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Study of STAT3 Protein Interactions Using an In Vitro Binding Assay

A Thesis Presented

by

Julie Armstrong

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Julie Armstrong

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Nancy C Reich Marshall, Ph.D., Thesis Advisor
Professor, Department of Molecular Genetics and Microbiology

Michael J. Hayman, Ph.D.
Professor, Department of Molecular Genetics and Microbiology

Laurie T. Krug, Ph.D.
Assistant Professor, Department of Molecular Genetics and Microbiology

This thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Thesis

STAT3 Protein Interactions Using an In Vitro Binding Assay

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Transcription factors function as regulators of gene expression by controlling the activation of target genes. As a member of the STAT (Signal Transducer and Activators of Transcription) family, STAT3 regulates gene expression responsible for controlling differentiation, cell survival, proliferation, migration, and apoptosis. Activation of STAT3 conventionally occurs through tyrosine phosphorylation by JAK kinases stimulated following cytokine binding receptors. Cytokines like IL-6 bind to cell surface receptors, leading to the activation of associated JAK kinases. JAKs phosphorylate the cytoplasmic domains of the receptors, and STAT3 is recruited through its SH2 domain resulting in its phosphorylation by JAKs. It has been determined that the phosphorylation state of STAT3 does not impact its ability to enter the nucleus, but its precise mechanism of nuclear import remains to be completely understood. The

importin α family is known to bind conventional nuclear localization signals (NLS) in proteins destined for the nucleus. Importin α associates with importin β, which mediates interaction with the nuclear pore complexes to transport the cargo.

The importin α family contains several members, and the purpose of this study is to determine if there is an interaction present between STAT3 and members of the importin α family, and to identify the region of STAT3 recognized. The approach taken involved preparing tyrosine phosphorylated STAT3 (pTyr-STAT3) or unphosphorylated STAT3 (USTAT3) from bacteria. Recombinant clones were generated and STAT3 protein was expressed containing a Histidine and Maltose Binding Protein tag. Different members of the importin alpha family including importin α1, α3, and α5 tagged with glutathione-S-transferase (GST) were expressed in bacteria cells and purified using glutathione beads. The STAT3 proteins (USTAT3 and pTyr-STAT3) and purified GST-Importins were used in an *in vitro* binding assay. STAT3 was pulled down with amylose beads and analyzed for associated importins with GST antibodies. Multiple importin α proteins were able to interact with both USTAT3 and pTyr STAT3. The phosphorylation of STAT3 impacts which importin α will recognize the protein.

I also cloned STAT3 mutants which contain N-terminal deletions. The STAT3 mutants include amino acids encoding 127-770, 320-770, 495-770, and 585-770. These mutants were used to determine which region of the STAT3 is needed for binding to the importin α protein. The DNA binding region of recombinant STAT3 appeared necessary for pTyr STAT3 and importin α1 and α5 interaction.

STAT3 is also suspected of playing a role in the ability of gammaherpesvirus to establish latency. Following lytic replication, gammaherpesviruses are maintained in host B cells as viral DNA episomes. Latent viral gene expression is known to be able to promote the development of various cancers. The latent virus can subsequently reactivate, and this process requires the viral protein, replication and transcription activator (RTA). I tested the murine gammaherpesvirus 68 (MHV68) RTA protein for its ability to bind recombinant STAT3 protein in an in vitro binding assay. My results indicate that RTA binds STAT3 with a preference for tyrosine phosphorylated STAT3. Binding to tyrosine phosphorylated STAT3 N-terminal deletions demonstrated RTA association requires the DNA binding domain of STAT3.

My results demonstrate STAT3 interaction with the transport protein, importin α, and a viral protein, RTA. Understanding the interface of STAT3 with these proteins could lead to the development of potential therapeutics to inhibit the role of STAT3 in proliferative or viral diseases.

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List of Abbreviations

aa   amino acid

BSA   Bovine Serum Albumin

DTT   Dithiothreitol

EBV   Epstein Barr Virus

GST   Glutathione-S-Transferase

His   Histidine

HEPES   2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid

HSV-1   Herpes Simplex Virus 1

IBB   Importin β Binding Domain

IL-6   Interleukin 6

IPTG   Isopropyl β-D-1-thiogalactopyranoside

JH   JAK Homology

JAK   Janus Kinases

KSHV   Kaposi's Sarcoma Associated Herpesvirus

LB   Luria Broth

MBP   Maltose Binding Protein

MHV68   Murine gammaherpevirus 68

NLS   Nuclear Localization Sequence

NP-40   octylphenoxypolyethoxyethanol

PAGE   Polyacrylamide gel electrophoresis

PCR   Polymerase chain reaction

PMSF   Phenylmethylsufonylfluoride

pTyr-STAT3 Tyrosine phosphorylated STAT3

RRE   RTA Responsive Element

RTA   Replication and Transcription Activator

SDS   Sodium dodecyl sulfate

SH2   Src Homology 2 Domain

STAT   Signal Transducer and Activator of Transcription

STAT3   Signal Transducer and Activator of Transcription-3

TAD   Transactivation Domain

Tris   tris(hydroxymethyl)aminomethane

Tween 20   Polyoxyethylene (20) sorbitan monolaurate

U-STAT3   Unphosphorylated STAT3

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The work presented in this thesis would not have been possible without help from several people. I would like to thank Dr. Nancy Reich for the guidance and support she has given me over the past two years. She welcomed into her lab and provided me with the resources necessary to be successful.

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I would not have made it this far without the love and support of my family. They have provided me with the strength and encouragement I needed to get to where I am today and for that I am grateful.

Chapter 1

Introduction

Cytokines

Cytokines play an important role in many different biological processes including embryonic development, defense against diseases, and response to infections (Dinarello CA 2007). Cells secrete cytokines which can act on nearby cells or as autocrine factors to stimulate specific signaling pathways that regulate gene expression (Dinarello CA 2007). One specific cytokine, Interleukin 6 (IL-6), is known to be involved in inflammation and immune response to infection, as well as the regulation of processes like metabolism (Scheller J. et al 2011). IL-6 functions by activating a common receptor, gp130, in complex with the IL-6 receptor (IL-6R) and transduces a signal within the cell to the nucleus. IL-6 has a four helix bundle structure which contains 3 different receptor binding sites. One binding site recognizes the IL-6R subunit, and the other two binding sites recognize different sites of the gp130 receptor subunit (Scheller J et al 2011). These binding sites bring two receptors, IL-6R and gp130, in close association, which is necessary for transduction of the signal in response to IL-6 binding. The gp130 receptor is a type 1 membrane protein which has extracellular, intracellular, and cytoplasmic domains allowing for transduction of the signal from the outside to the inside of the cell. IL-6 binding activates the gp130/IL-6 receptor by stimulating the receptor associated Janus kinases (JAKs) (Scheller J. et al 2011). IL-6 binding results in the juxtaposition of JAKs on each subunit so that they are

able to activate each other through cross phosphorylation, and then to phosphorylate other proteins to promote signaling (Scheller J et al 2011). An essential part of signaling by IL-6 and many cytokines involves the JAK tyrosine phosphorylation of transcription factors called signal transducers and activators of transcription (STATs) (Scheller J et al 2011) (Figure 1B). It is the action of the STATs that mediates induction of genes that regulate many of the cytokine-induced biological responses.

Janus Kinases

In order for the IL-6 signal to be transduced, JAKs must be activated by IL-6 binding to gp130 receptors. The JAK family contains four members Jak1, Jak2, Jak3, and Tyk1 (Ghoreschi K et al 2009). The JAKs are physically associated with the cytokine receptors near the plasma membrane (Yamaoka K 2004). The JAKs tyrosine-phosphorylate proteins involved in cell growth, survival, and differentiation including members of the STAT family. JAKs contain seven JAK homology (JH) domains with the JH1 domain being the catalytically active kinase. The next region, JH2, is the catalytically inactive pseudokinase region which is also important for the regulation of the protein. The FERM domain present within these kinases provides the binding site for the cytokine receptors (Ghoreschi K et al 2009). The binding of receptors to the FERM domain leads to phosphorylation of cytokine receptors and the creation of docking sites for other signaling molecules with an SH2 domain (Yamaoka K 2004). STAT molecules contain an SH2 domain allowing them to bind to the cytokine receptors and become tyrosine-phosphorylated by JAKs.

A.STAT3 Structure

pTyr705

| N | Coiled Coil | DNA Binding | Linker | SH2 | TAD |

1　　　　138　　　　　　　　319　　　　　　465　　　　　　688　　　　770
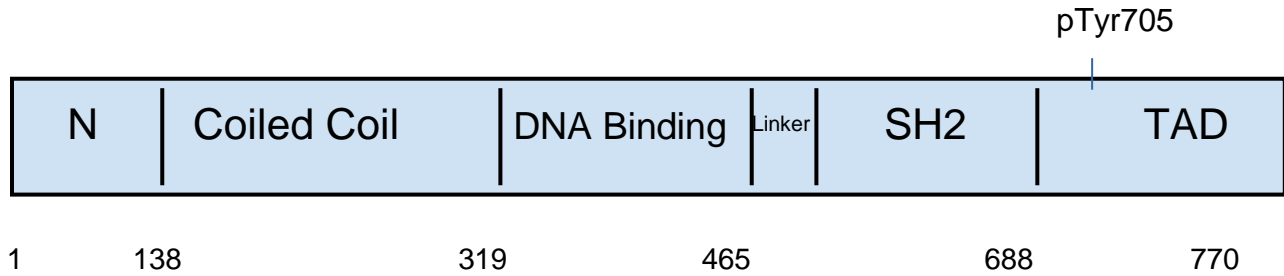
B. STAT Signaling Pathway
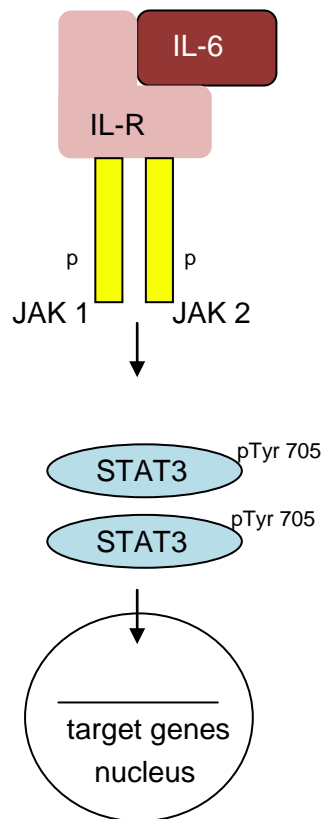
Figure 1: *STAT3 (Signal Transducer and Activator of Transcription-3) structure and signaling.* A) The STAT3 structure contains a coiled coil domain near the N' terminus of the protein followed by a DNA binding domain, Src Homology 2 (SH2) domain, and transactivation domain (TAD). The phosphorylated tyrosine residue amino acid 705 is shown in the diagram. B) The diagram depicts the phosphorylation of STAT3 residue by Janus kinases (JAKs) which are themselves phosphorylated following activation by cytokines like IL-6 binding to receptors. Phosphorylated STAT3 (pTyr STAT3) can enter the nucleus and bind target genes.

