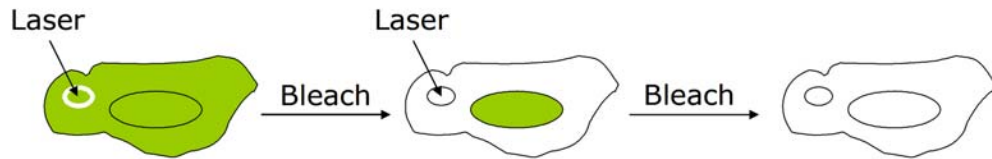


Figure 45: Principle of the FLIP photobleaching technique

In the FLIP technique, a region in the cell (white circle) expressing a fluorescently tagged protein is subjected to a continuous high intensity laser beam resulting in the bleaching of fluorescence in that region. Since the laser is constantly passing through the cell, if the fluorescent protein is mobile then the loss in fluorescence will spread throughout the cell.

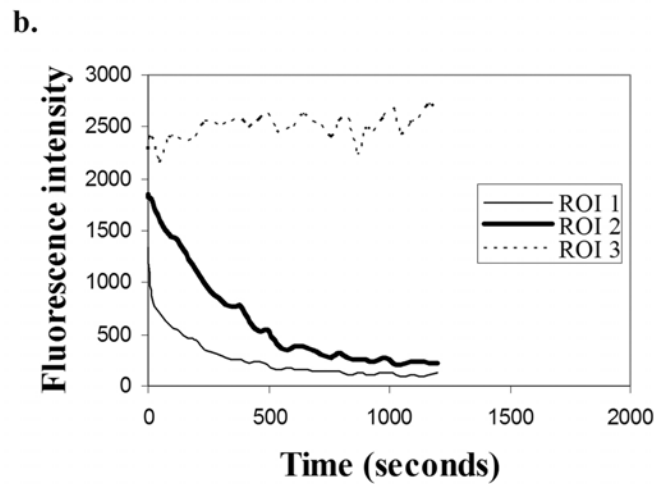
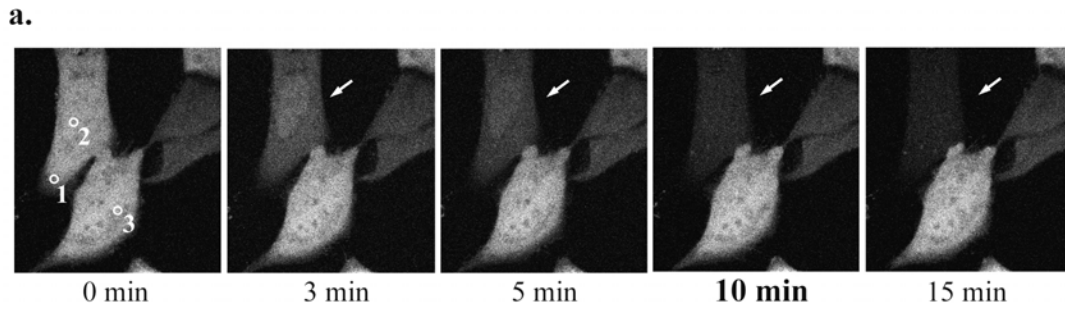
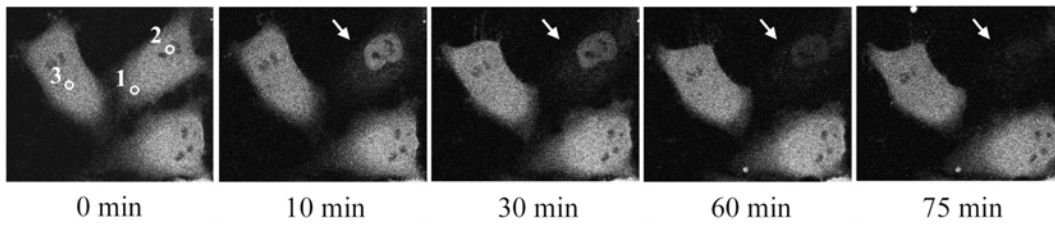


Figure 46: Cytoplasmic FLIP in cells expressing GFP (Iyer and Reich, 2007)

a. ROI 1 in the cytoplasm of a cell expressing GFP was subjected to continuous high-intensity laser pulses. The fluorescence intensity of the cell was monitored with time.

b. Quantitative measurement of relative fluorescence in the cytoplasmic focal point of the bleached cell (ROI 1) relative to a site in the nucleus (ROI 2) of the bleached cell or a site in an adjacent control cell (ROI 3) shown in (a).

a.



b.

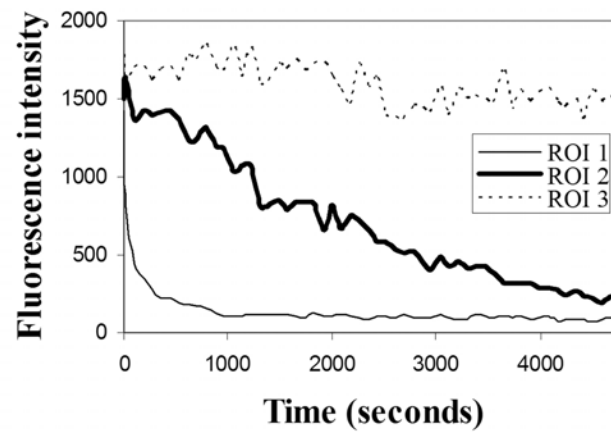


Figure 47: Cytoplasmic FLIP in cells expressing unphosphorylated STAT5a-GFP (Iyer and Reich, 2007)

a. ROI 1 in the cytoplasm of a cell expressing unphosphorylated STAT5a-GFP was subjected to continuous high-intensity laser pulses. The fluorescence intensity of the cell was monitored with time.

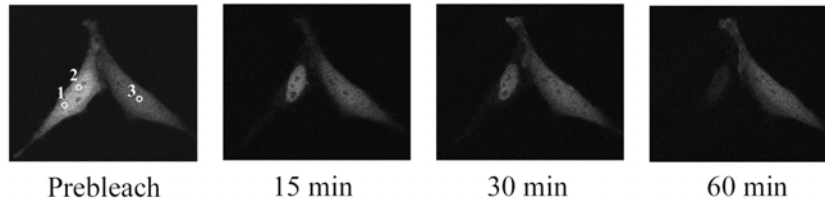
b. Quantitative measurement of relative fluorescence in the cytoplasmic focal point of the bleached cell (ROI 1) relative to a site in the nucleus (ROI 2) of the bleached cell or a site in an adjacent control cell (ROI 3) shown in (a).

was a result of protein degradation, a similar experiment was performed in the presence of the proteasome inhibitor MG132 (data not shown). The results obtained indicated that the inhibitor had no effect on the loss of fluorescence intensity of STAT5a-GFP in the nucleus. Thus unphosphorylated STAT5a is constitutively exported out of the nucleus.

To determine whether the nuclear export of STAT5a was independent of tyrosine phosphorylation, the nuclear export of a mutant of STAT5a, STAT5a-RY-GFP, was studied by performing a cytoplasmic FLIP (Figure 48). This mutant of STAT5a does not have the ability to be phosphorylated and is unable to associate with other STATs *via* reciprocal phosphotyrosine and SH2 domain interactions. A small region in the cytoplasm of HeLa cells expressing STAT5a-RY-GFP was subjected to a continuous high intensity laser beam. As shown in figure 48, the fluorescence of the cytoplasm was lost in 15 minutes while the nuclear fluorescence intensity disappeared by 70 minutes. This pattern was similar to wild type unphosphorylated STAT5a. Thus the constitutive nuclear export of STAT5a is independent of tyrosine phosphorylation.

CRM1, also known as exportin1, is one of the most well characterized exportins that can mediate the nuclear export of proteins. The export activity of CRM1 can be inhibited by the antibiotic Leptomycin B (LMB) (Kudo et al., 1998). To evaluate the role of CRM1 on the nuclear export of unphosphorylated STAT5a, I analyzed the nuclear export of STAT5a in living cells in the presence of LMB (Figure 49). HeLa cells expressing unphosphorylated STAT5a were subjected to cytoplasmic FLIP in the presence of LMB. As shown in figure 49, continuous bleaching of a small region in the cytoplasm results in a decrease in the cytoplasmic fluorescence of STAT5a by 10 minutes. The nuclear fluorescence also decreased with time and could not be detected by 50 minutes of cytoplasmic FLIP. Thus the presence of LMB did not affect the nuclear export of unphosphorylated STAT5a. These results suggest that CRM1 does not play a significant role in the nuclear export of unphosphorylated STAT5a.

a.



b.

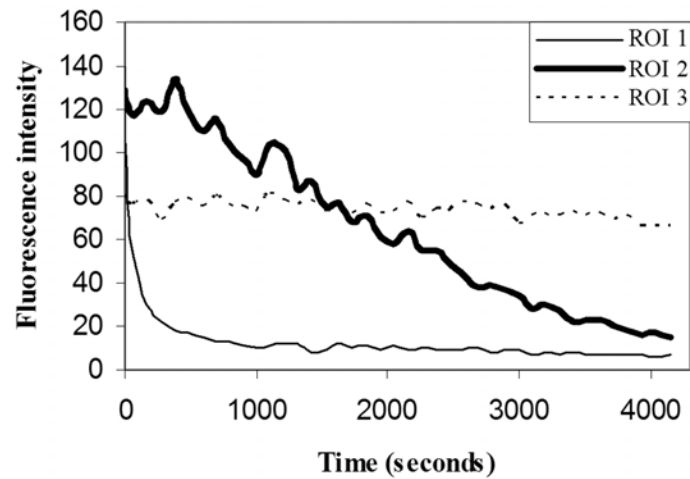
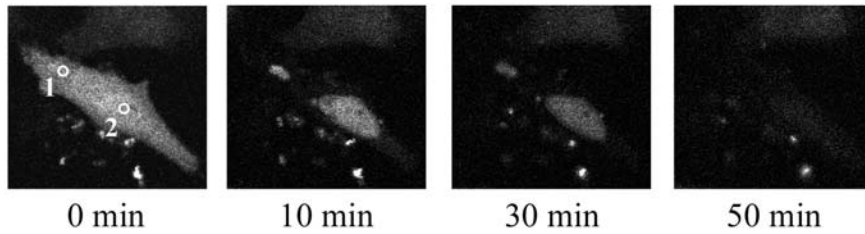


Figure 48: Cytoplasmic FLIP in cells expressing STAT5a-RY-GFP

a. ROI 1 in the cytoplasm of a cell expressing STAT5a-RY-GFP was subjected to continuous high-intensity laser pulses. The fluorescence intensity of the cell was monitored with time.

b. Quantitative measurement of relative fluorescence in the cytoplasmic focal point of the bleached cell (ROI 1) relative to a site in the nucleus (ROI 2) of the bleached cell or a site in an adjacent control cell (ROI 3) shown in (a).

a.



b.

