Analysis of CrkL and Stat5 interactions in Bcr-Abl-expressing and cytokine stimulated cells

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LIST OF ABBREVIATIONS

CML, Chronic Myelogenous Leukemia

Stat, Signal Transducer and Activator of Transcription

GM-CSF, Granulocytic Macrophage-Colony Stimulating Factor

IL-3, Interleukin-3

IL-5, Interleukin-5

EMSA, electrophoretic mobility shift assay

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ABSTRACT

The study of leukemia includes determining the difference between normal blood cells and abnormal (leukemic) cells. The erroneous activation of endogenous pathways may account for the transformation of normal cells to a leukemic state. Cytokines are soluble factors that mediate cell-cell communcation, proliferation, differentiation, and survival of hematopoietic cells. Cytokine signaling activates tyrosine kinases to phosphorylate downstream effector molecules. Cells may become leukemic by activating intracellular kinases and therefore activating cytokine signaling pathways. The Bcr-Abl fusion protein is a constitutively activated tyrosine kinase that is the causative abnormality of chronic myelogenous leukemia (CML). To determine proteins important in Bcr-Abl transformation and cytokine signaling, proteins that were tyrosine phosphorylated in Bcr-Abl-expressing or cytokine stimulated cells were studied. Examination of CML patient samples for novel tyrosine phosphorylated proteins demonstrated that CrkL is the major tyrosine phosphorylated protein in CML neutrophils as compared to normal neutrophils. CrkL, a member of the Crk family of oncoproteins, is an SH2, SH3 domain containing adaptor protein. Crk is known to transform cells by activating intracellular tyrosine kinases and associating with a variety of cytoplasmic tyrosine phosphorylated proteins. Treatment of hematopoietic cells with stem cell factor or thrombopoietin results in tyrosine phosphorylation of CrkL, but minimal tyrosine phosphorylation is seen following treatment with IL-3, GM-CSF, or erythropoietin. Similar to Crkl, Signal Transducer and Activator of Transcription (Stat) 5 is constitutively tyrosine phosphorylated in Bcr-Abl expressing cells and inducibly tyrosine phosphorylated in cells stimulated with thrombopoietin. However, Stat5 tyrosine phosphorylation is also seen in cells stimulated with IL-3, GM-CSF, and erythropoietin. Here we show that in GM-CSF stimulated or Bcr-Abl-expressing

cells, CrkL interacts with tyrosine phosphorylated Stat5. Additionally, following cytokine stimulation or in the presence of Bcr-Abl, CrkL is found in the nucleus, can be detected in a Stat5/DNA complex, and increases transcriptional activation from a Stat responsive reporter construct. This suggests a novel role for CrkL, functioning as a nuclear adaptor protein that can associate with and activate Stat proteins in Bcr-Abl-expressing or cytokine stimulated cells. Through these studies we have identifed an interaction induced by constitutive activation of a pathway normally activated by cytokine stimulation. This may indicate a role for the Stat5/CrKl interaction in leukemogenesis.

CHAPTER I

Introduction

Hematopoiesis and Cytokines

Hematopoiesis is the development of a pluripotent stem cell through distinct lineages to form blood cells (1). A pluripotent stem cell has self-renewal abilities and the potential to develop into different differentiated cell types, each with specific functions and morphology. As a progenitor cell differentiates into one lineage, the ability to establish any other lineage is lost. There are two basic developmental cascades in hematopoiesis, the myeloid and lymphoid lineages. The lymphoid cascade produces B and T cells. The myeloid pathway produces erythrocytes, megakaryocytes, platelets, monocytes, macrophages, neutrophils, basophils and eosinophils (2).

A broad spectrum of cytokines, soluble factors that mediate cell-cell communication, collaborate to control the differentiation, proliferation, maturation, and survival of hematopoietic cells (3). Cytokines were identified by their ability to enable growth in tissue culture of immature progenitor cells from bone marrow (4-7). In tissue culture, hematopoietic cells may be dependent on cytokines for growth and survival and deprivation of cytokines induces apoptosis (8, 9). Cytokines are produced by activated T cells, macrophages, and stromal cells (10). Cytokines target specific receptors on the surface of developing blood cells, expression of which is regulated by environmental signals (11). Therefore, receptor expression determines the cytokine responsiveness of a cell. Receptors couple ligand binding to tyrosine phosphorylation of effector molecules, ultimately coordinating a genetic program through positive and negative regulation of transcription (12). Thus, the cytokine receptors couple an extracellular signal to a nuclear response.

Cytokines are categorized by receptor homology in which family members may have redundant signaling. For example, IL-3, IL-5, and GM-CSF receptors have unique ligand binding alpha chains which dimerize following ligand binding with a common signal transducing beta chain. IL-3 supports the growth of progenitor cells of all lineages. GM-CSF induces myeloid lineage development to mature neutrophils, monocytes, and eosinophils (13). Whereas IL-3 and GM-CSF stimulate multiple hematopoietic lineages, IL-5 stimulates a more restricted set including eosinophils and B cells (14). The restricted action of IL-5 is likely due to the restricted expression of the receptor on eosinophils and B cells (15). Redundancy can be demonstrated in that stimulation of eosinophils by either GM-CSF, IL-3, or IL-5 induces almost identical biological effects (16). Although GM-CSF and IL-3 stimulate multiple lineages, mice deficient in the IL-3/IL-5/ GM-CSF family of receptors have a normal hematopoietic constitution except for a reduced number of peripheral eosinophils (17). These data suggest that there may be redundancy between families as well as within a family of cytokine receptors. The cytokine families are categorized in Appendix I, Table 3.