STAT Family Signaling

The STAT family members are responsible for activating many genes in response to growth factor or cytokine binding. The members of the STAT family include STAT1, STAT2, STAT3, STAT4, STAT5, and STAT6. STAT family proteins all have similar structures which contain an N terminal domain, coiled-coil domain, DNA binding domain, SH2 domain, and Transcriptional Activation Domain (TAD) (Figure 1A). STAT3 was first discovered by its activation in response to IL-6. STAT3 is phosphorylated on tyrosine residue at amino acid 705 resulting in a conformational change that is important for the protein to recognize and bind DNA targets to induce expression (Liu L et al 2005). It induces the transcription of genes involved in differentiation, proliferation, and maintenance of pluripotency (Levy DE 2002). The importance of STAT3 is linked to its role in several cancers. STAT3 has been found to be constitutively phosphorylated in transformed cells and is important for malignant transformation (Levy DE 2002). The constitutive activation of STAT3 is required for the growth of cells associated with head and neck cancer and multiple myelomas (Yu H et al. 2004). STAT3 is constitutively active in many other diverse types cancer including breast and prostate cancer, leukemias, and lymphomas (Yu H et al 2004). Therefore STAT3 is a potential therapeutic target in the fight against cancer.

Nuclear Trafficking

The nuclear membrane regulates traffic into the nucleus by preventing large molecules from entering the nucleus without help from transport machinery. In order for STAT proteins to enter the nucleus they need to be bound by protein carriers from the karyopherin family (Liu L et al 2005). The karyopherin family includes importins and

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exportins which regulate the movement of molecules across the nuclear membrane (Goldfarb DS et al 2004). An importin α / importin β heterodimer plays a critical role in nuclear import. Importin α acts as an adapter and binds to the nuclear localization sequence (NLS) found within proteins. Importin α consists of six members, importin-α1, importin-α3, importin-α4, importin-α5, importin-α6, and importin-α7. There is evidence that there is some specificity for particular importin-α recognition of NLS cargo. Classical NLS sequences are a stretch of basic amino acids recognized by importins. Importin-α also binds to importin β which interacts with the nuclear pore to import cargo (Goldfarb DS et al 2004). The structure of the importin α protein contains an importin β binding (IBB) domain upstream of 10 armadillo domains (Goldfarb DS et al 2004) (Figure 2A). Importin α is negatively regulated by self-association of the IBB with its armadillo repeats. Following IBB binding to importin β, the armadillo repeats become available to bind other NLS-containing cargo. Importin β mediates the transport of the trimer through the nuclear pore complex. Inside the nucleus there is a high concentration of Ran-GTP that binds importin β resulting in a conformational change, and the dissociation of the importin α/β transport complex (Figure 2B). Free importin α is recycled out of the nucleus by the specific exportin CAS that binds the carboxyl region of importin α and exports it out of the nucleus (Goldfarb DS et al 2004).

The nuclear localization of STAT family member STAT1 was shown to correlate with its tyrosine phosphorylation and dimerization (McBride KM et al 2002). The import of STAT1 uses the importin α/β complex for translocation into the nucleus (McBride KM et al 2002). Studies found that STAT1 translocates to the nucleus once cells are stimulated with interferon gamma. The importin α5 specifically recognizes the tyrosine

phosphorylated STAT1 homodimer. An important residue near the DNA binding domain of STAT1, amino acid leucine at position 407, was mutated to an alanine and this prevented the nuclear localization of the protein (McBride KM et al 2002). The mutation also inhibited binding to importin α5 (McBride KM et al 2002).

Unlike STAT1, both tyrosine phosphorylated STAT3 (pTyrSTAT3) and unphosphorylated STAT3 (U-STAT3) are found in the nucleus and cytoplasm. The nuclear localization sequence of STAT3 is still undefined, however previous studies have shown that amino acid residues 150-163, within the coiled coil domain, are needed for import. When these amino acids are deleted from STAT3, cellular localization assays show that the protein is mostly cytoplasmic. The deletion of this key stretch of amino acids results in the loss of the ability for importin α to bind the protein and transport it through the nuclear pore with help from importin β (Liu L et al 2005) (Reich NC et al 2006).

The specific nature of the NLS of STAT3 has yet to be determined, although the coiled coil domain is necessary (Liu L et al 2005). U-STAT3 has been shown to bind importin α3 (Liu et al 2005), while another group found that pTyrSTAT3 can bind importin α1, α3, and α5 (Ushijima R et al 2005). Nuclear import of STAT3 is critical for its function in transcription, and for this reason the mechanisms that regulate its import need to be understood.

My study used an in vitro approach with recombinant STAT3 to determine whether it could bind importin α, and if there was a preference for a particular importin α. Bacterial STAT3 proteins that were unphosphorylated or tyrosine phosphorylated

A.

IBB	Armadillo Domains	CAS Binding
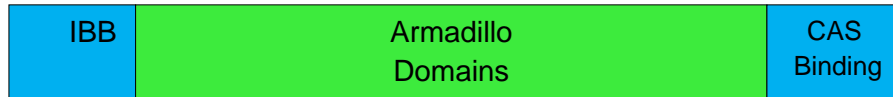
B.
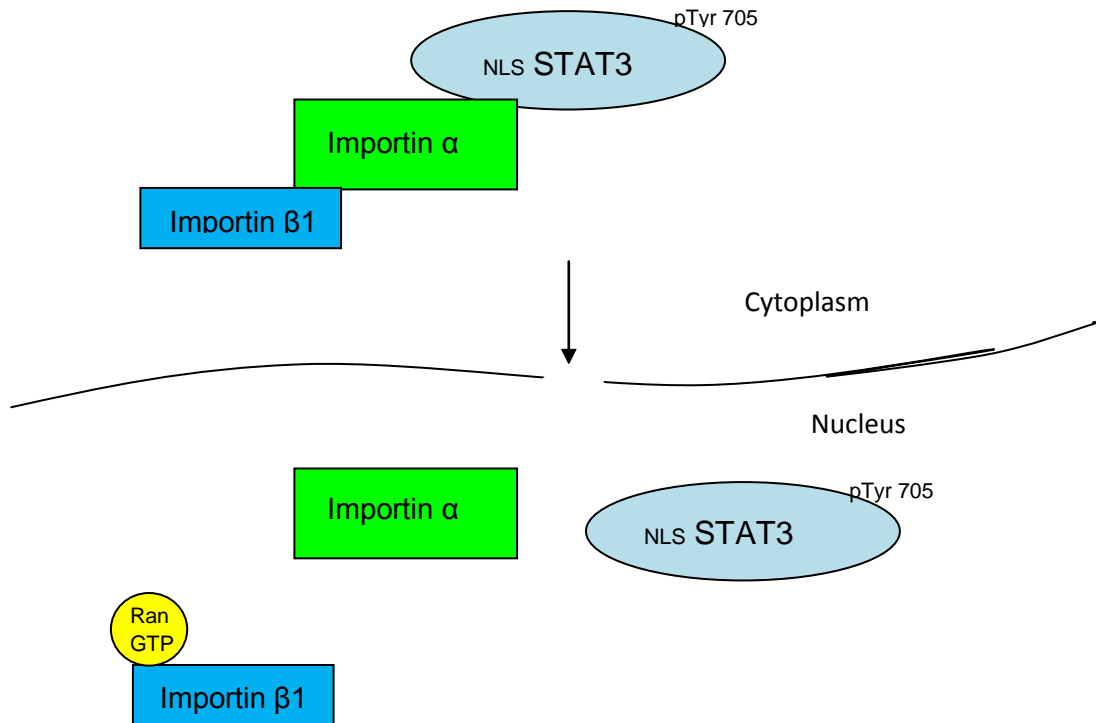
Figure 2: *Translocation of STAT3 protein into the nucleus.* A) The structure of the importin α proteins contains the IBB domain, ten armadillo repeats, and CAS binding domain. (B) The diagram depicts the translocation of the STAT3 protein using the importin α/β transport complex. Importin α binds the nuclear localization sequence (NLS) of STAT3 and also binds to importin β1. Importin β1 binds to the IBB domain of the importin α protein and carries the cargo through the nuclear pore. In the nucleus a high level of Ran GTP which binds B1 causing dissociation of the transport complex and release of the cargo.

were reacted with recombinant Importin α proteins in an in vitro binding assay to evaluate which importin α protein preferred to bind specific forms of STAT3.

The Role of STAT3 in Gammaherpesvirus

Herpesviruses have the ability to persist in a dormant or latent state for the lifetime of the host. Latency allows the virus to evade the immune system due to limited viral protein expression. In addition, expression of viral latency genes can promote lymphoproliferation, lymphoma, or sarcoma. The latent virus can reactivate at a later time leading to viral gene expression, production of virus, and detrimental effects on the host.

Two human gammaherpesvirus are Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV). EBV and KSHV have double stranded DNA genomes and can establish latency within its host, particularly in B lymphocytes (Guito J et al 2015). The DNA of these viruses is packaged in a protein capsid structure, tegument matrix made up of a combination of viral and host proteins, and surrounded by a glycolipid envelope (Guito J et al 2015). It has been challenging to understand the ability of EBV and KSHV to establish latency due to the inability of human gammaherpesvirus to infect other hosts (Barton E et al 2011). Therefore a mouse model of the infection using murine gammaherpesvirus 68 (MHV68) has been used to study latency (Barton E et al 2011, Krug L et al 2014).

The infection of mice with MHV68 illustrates the complexity of the gammaherpesvirus life cycle. The acute phase initiates with replication of the virus within mucosal epithelial cells and expression of the viral proteins. The virus gains access to the B cells and leads to latency expansion which involves the proliferation and

differentiation of B lymphocytes (Barton E et al 2011). During this phase there is expression of viral proteins that regulate B cell signaling pathways and allow the infected cells to evade the immune system. Eventually the virus within B cells becomes latent with expression of only a few viral proteins needed for maintenance of the viral DNA as an episome. The episome is tethered to the host chromosome while it maintains latency. Reactivation of the latent viral DNA and replication of the virus within B cells are the last two phases of the MHV-68 pathogenesis (Barton E et al 2011). In order for the virus within these cells to switch from the latent phase to the lytic phase, a virally encoded immediate early gene, replication and transcription activator (RTA) is needed (Barton E et al 2011). The role of RTA is to induce the transcription of the viral genes that cause the virus to enter the lytic phase (Hong Y et al 2011). Once the virus enters the lytic phase it will be able to replicate and disseminate. The structure of RTA includes a DNA binding domain located at the N terminus and a transactivation domain found at the C terminus (Hong Y et al 2011) (Figure 9). To activate the transcription of viral genes, RTA can bind to viral gene promoters through an RTA-responsive element (RRE) (Hong Y et al 2011). Some of the viral genes that are activated by MHV68 RTA binding to the RRE include viral ORF57 and ORF72 genes (Hong Y et al 2011). RTA of KSHV has also been shown to bind DNA indirectly by its interaction with other DNA-binding proteins such as RBP-Jk (Guito J et al 2015).