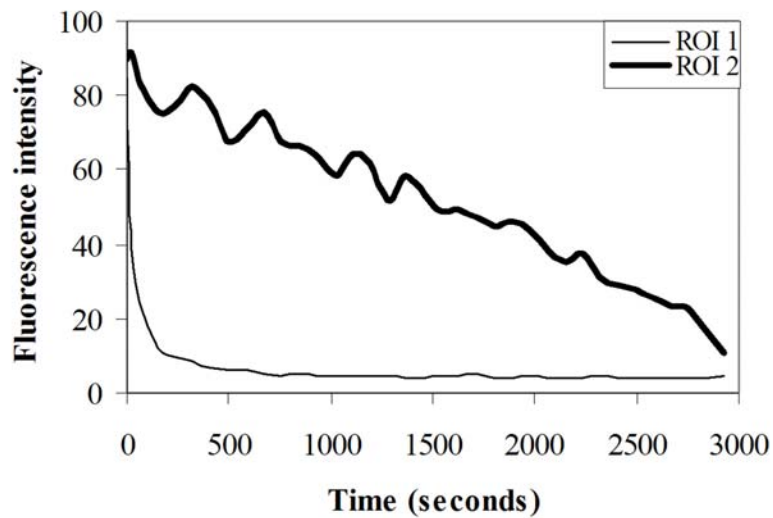


Figure 49: Live cell imaging to evaluate nuclear export of STAT5a-GFP in the presence of LMB

a. ROI 1 in the cytoplasm of a cell expressing STAT5a-GFP and treated with 10 nM LMB, was subjected to continuous high-intensity laser pulses.

b. Quantitative measurement of relative fluorescence in the cytoplasmic focal point of the bleached cell (ROI 1) relative to a site in the nucleus (ROI 2) of the bleached cell.

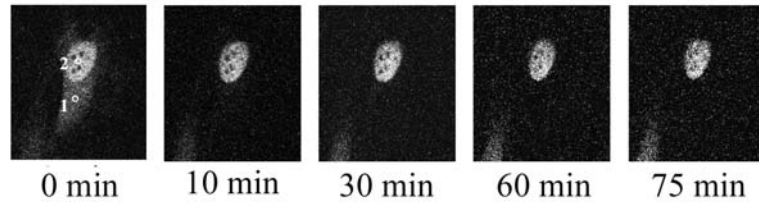
Nuclear export of tyrosine phosphorylated STAT5a

Cytokine stimulation results in the tyrosine phosphorylation and nuclear accumulation of STAT5a (Figures 17 and 18). I have already demonstrated that following tyrosine phosphorylation, the mobility of STAT5a is reduced in the nucleus due to DNA binding (Figures 43 and 44). Since tyrosine phosphorylated STAT5a is retained in the nucleus by DNA binding, it can be assumed that the nuclear export of tyrosine phosphorylated STAT5a would be slower than unphosphorylated STAT5a. As depicted in figure 47, the nuclear fluorescence of unphosphorylated STAT5a was lost by 75 minutes of performing FLIP in a small region in the cytoplasm. To study the nuclear export of tyrosine phosphorylated STAT5a, I used a constitutively active mutant of STAT5a which shows a higher basal level of phosphorylation in the absence of cytokine stimulation. This mutant was used to ensure that majority of the STAT5a molecules were tyrosine phosphorylated.

Two amino acids, H298 and S711 were mutated to R and F respectively to obtain the constitutively active form of STAT5a (STAT5a-HS-GFP) (Kawashima et al., 2006). It has been hypothesized that this mutant adopts a conformation that is resistant to dephosphorylation. FLIP was performed in a small region in the cytoplasm of cells expressing STAT5a-HS-GFP following treatment with growth hormone (GH) (Figure 50). The results indicated that even though there was a steady decrease in the nuclear fluorescence of STAT5a, fluorescence in the nucleus could still be detected after 75 minutes of performing the FLIP. Thus it can be concluded that phosphorylated STAT5a is also exported out of the nucleus but there is a decrease in the rate of export in comparison to unphosphorylated STAT5a.

To determine whether this nuclear export of tyrosine phosphorylated STAT5a was mediated by CRM1, I performed a similar cytoplasmic FLIP in cells treated with GH and LMB. Unfortunately, the experiment could not be completed due to the sudden death of cells by apoptosis during the FLIP analysis (data not shown). This is surprising because such cell death was not observed when the cells were left untreated or treated with GH or LMB alone. Thus this technique could not be used successfully to evaluate the contribution of CRM1 on the nuclear export of

a.



b.

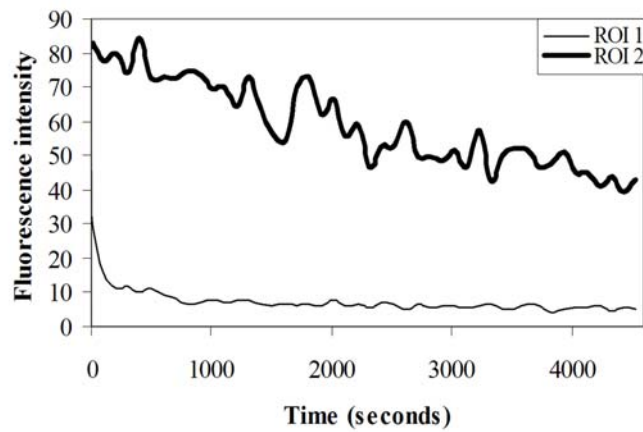


Figure 50: Live cell imaging to evaluate nuclear export of tyrosine phosphorylated STAT5a-HS-GFP (Iyer and Reich, 2007)

a. ROI 1 in the cytoplasm of a cell expressing STAT5a-HS-GFP was subjected to continuous high-intensity laser pulses.

b. Quantitative measurement of relative fluorescence in the cytoplasmic focal point of the bleached cell (ROI 1) relative to a site in the nucleus (ROI 2) of the bleached cell.

phosphorylated STAT5a. Hence it is not clear if CRM1 can mediate the export of tyrosine phosphorylated STAT5a.

Nuclear export of STAT5a following GH removal

In resting cells, there is an equilibrium between the activity of cellular kinases and phosphatases. Binding of a cytokine to its cognate receptor leads to the activation of cellular tyrosine kinases. This in turn results in an increase in the kinase activity and thereby the tyrosine phosphorylation of STAT5a. It should be noted that even in the presence of cytokines, cellular tyrosine phosphatases can continuously dephosphorylate tyrosine phosphorylated STAT5a but the rate of dephosphorylation is significantly lesser than the rate of phosphorylation. This difference in rates results in an overall increase in the population of phosphorylated STAT5a in the cell. As long as the cytokine is present, the kinases are active and STAT5a is continuously phosphorylated (Figure 35). Once the cytokine is removed, the tyrosine kinases are no longer activated and hence subsequent phosphorylation of STAT5a ceases. This results in a steady decrease in the rate of phosphorylation. Since phosphorylated STAT5a can be continuously dephosphorylated by phosphatases there is a steady decrease in the population of phosphorylated STAT5a.

This reduction in tyrosine phosphorylation of STAT5a after GH withdrawal was evaluated by performing Western Blotting on cell lysates (Figure 51). HeLa cells were co-transfected with STAT5a-GFP and GH receptor and stimulated with GH for 45 minutes. The GH was then removed and cells were harvested at various time points and subsequently lysed. The phosphorylation of STAT5a was then determined by Western Blotting by using antibodies that detect specific phosphorylation of STAT5a on tyrosine 694. Phosphorylation was apparent 1.5 hours after GH withdrawal but there was a significant decrease in phosphorylation by 2 hours. By 4 hours, a very low level of phosphorylation was detected. Thus, following the removal of GH, there is a steady decrease in the levels of phosphorylated STAT5a.

Unphosphorylated STAT5a is present in the nucleus and cytoplasm while tyrosine phosphorylated STAT5a accumulates in the nucleus. Since following GH

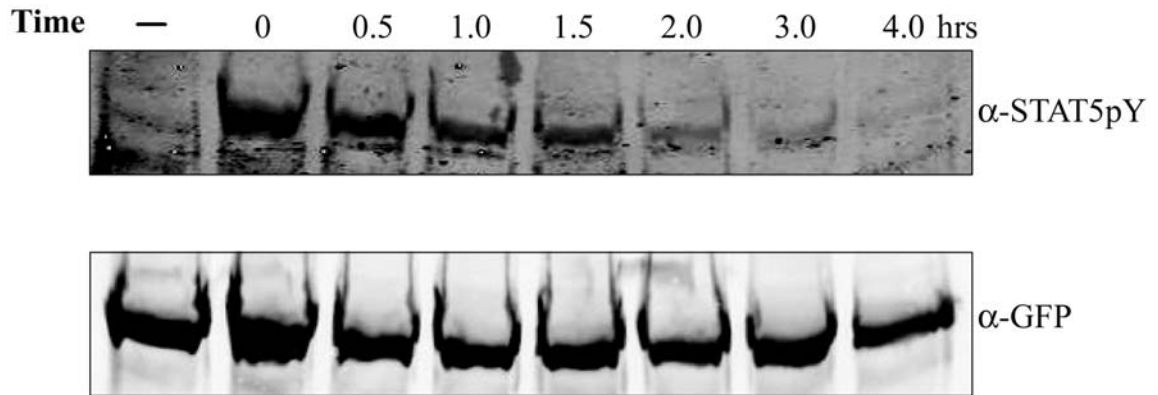


Figure 51: Phosphorylation kinetics of STAT5a-GFP after GH removal

HeLa cells expressing STAT5a-GFP were serum deprived and treated with 1 ng/mL GH for 45 minutes. After 45 minutes, the GH was removed and cells were harvested at different times after GH removal and lysed. Western blot analysis of protein lysates was performed with antibodies to phosphorylated STAT5a (α -STAT5pY) or GFP (α -GFP).

removal there is a steady decrease in the phosphorylation levels of STAT5a, it would be predicted that there would be a decrease in the nuclear accumulation of STAT5a.

To test this possibility, HeLa cells expressing STAT5a-GFP were stimulated with GH for 45 minutes. After 45 minutes, the GH was removed and the cellular localization of STAT5a was evaluated with time. As shown in figure 52, STAT5a-GFP accumulated in the nucleus following GH treatment. Following GH removal, STAT5a appeared nuclear for 2 hours. By 3 hours of GH withdrawal, considerable fluorescence was present in the cytoplasm and by 4 hours, the localization was similar to unphosphorylated STAT5a. As shown in figure 51, the phosphorylation of STAT5a was significantly reduced by 3 hours and was barely detectable by 4 hours of GH withdrawal. Thus there is some correlation between the phosphorylation levels and nuclear accumulation of STAT5a.

To evaluate whether CRM1 mediated the export of STAT5a following the removal of GH, a similar experiment was performed in the presence of LMB. Cells were treated with GH and LMB for 45 minutes. After treatment, the GH was removed and the localization pattern of STAT5a was analyzed in the presence of LMB. Figure 53 shows the localization pattern of STAT5a following GH withdrawal and in the presence of LMB. In the presence of LMB, STAT5a appeared nuclear even after 4 hours of GH withdrawal. After 6 hours of removal of GH, there was a considerable decrease in the nuclear fluorescence but the overall localization pattern was still not similar to unphosphorylated STAT5a. Thus in the presence of LMB, STAT5a was nuclear for a longer period of time after GH withdrawal. It is possible that this extended nuclear localization of STAT5a in the presence of LMB was either due to a delay in the dephosphorylation of STAT5a following GH removal or due to an inhibition of nuclear export.