Although cytokine receptors lack an intrinsic kinase domain, the induction of tyrosine phosphorylation is critical to their function. Cytokine receptors associate with distinct kinases that couple ligand binding to intracellular phosphorylation events. For example, following ligand binding Janus kinases are activated and phosphorylate sites on the receptor complexes. These phosphorylation events allow the recruitment and activation of specific effector proteins, such as Signal Transducers and Activators of Transcription (Stat) proteins, that results in transcriptional activation. The JAK/Stat signaling pathway is reviewed in detail in Appendix I.

Hematopoietic cells may become malignant by constitutive activation of pathways regulated by cytokines. This could lead to excessive proliferation, a block to differentiation, or inhibition of programmed cell death that are characteristic of transformed cells. As cytokine signaling is controlled by tyrosine phosphorylation, a mechanism for cytokine independence and malignant transformation is the aberrant activation of specific tyrosine kinases (18). It follows that defining the pathways normally activated by cytokines that are constitutively activated in leukemic cells would lead to an improved understanding of the disease.

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a malignancy of a pluripotent hematopoietic stem cell. All myeloid lineages are involved, however, significant expansion of granulocyte (neutrophil) and megakaryocyte (platelet) lineages are most common. CML accounts for 20% of all leukemias (19). The disease proceeds from a chronic phase to an accelerated phase followed by a blastic phase. Chronic phase is characterized by excesive numbers of myeloid cells that diferentiate and function normally. The chronic phase tyically lasts 4-6 years. With disease progression, to accelerated phase and blast crisis, myeloid cells lose the capacity for terminal differentiation. Disease progression is accompanied by increased genetic instability at a cytogenetic level (20). Although bone marrow transplant is a curative therapy, less than a third of patients with CML are eligible for this procedure. This is either due to an inability to find a human leukocyte antigen (HLA) matched donor or concerns about mortality from this procedure in older patients.

Bcr-Abl

The major distinguishing feature of CML is a specific chromosomal translocation, known as the Philadelphia (Ph) chromosome, that is identifiable throughout the

course of the disease (21, 22). The Ph chromosome is a somatic mutation that results from a reciprocal translocation between chromosomes 9 and 22 and fuses breakpoint cluster region (Bcr) sequences on chromosome 22q11 with c-Abl sequences from chromosome 9q34 (22-24). As a consequence of this chromosomal translocation, a fusion mRNA and protein, Bcr-Abl, is created. Specifically, Bcr exons 1 through 3 replace the first exon of c-Abl to form a fusion protein of 210 kilodaltons (kDa). An alternate form of Bcr-Abl that includes only Bcr exon 1 has been identified (185 kDa, p185), however, virtually all CML patients express the p210 form of Bcr-Abl(25, 26).

Several experiments suggest that Bcr-Abl is the causative agent of CML. In bone marrow reconstitution studies using bone marrow infected with p210 Bcr-Abl retrovirus, transplanted mice develop a CML-like syndrome along with other leukemias (27, 28). Consistent with this, transgenic mice expressing the p185 form of Bcr-Abl develop acute myeloid or lymphoid leukemia, similar to CML blast crisis or acute lymphoblastic leukemia associated with the expression of p185 Bcr-Abl (29).

Additionally, Bcr-Abl expression transforms fibroblasts and immature hematopoietic cells and induces IL-3 independent growth of myeloid cells (30-33). Thus, retroviral expression of Bcr-Abl in bone marrow or bone marrow derived cell lines (BaF3) grow regardless of the presence of IL-3 and induce nonregressing, lethal tumors in nude mice when injected subcutaneously. As noted above, CML disease progression is accompanied by genetic instability. Similarly, Bcr-Abl transfected BaF3 cells have a higher mutation rate in comparison to untransfected cells (20).

The mechanism of Bcr-Abl transformation is not known. The Bcr-Abl fusion protein has elevated protein tyrosine kinase activity as compared to c-Abl (34, 35). The

importance of the kinase activity of Bcr-Abl is illustrated by mutants of the kinase domain ATP binding pocket that lack kinase activity and are non-transforming (36). Bcr-Abl activates pathways that increase proliferation, inhibit apoptosis, and result in adhesion defects in hematopoietic progenitor cells. Bcr-Abl, through phosphorylation or protein-protein interactions, leads to the activation of a variety of signaling pathways. Many of these proteins have roles in multiple pathways and interact between pathways to create the complicated signal transduction observed in Bcr-Abl expressing cells. Figure 1 is a diagram of some of these interactions. Many of the pathways activated by Bcr-Abl are also activated by cytokine stimulation.

Bcr-Abl activation induces Ras activation with Ras becoming GTP loaded (37). Expression of a dominant negative ras in 32D cells expressing Bcr-Abl or K562 cells, derived from a patient with chronic myelogenous leukemia, resulted in increased apoptosis, suggesting that Bcr-Abl inhibition of apoptosis may be ras dependent (38). Expression of dominant negative ras inhibited transformation of mouse bone marrow cells by Bcr-Abl (39). Ras activation results in activation of Map kinases, JNK and ERK kinases. JNK activation results in activation of Jun transcription factor, and a dominant negative Jun impairs Bcr-Abl transformation (40). Interestingly, bcl-2 gene transcription is activated by Ras in Bcr-Abl expressing cells, whereas a truncated Bcr-Abl lacking Bcr sequences that are necessary to activate Ras fails to activate transcription of bcl-2 (41). Ras activation is commonly associated with proliferation, however, Bcl-2 is involved in apoptotic pathways, suggesting that Ras may also be involved in survival effects. ERK activity has been implicated in the proliferation and transformed phenotype of K562 cells, although the mechanism is not known (42).