The ability of herpesvirus to reactivate after established latency is an important characteristic of these viruses. One study evaluated the contribution of the host STAT3 transcription factor on the ability of latent herpes simplex virus 1 (HSV-1) to undergo reactivation. When an inhibitor of STAT3 phosphorylation was introduced to cells

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containing HSV-1, an increase in expression of viral genes was observed (Du T et al 2013). This result indicates STAT3 is needed for maintenance of viral latency. STAT3 has also been shown to promote latency in vitro by EBV (Koganti S et al 2015). The relationship between STAT3 and KSHV RTA has been investigated in tissue culture, and a physical interaction was proposed to induce the activity of STAT3 (Gwack Y et al 2002).

I have used an in vitro approach to determine if there is a physical interaction between MHV68 RTA and recombinant STAT3. Both recombinant unphosphorylated STAT3 (U-STAT3) and tyrosine-phosphorylated STAT3 (pTyr STAT3) were used in an in vitro binding assay with mammalian cell lysates expressing MHV68 RTA. My results demonstrate a specific physical interaction between RTA and pTyr-STAT3. Recombinant STAT3 deletion mutants were also generated and results of binding assays indicate the DNA binding domain is needed for the interaction with RTA.

Chapter 2

Materials and Methods

Generation of Recombinant Plasmids:

STAT3

The STAT3 gene and gene fragments were cloned into the bacterial expression vector T7HMBP (Gift from Dr. Miguel Garcia Diaz). The protein produced is tagged with the T7 epitope, $(His)_6$, and maltose binding protein (MBP). Oligonucleotide primers were used to amplify genes with polymerase chain reaction (PCR) as shown in table 1. PCR products were ligated to HindIII-EcoRI linearized T7HMBP vector. The fragments were transformed into RapidTrans TAM1 competent bacterial cells (Active Motif) and selected for on agar plates with 100 ug/ul kanamycin (STAT3) or a combination of 12.5 ug/ul tetracycline and 50 ug/ul kanamycin. Single colonies were tested by restriction enzyme digestion for plasmids and followed by DNA sequence confirmation (SBU DNA Sequencing Facility).

The same method was used to clone STAT3 proteins with various deletions at the N terminus into the T7HMBP vector producing N terminal STAT3 deletion mutants tagged with $(His)_6$ and MBP. The STAT3 mutants included the amino acids 127-770 (Coiled coil domain, DNA binding domain, Linker, SH2 domain, and TAD domain), 320-770 (DNA binding domain, Linker, SH2 domain, and TAD domain), 495-770 (Linker, SH2 domain, and TAD domain), 585-770 (SH2 domain and TAD domain).

The STAT3 fragment 127-722, generated by inserting a stop codon after amino acid 722 through site directed mutagenesis using STAT3 127-770 as a template, was prepared by Jane Foreman.

A. PCR Oligonucleotide Primers

Location (Amino Acid)	Primer (5'->3')
1	CGGAATTCGCCACCATGGCTCAGTGGAACCAGCTG
127	GGAATTCGGCCAGGCCAACCACCCA
320	GGGGAATTCGCCTTCGTGGTGGAGCGG
495	GGGGAATTCAAGCCGCCAATTGGAACC
585	GGGGAATTCATCATGGGTTTCATCAGC
770	CCCAAGCTTTCACATGGGGGGAGGTAGCACA

B.PCR and Site Directed Mutagenesis Primers for STAT3 127-722

	Primer (5'->3')	PCR/Mutagenesis
STAT3 127-770 Forward	GGAATTCGGCCAGGCCAACCACCCA	PCR
Reverse	CCCAAGCTTAATGGTATTGCTGCAGGT	PCR
STAT3 127-722 Forward	AGCAATACCATTTGAAAGCTTGCGGCC	Mutagenesis
Reverse	GGCCGCAAGCTTTCAAATGGTATTGCT	Mutagenesis

Table 1*: Oligonucleotides used for PCR*. These primers were used in PCR cloning of STAT3 and various STAT3 N Terminal mutants. A) The clones include STAT3 1-770, STAT3 127-770, STAT3 320-770, STAT3 495-770, and STAT3 585-770. These primers include EcoRI sites at the 5' end (1, 127, 320, 495, and 585) and HindIII sites at the 3' end. B) The STAT3 fragment 127-722 was cloned using STAT3 127-770 as a template and inserting a stop codon after amino acid 722 using site directed mutagenesis. STAT3 fragments that were generated using these primers were ligated into a vector linearized with EcoRI and HindIII.

Importin α and β

Importin genes α1, α3, and α5 without the IBB domain were amplified by PCR with oligonucleotide primers, then ligated with the pGEX-KG vector. The proteins that were produced were tagged with a GST tag (Shin HY et al 2013).

Production of Recombinant Protein:

U-STAT3/ GST-Importin α/MBP

Plasmids were transformed into BL21 (DE3) bacterial cells (NEB) and selected for on agarose plates with 100 ug/ul kanamycin (STAT3/MBP) or 100 ug/ul ampicillin (GST-Importin α). Single colonies were grown in LB with kanamycin overnight at 37^oC and a 1/50 dilution was used to inoculate fresh LB. The culture was grown until the OD_{600} was between 0.4-0.8. The expression of the protein was induced with 0.2 mM IPTG and kept at 25^oC overnight before the cells were collected by centrifugation. The cell pellets were lysed in buffer containing 20 mM HEPES (pH 8.0), 500 mM KCl, 5% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, and 1X Protease Inhibitor (Roche) by sonication with a 1 sec pulse and 10 sec rest. The supernatant was run on 8% SDS PAGE gel and transferred to immobilon membrane for 1.5 hours. The membrane was blocked with 5% milk for 30 minutes and incubated with the primary antibody of anti-STAT3 (1:1000 Roche). The membrane was washed three times with 1X-TBS 0.1% Tween 20 and incubated with secondary antibody cocktail (anti rabbit 1:2500) for 1 hour at 4^oC. The membrane was washed another three times before being scanned with the LICOR Odyssey CLx scanner.

pTyr-STAT3/MBP

Plasmids were transformed into TKB1 competent cells (Agilent) which express the Elk tyrosine kinase and were selected on an agar plate with a combination of 50 ug/ul kanamycin and 12.5 ug/ul tetracycline. Single colonies were picked and grown in 2XYT media supplemented with kanamycin and tetracycline. A 1/50 dilution of the culture was made into LB which was grown until the O.D. 600 was between 0.4-0.8. The expression of the proteins and Elk kinase were induced with 0.2 mM IPTG and the cultures were grown at room temperature overnight before the cell pellets were collected by centrifugation (modified by Becker S, Corthals GL et al 1998) (Figure 3). To express STAT3 320-770 and 495-770 proteins the cultures were incubated at 16°C after the addition of IPTG. The expression of the protein was checked as described above using the pTyr-STAT3 (Cell Signaling 1:1000) primary antibody and anti-rabbit secondary antibody.

Purification of Recombinant Proteins by Binding to Amylose Beads

The expression of U-STAT3 and pTyr-STAT3 proteins was verified by immunoblot. The bacterial cell pellets were lysed in buffer containing 20 mM HEPES (pH 8.0), 500 mM KCl, 1% Triton X -100, 5% glycerol, and 1 mM EDTA with 1X bacterial protease inhibitor (Roche). The supernatant was cleared by centrifugation for 15 minutes at 14,000 rpm in 4°C. The cleared supernatant was then added to amylose beads pre-equilibrated in lysis buffer for 1 hour at 4°C. The beads were washed with lysis buffer three times and the bound protein complexes were eluted off the beads with the addition of sample buffer. The bound protein samples were separated on an 8%

A. Cloning STAT3 in pT7TEV-HMBP Vector
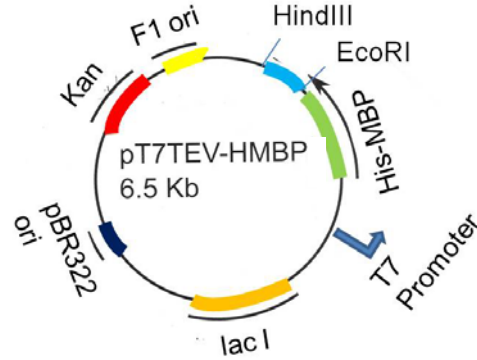
B. In Vitro Phosphorylation of STAT3
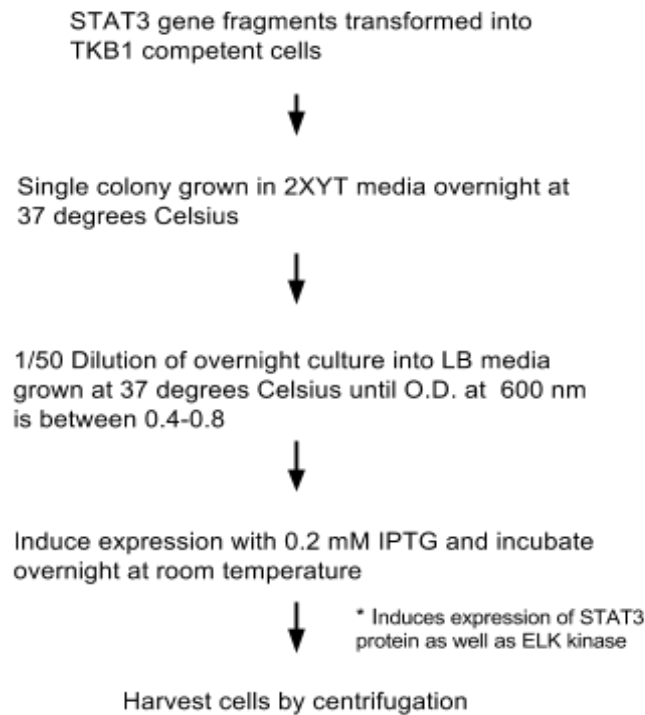
Figure 3. *Strategy for the expression and tyrosine phosphorylation of recombinant STAT3.* A) Fragments of the STAT3 gene were amplified by PCR with oligonucleotides containing HindIII and EcoRI sites at the end and ligated into the pT7TEV-HMBP vector linearized by HindIII and EcoRI. B) The flow chart describes how the STAT3 clone was phosphorylated using TKB1 competent cells which contain the elk kinase. The induction of protein expression with isopropyl β-D-1-thiogalactopryanoside (IPTG) also induced the expression of the elk kinase resulting in expression and phosphorylation of the protein.