To determine if LMB treatment affected the tyrosine phosphorylation of STAT5a after GH withdrawal, the phosphorylation level of STAT5a was evaluated after removal of GH in the presence of LMB (Figure 54). The results showed that phosphorylation was apparent 1.5 hours after GH withdrawal and there was a

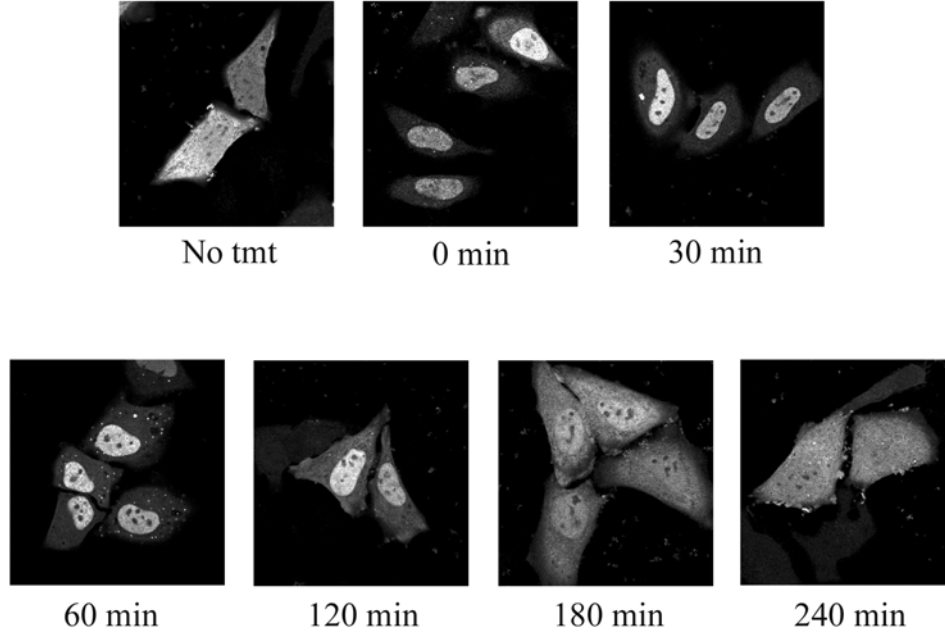


Figure 52: Localization kinetics of STAT5a-GFP after GH removal

HeLa cells expressing STAT5a-GFP were serum deprived and treated with 1 ng/mL GH for 45 minutes. The GH was then removed and the localization of STAT5a was evaluated with time using confocal microscopy.

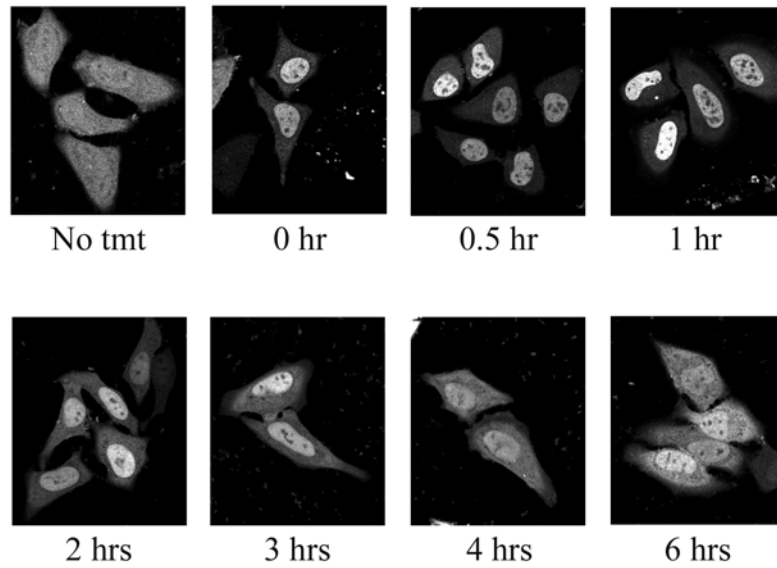


Figure 53: Localization kinetics of STAT5a-GFP in the presence of LMB after GH removal

HeLa cells expressing STAT5a-GFP were serum deprived and treated with 1 ng/mL GH and 10 nM LMB for 45 minutes. The GH was then removed but LMB was left behind and the localization of STAT5a was evaluated with time using confocal microscopy.

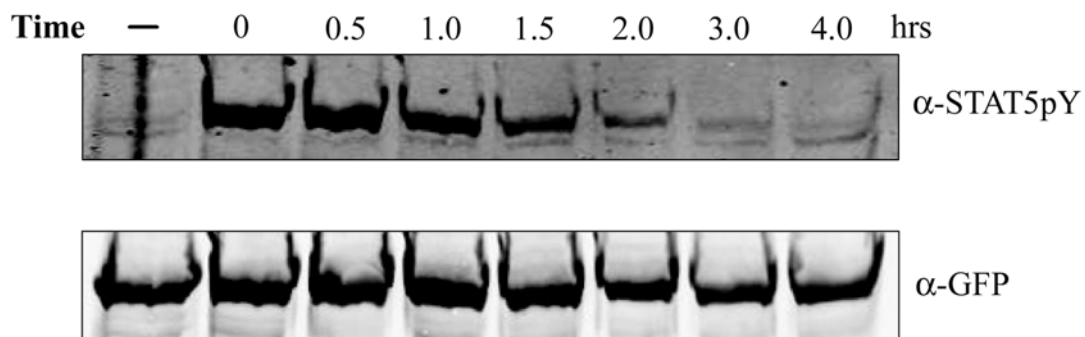


Figure 54: Phosphorylation kinetics of STAT5a-GFP after GH removal and in the presence of LMB

HeLa cells expressing STAT5a-GFP were serum deprived and treated with 1 ng/mL GH and 10 nM LMB for 45 minutes. After 45 minutes, the GH was removed but LMB was left behind. Cells were harvested at different times after GH removal and lysed. Western blot analysis of the cell lysates was performed with antibodies to phosphotyrosine 694 STAT5 (α -STAT5pY) or GFP (α -GFP).

significant decrease in phosphorylation after 2 hours of GH withdrawal. By 4 hours of a very low level of phosphorylation of STAT5a was detected. This pattern of phosphorylation was very similar to the results obtained in the absence of LMB (Figure 50) indicating that presence of LMB had no effect on the dephosphorylation of STAT5a after GH withdrawal. Hence, it can be concluded that STAT5a appeared nuclear for a longer period of time in the presence of LMB due to an inhibition of nuclear export.

Since, in the presence of LMB, STAT5a appeared nuclear after 4 hours of GH withdrawal even though the tyrosine phosphorylation of STAT5a at this time was very weak, it is possible that CRM1 plays a role in the nuclear export of dephosphorylated STAT5a. I have already shown that CRM1 does not play a significant role in the nuclear export of unphosphorylated STAT5a (Figure 49). To explain this observation, we might have to consider the possibility that dephosphorylated STAT5a is present in a conformation that is different from unphosphorylated STAT5a. As explained in Chapter 1, both unphosphorylated and phosphorylated STATs are dimeric but show very different conformations (Figures 8 and 9). Once phosphorylated STATs are dephosphorylated, they would again have to go through extensive spatial rearrangement to return to the conformation of the unphosphorylated protein (Zhong et al., 2005). Thus, it is possible that CRM1 can recognize the conformation adopted by dephosphorylated STAT5a and mediate its nuclear export. Since not much is known about the various conformation adopted by STAT5a, this hypothesis would be difficult to prove. Hopefully, in the future more information will be available so that experiments can be designed to test this hypothesis.

Effect of DNA binding on the nuclear export of STAT5a

In chapter 3, I have shown that DNA binding plays a critical role in the nuclear accumulation of tyrosine phosphorylated STAT5a. This was determined by evaluating the cellular localization of STAT5a-KR-GFP, a mutant of STAT5a that was defective in DNA binding (Figure 41). In response to GH, STAT5a-KR-GFP

failed to show the nuclear accumulation that was observed with wild type STAT5a (Figure 41). Following tyrosine phosphorylation, this mutant also failed to show a decrease in mobility in the nucleus of cells that was observed with phosphorylated wild type STAT5a (Figures 43 and 44). It is possible that since STAT5a-KR-GFP was unable to bind DNA, it could be exported out of the nucleus which would result in a lack of nuclear accumulation.

To determine whether this mutant was exported out of the nucleus by CRM1 following tyrosine phosphorylation, I determined its cellular localization in the presence and absence of GH stimulation, before and after LMB treatment (Figure 55). No difference in the localization pattern of unphosphorylated STAT5a-KR-GFP was observed with treatment with LMB suggesting that CRM1 was not involved in the nuclear export of unphosphorylated STAT5a-KR-GFP. This result reinforces the notion that CRM1 does not play a significant role in the nuclear export of unphosphorylated STAT5a. However, when cells were treated with GH, nuclear accumulation of STAT5a-KR-GFP was observed only in the presence of LMB. Thus, in the presence of LMB, phosphorylated STAT5a-KR-GFP showed a nuclear accumulation that was not observed in the absence of LMB. This would suggest that CRM1 was able to mediate the nuclear export of tyrosine phosphorylated STAT5a-KR-GFP. Further experiments need to be performed to determine whether CRM1 can mediate the nuclear export of phosphorylated wild type STAT5a.

Identification of Nuclear Export Signals in STAT5a

Nuclear export signals (NESs) that are recognized by CRM1 usually conform to the consensus sequence LxxxLxxLxL, where L is leucine and x is any amino acid (Fornerod et al., 1997). There are many leucine rich sequences in STAT5a that loosely resemble NESs. Instead of analyzing all these putative NESs, I decide to test candidate NESs in STAT5a based on studies performed with other STAT members. NESs have been identified in some STATs like STAT1, 2 and 3 (Banninger and Reich, 2004; Bhattacharya and Schindler, 2003; McBride et al., 2000). The NES in STAT1 is present in the DNA binding domain (amino acids 399-410) and the

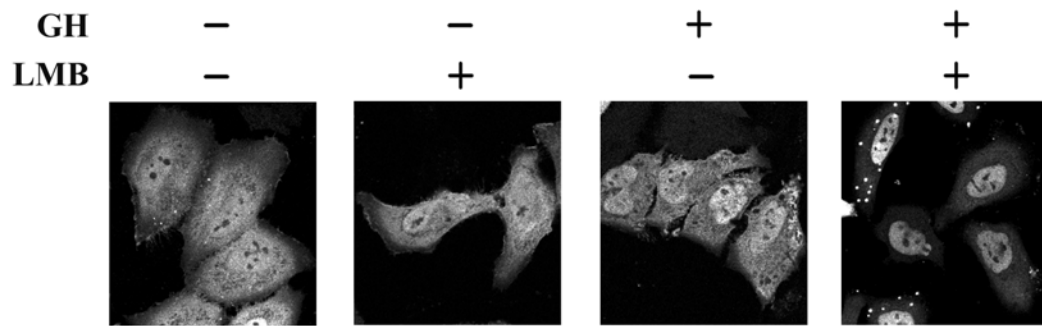


Figure 55: Localization of unphosphorylated and phosphorylated STAT5a-KR-GFP in the presence of LMB

HeLa cells expressing STAT5a-KR-GFP were untreated (-) or treated (+) with 1 ng/mL of GH for 45 minutes, 10 nM LMB for 45 minutes or a combination of both. The cellular localization was then determined by confocal microscopy.

sequence of this region is SLAAEFRHLQLK (the hydrophobic residues are underlined). Alignment of the primary amino acid sequence of STAT5a with STAT1 revealed that the amino acids 409-422 of STAT5a corresponded to amino acids 399-410 of STAT1. The sequence of amino acids in this region of STAT5a is TGTLSAHFRNMSLK (hydrophobic residues that are conserved between STAT1 and STAT5a are underlined).