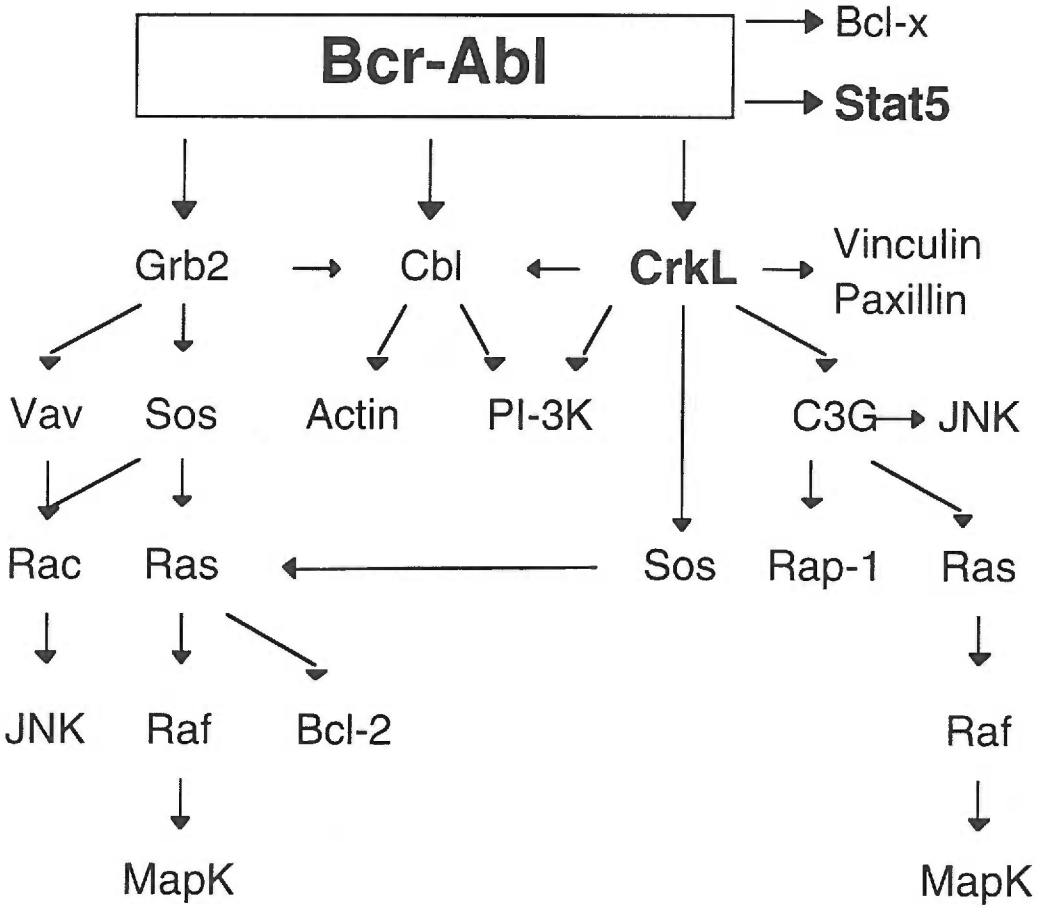


Figure 1. Diagram of Bcr-Abl signal transduction pathways. The adaptor proteins include CrkL, Cbl, Grb2, and Shc. Cytoskeletal proteins include actin, paxillin, and vinculin. Guanine nucleotide exchange factors C3G and Sos activate GTPases Rap-1 and Sos, respectively. The tumor suppressor Rap-1 has been implicated in cell adhesion wheras Sos activates the Ras pathway to regulate proliferative, anti-apoptotic and cytoskeletal signaling. Kinases activated include phosphatidylinositol 3-kinase (PI-3K), the dual function mitogen activated protein kinases (MapK), and the serine/threonine Ras activated kinase (Raf). Transcription factor Stat5 is activated. Bcl-2 and Bcl-x are mitochondrial membrane anti-apoptotic proteins. Although the arrows indicate pathways, these protein interactions may not be direct.

Bcr-Abl expression induces an increase in spontaneous motility, membrane ruffling, and filapodia extensions and retractions suggesting an involvement of Bcr-Abl in cellular motility (43). Bcr-Abl localizes to the cytoskeleton and mutants that fail to localize to the cytoskeleton have reduced transforming potential. (44-47) Bcr-Abl phosphorylates focal adhesion proteins (45). Immunofluescence has demonstrated that Bcr-Abl expression disrupts the normal array of F-actin filaments resulting in altered morphology (nonspherical, abnormal extensions with actin), spontaneous motility and membrane ruffling (43). Bcr-Abl expressing cells have decreased ability to adhere to stromal layers and increased cell motility on fibronectin and collagen IV (43, 48).

The Abl domains of Bcr-Abl include a Src homology (SH) 3 motif, an SH2 motif, a kinase domain, a nuclear localization signal, and a unique carboxy terminus which contains DNA and actin binding motifs. Wild type c-Abl has two alternate exon 1, referred to as 1A and 1B. Deletion of the amino terminus resulted in an increase in autokinase activity, suggesting that the variable amino terminal region has negative regulatory effects on c-Abl kinase activity (49). The Abl SH2 domain favors binding to the specific sequence pTyr-X-X-Pro, where X signifies any amino acid (50). The specificity of SH2 domains for tyrosine phosphate is determined by conserved residues in the SH2 binding pocket, whereas additional amino acids in the pocket determine specificity for the amino acids surrounding the phosphorylated tyrosine (51). Interestingly, point mutations in the Abl SH2 domain inhibit transforming ability of Bcr-Abl in fibroblasts, but do not impair the ability of Bcr-Abl to induce growth factor independence of myeloid cells (36, 52, 53).

The Abl SH3 interacts with proline rich regions on target proteins and regulates kinase activity. The Abelson murine leukemia virus, a transforming retrovirus, contains

a fusion of viral gag sequences to c-Abl sequences lacking the amino terminus (SH3 domain). This Abl variant (v-Abl) has increased tyrosine kinase activity in comparison to c-Abl (54, 55). c-Abl with a deleted SH3 domain is also an activated kinase that can transform fibroblasts and hematopoietic cells (56, 57).