SDS Page gel and transferred to immobilon membrane for 1.5 hours. The immobilon membrane was then blocked with 5% milk and the primary antibody cocktail of anti-pTyr-STAT3 (1:1000 Cell Signaling) and anti-STAT3 (1:1000 Roche) was added overnight at 4C. The membrane was washed three times with 1X TBS-0.1% Tween-20 wash buffer for ten minutes. The secondary antibody cocktail which included anti-mouse (1:2500 Rockland) and anti-rabbit (1:2500 Invitrogen) was incubated for 1 hour at room temperature. The membrane was washed three times again with 1X TBS-0.1% Tween-20 wash buffer and scanned using the LICOR Odyssey CLx Infrared Scanner.

GST Importin purification

Importin α 1, α3, and α5 were cloned into the pGEX-KG vector providing a GST tag for purification by glutathione beads (Sigma). The recombinant proteins were transformed into DH5α (Invitrogen) cells and a single colony was grown in LB supplemented with 100 ug/ml of ampicillin. The cultures were harvested and the cell pellets were resuspended in column buffer containing 1X PBS, 50 mM EDTA, 1% Triton-X 100, 1 mM PMSF and 1X bacterial protease inhibitors (Sigma) with 1 mg/ml lysozyme and incubated on ice for 30 minutes. The suspension was then sonicated with a 10 sec pulse and 20 sec rest. The cell lysate was cleared by centrifugation at 12,000 rpm for 30 minutes in 4°C. The supernatant was added to 1 mL of GSH beads slurry equilibrated in column buffer and incubated at 4°C for two hours. The beads were spun down by centrifuging at 2,500 rpm for five minutes and washed by incubating with 4 ml column buffer for ten minutes three times. After the last addition of column buffer the beads were spun down again by centrifuging at 2,500 rpm for five minutes and the supernatant was removed. The recombinant proteins were eluted off the beads with 2

ml of 40 mM glutathione in 10 mM TE buffer for 10 minutes. The beads were centrifuged for fifteen minutes at 2,500 rpm in 4°C and the supernatant was collected. The proteins were eluted off the beads three times and all three supernatants were combined and put into a dialysis bag to dialyze twice in buffer containing 20 mM Hepes pH 8.0, 50 mM NaCl, 1 mM EDTA, 15% glycerol, 0.2 mM PMSF and 1.0 mM β-mercaptoethanol.

In Vitro Binding Assay: Binding GST-Importins to HMBP-STAT3 proteins

Recombinant STAT3 bacterial pellets, USTAT3 127-722 and PSTAT3 127-722, and MBP cell pellets were resuspended in column buffer containing 20 mM HEPEs (pH 8.0), 500 mM KCl, 5% glycerol, 1% Triton X 100, 1 mM EDTA, 1 mM PMSF, and 1X bacteria protease inhibitor (Roche). The cells were lysed by sonication with a 1 sec pulse and 10 sec rest and centrifuged for fifteen minutes at 14,000 rpm in 4°C. Equal amounts of supernatant were combined with 15 ug of Importin α proteins overnight at 4°C. Next, 15 ul of amylose beads pre-equilibrated in column buffer were added for one hour. The beads were collected by centrifugation and the supernatant was removed before the beads were washed once with column buffer and 0.005% BSA then twice with column buffer. The bound proteins were eluted with sample buffer and run on 8% SDS PAGE gel then transferred to immobilon membrane for one hour and thirty minutes. The membrane was blocked for thirty minutes with 5% milk and incubated with the primary antibody, anti-GST(1:1000 Sigma), overnight at 4°C. The membrane is washed three times in 1X TBS-0.01% Tween 20 buffer for ten minutes. Secondary antibody, anti-mouse, (1:2500 Rockland) was incubated for one hour at room temperature. The membrane is washed three times with 1X TBS-0.1% Tween 20 three

In Vitro Binding Assay

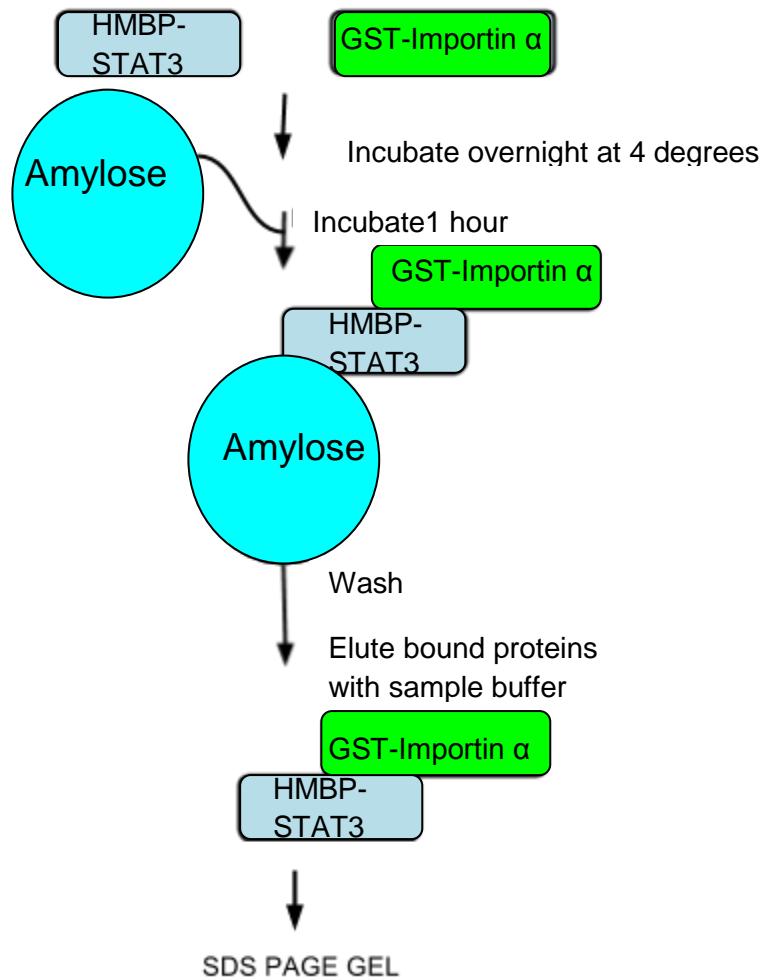Figure 4: *In vitro binding assay protocol.* The flow chart describes how the in vitro binding assay was used to determine the association between MBP-STAT3 and GST-Importin α proteins. The recombinant MBP-STAT3 and purified GST-Importin α proteins were incubated overnight together. Recombinant STAT3 was pulled down using amylose beads and the associated Importin α proteins were detected using a GST antibody.

times for ten minutes each. The membrane is scanned with the LICOR Odyssesy CLx

Infrared scanner to detect the GST protein. The GST-Importins were also detected with

HRP conjugated secondary antibodies (anti-rabbit 1:10,000, GE Healthcare Life

Sciences) and developed using autoradiography film. Phosphorylation of the input was

detected using anti pTyr STAT3 antibody (1:1000 Cell Signaling). The STAT3 protein

was visualized by coomassie stain.

The above assay was used to determine what region of pTyr STAT3 is needed

for the interaction with importin α proteins. The pTyr STAT3 mutants previously

described were used in this assay with importin α1 or α5.

Recombinant HMBP-STAT3 Interaction with RTA-FLAG

To study the interaction between recombinant STAT3 and RTA-FLAG, 293T cells

plated in a 10 cm tissue culture plate were transfected with 4 ug RTA-FLAG using Mirus

LTI transfection reagent. The RTA FLAG plasmid, cloned into the pcDNA™5/FRT/TO

vector, was a gift from Dr. Laurie Krug. The cells were incubated for 36 hours before

they were harvested. Recombinant full length STAT3 was expressed as described

previously and resuspended in binding buffer containing 20 mM HEPEs (pH 8.0), 200

mM KCl, 5% glycerol, 1% Triton-X100, 1 mM EDTA with 1 mM PMSF and 1X Protease

Inhibitor (Roche). The recombinant STAT3 was added to amylose beads pre-

equilibrated in binding buffer for one hour at 4°C. The mammalian cell pellets,

containing 340 ug of protein including RTA, were lysed in lysis buffer containing 20 mM

HEPEs (pH 7.4), 100 mM NaCl, 0.5% NP-40, 1% Triton X-100 with 1 mM PMSF and 1X

Mammalian Cell Protease Inhibitor (Sigma) incubating for 1 hour at 4°C. The beads

were then washed once in binding buffer and 0.1% BSA for five minutes, and twice with

lysis buffer for five minutes each time. After the beads were washed and equilibrated in lysis buffer, mammalian cell lysate containing RTA FLAG was added to the beads to incubate overnight at 4^oC. The beads were then washed six times with lysis buffer and the bound proteins were eluted off the beads with sample buffer. The samples were loaded onto an 8% SDS PAGE gel and transferred to immobilon for one hour and a half. The membrane was blocked in 3% BSA and the primary antibody, anti-FLAG (1:500 Sigma) was incubated overnight at 4^oC. The membranes was washed three times in 1X TBS-0.1% Tween 20 buffer for ten minutes. The secondary antibody, anti-mouse (1:2500 Rockland or 1:10,000 Invitrogen) was added for one hour at room temperature. The membrane was washed three times again before being scanned by the LICOR Infrared Imaging scanner or developed on Amersham Hyperfilm (GE Healthcare). The phosphorylation of the input was detected using anti pTyr STAT3 antibody (1:1000, Cell Signaling) and the STAT3 protein was visualized with coomassie stain.

The above in vitro binding assay was also used to determine which region of pTyr- STAT3 is involved in interacting with RTA-FLAG using STAT3 N terminal deletion mutants previously described.

Chapter 3

Results

STAT3 Interaction with Nuclear Import Transport Proteins:

To gain entrance into the nucleus, large proteins must be escorted by transport receptors called importins (Goldfarb DS et al 2004). The common receptor is a heterodimer of importin-β and importin-α. Importin β physically interacts with proteins lining the nuclear pore complexes, and it is bound to one of several importin-αs that directly binds cargo. To determine if one or more of the importin-αs recognize STAT3, I developed an in vitro binding assay.

Expression and Phosphorylation of Recombinant STAT3

In order to evaluate the mechanism by which STAT3 is imported to the nucleus, our objective was to test direct binding of STAT3 to various importin-alpha proteins. For this reason I developed an in vitro binding assay of partially purified proteins expressed in bacteria. The full-length STAT3 gene was amplified with oligonucleotide primers and polymerase chain reaction (PCR) to generate full length STAT3 1-770 a.a., and 127-722 a.a. (primers shown in Table 1). The 127-722 a.a. fragment was crystallized previously and was therefore likely to express stable protein (Becker S et al 1998).