I decided to test the nuclear export activity of this stretch of amino acids. A fragment of STAT5a coding for the amino acids 409-422 was linked to GFP and its cellular localization was evaluated. GFP has a molecular mass of 27 kDa and addition of another 13 amino acids to GFP would not result in a substantial increase of molecular mass. Thus the resulting protein should be small enough to pass through the NPC by passive diffusion. STAT5a (409-422)-GFP showed a cytoplasmic localization and not a nuclear presence suggesting that the amino acids 409-422 can function as an NES (Figure 56). To explore the possibility that this NES is recognized by CRM1, I evaluated the localization of the protein following LMB treatment (Figure 56). After LMB treatment, STAT5a(409-422)-GFP showed a localization similar to GFP and was nuclear indicating that its nuclear export was mediated by CRM1.

It has been shown that leucines or, in some instances, hydrophobic residues in NESs play critical roles in the recognition of NESs by CRM1 (Fornerod et al., 1997; Fukuda et al., 1997). To determine if the residues L412 and F416 are required for NES activity, I mutated these residues to alanines and generated the constructs STAT5a(409-422L)-GFP, STAT5a(409-422F)-GFP and STAT5a(409-422LF)-GFP. The localization of the mutants STAT5a(409-422L)-GFP and STAT5a(409-422F)-GFP were not as cytoplasmic as STAT5a(409-422)-GFP but did not show a significant nuclear presence as was observed with GFP. Thus these mutations did not significantly impair nuclear export. Treatment with LMB had no effect on the cellular localization of these mutants. To determine whether mutating both these residues had an effect on nuclear export, I analyzed the cellular localization of the double mutant STAT5a(409-422LF)-GFP. As shown in figure 56, STAT5a(409-422LF)-GFP

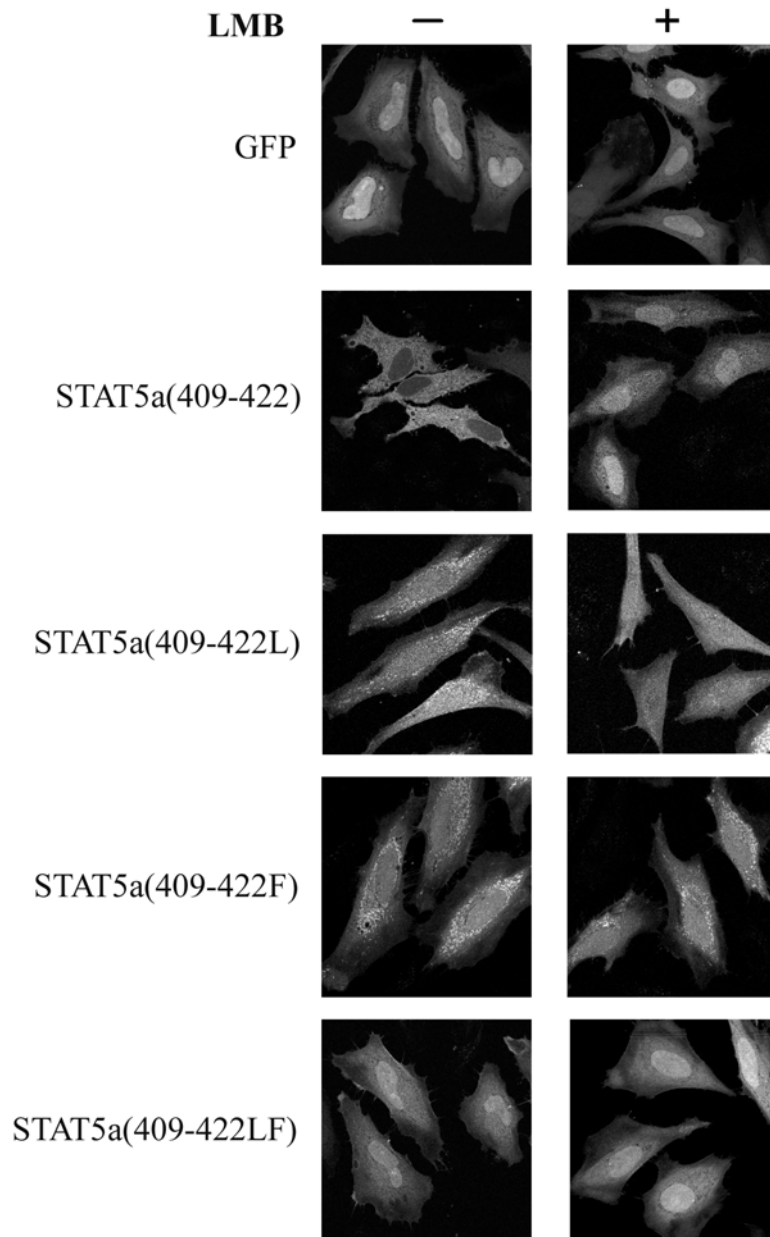


Figure 56: Cellular localization of GFP, STAT5a(409-422)-GFP and mutants of STAT5a(409-422)-GFP in the presence of LMB

HeLa cells expressing GFP, STAT5a(409-422)-GFP or point mutants of STAT5a(409-422)-GFP were left untreated (-) or treated (+) with 10 nM LMB for 45 minutes. Localization patterns were evaluated by confocal microscopy.

showed a stronger nuclear presence than the single mutants or STAT5a(409-422)-GFP. Its localization pattern was similar to GFP indicating that the NES activity was completely lost. Thus these residues play critical roles in nuclear export.

The contribution of these residues on nuclear export of full length STAT5a was tested by mutating them to alanines in wild type STAT5a. However, when these residues were mutated in the full length protein, the protein failed to be tyrosine phosphorylated in response to any cytokine that was used. HeLa cells or COS1 cells were transfected with mutant protein STAT5a-L412A-F416A-GFP (STAT5a-LF-GFP), and phosphorylation was analyzed following IFN α , GH, or EGF treatment by Western blotting. In all cases, no signal was detected by the antibody specific to tyrosine 694 of STAT5 even though the protein was expressed in the cells (data not shown). The mutations apparently induced some change in STAT5a so that it no longer responded to the JAKs or EGF kinases. These results make it difficult to analyze the contribution of these residues on the nuclear export of STAT5a after phosphorylation. However, experiments can be designed to determine if the mutations have any effect on the nuclear export of unphosphorylated STAT5a.

One study reported the presence of multiple nuclear export signals in STAT3 (Bhattacharya and Schindler, 2003). One of these NESs was also in the DNA binding domain between the amino acids 524-533. The sequence of this region is QLTTLAEKLL (hydrophobic residues are underlined) and this corresponded to the amino acids 529-538 of STAT5a which encoded the sequence NLVFLAQKLF (conserved hydrophobic residues are underlined). I evaluated the cellular localization of a fragment encoding the amino acids 529-540 tagged to GFP, STAT5a(529-540)-GFP, in HeLa cells (Figure 57). The molecular mass of this protein is ~28 kDa, hence it can easily diffuse through the NPC and enter the nucleus. STAT5a(529-540)-GFP showed a cytoplasmic localization but the distribution of the protein in the cytoplasm was not homogenous. Sometimes this non-homogenous localization of proteins is due to their aggregation. Thus the cytoplasmic localization of STAT5a(529-540)-GFP could be due to the nuclear export of the protein or due to aggregation of the protein.

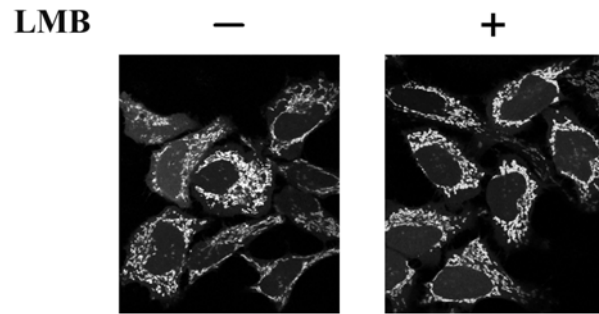


Figure 57: Cellular localization of STAT5a(529-540)-GFP in the presence of LMB

HeLa cells expressing STAT5a(529-540)-GFP were untreated (-) or treated (+) with 10 nM LMB for 45 minutes. Localization pattern was evaluated by confocal microscopy.

To determine whether STAT5a(529-540)-GFP was exported out of the nucleus by CRM1, I evaluated the cellular localization of this protein following LMB treatment. Surprisingly, the protein remained cytoplasmic even after LMB treatment. This result would suggest that if this region had the ability to function as an NES, the nuclear export was independent of CRM1. There still exists the possibility that this peptide is structurally unstable which promotes its aggregation. Hence further experiments need to be performed to determine whether this region can function as an NES. Experiments can be performed by mutating the hydrophobic residues and determining if they have any effect on the nuclear export of full length unphosphorylated or phosphorylated STAT5a.

I also determined the position of the amino acids 409-422 and 524-540 in the structure of unphosphorylated STAT5a. The amino acids 409-422 form one sheet of a β -sheet while the amino acids 529-540 form an α -helix (Figure 58). From their position in the structure, it is not clear if they are accessible to the solvent where they can interact with other proteins. The structure of phosphorylated STAT5a is not known but since the structure of unphosphorylated STAT1 and STAT5a is very similar (Figure 8), it is possible that the structure of phosphorylated STAT5a is similar to phosphorylated STAT1. The position of the amino acids 409-422 in STAT5a correspond to the position of the amino acids 399-410 of STAT1. In figure 57, the position of the amino acids 399-410 of STAT1 are shown in the structure of phosphorylated STAT1 bound to DNA. It has been hypothesized that this region is masked when phosphorylated STAT1 binds to DNA but is exposed once STAT1 dissociates from DNA (McBride et al., 2000). This might be the case with STAT5a too and further experiments will have to be performed to test this hypothesis.