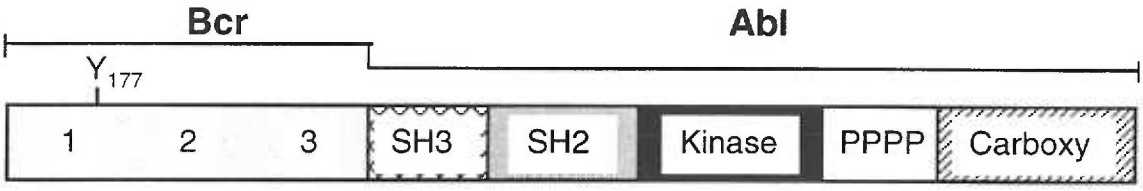


Figure 2. Domains of Bcr-Abl. Bcr is labeled as 1, 2, and 3, for the corresponding exons. Abl domains include the SH3, SH2, kinase, proline rich region labeled PPPP, and the carboxy terminus region that includes the actin binding domain, nuclear localization signal, and DNA binding sequence.

c-Abl activated by SH3 domain deletions versus fusion to Bcr sequences induces different disease phenotypes. Retroviral expression of c-Abl with an SH3 deletion in bone marrow cells did not result in the growth of myeloid colonies in vitro (58). Transplantation of these cells into mice induced only lymphoid malignancies (58). In contrast, Bcr-Abl lacking a major portion of the SH3 domain retained the ability to induce a myeloproliferative disease in mice (58). The c-Abl SH3 deletion mutant resulted in decreased phosphorylation of Stat5 in NIH3T3 cells in comparison to Bcr-Abl or Bcr-Abl lacking the SH3 domain (58). This suggests that Stat5 activation might be one of the pathways responsible for the differing phenotypes of these Abl mutants.

The Arg tyrosine kinase has high homology to Abl in the kinase, SH2, and SH3 domains, but is divergent in the carboxyl terminus amino acid sequence (59). Both Abl and Arg proteins are widely expressed (59). Similar to Abl, Arg is expressed in 1A and 1B forms that differ in their amino terminal sequences (60). However, in contrast to Abl, Arg is localized largely to the cytoplasm (60). In contrast to c-Abl, a gag-Arg fusion analogous to v-Abl is not transforming (61). However, if the entire carboxy terminus of Abl is swapped for Arg in the gag fusion, the resulting protein is transforming (61). This suggests a significant functional difference between c-Arg and c-Abl due to carboxy terminal sequences.

The ubiquitously expressed c-Abl non-receptor tyrosine kinase is localized both to the nucleus and cytoplasm in hematopoietic cells and binds to DNA (62, 63). This is in contrast to Bcr-Abl which is exclusively localized to the cytoplasm (62). Although the biological functions of c-Abl are not fully understood, there is some evidence that indicate a role for c-Abl in cell cycle control. Thus, overexpression of c-Abl causes cell cycle arrest in NIH3T3 fibroblasts in G1 phase. (57, 64)

Mitotic checkpoints control the G1 to S transition and the G2 to M transition to prevent premature commitment to mitosis. The cell cycle dependent kinases and structural proteins (cyclins) are expressed in succession to control the cell cycle. Upon phosphorylation of the retinoblastoma (Rb) protein by cell cycle dependent kinases, Rb releases the transcriptional factor E2F which then is available to activate transcription of genes necessary for the G1 to S transition. The retinoblastoma protein binds to the ATP-binding pocket of the c-Abl kinase domain resulting in kinase inhibition in the Molt-4 human T-cell leukemia cell line (65). Abl activation in S phase correlates with phosphorylation and release of Rb from the Abl complex suggesting that Abl may be regulated by cell cycle proteins (65).

In M phase, the chromosomes condense, are segregated, and decondense, the mitotic spindles are assembled and dissembled, cytokinesis occurs, and the nuclear membrane is disassembled and reassembled. Phosphorylation of histone H1 is important for chromosome condensation and, therefore, initiation of M phase. Cell division cycle (cdc)2 kinase associates with histone H1 and this association is decreased by activation of c-Abl (66). c-Abl binds DNA through a motif in the carboxyl terminus in which serine phosphorylation by cdc2 kinase abolishes DNA binding (67). This suggests that c-Abl may be a negative regulator of M phase by inhibiting chromosome condensation.

In human U-937 cells, NIH3T3 fibroblasts, and MCF-7 breast cancer cells, c-Abl is activated in response to DNA damage from ionizing radiation or alkylating agents (66, 68). Partial inhibition of a G1 arrest occurred following irradiation of c-Abl -/- mouse embryonic fibroblasts as compared to wild type cells (66). Therefore, DNA damage induces the activation of c-Abl which leads to cell cycle arrest. In contrast, hematopoietic cells from untreated chronic phase CML patients were exposed to gamma radiation and were found to have a reduced number of G2 arrested and S phase cells (69). Therefore, expression of Bcr-Abl leads to a lack of cell cycle arrest induced by DNA damage which may contribute to the propensity for acumulation of mutations in CML patients as the disease progresses. Additionally, gamma irradiated p210 expressing 32D myeloid cells had increased cell survival due to an inhibition of gamma-irradiation induced programmed cell death (70).

c-Abl null mice are viable, but are smaller and have decreased survival after birth as compared to control mice (71). 18 day embryos showed no difference between null and wild type in body weight and cellularity of thymuses and spleens, however, 85% of the homozygous null mice have lymphopenia. c-Abl expression is highest

in the thymus and spleen. Within the first three weeks of life, hepatocytes from null mice contain fatty vacuoles associated with runting and wasting, atrophied thymuses, and occasionally atrophied spleens. The null thymus had a severe decrease in thymocytes. Abl null mice also have reduced B cell precursors in the bone marrow. B cell progenitor cell lines derived form bone marrow cultures of Abl null mice had an increased rate of cell death after growth factor deprivation and increased susceptibility to apoptosis following glucocorticoid treatment (72). This suggests that the Abl null cells have an increased sensitivity to apoptosis.