The STAT3 transcription factor recombinant proteins were generated through PCR cloning. The recombinant proteins were expressed using two different competent cells in order to generate USTAT3 along with pTyr STAT3. STAT3 fragments were ligated into the pT7HMBP bacterial expression vector and cloned in frame with an N-terminal tag; Histidine repeats and the Maltose Binding Protein. The STAT3 clones were transformed into RapidTrans TAM1 competent cells and the colonies were evaluated for

the correct placement of the insert into the vector. The DNA was then sequenced to confirm the cloning was correct. Plasmid DNA was then transformed BL21 competent cells and expressed with IPTG to generate unphosphorylated STAT3 (U-STAT3), or transformed into TKB1 competent cells which express the elk1 tyrosine kinase in order to prepare tyrosine phosphorylated STAT3 (pTyr-STAT3) (Figure 3). STAT3 protein was isolated from the transformed bacteria that was either unphosphorylated or tyrosine phosphorylated. The expression and phosphorylation of recombinant STAT3 was confirmed using Western blot analysis with antibodies to STAT3 (Figure 5A), or antibodies to anti-tyrosine phosphorylated STAT3 (Figure 5B). The tagged STAT3 proteins expressed well in both bacteria. STAT3 was tyrosine phosphorylated only in the TBK1 cells.
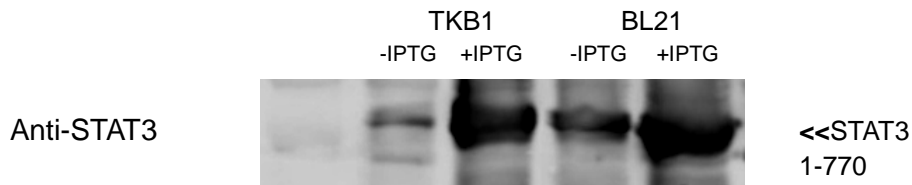
Expression of GST-importin-alphas

Bacterial expression plasmids encoding importin genes α1, α3, and α5 tagged with glutathione–S-transferase (GST) and lacking the inhibitory amino terminal importin-beta binding (IBB) domain were generated previously (Liu L et al 2005). I transformed DH5α bacteria with the GST-importin α and purified the proteins by binding to glutathione beads. Protein was quantified, and evaluated by gel electrophoresis (data not shown).

Preferential in vitro binding of STAT3 with specific importin-alphas

The most common mechanism for nuclear import is mediated by the transport receptor heterodimer, importin α/β (Goldfarb DS et al 2004). Previous studies of our laboratory found that importin α3 binds to U-STAT3, and importin α3 is necessary for U-STAT3 entry into the nucleus (Liu L et al 2005). Studies of another group indicated that

A. STAT3 Protein Expression

Anti-STAT3   **<<**STAT3 1-770

TKB1   BL21
-IPTG +IPTG   -IPTG +IPTG

B. STAT3 Tyrosine Phosphorylation

Anti-
pTyr STAT3   **<<**STAT3 1-770

TKB1   BL21
-IPTG +IPTG   -IPTG +IPTG
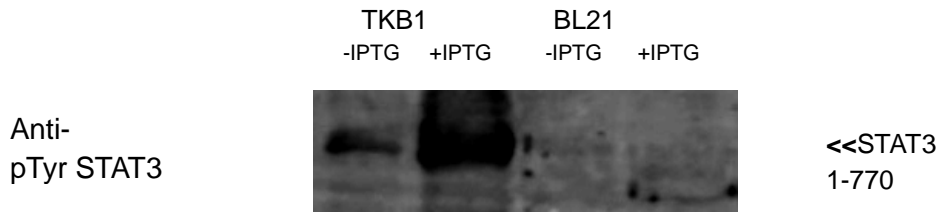
Figure 5 : *Protein expression and tyrosine phosphorylation of recombinant STAT3.* A) Unphosphorylated (USTAT3) and phosphorylated STAT3 (pTyr STAT3) were expressed and bacterial pellets were lysed and analyzed on a 8% SDS PAGE gel. Proteins were transferred to immobilon membrane for Western blot. The expression of the STAT3 proteins was detected using STAT3 antibody. B) The phosphorylation of the STAT3 was detected using an anti pTyr STAT3 antibody. pTyr STAT3 expressed in TBK1 cells, and U-STAT3 expressed in BL21 cells.

pTyr-STAT3 was recognized by various members of the importin α family including importin α1/α3/α5. For this reason my in vitro binding assay included a member from each of the subfamilies. Each of these importin α proteins belong to a different importin α subfamily to determine if a specific importin α subfamily preferentially interacted with STAT3.

I used an in vitro binding assay to investigate whether one of the importin alpha proteins preferred to bind U-STAT3 or pTyr-STAT3. The in vitro binding assay used the STAT3 protein fragment that encodes amino acids 127-722 tagged with HMBP. Partially purified recombinant GST-importins and HMBP-STAT3 bacterial lysates were incubated overnight at 4^oC and then HMBP-STAT3 was pulled down by binding to amylose beads (Figure 6). Associated GST-importin α proteins were evaluated by a Western blot probed with a GST antibody.

My findings are shown in Figure 6. MBP protein, MBP-U-STAT3 or MBP-pTyr-STAT3 were bound to amylose beads and associated GST-importins are indicated by the Western blot. There was no detectable binding observed with the MBP bacterial lysate which served as a control for importin α1 and importin α3 (lanes 1 and 4). Importin-a1 was found to bind both U-STAT3 and pTyr-STAT3 relatively equally (lanes 2 and 3). Both importin-a3 and importin-a5 preferred to bind to U-STAT3 in comparison to pTyr-STAT3. While it appears that importin α5 can bind pTyr STAT3 there is also some binding to the MBP control lysate as well. It was uncertain whether the binding between importin α5 and pTyr STAT3 was more than MBP. A follow up experiment indicated stronger binding interaction of importin α5 with pTyr STAT3 in comparison to the MBP (data not shown). Therefore the binding of importin α5 to pTyr STAT3 was

pursued further. When the membrane was incubated with anti pTyr STAT3 antibody the input protein was confirmed to be phosphorylated (Figure 6B). These results indicate that more than one importin-alpha can specifically bind to STAT3 in this assay. In addition, the phosphorylated state of STAT3 may influence its association with particular importins.

Domains of STAT3 recognized by importin-alphas

Tyrosine-phosphorylated dimers of STAT3 have a parallel conformation in which the phosphotyrosine of one monomer interacts with the SH2 domain of the other monomer. This is evident in the crystal structure of STAT3 bound to DNA (Becker S et al 1998). In comparison U-STAT3 crystal encoding amino acids 127-688, lacking the tyrosine residue which can be phosphorylated, was found to be in an anti-parallel conformation (Ren Z et al 2008). The phosphorylation of STAT3 appears to result in a conformational change of the protein from an anti-parallel structure to a parallel structure (Figure 7A).

Since there is a preference of some of the importin-alphas tested for binding to U-STAT3 or pTyr-STAT3, the importins may recognize a different domain of pTyr-STAT3. To identify the region of pTyr STAT3 needed for interaction with GST-Importin α1 or α5, I generated N-terminal deletions of STAT3 and cloned the STAT3 fragments into the HMBP vector. PCR primers used for cloning are shown in Table 1. These fragments were expressed in the TBK1 bacteria so that they would all be tyrosine phosphorylated. A linear depiction of the new deletion mutants is shown in Figure 7A.
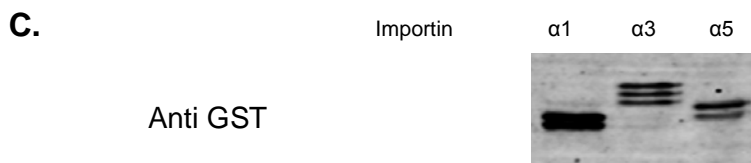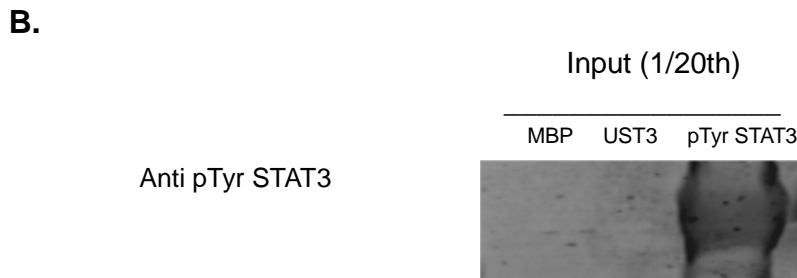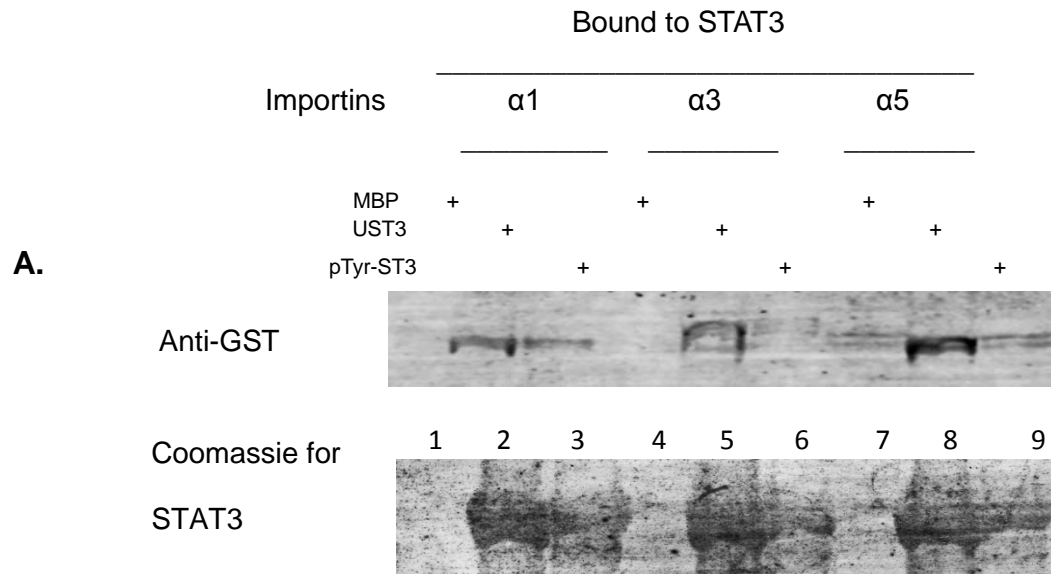
Bound to STAT3

Importins		α1			α3			α5	
MBP	+			+			+		
UST3		+			+			+	
pTyr-ST3			+			+			+

A.

Anti-GST

Coomassie for

STAT3

1 2 3 4 5 6 7 8 9

B.

Input (1/20th)

MBP   UST3   pTyr STAT3

Anti pTyr STAT3

C.

Importin   α1   α3   α5

Anti GST

Figure 6. *The Interaction between HMBP-STAT3 and GST-Importin α proteins*.   A) Bacteria expressing USTAT3, or pTyr STAT3, or MBP were lysed and incubated with purified GST-Importin α proteins overnight.   Amylose beads are used to pull down STAT3 and the associated GST-Importin α proteins were detected by a Western blot probed with anti GST.   MBP bacteria lysate was used as a control (lanes 1, 4, and 7) , U-STAT3 was in lanes 2, 5, and 8 and pTyr STAT3 results were shown in lanes 3, 6, and 9.   A coomassie stain was used to show the STAT3 protein bound to amylose beads. Anti-pTyr STAT3 was used to demonstrate phosphorylation of pTyr STAT3 input. The Importin α input was detected with using a GST antibody.