Summary

The current chapter explores the nuclear export of STAT5a both before phosphorylation and after tyrosine phosphorylation. Unphosphorylated STAT5a is constitutively exported out of the nucleus into the cytoplasm and this nuclear export is independent of tyrosine phosphorylation. Studies performed with the inhibitor of

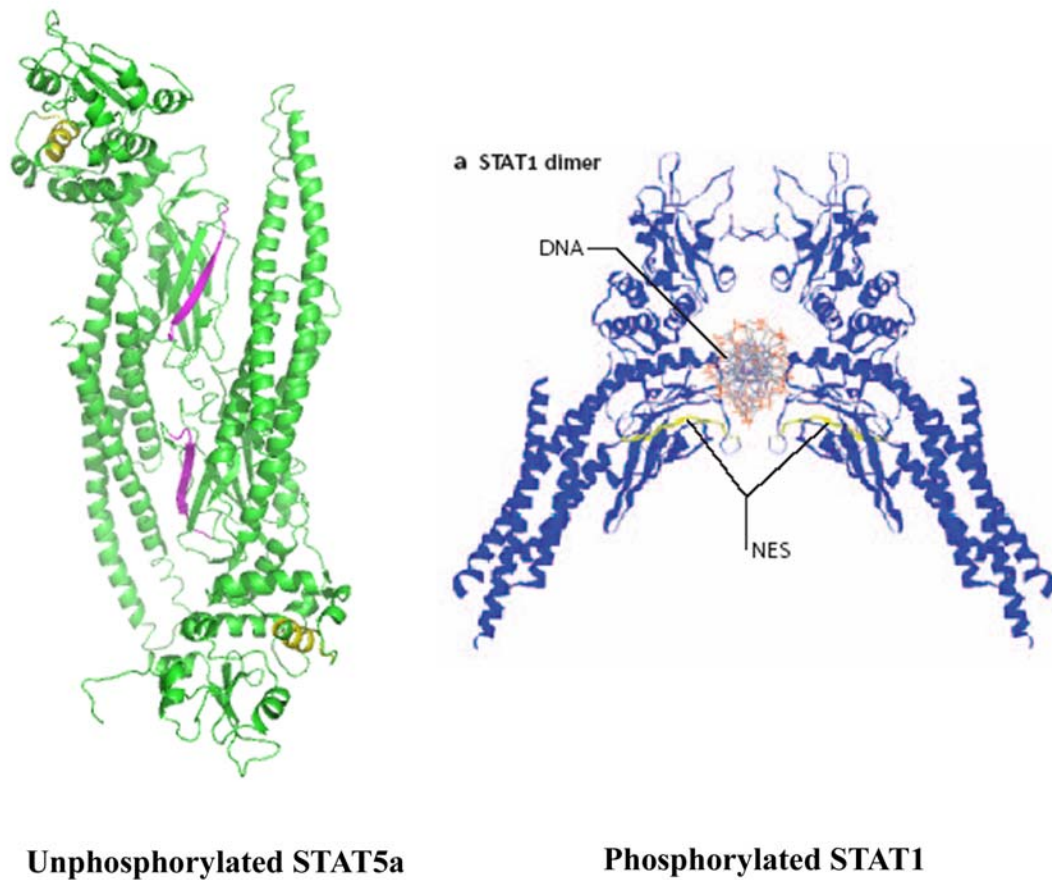


Figure 58: Position of NESs in the structure of STATs

The amino acids 409-422 and 529-540 of STAT5a are highlighted in magenta and yellow respectively in the structure of unphosphorylated STAT5a (left). The NES of STAT1 that corresponds to the amino acids 409-422 of STAT5a are shown in yellow in the structure of phosphorylated STAT1 bound to DNA (right) (McBride et al., 2000).

CRM1, LMB, indicated that CRM1 did not play a significant role in the nuclear export of unphosphorylated STAT5a. Thus, the mechanism by which unphosphorylated STAT5a is exported from the nucleus is unknown. Cytokine stimulation results in the tyrosine phosphorylation and nuclear accumulation of STAT5a. Studies performed in living cells revealed that there is a decrease in the nuclear export of phosphorylated STAT5a but it could not be determined if CRM1 had the ability to mediate the export of phosphorylated STAT5a. On removal of the cytokine, STAT5a appears back to the cytoplasm presumably due to dephosphorylation and nuclear export. This nuclear export of STAT5a can be delayed in the presence of LMB without affecting its dephosphorylation kinetics. Thus, CRM1 might play a role in the nuclear export of STAT5a following ligand stimulation.

Studies performed with a DNA binding mutant of STAT5a (STAT5a-KR-GFP) revealed that CRM1 can mediate the nuclear export of this mutant following cytokine stimulation. The nuclear export activity of two candidate NES sequences in the DNA binding domain of STAT5a was also studied. One of these sequences can be recognized by CRM1 while the other one cannot be recognized by CRM1. The positioning of the NES might suggest that it is accessible to proteins that mediate nuclear export when STAT5a is not bound to DNA. These findings should lay a foundation for further studies that involve targeting the activity of STAT5a.

Chapter 5

Discussion and Concluding remarks

STAT proteins share a similar arrangement of functional domains and a similar mode of activation by tyrosine phosphorylation. As signaling molecules and transcription factors, they must have the capability of moving between the cytoplasm and nucleus of cells. Cytoplasmic presence is critical for the STATs to sense signals coming from the cell surface while nuclear presence following tyrosine phosphorylation allows the molecules to regulate the expression of target genes. STATs play important roles in a variety of physiological processes that include immunity and growth and development. There is mounting evidence that some STATs, when activated constitutively, promote the transformation of cells and support tumor growth. In such scenarios where the transcriptional activity of STATs results in carcinogenesis, the goal is to inhibit the function of STATs. By understanding the process of nuclear import and export, various therapeutic drugs can be designed to intervene with this nucleocytoplasmic transport and thus regulate the transcriptional activity of constitutively active STATs.

Cellular localization of unphosphorylated STATs

The cellular trafficking of some STATs has already been elucidated and studies indicate that individual STATs show some similarities and differences (Reich and Liu, 2006). The localization of STAT1 and STAT2 is predominantly cytoplasmic before cytokine stimulation, whereas STAT3 is predominantly nuclear. Studies to evaluate the localization of the STAT5 proteins, STAT5a and STAT5b, have reported differences with respect to the presence of STAT5 in the nucleus before cytokine stimulation (Herrington et al., 1999; Kawashima et al., 2006; Marg et al., 2004; Zeng et al., 2002). Some of the differences reported may be due to the cell fractionation techniques employed, immunofluorescence techniques, antibody cross-reactivity or to

interpretations of static images. Following cytokine stimulation, all the STATs show a nuclear presence.

My studies indicate that latent STAT5a has a constitutive nuclear presence although it is not as prominent as STAT3 (Iyer and Reich, 2007; Liu et al., 2005). The localization of unphosphorylated STAT5a tagged with GFP or the V5 epitope was evaluated using fluorescence microscopy or indirect immunofluorescence respectively (Figure 18 and 19). In both cases unphosphorylated STAT5a was present in the nucleus and cytoplasm of cells. The constitutive nuclear presence of latent STAT5a was observed in multiple cell lines (Figure 20) and was independent of tyrosine phosphorylation (Figure 21). The nuclear import of unphosphorylated STAT5a was evaluated by studying the mobility of STAT5a in living cells using the Fluorescence Recovery After Photobleaching (FRAP) technique (Figure 22). FRAP analysis revealed that unphosphorylated STAT5a was constitutively imported into the nucleus of living cells (Figure 25). Furthermore, this constitutive nuclear import was independent of tyrosine phosphorylation (Figure 27).

Nuclear transport occurs through the nuclear pore complex (NPC). Macromolecules whose molecular mass is below 50 kDa can freely pass through the NPC by diffusion while larger molecules like STAT5a are imported by active transport (Figure 13). In most instances, this active transport is mediated by proteins like importins that function as soluble shuttling receptors. The proteins that are imported by these importins possess nuclear localization signals (NLSs). Classical NLSs usually contain one or two stretches of basic residues and these basic residues play critical roles in nuclear import (Lange et al., 2007). I scanned the primary amino acid sequence of STAT5a for potential classical NLSs but none of the candidates identified were required for import (Figure 28). This would suggest that the NLS of STAT5a might not be a non-classical NLS.

An unconventional NLS mediates the nuclear import of unphosphorylated STAT5a

Analysis of various deletions of STAT5a revealed a region in the coiled coil domain to be necessary for the nuclear import of unphosphorylated STAT5a (Figure 30). This region was mapped to the amino acids 142-LQINQTFE-149. This particular sequence and the amino acids in the vicinity of this sequence were not rich in basic residues. Thus this sequence did not resemble any classical NLS and can be thought to be an unconventional NLS. Deletion of the amino acids 142-149 resulted in the cytoplasmic localization of STAT5a indicating that these residues were required for nuclear import (Figures 30 and 31)

One of the characteristics of classical NLSs is that they can function as an NLS outside the context of the parent protein. I analyzed the localization of the amino acids 138-155 of STAT5a linked to GFP (data not shown). This region included the amino acids 142-149 which were necessary for the nuclear import of STAT5a. It was observed that these amino acids could not drive the nuclear import of GFP. Thus, these amino acids could not function as an NLS outside the context of STAT5a. These observations might suggest that the amino acids 142-149 form a conformation that can function as a recognition site for proteins that mediate nuclear import. For this region to function as a recognition site for other proteins, it must be accessible. Since the crystal structure of unphosphorylated STAT5a has been resolved (Neculai et al., 2005), I determined the location of these amino acids in the structure. The amino acids 142-149 form part of the first α -helix of the coiled coil domain (Figure 33). This region of the α -helix is on the outer surface of the molecule thereby allowing access to any molecules that mediate nuclear import. Thus these amino acids function as an unconventional NLS.

Numerous studies have reported the presence of unconventional NLSs that play important roles in the nuclear import of some proteins. Studies with STAT1 have indicated that amino acid L407 is necessary for the nuclear import of phosphorylated STAT1 (McBride et al., 2002). Mutation of this residue resulted in the inability of STAT1 to enter the nucleus in response to IFN γ . In the case of STAT3, the sequence

150-DVRKRQDLEQKM-162 (basic residues are underlined) was shown to be critical for nuclear import (Liu et al., 2005). Even though the region showed the presence of basic amino acids, none of the basic residues were required for nuclear import. Deletion of these amino acids in the full length protein rendered the protein cytoplasmic suggesting that these residues were required for nuclear import. The amino acids 150-162 of STAT3 reside in the coiled coil domain but the position of the region and the sequence of amino acids are distinct from the region required for STAT5a nuclear import. Thus unconventional NLSs play important roles in the nuclear import of other STATs.

Unconventional NLSs have also been characterized in non-STAT proteins. In one study, the amino acids EVYLDRSLLTL were shown to play an important role in the nuclear import of the tyrosine kinase Syk (Zhou et al., 2006). The arginine residue was not required for nuclear import. Deletion of these amino acids rendered the kinase cytoplasmic suggesting that these amino acids were required for the nuclear import of Syk. In the case of the serine/threonine kinase, Receptor-interacting protein 3 (RIP3), the amino acids NPVTGRPLVNIYNCSGVQVGDNNYLTMQQT have been shown to mediate its nuclear localization (Yang et al., 2004). The influenza A virus nucleoprotein, NP, also possesses an unconventional NLS that is indispensable for the nuclear import of the protein. The sequence of this NLS is TKGTKRSYEQM (Wang et al., 1997). Even though this sequence did not resemble a classical NLS, the basic residues were shown to be critical for nuclear import. From all these examples, it is clear that not all NLSs conform to classical NLSs.