Schwartzberg et. al. developed a mouse that was homozygous for an Abl (Abl M1) lacking the carboxy terminal domain but that retains the ability to autophosphorylate and potentially phosphorylate substrates. (73) Abl m1 homozygous mice were fertile (30-80% were runted) and had normal embryonic development, but had decreased viability postpartum and increased susceptibility to bacterial infection. M1 mice had deficiencies in lymphopoieses with decreased early bone marrow B cell progenitors, although mature bone marrow B cells appeared normal in numbers. During embryogenesis, the percentage of immature B cells in the spleen were identical to wild type, although the overall number was decreased. At one week of age, the proportion of pre-B cells was reduced in the spleen. This may be due to a difference in the requirement of Abl in embryonic versus adult hematopoiesis. A small percentage of null mice had small thymuses with decreased early thymocytes and peripheral mature T cells, although highly variable. Additional abnormalities included kidney shape spleens, shorter skulls, and early opening of the eyes resulting in scarring and permanent damage. The Abl M1 mice have a remarkably similar phenotype to the Abl null mice, suggesting an important role for the carboxyl terminus for the function of c-Abl.

To determine if the Abl family member Arg may have a redundant role in development, Koleske et. al. developed Arg knockout mice (74). The Arg null mice were obtained at expected Mendelian rates. Although Arg is highly expressed in the brain, null mice had no gross histological abnormalities of the brain. Blood cells were normal in blood, spleen, bone marrow, and thymus. Arg -/- mice displayed multiple behavioral phenotypes including difficulty completing motor skills tests, decreased mating and aggression behaviors, and abnormal reflexes such as not being startled by acoustic stimuli (74). To determine processes that depend on both Arg and Abl, mice were mated to produce double knockouts (74). The double null resulted in an embryonic lethal phenotype with defective neural tube development, excessive apoptosis in multiple tissues of the body, and hemorrhage into the pericardial sac (74). Double null neuronal cells had disordered actin distribution in neuroepithelium, but normal patterns of phosphotyrosine staining. The G1/S checkpoint was intact in double null cells. Therefore, a lack of Abl leads to deficiencies in lymphoid development, a lack of Arg leads to neurological defects, and double null mice displayed hemorrhaging and neural tube defects, but no obvious abnormalities of blood cell number or percentage. Further distinctions were made in null mice containing a single allele of the alternate gene. Abl-/- Arg+/- mice died at embryonic day 15.5-16 and displayed hemorrhaging in the pericardial space (74). Abl +/- Arg-/- mice had decreased survival prior to birth, were runted at birth, but had normal viability following birth (74).

c-Abl is highly conserved as determined from sequence comparison from mouse, human, fruit fly, and nematode, although the carboxyl terminal region is less conserved. Fruit flies with homozygous deletions that include the Abl locus resulted in three categories of mutants: larval lethal, pupal lethal, and an adult with a range of defects in eye development, fertility, and viability (75). The mechanism for these

phenotypes is not yet defined, but the more severe phenotypes were associated with deletions that included more centromeric regions suggesting the involvement of another gene in the phenotypes observed.

Although Abl has many protein-protein interactions and is highly conserved, some function of Bcr is necessary for transformation. This has been demonstrated in that c-Abl lacking exon 1 is not transforming, regardless that exon 1 of c-Abl is deleted in Bcr-Abl, (56). Bcr exon 1 has multiple activities, including a function necessary for transformation that involves non-phosphotyrosine dependent binding to the Abl SH2 regulatory domain (76). Bcr has a coiled-coil oligomerization domain which is necessary for Bcr-Abl mediated transformation of Rat-1 fibroblasts and induction of interleukin-3 independence in lymphoid cells (44). Disruption of this domain prevents tetramerization of Bcr-Abl and abolishes the transforming ability of Bcr-Abl (77). Bcr has a tyrosine at amino acid 177 which is a site for Grb2 binding, a protein that links to Ras nucleotide exchange factor Sos (78-80). Exon 1 also contains a serine/threonine kinase activity that is not necessary for transformation by Bcr-Abl (81, 82). Bcr exon 2 has homology to a rho/rac guanine nucleotide exchange factor. However, both Bcr exons 2 and 3 are not necessary for transforming ability as Bcr-Abl lacking either one of these exons is still transforming.

To determine the role of c-Bcr in myeloid development, Voncken et. al. created a c-bcr knockout mouse (83). The c-Bcr knockout mice were obtained in normal Mendelian ratios and males and females were fertile. The null mice had normal peripheral neutrophil numbers, however, following endotoxemia induced by lipopolysacharide (LPS), null mice neutrophil numbers were increased as compared to wild type mice. Following LPS exposure, wild type mice neutrophil numbers did not change within the first 24 hours, whereas null mice doubled peripheral neutrophils

within 6 hours. Endotoxemia induced by LPS also led to more severe septic shock and increased tissue injury due to inflammation in the null mice. Additionally, Bcr null neutrophils have increased superoxide production (the major defense against microorganisms). This suggests that c-Bcr has a role in neutrophil response to infection.