STAT3 mutants lacking the N-terminus (127-770 a.a.), lacking the N-terminus and coiled coil domain (320-770 a.a.), lacking the N-terminus, coiled-coil domain and DNA binding domain (495-770 a.a.), or lacking all of STAT3 except the SH2 domain and transcriptional activation domain (TAD) (585-770 a.a.) are shown (Figure 7B). Based on sequencing results, the recombinant clones were correct and in frame with MBP.

GST-Importin alpha proteins were incubated with pTyr STAT3, and complexes were collected by MBP-STAT3 binding to amylose. Importins associated with STAT3 were eluted off the beads and evaluated by Western blot probed with anti GST (Figure 8). Importin-α1 was evaluated in lanes 1-6, and binding was found to be greatest with pTyr-STAT3 127-722 (lane 1). Importin-α1 also binds to pTyr-STAT3 127-770 (lane 2) and 320-770 (lane 3) although less well. The core fragment of STAT3 (127-722 a.a.) that was previously crystallized as a tyrosine-phosphorylated dimer appeared to be most stable of the recombinant STAT3 proteins. For this reason it may be able to bind the importins more efficiently in the assay. It is also possible that the longer pTyr-STAT3 127-770 has an altered conformation when fused to MBP and the importin binding site is not as accessible. However, all the STAT3 fragments were accurately phosphorylated on tyrosine 705 in the bacteria. By using LICOR Odyssey software to quantify pTyr STAT3 bound to amylose beads and associated GST-Importin alpha the deletion of the coiled coil domain (pTyr-STAT3 320-770, lane 4) most likely has no significant effect on interaction between the importins and pTyr STAT3. There was no detectable binding to pTyr-STAT3 495-770 that encodes a linker, SH2 domain, and TAD (lane 5) or pTyr-STAT3-585-770 (lane 6). Binding reactions with GST-importin-α5 appear to also require the DNA binding domain (lane 10). The input STAT3 proteins were shown by a

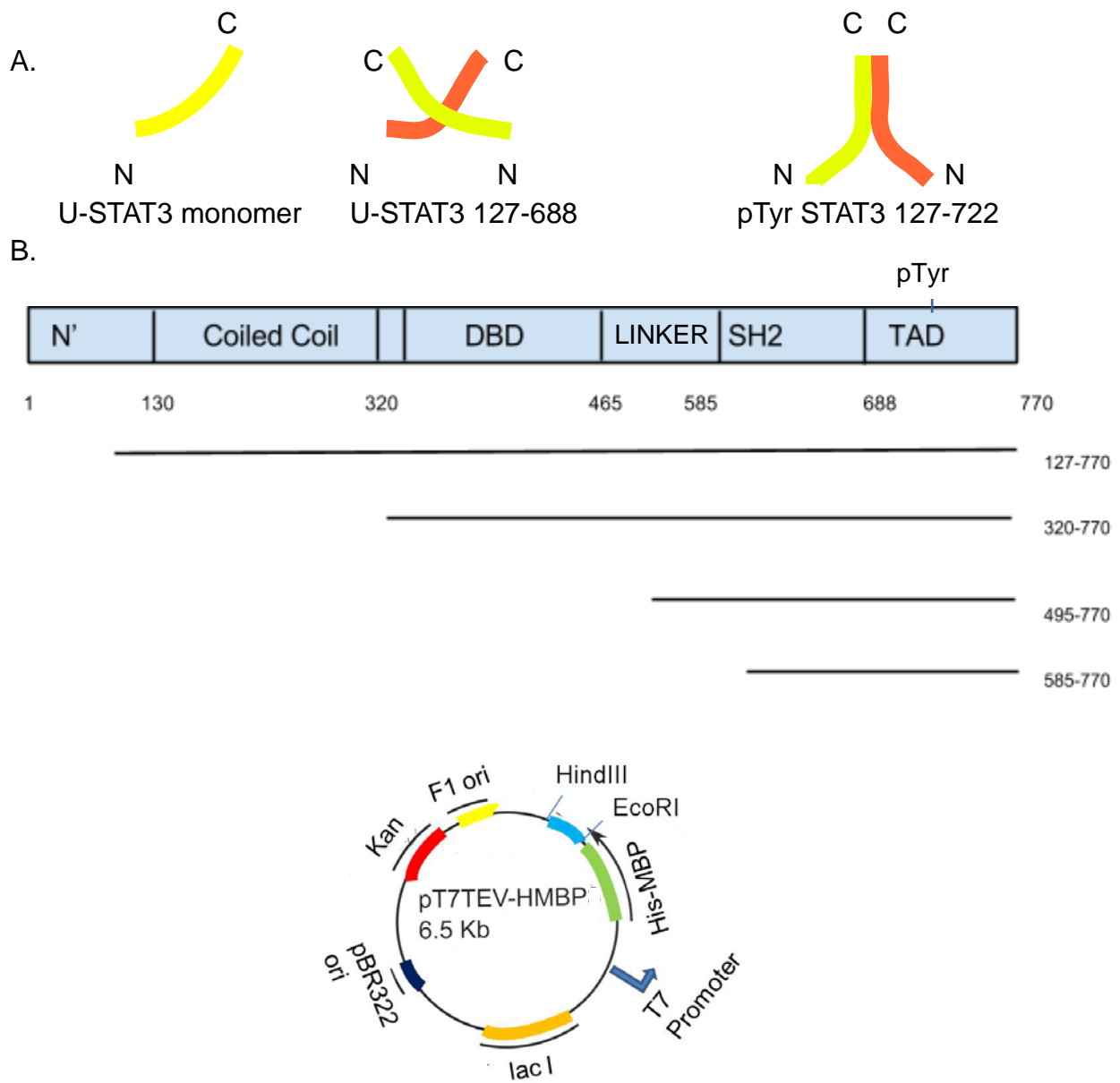Figure 7. *Cloning of recombinant phosphotryosine STAT3 mutants.* A)The conformation of STAT3 proteins is dependent on the phosphorylation state of the protein. The U-STAT3 protein is an anti-parallel state while pTyr-STAT3 is an a parallel conformation. B) Cloning of the pTyr STAT3 N terminal mutants involved the PCR amplification of STAT3 fragments that are ligated into the pT7TEV-HMBP vector linearized with EcoRI and HindIII.
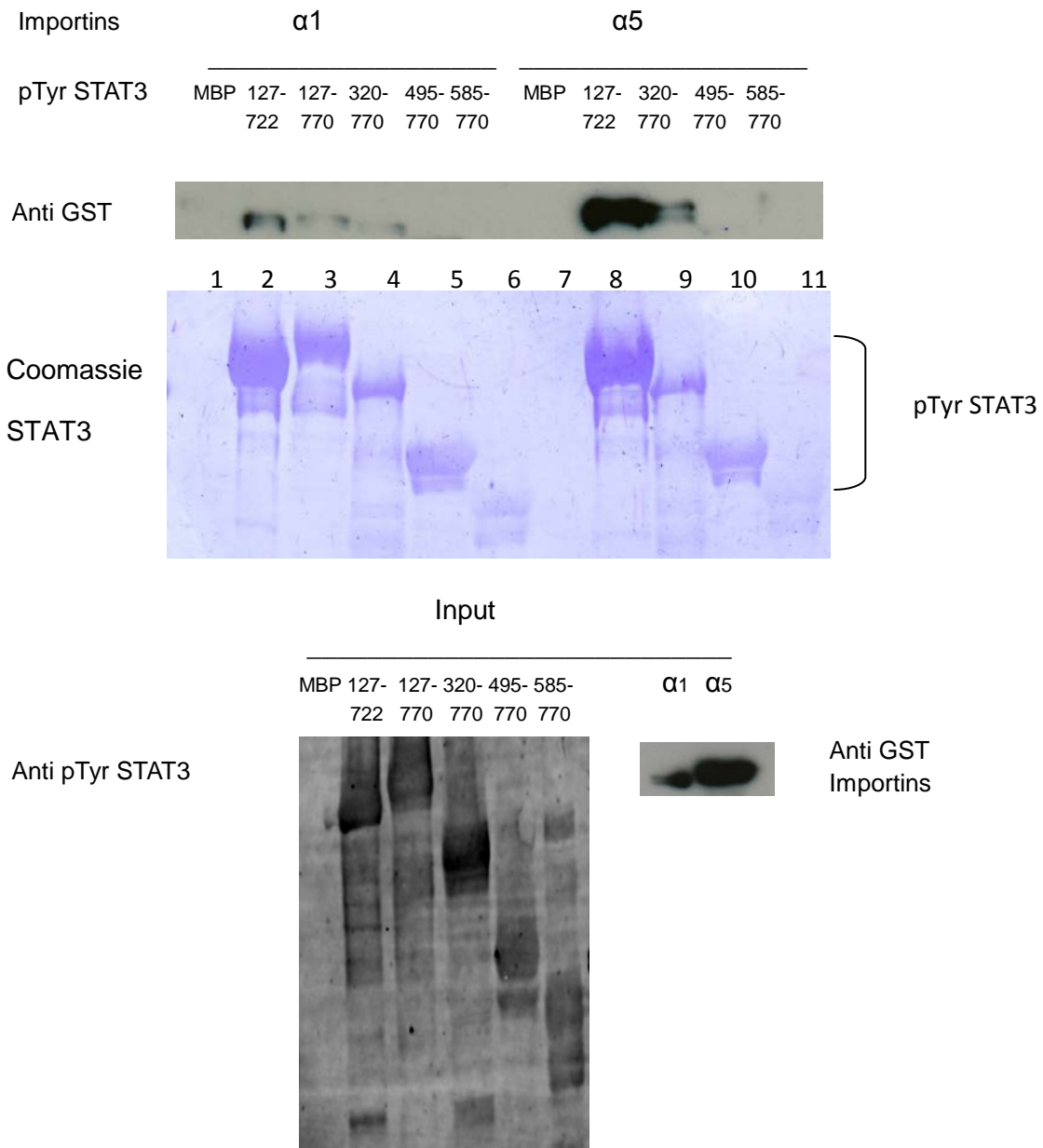
Figure 8: *Mapping the region of pTyr STAT3 needed for the interaction with Importin αs*. The results of the in vitro binding assay are shown using the Western blot probed with GST. The results include control lysate, MBP, shown in lanes 1&7, pTyr STAT3 127-722 (lane 2&8), pTyr STAT3 127-770 (lane 3), pTyr STAT3 320-770 (lane 4&9), pTyr STAT3 495-770 (lane 5&10), and pTyr STAT3 (lane 6 & 11). The Coomassie stain depicted the pTyr STAT3 mutants bound to amylose. Input GST importins are shown below as tyrosine phosphorylation of pTyr STAT3 input.