Mechanism of nuclear import of unphosphorylated STATs

Since unphosphorylated STAT5a has the ability to enter the nucleus, the next logical step would be to determine the mechanism of nuclear import. The mechanism of nuclear import of unphosphorylated STAT5a is not known but the mechanism of nuclear import of other STATs has been studied. Unphosphorylated STAT1 can directly interact with the phenylalanine-glycine (FG) dipeptidyl repeats of the nucleoporins, Nup153 and Nup214 (Marg et al., 2004). It has been hypothesized that

STAT1 can thus enter into the nucleus in the absence of any transport factors by directly interacting with the nucleoporins. In the case of unphosphorylated STAT3, the nuclear import was mediated by a member of the importin- α family, importin- α 3 (Liu et al., 2005). Unphosphorylated STAT3 was able to bind to the armadillo repeats of importin- α 3 and silencing the expression of importin- α 3 resulted in a defect in the nuclear import of unphosphorylated STAT3.

It is possible that unphosphorylated STAT5a can enter the nucleus by directly interacting with the nucleoporins or its import can be mediated by members of the importin- α family of proteins. So far it is not clear which case is true. My preliminary experiments in the form of protein binding assays were unable to detect any significant binding between unphosphorylated STAT5a and the importin- α family members (data not shown). Thus, it is possible that unphosphorylated STAT5a can mediate its own nuclear import by interacting directly with the nucleoporins of the NPC. Further experiments will have to be designed and performed to elucidate the mechanism of nuclear import of unphosphorylated STAT5a.

Significance of nuclear presence of unphosphorylated STATs

Since latent STAT5a is present in the nucleus, it may have a specific nuclear function. So far no function has been assigned to unphosphorylated STAT5a in the nucleus, however, there have been reports that two other STATs, unphosphorylated STAT1 and STAT3, play a role in the nucleus (Chatterjee-Kishore et al., 2000; Yang et al., 2005; Yang et al., 2007). Microarray experiments were performed comparing gene expression in cells expressing a tyrosine mutant of STAT1 (STAT1-Y701F) and cells lacking STAT1. The comparisons revealed that unphosphorylated STAT1 regulated the constitutive expression of many genes including MHC class I, β -microglobulin, and low molecular mass polypeptide 2 (LMP2) (Chatterjee-Kishore et al., 2000). The promoter of LMP2 contains binding sites for STAT1 and interferon regulatory factor 1 (IRF1) in the form of a partially overlapping GAS site and interferon consensus sequence 2 (ICS-2) respectively. Chromatin immunoprecipitation revealed that unphosphorylated STAT1 and IRF1 bind

constitutively to the promoter of LMP2. Thus even in the unphosphorylated state, STAT1 appears to mediate gene expression.

In a similar fashion, microarray experiments were performed comparing gene expression in cells expressing a tyrosine mutant of STAT3 (STAT3-Y705F) and cells lacking STAT3 (Yang et al., 2005; Yang et al., 2007). It was observed that, similar to unphosphorylated STAT1, unphosphorylated STAT3 also appeared to mediate the expression of a subset of genes. Thus it is possible that the population of unphosphorylated STAT5a resident in the nucleus can also regulate gene expression. To explore this possibility, experiments can be performed in cell lines that lack both endogenous STAT5a and STAT5b. Since STAT5b can complement deficiency of STAT5a to a considerable extent, the experiments should be performed in cells that lack both proteins.

Mice that lack endogenous STAT5a and STAT5b have been generated and embryonic fibroblasts obtained from these mice can be used. As it was done for unphosphorylated STAT1 and STAT3, microarray analysis can be performed in these STAT5^{-/-} MEFs and in STAT5^{-/-} MEFs stably expressing STAT5a-Y694F. A comparison of the results obtained should reveal if unphosphorylated STAT5a can regulate gene expression. This should provide valuable information on the role of unphosphorylated STAT5a in the nucleus. It is also possible that the nuclear presence of unphosphorylated STAT5a enables it to be a substrate for nuclear kinases and this might necessary to bring about efficient gene regulation and a strong transcriptional response. Further experiments will have to be performed to test this possibility.

Cellular localization of phosphorylated STAT5a

On activation by tyrosine phosphorylation, STAT5a accumulates in the nuclear (Figure 18 and 34). This nuclear accumulation of phosphorylated STAT5a was observed in different cell lines and in response to different cytokines (Figure 34). This feature of nuclear presence following activation by tyrosine phosphorylation is a common feature shared by all the STAT family members (Reich and Liu, 2006).

Since the amino acids 142-149 are required for the nuclear import of unphosphorylated STAT5a (Figure 30), I evaluated the cellular localization of STAT5a(Δ 142-149)-GFP following tyrosine phosphorylation. This mutant was efficiently tyrosine phosphorylated in response to EGF but showed no phosphorylation in response to GH or IFN α (Figure 32). Since GH and IFN α receptors do not possess intrinsic kinase activity, they rely on the kinase activity of JAKs to phosphorylate STAT5a. It is possible that deletion of the amino acids 142-149 disrupts the interaction between STAT5a and the receptor or the JAKs. However, this deficiency of STAT5a(Δ 142-149)-GFP did not impact my studies since I could use EGF to phosphorylate this mutant. Following EGF treatment, STAT5a(Δ 142-149) gained the ability to bind DNA (Figure 32) indicating that this mutant had retained the functional characteristics of wild type STAT5a. The cellular localization of STAT5a(Δ 142-149)-GFP was cytoplasmic even after phosphorylation by EGF treatment (Figure 37). Thus the amino acids 142-149 are required for the nuclear import of both unphosphorylated and phosphorylated STAT5a.

Recently, one study reported that the amino acids 341-365 of STAT5a were also critical for the nuclear import of phosphorylated STAT5a (Kawashima et al., 2006). These amino acids reside in the DNA binding domain of STAT5a and their sequence does not resemble a classical NLS. I evaluated the localization of a mutant of STAT5a that lacked these amino acids (Figure 29). The mutant protein appeared cytoplasmic in resting cells which initially indicated that the amino acids were required for nuclear import. However, cytoplasmic localization of a protein does not necessarily imply a lack of nuclear import. It is possible that the protein shuttles between the nucleus and cytoplasm and the rate of nuclear export exceeds the rate of import thereby resulting in a cytoplasmic localization. This was indeed the case with STAT5a(Δ 341-365) as when the cells were treated with the CRM1 exportin inhibitor, LMB, the protein showed a nuclear presence. This phenomenon was observed in multiple cell lines (Figure 29). Thus it can be concluded that the 341-365 a.a. deletion mutant is imported to the nucleus and is exported effectively. To ensure that this was not the case with STAT5a(Δ 142-149), its cellular localization was evaluated

following LMB treatment. The results showed that STAT5a(Δ 142-149) remained cytoplasmic following LMB treatment suggesting that it cannot be imported into the nucleus (Figure 31).

Effect of tyrosine phosphorylation and DNA binding on nuclear accumulation of phosphorylated STAT5a

The nuclear import of unphosphorylated STAT5a is independent of tyrosine phosphorylation (Figure 27). The role of tyrosine phosphorylation on the nuclear accumulation of STAT5a following cytokine stimulation was determined by evaluating the cellular localization of a mutant of STAT5a, STAT5a-RY-GFP. This mutant of STAT5a does not have the ability to be phosphorylated and is unable to bind DNA (Figure 38). It also cannot associate with other STATs *via* reciprocal phosphotyrosine and SH2 domain interactions. The cellular localization of STAT5a-RY-GFP revealed that it failed to accumulate in the nucleus following cytokine stimulation (Figure 39). This would suggest that tyrosine phosphorylation was necessary for the nuclear accumulation of STAT5a. However, there is a gain in the ability to bind DNA following phosphorylation. Hence, either tyrosine phosphorylation or DNA binding could be responsible for the nuclear accumulation of STAT5a following cytokine stimulation.

To distinguish between these two possibilities, I performed localization studies with a DNA binding mutant of STAT5a, STAT5a-KR-GFP. Unphosphorylated STAT5a-KR-GFP showed a constitutive nuclear presence that was similar to unphosphorylated wild type STAT5a indicating that DNA binding was not required for the constitutive nuclear import of unphosphorylated STAT5a (Figure 41). Following GH stimulation, STAT5a-KR-GFP was efficiently phosphorylated but did not have the ability to bind DNA (Figure 41). The localization pattern of the mutant following GH treatment revealed that it did not accumulate in the nucleus in a manner that was observed for phosphorylated wild type STAT5a. Thus the ability to bind DNA and not tyrosine phosphorylation is required for the nuclear accumulation of phosphorylated STAT5a.

This dependence of DNA binding on the nuclear accumulation of phosphorylated STATs has also been observed in STAT1. A DNA binding mutant of STAT1 was created by mutating two glutamate residues (E428 and E429) in the DNA binding domain of STAT1 (Horvath et al., 1995). Wild type STAT1 shows a cytoplasmic localization and becomes nuclear following IFN γ treatment. The STAT1 DNA binding mutant was also cytoplasmic prior to cytokine stimulation and failed to accumulate in the nucleus following IFN γ treatment (McBride et al., 2000). Thus nuclear accumulation of phosphorylated STAT1 depends on its DNA binding ability.

If nuclear accumulation of tyrosine-phosphorylated STAT5a is a consequence of DNA binding, the movement of STAT5a in the nucleus would be expected to reduce significantly. This was tested by performing FLIP analysis in a small region in the nucleus of living cells expressing STAT5a before and after GH treatment (Figure 43). A small region in the nucleus of living cells was subjected repeatedly to a high energy laser. Unphosphorylated STAT5a was found to rapidly move within the nucleus since photobleaching at a single focal point in the nucleus led to rapid bleaching of fluorescence in the entire nucleus (Figures 43a and 43b, top panel). On the other hand, phosphorylated STAT5a showed significantly reduced movement in the nucleus as evident by the occurrence of photobleaching primarily at the focal point of the laser beam (Figure 43a and 43b, bottom panel). When a similar analysis was performed with the STAT5a-KR DNA binding mutant, a reduced mobility was not observed in the nucleus following tyrosine phosphorylation (Figure 44). Thus, the reduction in mobility of phosphorylated STAT5a within the nucleus can be attributed to DNA binding.

Mechanism of nuclear import of phosphorylated STAT5a

The mechanism of nuclear import of several phosphorylated STATs has been elucidated (Ma and Cao, 2006; McBride et al., 2002; Sekimoto et al., 1997). Phosphorylated STAT1 is imported into the nucleus by a member of the importin- α family, importin- α 5 (Sekimoto et al., 1997). Phosphorylated STAT1 was shown to interact with the carboxyl terminus of importin- α 5. This is an unconventional

interaction because conventional NLSs in proteins usually bind to the armadillo repeats of importins (Chook and Blobel, 2001). Phosphorylated STAT3 can be imported by importin- α 5 and importin- α 7. Studies have revealed that phosphorylated STAT3 can bind to the armadillo repeats as well as carboxyl terminus of importin- α 5 (Ma and Cao, 2006). Thus the nuclear import of both phosphorylated STAT1 and STAT3 can be mediated by importin- α s.

In the case of phosphorylated STAT5a, a recent study indicated that the protein, male germ cell RacGAP, was able to mediate the nuclear import of phosphorylated STAT5a in co-operation with importin- α 1 and importin- α 5 (Kawashima et al., 2006). My preliminary experiments indicate that phosphorylated STAT5a is able to bind to importin- α 5 but not to importin- α 1. Thus the nuclear import of phosphorylated STAT5a requires multiple players.