This thesis investigates proteins that may be important in both cytokine and Bcr-Abl signaling. CrkL is the major tyrosine phosphorylated protein in Bcr-Abl-expressing cells. CrkL is also tyrosine phosphorylated in hematopoietic cells following stimulation with a variety of cytokines. Thus, CrkL is a clear example of overlap between the two systems. We hypothesize that the CrkL adaptor protein may mediate interactions between Bcr-Abl and tyrosine phosphorylated effector molecules necessary for transformation. As Bcr-Abl induces IL-3 independence in myeloid cells, indicating an activation of cytokine signaling pathways, it follows that CrkL interactions detected in Bcr-Abl expressing cells may also be observed following cytokine stimulation. In examining signaling pathways mediated by CrkL in cytokine stimulated or Bcr-Abl-expressing cells, we discovered that CrkL interacts with Signal Transducer and Activator of Transcription (Stat)5. Similar to CrkL, Stat5 is also tyrosine phosphorylated following cytokine stimulation or in Bcr-Abl-expressing cells. The inducible phosphorylation and association of Stat5 with CrkL suggested a role in signaling, therefore, this interaction was further investigated.

CrkL

Adaptor proteins contain SH3 and SH2 domains and have no catalytic functions. As stated earlier, SH2 domains target binding to phosphotyrosine with specificity depending on amino acids surrounding the tyrosine. SH3 domains bind proline rich

sequences. Although adaptor proteins have no intrinsic enzymatic activities, they function by recruiting effector molecules to signaling complexes (84).

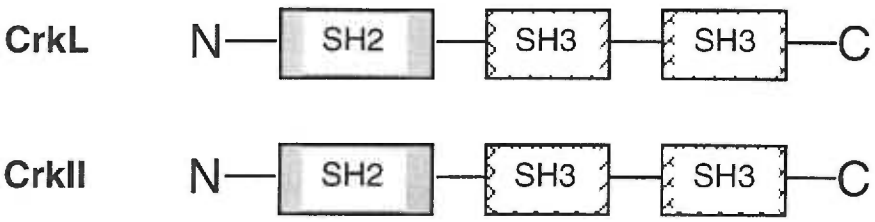


Figure 3. Domains of CrkL. The SH2 and SH3 domains are as labeled. The N indicates the amino terminus and the C indicates the carboxy terminus. There is 60% homology between the SH2 and SH3 domains of CrkL and CrkII. CrkL is 39 kDa, CrkII is 42 kDa.

v-Crk was originally identified in the avian sarcoma virus CT10 and indicates that c-Crk is a proto-oncogene. (85) Although v-Crk lacks kinase activity, transformation by CT10 results in an increase in both Crk and intracellular tyrosine phosphorylation. (85-87). Crk has two isoforms, Crk II which includes both amino and carboxy terminal SH3 domains and Crk I that includes only the amino terminal SH3 domain. Expression of Crk I in Rat 3Y1 cells allows proliferation of cells in soft agar and induces tumors in nude mice (88). CrkL was initially characterized by cross hybridization of mouse genomic DNA fragments to human chromosome 22 by fluorescence in situ hybridization. Binding of a probe indicated conservation in sequence and possible coding domains (89). ten Hoeve, et. al. identified a strong cross-hybridization and purified the human cDNA homologous to the mouse fragment. Sequencing of the cDNA revealed a Crk-like gene, CrkL. The CrkL gene is located on human chromosome 22q11 whereas Crk is located on 17p13. The Crk

family members are ubiquitously expressed and contain strong homology in the SH2 and SH3 domains. The biological function of CrkL and Crk are not well defined and mouse knockouts have not yet been reported.

Examination of CML patient samples for novel tyrosine phosphorylated proteins demonstrated that CrkL is the major tyrosine phosphorylated protein in CML neutrophils as compared to normal neutrophils (90-92). In contrast, Crk-II is not tyrosine phosphorylated in Bcr-Abl expressing cells (90, 92-94). Although phosphotryptic mapping indicated that CrkL is tyrosine phosphorylated on multiple residues when overexpressed alone or co-expressed with Bcr-Abl, tyrosine 207 of CrkL is the major Bcr/Abl phosphorylation site (95, 96). CrkL is phosphorylated following cytokine stimulation with IL-2 (97), steel factor (stem cell factor) (98), erythropoietin (99), IL-3 (99), and thrombopoietin (93) suggesting that processes involving CrkL in Bcr-Abl expressing cells may also occur in cytokine stimulated cells.

CrkL has been shown to bind directly to Bcr-Abl (100) and is known to associate with a variety of tyrosine phosphorylated proteins (87, 101). The SH2 domain of CrkL has been shown to bind tyrosine phosphorylated CBL, HEF1, CAS and Paxillin (102-104). These proteins mediate cytoskeletal signaling of cell adhesion and motility through integrin binding to the extracellular matrix. Bcr-Abl expression induces an increase in spontaneous motility, membrane ruffling, and filapodia extensions and retractions suggesting an involvement of Bcr-Abl in cellular motility (43). Interestingly, mutation of tyrosine 207 of CrkL inhibits all CrkL tyrosine phosphorylation but does not inhibit fibroblast transformation and Paxillin binding indicating that tyrosine phosphorylation may not be necessary for CrkL function (95).

The consensus binding sequence for the CrkL SH3 domain appears to be the same as Crk, Pro-X-Leu-Pro-X-Lys, in which X is any amino acid (103, 105, 106) The amino-terminal SH3 domain of CrkL targets proline rich sequences in C3G, SOS, p85 regulatory subunit of PI3-K, c-Abl and Bcr-Abl (96, 103, 107). Although far western analysis has shown that the amino terminal CrkL SH3 domain binds directly to a proline-rich region in the C-terminus of BCR-ABL, direct binding of CrkL to Bcr-Abl is not required for BCR-ABL transformation (100, 108). Many of the CrkL interacting factors are involved in multiple pathways and are activated in Bcr-Abl expressing cells. For example, PI3-kinase activates the serine kinase AKT which phosphorylates and inactivates BAD. Serine phosphorylated BAD binds 14-3-3 which is subsequently prevented from contributing to apoptosis (103). PI3-kinase has also been implicated in the proliferation of Bcr-Abl-expressing cells as wortmanin, a specific inhibitor of PI3-kinase, inhibits growth of these cells (109). C3G and SOS are guanine nucleotide exchange factors that activate p21Rap1 and p21Ras, respectively. Therefore, Ras can link to Bcr-Abl through either Grb2 or CrkL.