Coomassie-stained membrane, and the associated importin–α1 or importin-α5 were detected with anti-GST antibody (Figure 8). The STAT3 input was also confirmed to be phosphorylated using a pTyr STAT3 antibody (Figure 8).

The recognition of pTyr-STAT3 by importin-αs appears to require the DNA-binding domain.

Chapter 4

STAT3 Recognition by the MHV68 Replication and Transcription Activator (RTA)

Human gammaherpesviruses such as Epstein Barr Virus (EBV) or Kaposi's sarcoma-associated herpesvirus (KSHV) establish life-long infections in their host (Barton E et al 2011). The viruses can remain latent in cells as DNA episomes and promote cancer, or reactivate to produce virus and cause various pathologies. Viral latency as well as acute infection are health challenges. EBV and KSHV are species specific pathogens, and for this reason the murine gammaherpesvirus 68 (MHV68) is used as a model to evaluate various viral genes that are necessary for pathogenesis in mice, and to infect mice with genetic alterations to identify host determinants of disease (Barton et al 2011).

One of the viral proteins that is necessary for KSHV and MHV68 acute infection is the replication and transcription activator (RTA) (Guito J et al 2015). RTA induces expression of viral genes needed for replication. RTA has an N-terminal DNA binding domain and a C-terminal TAD. In addition, KSHV RTA has been shown to bind and activate the host transcription factor STAT3 (Gwack Y et al 2002). For this reason we investigated the ability of the MHV68 RTA protein to bind to recombinant U-STAT3 or pTyr-STAT3 in an in vitro binding assay.

30

Preferential Binding of MHV68 RTA to Tyrosine-Phosphorylated STAT3.

I established an in vitro binding assay using recombinant STAT3, either unphosphorylated or tyrosine-phosphorylated, and RTA expressed in mammalian cells. The mammalian RTA expression vector was a gift of Dr. Laurie Krug. This construct codes for MHV68 RTA tagged with the FLAG epitope at the C-terminus. The RTA-FLAG was transfected into 293T cells with Mirus Trans-IT reagent, and cells were harvested 36 hours post transfection and lysed in buffer containing 20 mM Hepes (7.4), 100 mM NaCl, 1% Triton X 100, 0.5% NP-40, 1 mM PMSF, and 1X Mammalian Protease Inhibitor (Sigma). The bacterial lysate expressing recombinant HMBP tagged U-STAT3 or pTyr-STAT3 was prepared as described in Chapter 3.

An in vitro binding assay was performed using 340 ug of 293T protein lystate containing RTA-FLAG incubated with bacterial U-STAT3 or pTyr-STAT3. The MBP-tagged STAT3 proteins were collected on amylose beads, and associated RTA was detected by Western blot with anti-FLAG antibodies (Figure 9). RTA was readily detected in the 293T cell lysate (lane 1), and there was no RTA binding to MBP (lane 5), and only a modest binding to U-STAT3 (lane 6). However, RTA was found to clearly and selectively bind to pTyr-STAT3 (lane 7).

The results clearly indicate MHV68 RTA ability to recognize STAT3 in vitro, and RTA has a preference for binding the tyrosine-phosphorylated form of STAT3.

Domain of Tyrosine-Phosphorylated STAT3 recognized by MHV68 RTA.

To identify the regions of pTyr STAT3 important for the interaction with RTA in the in vitro binding assay, I tested the N-terminal deletion mutations of STAT3 described

in Chapter 3. FLAG-RTA was expressed in 293T cells and lysate was incubated with HMBP-pTyr-STAT3 proteins expressed in the TBK1 cells. As shown for full length STAT3 in Figure 10, there was no binding to MBP control (lane 8), but clear binding to MBP-pTyr-STAT3 full length (1-770 a.a.) (lane 9). Deletion of STAT3 N-terminus (lane 10) and the N-terminus with the coiled coil domain (lane 11) reduced the binding of RTA, but it was still detectable. Deletion of the N-terminus, coiled coil domain, and the DNA binding domain eliminated binding by RTA (lane 12). Unexpectedly, it seems that the RTA binding requires the same region of STAT3 as importin-alpha binding (Figure 8).
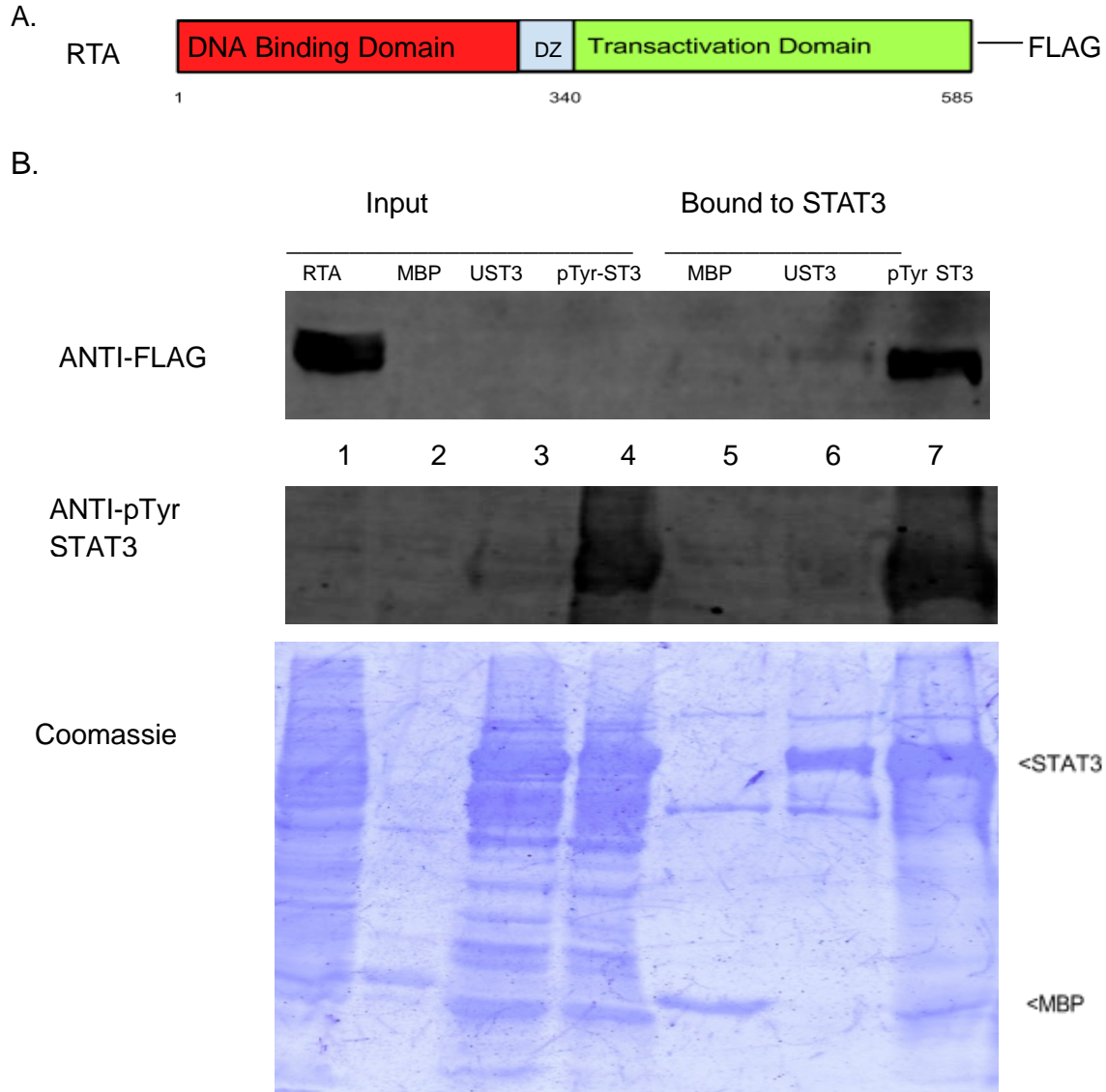
Figure 9. *Binding of RTA-FLAG to recombinant STAT3 proteins*. A) The structure of the murine gammaherpesvirus 68 protein, RTA, includes a DNA Binding Domain, dimerization domain (DZ), and transactivation domain (TAD). B) The RTA protein was expressed by the transfection of 293T cells. Recombinant STAT3, U-STAT3 and pTyr-STAT3 were expressed in bacteria. The results of the in vitro binding assay with RTA FLAG and HMBP-STAT3 are shown using a Western blot probed with anti FLAG antibody. The blot shows MBP control (lane 5), U-STAT3 1-770 (lane 6), and pTyr STAT3 1-770 (lane 7). The pTyr STAT3 input was shown to be phosphorylated using an anti-pSTAT3 antibody (middle panel). The STAT3 input is shown with Coomassie stain.
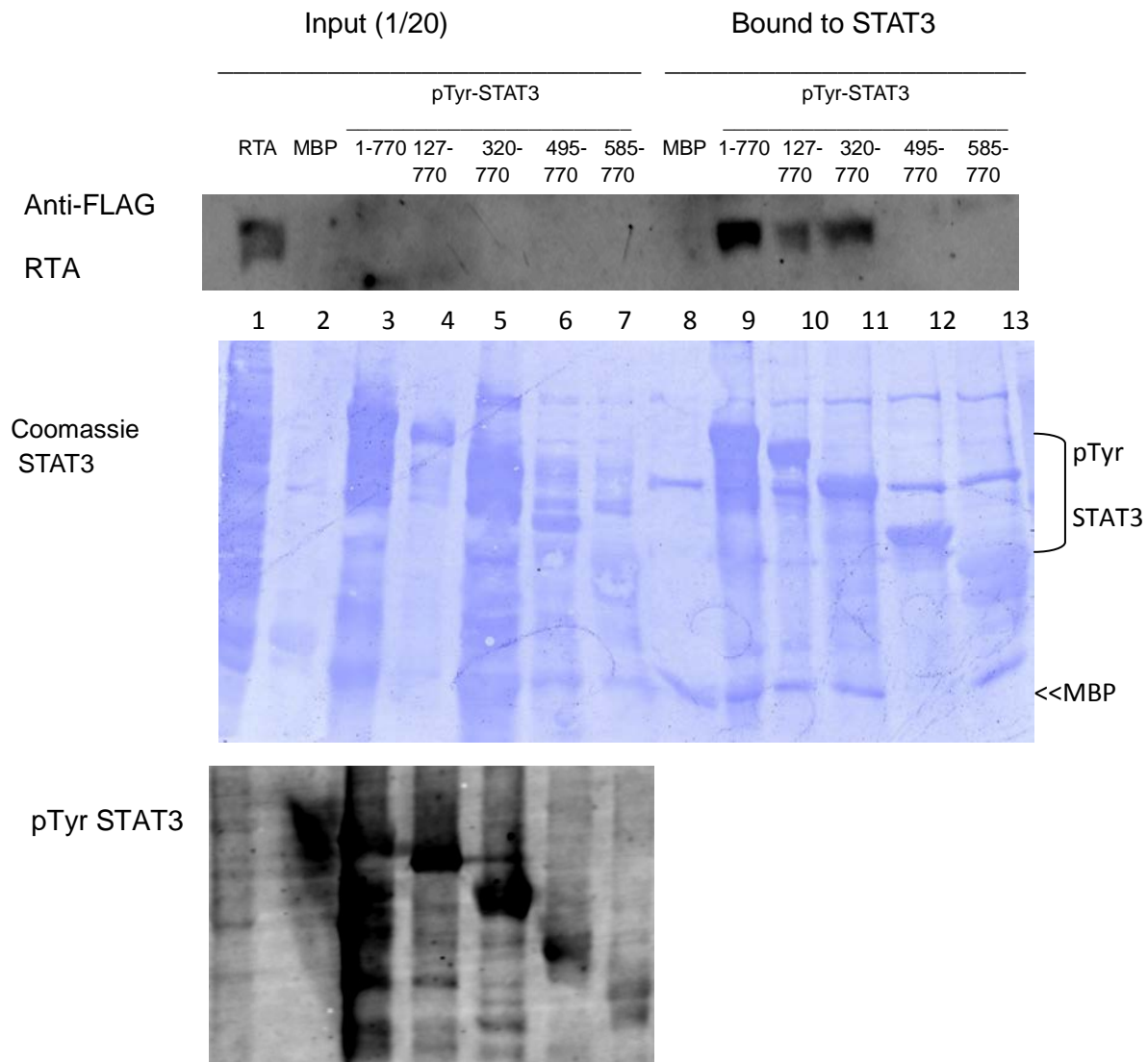
Figure 10. *The Domains of pTyr-STAT3 involved in the interaction with RTA.* The domains of pTyr STAT3 were mapped to determine which is needed for the interaction with RTA. The pTyr STAT3 mutants bound to amylose beads were incubated with RTA. RTA associated with the pTyr STAT3 mutants as detected on a Western blot probed with an anti-FLAG antibody. The RTA input was detected with anti-Flag antibody (lane 1). The results include MBP control (lane 8), pTyr STAT3 1-770 (lane 9), pTyr STAT3 127-770 (lane 10), pTyr STAT3 320-770 (lane 11), pTyr STAT3 495-770 (lane 12), and pTyr STAT3 585-770 (lane 13). The STAT3 input was detected with Coomassie (lanes 3-7) and phospho STAT3 antibody.