Nuclear export of unphosphorylated and phosphorylated STAT5a

Unphosphorylated STAT5a was found to be constitutively exported out of the nucleus since photobleaching at a single focal point in the cytoplasm resulted in the loss of fluorescence in the entire nucleus (Figure 47). This constitutive nuclear export of unphosphorylated STAT5a was independent of tyrosine phosphorylation (Figure 48). Due to the large size of STAT5a, it is likely that the nuclear export would be mediated by exportins. CRM1 is an exportin that has been shown to mediate the export of numerous proteins (Fukuda et al., 1997; Ossareh-Nazari et al., 1997). The discovery of the drug Leptomycin B that can specifically inhibit the export activity of CRM1 has aided immensely in studying CRM1-mediated export (Kudo et al., 1999; Kudo et al., 1998).

To determine whether CRM1 was able to mediate the export of unphosphorylated STAT5a, I analyzed the nuclear export of unphosphorylated STAT5a in living cells after treatment with LMB (Figure 49). There was no change in the rate of nuclear export of unphosphorylated STAT5a following LMB treatment. These results have led me to conclude that CRM1 did not play a significant role in the nuclear export of unphosphorylated STAT5a.

Thus the mechanism of nuclear export of unphosphorylated is not known. Experiments are being performed to determine what region of unphosphorylated STAT5a is required for its nuclear export. One particular deletion mutant that comprises of the first 330 amino acids of STAT5a linked to two GFP molecules; STAT5a1-330-GFP-GFP, shows a predominantly nuclear localization (Figure 30). The predominant nuclear presence might be due to the absence of an NES and this can be determined by performing a FLIP in a small region in the cytoplasm of cells expressing this mutant. If there is a significant decrease in the nuclear export of this mutant in comparison to full length unphosphorylated STAT5a, it would suggest that the NES was absent in the region 1-330. Further deletion constructs can then be created to determine where the NES of unphosphorylated STAT5a resides. Once the region required for nuclear export is mapped, it can be used to identify proteins that can interact with it. The identity of such proteins will provide a clue on the mechanism of nuclear import of STAT5a.

Nuclear export of phosphorylated STAT5a

Tyrosine phosphorylated STAT5a accumulates in the nucleus and this nuclear accumulation is due to DNA binding. I have also shown that the mobility of tyrosine phosphorylated STAT5a is reduced in the nucleus. It is possible that this reduced mobility of phosphorylated STAT5a in the nucleus would result in its nuclear retention. Nuclear retention would in turn lead to a decrease in nuclear export of phosphorylated STAT5a. To determine whether the nuclear export of phosphorylated STAT5a was slower than unphosphorylated STAT5a, a FLIP analysis was performed in living cells expressing a constitutively active mutant of STAT5a, STAT5a-HS-GFP. This mutant of STAT5a is believed to adopt a conformation that is resistant to dephosphorylation (Onishi et al., 1998). FLIP was performed in a small region in the cytoplasm of cells expressing STAT5a-HS-GFP following treatment with growth hormone (Figure 50). Nuclear export could be detected even though the rate of nuclear export was slower than unphosphorylated STAT5a. Thus phosphorylated

STAT5a is retained in the nucleus and exhibits a nuclear export slower than unphosphorylated STAT5a.

The role of CRM1 in the nuclear export of phosphorylated STAT5a was determined by performing a FLIP analysis in the presence of LMB. Unfortunately, the analysis could not be completed due to the death of cells during FLIP analysis. Hence this method cannot provide information on the role of CRM1 on the nuclear export of phosphorylated STAT5a.

Effect of DNA binding on the nuclear export of phosphorylated STAT5a

A DNA binding mutant of STAT5a, STAT5a-KR-GFP, fails to accumulate in the nucleus following GH stimulation (Figure 41). Since DNA binding has been shown to be required for nuclear accumulation of phosphorylated STAT5a, it is possible that in the absence of DNA binding phosphorylated STAT5a is exported out of the nucleus. This would account for the lack of nuclear accumulation of phosphorylated STAT5a-KR-GFP. This possibility was tested by analyzing the cellular localization of phosphorylated STAT5a-KR in the presence of LMB. STAT5a-KR-GFP failed to accumulate in the nucleus of cells following GH treatment but showed a predominantly nuclear presence when the cells were treated with GH and LMB (Figure 54). Treatment with LMB alone did not affect the cellular localization of STAT5a-KR-GFP.

These results suggest that when phosphorylated STAT5a is unable to bind DNA it is efficiently exported out of the nucleus by CRM1. This in turn results in a failure of nuclear accumulation. When this export is inhibited by LMB, STAT5a-KR-GFP can accumulate in the nucleus. This result would also suggest that CRM1 was able to mediate the nuclear export of phosphorylated STAT5a.

Experiments can be designed to detect the interaction between CRM1 and phosphorylated STAT5a *in vitro* by performing protein-protein binding assays using purified proteins. *In vivo* interactions can also be studied by performing co-immunoprecipitation experiments. Another emerging technology to study protein-protein interactions *in vivo* is a technique called Bimolecular Fluorescence

Complementation (BiFC) (Hu and Kerppola, 2003). This principle behind this technique involves splitting the yellow fluorescence protein (YFP) into two halves, an amino terminal (YN) half and carboxyl terminal (YC) half. These halves of YFP do not have the ability to fluoresce. The proteins whose interaction needs to be studied, for example CRM1 and STAT5a, are tagged with YN and YC respectively to obtain the fusion proteins CRM1-YN and STAT5a-YC. If the two proteins interact then it will bring the YFP fragments in close proximity resulting in the regeneration of YFP and thus the ability to fluoresce. Hence if fluorescence is detected in cells expressing CRM1-YN and STAT5a-YC, it would indicate that the two proteins are able to interact with each other. This technique has been used with success in detecting the interaction between proteins like Jun and Fos; IRF8 and PU.1; Myc, Max and Mad; etc (Grinberg et al., 2004; Hu et al., 2002; Laricchia-Robbio et al., 2005). Thus this technology may be able to give us information on the interaction between CRM1 and phosphorylated STAT5a.

Nuclear export of STAT5a following GH removal

GH stimulation results in the nuclear accumulation of STAT5a but following removal of GH the protein returns to the cytoplasm by 3 hours and localizes in a pattern similar to unphosphorylated STAT5a by 4 hours of GH withdrawal (Figure 52). This reduction in nuclear accumulation could be correlated with the phosphorylation levels of STAT5a following GH withdrawal. There was a significant decrease in phosphorylation levels of STAT5a by 3 hours of GH withdrawal and by 4 hours, a very low level of phosphorylation was detected (Figure 51). Thus, following the removal of GH, there is a steady decrease in the nuclear accumulation and levels of phosphorylated STAT5a.

This reduction in nuclear accumulation of STAT5a was delayed in the presence of LMB without altering the phosphorylation kinetics (Figures 53 and 54). STAT5a appeared nuclear even after 4 hours of GH withdrawal in the presence of LMB. After 6 hours of removal of GH, there was a considerable decrease in the

nuclear accumulation of STAT5a but the overall localization pattern was still not similar to unphosphorylated STAT5a.

These results suggest that CRM1 was able to mediate the nuclear export of STAT5a that was dephosphorylated, as by 4 hours I could still detect greater nuclear presence of STAT5a in the presence of LMB although the phosphorylation was very low at this time. I have already demonstrated that CRM1 does not play a significant role in the nuclear export of unphosphorylated STAT5a. To explain these observations, two possibilities have to be considered.

The first possibility is that at 4 hours, there exists a small population of phosphorylated STAT5a that cannot be detected by Western blotting due to the limits of sensitivity of the method. This small population of phosphorylated STAT5a is recognized by CRM1 and the export of these molecules is blocked in the presence of LMB. This results in the nuclear presence of these molecules for a longer period of time. This possibility can be tested by performing kinetic immunofluorescence experiments using antibodies against phosphorylated STAT5a on cells expressing STAT5a following GH removal. This experiment would be able to reveal the phosphorylation level of STAT5a on a cellular level over a period of time after GH removal. The limiting factor with this assay system is the efficiency and sensitivity with which the phosphoSTAT5 antibody can be used for immunofluorescence as these antibodies usually provide very weak signals (unpublished observations).

The second possibility is that dephosphorylated STAT5a is present in a conformation that is different from unphosphorylated STAT5a. As explained in Chapter 1, both unphosphorylated and phosphorylated STATs are dimeric but exhibit different conformations (Figure 8 and 9). Phosphorylation results in an extensive spatial rearrangement of the monomers relative to each other. In addition, the structures of phosphorylated STATs differ depending on whether they are bound to DNA or not (Figure 9). This was revealed by comparing the structures of phosphorylated *Dictyostelium* STAT unbound to DNA and phosphorylated STAT1 and STAT3 bound to DNA (Becker et al., 1998; Chen et al., 1998; Soler-Lopez et al.,

2004). Thus, depending on what state they are in, STATs can undergo extensive conformational changes.

Conversely, once STATs are dephosphorylated, they again will have to go through extensive spatial rearrangement to return to the conformation of the unphosphorylated protein (Zhong et al., 2005). Thus, it can be hypothesized that CRM1 can recognize and interact with the conformation adopted by dephosphorylated STAT5a which has not yet returned to the conformation of unphosphorylated STAT5a. This hypothesis would be difficult to prove as there is not much information available on how long it takes the dephosphorylated STAT to return to the conformation of the unphosphorylated STAT. Also, although the structure of unphosphorylated STAT5a is known, there is limited information available on the structural aspects of phosphorylated STAT5a. Hopefully more information will be available in the future so that experiments can be designed to test this hypothesis.

Recognition of NESs in STAT5a

Since the results obtained in Chapter 4 suggest that CRM1 plays a role in the nuclear export of STAT5a, it is likely that STAT5a would possess a nuclear export signal that can be recognized by CRM1. CRM1 is known to recognize leucine rich nuclear export signals that conform to the sequence LxxxLxxLxL, where L is leucine and x is any amino acid (Fornerod et al., 1997). Scanning the primary amino acid sequence of STAT5a to identify leucine rich NESs revealed that there were many sequences that loosely conformed to classical NESs. I then decided to test candidate NESs in STAT5a based on studies performed with other STAT members. In some STATs like STAT1, 2 and 3, sequences have been identified that function as NESs (Banninger and Reich, 2004; Bhattacharya and Schindler, 2003; McBride et al., 2000).

The amino acids 399-SLAAEFRHLQLK-410 of STAT1 have been shown to function as an NES (McBride et al., 2000). Alignment of the primary amino acid sequence of STAT5a and STAT1 revealed that the amino acids 409-422 (TGTLSAHFRNMSLK) of STAT5a corresponded to amino acids 399-410 of STAT1.

This region of STAT5a was able to function as a NES as evidenced by the cytoplasmic localization of this peptide linked to GFP (Figure 56). Following treatment with LMB the protein showed a nuclear presence; therefore the nuclear export of this peptide was mediated by CRM1. The interaction between the peptide and CRM1 was mediated by the amino acids L412 and F416A. Mutation of both these amino acids rendered the peptide nuclear indicating that it was no longer being exported from the nucleus (Figure 56). Thus the region 409-422 of STAT5a behaved like an NES that could be recognized by CRM1.