Overexpression of CrkL in fibroblasts induces transformation and in hematopoietic cells increases adhesion to fibronectin (95). In both fibroblasts and hematopoietic cells, individual mutations or deletions of CrkL SH2 and SH3 amino terminal domain abrogated transformation and adhesion, respectively, indicating that interactions with other proteins such as Cbl and Paxillin (SH2 domain) and Abl, Sos, and C3G (N-terminal SH3 domain) are essential for biological activity (95).

Signal Transducer and Activator of Transcription 5

Signal transducers and activators of transcription (Stat) proteins are activated by a variety of cytokines (see appendix I, table 5). The Stat proteins were originally

identified through studies of proteins that bind to interferon (IFN) response elements, IFN alpha response elements (ISRE) and IFN gamma activation sites (GAS) (110-116). The two proteins first identified, termed Stat1 and 2, are distinct but highly homologous. This indicated the existence of a family of Stat proteins and now there are seven members of the Stat family. Stat5 is the predominant Stat protein activated by Bcr-Abl. Stat5 was originally identified in studies of lactation in sheep in which a prolactin inducible DNA binding activity termed mammary growth factor (MGF) was found (117). MGF was purified from mammary gland tissue of lactating sheep through it's ability to bind to a beta casein promoter (117). MGF was highly homologous to Stat family proteins and was named Stat5a. The sheep cDNA for Stat5a was used to screen a murine MC/9 and a mouse mammary tissue library (118, 119). Two highly homologous genes were identified in these studies, termed Stat5a and Stat5b (118, 119). Stat5b was independently cloned by purifying proteins that bind to the IL-6 responsive 2-macroglobulin promoter (120). The Stat5a and Stat5b genes are closely positioned on human chromosome 17 (121). Stat5a and Stat5b have a similar, yet restricted pattern of expression in which the highest expression is in the mammary gland, but transcripts can also be detected in ovary, thymus, lung, adrenal gland, kidney, spleen, muscle, liver, and myeloid cells (117, 122, 123).

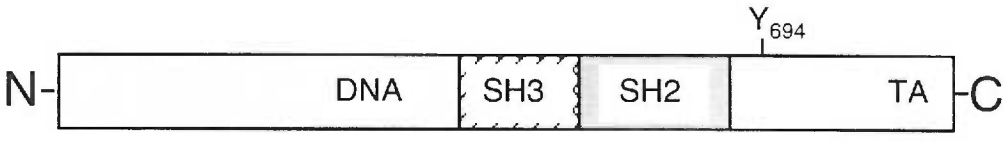


Figure 4. Domains of Stat5. The transactivation domain in noted as TA and the DNA binding domain is depicted as DNA. Tyrosine (Y) 694 is the activating tyrosine.

In cytokine signaling, Stat SH2 domains bind tyrosine phosphorylated cytokine receptors or associated proteins and are subsequently tyrosine phosphorylated by the kinases associated with the activated receptor. The carboxy terminus of all Stat proteins contain a conserved tyrosine that is a site of phosphorylation (124, 125). Tyrosine phosphorylated Stat proteins dimerize and translocate to the nucleus to activate transcription (126). However, a nuclear localization sequence in Stat proteins has not been well defined. Dimerization occurs by the SH2 domain of one Stat protein targeting the phosphorylated tyrosine on a second Stat protein. (127, 128) Consistent with this, mutations in the phosphotyrosine binding pocket of the SH2 domain or mutation of the carboxy terminal conserved tyrosine phosphorylation site prevent Stat dimerization (124). The SH2 domains of the Stat proteins differ in their recognition of tyrosine phosphorylated motifs, allowing for specificity in binding to and activation by different cytokine receptor complexes. Similarly, both homo- and heterodimerization of Stats can occur depending on the binding specificity of the SH2 domain and the sequences surrounding the phosphotyrosine (122, 129, 130).

Distal to the conserved tyrosine phosphorylation site of Stat proteins is a non-conserved carboxy terminal transactivation domain. The variability of sequences in this transactivation domain may contribute to the specificity of Stat transcriptional activation. Deletions in the carboxy terminus abolish transcriptional activity but do not prevent tyrosine phosphorylation, dimerization, or DNA binding of Stat proteins, thus creating a dominant negative mutant (131-135). Stat5 also has a serine residue in the carboxy terminal domain, whose phosphorylation is important for full transcriptional activity (136-140). Amino terminal mediated interactions have been observed between Stat dimer pairs allowing for oligomerization and increased transcriptional activation (141-143).

Crystal structure of Stat proteins demonstrates that Stat proteins utilize an immunoglobulin-like fold to bind DNA as a dimer much like Nf-kappaB (127, 128). The DNA binding domain of Stat proteins is located at amino acids 300-500 (144). This domain was delineated by comparing chimeric Stat proteins with differing DNA binding specificities (144, 145). The function of this domain has been confirmed by mutation of a number of conserved residues in this region that inactivate the DNA binding activity of Stat proteins without affecting tyrosine phosphorylation or dimerization (134, 144, 145). The glucocorticoid receptor can act as a transcriptional co-activator for Stat5 and enhance Stat5-dependent transcription (146). Other DNA binding factors that interact with Stat5 have yet to be identified.