Chapter 5

Discussion

One of the functions of the STAT family of transcription factors includes the transcriptional regulation of target genes in the nucleus. Tyrosine phosphorylated STAT3 is known to induce the transcription of genes involved in differentiation, proliferation, migration, and apoptosis (Dechow TN et al. 2004; Niu G et al. 2002; Qin Q et al. 2012; Sullivan NJ et al. 2009) The dysregulation of STAT3 expression can promote cancer and inflammatory related diseases. An understanding of the protein interactions with STAT3 which influence its ability to function as a transcriptional activator is important for developing methods to disrupt these interactions and block the activity of STAT3.

Developing a method to observe STAT3 binding in vitro provided an opportunity to determine which proteins interact with the transcription factor. Previous studies showed the importin alpha family interact with STAT3 and allow entry into the nucleus (Liu L et al 2005; Ma J et al. 2006; Ushijima R et al 2005). To elucidate the subset of importin alphas which interact with STAT3, I established an in vitro assay using recombinant STAT3 and importin alpha proteins. The STAT3 recombinant protein was cloned into a vector containing an MBP tag providing an affinity to amylose beads. The recombinant STAT3 protein was transformed into two different types of competent E.coli generating U-STAT3 and pTyr-STAT3. Recombinant importin proteins were cloned into a vector containing a GST tag providing for detection with an antibody against GST. Based on the in vitro binding assay results importin α1, α3, and α5 bind to STAT3 (Figure 6). The results of the binding assay show that Importin α1 was able to bind U-

STAT3 and pTyr-STAT3 to a relatively equal extent. Importin α3 has a preference to bind U-STAT3 rather than pTyr-STAT3. Importin α5 also shows a preference for binding U-STAT3 but can possibly bind pTyr-STAT3 to a lesser extent (Figure 6). While it was unclear at first whether importin α5 could bind to pTyr STAT3 future experiments proved that an interaction does exist. Previous studies used STAT3 protein expressed and phosphorylated in eukaryotic cells while my approach used recombinant STAT3 that was expressed and phosphorylated in bacteria. Phosphorylation of STAT3 is the only post translational modification that occurs when using bacteria cells but proteins that are expressed in eukaryotic cells undergo multiple post translational modifications which could be why the results obtained here were slightly different from those previously published.

The interaction between STAT3 and importin αs is present independent of the phosphorylation state of the protein. However, tyrosine phosphorylation of the protein influences which importin α interacts with STAT3. It has been established that STAT3 can enter the nucleus independent of tyrosine phosphorylation explaining why importins must be able to bind to U-STAT3 as well as pTyr-STAT3 (Liu L et al 2005). STAT3 was also shown to interact with different subclasses of the importin α family. The expression of different importin α proteins is dependent on the tissue (Kamei Y et al 2002). The ability of the STAT3 protein to interact with different subclasses of the importin α family allows STAT3 to be translocated into the nucleus in various tissues. Target genes that are activated by STAT3 can result in proliferation, survival, or apoptosis dependent on which tissue the protein is expressed in (Levy DE et al 2002). In order to activate target genes in different tissues the STAT3 would interact with the importin α that is expressed

within that tissue. Therefore it is important that STAT3 be able to interact with multiple members of the importin α family.

I mapped the domain of pTyr-STAT3 required for interaction with importin α proteins as well. Our results indicate that the DNA binding domain of pTyr STAT3 is important for binding importin α proteins. An in vitro binding assay with STAT3 N-terminal mutants and importin α1 and α5 was used to map the region. The disruption of the interaction between importin α and STAT3 would stop nuclear localization of the STAT3 protein and prevent the activation of target gene expression. Understanding what region of pTyr STAT3 is needed for the interaction with importin α allows for the design of a drug which disrupts this interaction. Lowering the expression of STAT3 might be an important therapeutic option for those suffering from cancer known to have STAT3 overexpressed.

Another protein interaction with STAT3 that we studied was with the murine gammaherpesvirus 68 protein, RTA. RTA is a viral protein produced early in gammaherpesvirus infection that is required for replication and reactivation of the latent virus. The STAT3 protein has also been shown to have a role in the gammaherpesvirus establishing latency. When the STAT3 gene is knocked out of B cells the virus cannot become latent (Krug et al 2014). Therefore understanding how STAT3 and RTA interact would be beneficial in elucidating the role of the protein in the gammaherpesvirus and potential therapeutic targets. Our results indicate that U-STAT3 has very little interaction with RTA while the pTyr-STAT3 establishes binding with RTA. RTA might interact with pTyr STAT3 in an attempt to regulate the activation of pTyr STAT3 target genes. Previous studies found that U-STAT3 can also bind DNA and turn

on the expression of target genes. However, USTAT3 and pTyr STAT3 activate different target genes (Nkansah E et al 2013). Therefore it is possible that RTA has a preference for regulating the activation of the target genes induced by pTyr STAT3.

Using mutants of the STAT3 protein which contained deletions of the regions starting from the N terminus in an in vitro binding assay with MHV68 RTA provided data that demonstrated the DNA binding domain was needed for the interaction with RTA. In the pTyr-STAT3 dimer the DNA Binding Domain of each monomer comes together forming a cleft for target DNA to bind. The binding of RTA to the DNA Binding Domain could influence how the target DNA is able to fit into the binding cleft in an effort to regulate transcriptional activation of target genes. The same region of pTyr STAT3 is required for binding to RTA or importin α. Therefore it is possible that when RTA binds pTyr STAT3 it prevents nuclear localization of the protein by blocking importin α binding.

The knowledge about protein interactions made with STAT3 prior to the transcriptional activation of target genes is valuable for determining how the protein can be a therapeutic target for the many diseases in which it is overexpressed. By targeting the STAT3 protein so that its expression is decreased, the influence STAT3 might have in the disease could be prevented. Preventing the ability of STAT3 to interact with importin α stops the nuclear localization of the protein and therefore the ability of the protein to act as a transcriptional activator. The interaction between pTyr STAT3 and RTA may be necessary for the ability of the gammaherpesvirus to establish latency. The deletion of the STAT3 gene prevented the ability of the gammaherpesvirus to become latent (Krug L et al 2014). The viral protein RTA is responsible for the reactivation of

gammaherpesvirus (Wu TT et al 2000). Therefore STAT3 must play an important role in the latency of gammaherpesvirus while the viral protein RTA is responsible for the reactivation of gammaherpesvirus (Wu TT et al 2000). Our results found that RTA and STAT3 physically interact and this interaction maybe important in determining the state of the virus. One possible model to explain the effects of the interaction between viral RTA and host pTyrSTAT3 is that RTA is inhibitory to the action of STAT3. RTA promotes lytic viral replication, whereas STAT3 would promote viral latency. Since RTA and importin-a proteins bind a similar region of pTyrSTAT3, the binding of RTA could prevent the nuclear localization of STAT3 and therefore the induction of target genes that are needed for viral latency. This model could be tested by immunofluorescence imaging to evaluate RTA and pTyrSTAT3 cellular localization. Another possibility is that the RTA-pTyrSTAT3 complex is cooperative. The RTA-pTyrSTAT3 complex could regulate a set of genes needed for the establishment of viral latency. These genes may be distinct from genes regulated by pTyrSTAT3, or they may be the same genes regulated more robustly by the RTA-pTyrSTAT3. To test the cooperative model, DNA-binding complexes of cells expressing pTyrSTAT3 or RTA-pTyrSTAT3 would be compared. Chromatin immunoprecipitation (ChIP) would be performed with anti-pTyrSTAT3 antibody, and the bound DNA would be evaluated by deep sequencing. The bound DNA could identify genes regulated similarly or distinctly. The mRNA expression of a subset of these genes could be quantified by RT-PCR. The interaction between RTA and pTyr-STAT3 could be an important therapeutic target in an effort to regulate the ability of the virus to undergo latency.

While the in vitro binding assay has shown that STAT3 interacts with two proteins, further experiments can be designed to support and extend these results. In order to determine whether these interactions occur in a more relevant model, immunoprecipitations of these protein complexes from mammalian cells can be performed. It is also important to understand which region of importin α and RTA are necessary to interact with the pTyr STAT3 protein. Mutants of these proteins with specific regions deleted can be generated by PCR cloning and used in the in vitro binding assay to determine which region is needed for binding to be detected by Western blot. The region of pTyr STAT3 needed for interaction with these proteins could also be identified.

The information learned from these experiments could be useful in understanding how STAT3 functions in normal cells, cancer cells, and cells infected with viruses. The function of STAT3 is important for learning how STAT3 can be targeted to treat cancer, infection and autoimmune disease.

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