In the case of STAT2, the NES was mapped to the carboxyl end of the protein (Banninger and Reich, 2004). Sequence alignments between STAT2 and STAT5a revealed that this region was not conserved in STAT5a. In the case of STAT3, the amino acids 524-533 were shown to behave as an NES (Bhattacharya and Schindler, 2003). There was a conservation between the amino acids 524-533 of STAT3 and 529-540 of STAT5a. When the corresponding amino acids in STAT5a were fused to GFP, the protein showed a cytoplasmic localization even in the presence of LMB (Figure 57). This would suggest that this region also displayed nuclear export activity but the export was CRM1-independent. It should however be noted that STAT5a(529-540)-GFP displayed a non-homogenous localization pattern. In some instances such non-homogenous distribution of the protein is due to aggregation. Hence additional experiments need to be performed to determine whether this region in STAT5a can function as an NES.

It is possible that a peptide can display nuclear export activity by itself but once it is in the context of a protein, it no longer acts as an NES. This can be due to several reasons including the positioning and/or structure of the peptide in the protein. This is why it is important to determine whether a peptide can function as an NES in the context of the protein. This is normally done by mutating the residues that are critical for nuclear export and evaluating the localization of the mutated protein. In the case of STAT5a, the residues that will be targeted are L412, F416, L530, L533, L537 and F538. These residues have been chosen based on the studies performed with STAT1 and STAT3. The localization of STAT5a bearing these mutations will be

evaluated in the presence and absence of LMB and cytokine stimulation. These studies should shed more light on the nuclear export of STAT5a.

Model for nucleocytoplasmic transport of STAT5a (Figures 59 and 60)

The aim of this dissertation is to characterize the manner in which STAT5a regulates its nucleocytoplasmic trafficking. Latent STAT5a is present in the nucleus and cytoplasm of cells. It is rapidly moving within these two compartments and has the ability to constitutively shuttle between the nucleus and cytoplasm of cells. Similar nucleocytoplasmic shuttling of unphosphorylated STATs between the nucleus and cytoplasm has been reported in other STATs like STAT1, 2 and 3 (Frahm et al., 2006; Koster et al., 2005; Koster and Hauser, 1999; Lillemeier et al., 2001; Pranada et al., 2004). Hence, this property of shuttling may be a common feature among the STAT family members.

A region in the coiled coil domain is critical for the nuclear import of unphosphorylated STAT5a. Deletion of the amino acids 142-149 from full length STAT5a renders the protein cytoplasmic and unable to be imported into the nucleus. Thus, these amino acids are necessary for the nuclear import of unphosphorylated STAT5a. From the studies presented in this dissertation it is apparent that the exportin CRM1 does not play a significant role in the nuclear export of unphosphorylated STAT5a. Thus, the exact mechanism by which unphosphorylated STAT5a is imported into and exported out of the nucleus remains unknown.

On activation by tyrosine phosphorylation, STAT5a accumulates into the nucleus. Studies have shown that the amino acids 142-149 are critical for the nuclear import of tyrosine phosphorylated STAT5a too. A mutant of STAT5a lacking these amino acids is unable to show any nuclear presence following activation by tyrosine phosphorylation. The nuclear accumulation of tyrosine phosphorylated STAT5a could be due to retention of phosphorylated STAT5a in the nucleus by DNA binding, decrease in the rate of nuclear export, increase in the rate of nuclear import of phosphorylated STAT5a or all of the above. I have been able to show that DNA binding reduces the mobility of phosphorylated STAT5a in the nucleus thereby

resulting in its nuclear accumulation. The nuclear import of phosphorylated STAT5a is mediated by male germ cell RacGAP (mgcRacGAP) in close association with importin- α 1 and importin- α 5. It is yet to be determined if there is an increase in the rate of nuclear import of phosphorylated STAT5a. Phosphorylated STAT5a is exported out of the nucleus but the rate of nuclear export is slower as compared to unphosphorylated STAT5a. Thus the retention of phosphorylated STAT5a in the nucleus by DNA binding results in a decrease in nuclear export. The results from studies presented here suggest that the exportin CRM1 can mediate the export of tyrosine phosphorylated and dephosphorylated STAT5a but not unphosphorylated STAT5a. Thus the nucleocytoplasmic trafficking of unphosphorylated and phosphorylated STAT5a are differentially regulated. These findings and further understanding of the mechanisms of STAT5a cellular trafficking should provide a foundation for studies that involve targeting the activity of STAT5a in various pathological conditions.

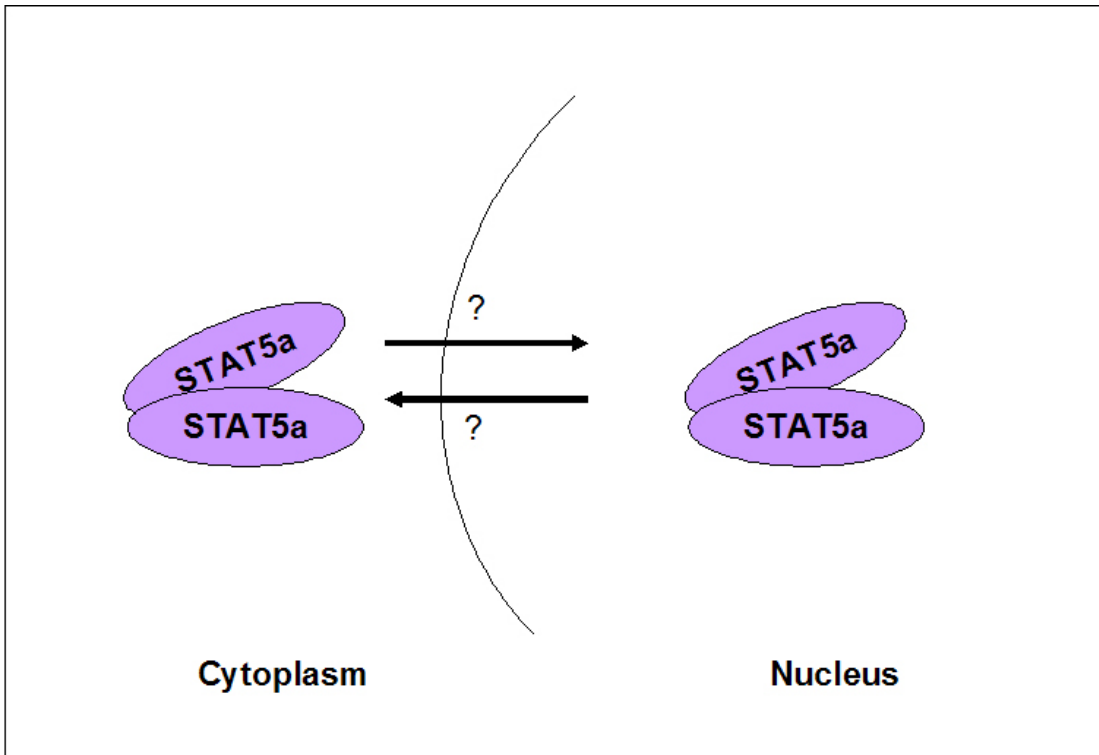


Figure 59: Nucleo-cytoplasmic shuttling of unphosphorylated STAT5a (Iyer and Reich, 2007)

Unphosphorylated STAT5a usually exists as a dimer and shuttles constitutively between the nucleus and cytoplasm. Since unphosphorylated STAT5a is present throughout the cell, it is assumed that the rate of import is similar to the rate of export. The mechanism of import and export remains unknown (?).

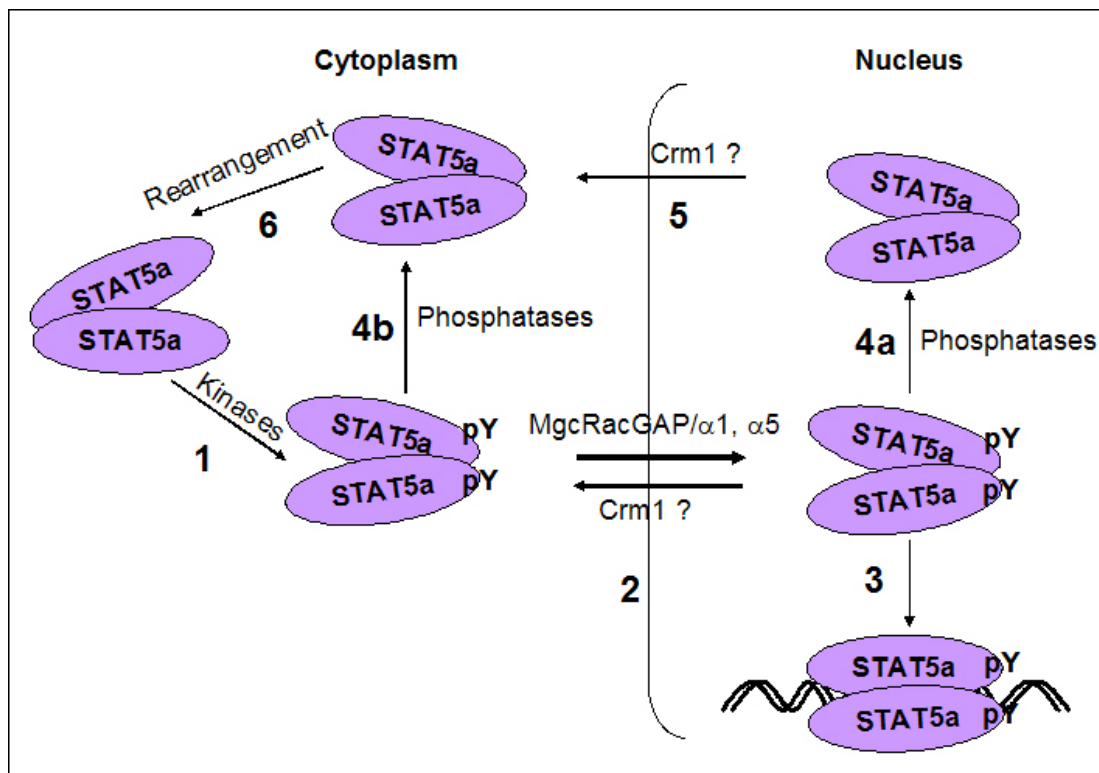


Figure 60: Subcellular trafficking of STAT5a following tyrosine phosphorylation (Iyer and Reich, 2007; Kawashima et al., 2006)

Latent STAT5a is phosphorylated by tyrosine kinases which results in a conformational change in the dimer (1). Phosphorylated STAT5a also has the ability to shuttle between the nucleus and cytoplasm but there is a decrease in the rate of export (2). Nuclear import of the molecule is mediated by MgcRacGAP in cooperation with importin- α 1 and importin- α 5. There is preliminary evidence showing nuclear export of STAT5a might be mediated by CRM1. In the nucleus, phosphorylated STAT5a can bind to target GAS sites and induce transcription (3). Phosphorylated STAT5a can also be dephosphorylated in the nucleus or cytoplasm by phosphatases (4a, 4b). This dephosphorylated STAT5a is hypothesized to be in a conformation different from unphosphorylated STAT5a. It is also hypothesized that it may be exported out of the nucleus by CRM1 (5). Eventually, the conformation returns to that of unphosphorylated STAT5a (6).

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