All Stat proteins have been shown to recognize highly related DNA sequences. These sequences have been identified by mutagenesis of response or promoter elements, oligonucleotide competition studies, or isolation of Stat binding sites from random oligonucleotide pools (122, 147). In particular, these experiments have shown that Stat proteins bind to a semipalindromic sequence TTNNNNNAA, known as a GAS sequence. However, the affinities of Stat proteins for different GAS elements varies depending on the nucleotides in or around the GAS sequence. Table 1 lists Stat5 binding sequences and indicates $TTC(N)_3GAA$ is the optimal binding site for Stat5. Although a fair number of genes containing this site have been identified, an extensive screen of genes activated by Stat5 has not been published.

Table 1. Stat5 response elements

Gene	Target sequence	Reference
Rat beta-casein	TTCTTGGAA	(117, 148-150)
sheep beta-casein	TTCTAGGAA	(151)
B cell lymphoma	TTCGGAGAA	(152)

(BCL)-X	TTCATTGAA	
cytokine inducible	TTCCTGGAA	(153)
protein (CIS)	TTCTTGGAA	
	TTCTAGGAA	
	TTCCGGGAA	
Fc gamma Receptor1	TTCCCAGAA	(148, 154)
c-Fos	TTCCCGTAA	(155)
IL-2 Receptor alpha	TTCTGAGAA	(156)
interferon regulatory	TTCCCCGAA	(157-159)
factor (IRF)-1		
oncostatin-M (OSM)	TTCGAAGAA	(160)
	TTCCCAGAA	
Pim.1	TTCCCAGAA	(161)
serine protease	TTCTACTAA	(162)
inhibitor (Spi)2.1		
p21 WAF/Cip1	TTCTGAGAA	(163)
	TTCTTGGAA	
Consensus	TTCXXXGAA	

Several mutations result in dominant negative Stat proteins. As expected, deletion of the carboxy terminal transactivation domain accomplishes this (133). Interestingly, there are several naturally occurring Stat isoforms that contain deletions of this carboxy terminal region, suggesting that these proteins may serve to regulate Stat activity (164, 165). Mutation of the carboxy terminal tyrosine phosphorylation site of Stat proteins also results in a dominant negative Stat protein (166). This mutant inhibits tyrosine phosphorylation of the endogenous Stat proteins suggesting that

this mutant may fail to dissociate from activated receptors or kinases thus preventing endogenous Stat proteins from binding and becoming tyrosine phosphorylated. Expression of a dominant negative Stat5 in wild type mouse fetal liver results in increased apoptosis cells and a decreased growth rate (152). Stat5 binds sequences in the initiation site of Bcl-xL, a factor that inhibits cell death upon growth factor withdrawal, and mediates erythropoietin induced transcriptional activation of Bcl-xL (152, 167). Cells expressing Bcl-xL have reduced apoptosis after erythropoietin withdrawal to a level similar to that seen in cells expressing a constitutively active Stat5 expressing cells.

A constitutively activated Stat5 mutant was identified by Onishi et al. using PCR driven random mutagenesis and a retrovirus mediated expression screening system (168). This mutant has two amino acid substitutions, one upstream of the DNA binding domain (H299R) and the other in the transactivation domain (S711F). Expession of this constitutively active Stat5 allowed HCD-57 cells to survive longer than parental or cells overexpressing wild type Stat5a (152).

Stat5 is tyrosine phosphorylated following stimulation of the appropriate cell types by interferon alpha/beta, interferon gamma, IL-10, IL-2, IL-4, IL-7, IL-9, IL-15, IL-6, G-CSF, LIF, IL-3, IL-5, GM-CSF, erythropoietin, prolactin, thrombopoietin, growth hormone, epidermal growth factor, PDGF, and stem cell factor (c-kit), and angiotensin II (see appendix I, table 5). Although activated by a multitude of cytokines, knockout mice phenotypes suggest a more discrete set of cytokines dependent on Stat5. Stat5a null mice indicate that Stat5a is the primary target of prolactin (169). In examining a potential role for Stat5a in responses to other cytokines, bone marrow macrophages from Stat5a deficient mice had decreased proliferation and gene expression in response to granulocyte/macrophage-colony stimulating factor (GM-

CSF) (170). Stat5b null mice indicate that Stat5b is the primary target of growth hormone (171). Additionally, Stat5b deficient mice have a modest decrease in both thymic and splenic cellularity (172). The Stat5a/b deficient mice were derived by double targeting of embryonic stem cells, since their genetic colocalization would not allow a purely genetic approach (173). Double homozygous deficient mice had more severe prolactin and growth hormone defects as well as defects in responses to cytokines that utilize the beta common family of cytokine receptors (IL-3, IL-5, GM-CSF) (170, 172, 174-176). Specifically, colony forming assays of bone marrow cells from Stat5a/b null mice demonstrated a decreased response to IL-3, IL-5 and GM-CSF with fewer colonies and smaller colony size. Adult red cell numbers, hemoglobin levels, hematocrits, and platelet count are normal, however, Stat5a/b null mice had a decrease in white cell numbers (173).

Although Stat5a/b double null adult mice had normal red blood cell counts, null embryo fetal livers had decreased erythroid colony forming progenitors as compared to wild type (152). Fetal liver cells had increased apoptosis of erythroid progenitors and erythropoietin was not as successful in rescuing fetal liver cells as compared to wild type, although there was some effect. This suggests that Stat5 mediates erythropoietin signaling to rescue colony forming units from apoptosis which is consistent with erythropoietin induction of Stat5 trancriptional activation of the Bcl-xL gene.

As stated earlier, Bcr-Abl is known to induce IL-3 independence in myeloid cells indicating an activation of cytokine signaling pathways by Bcr-Abl. Overexpression of the erythropoietin receptor (Epo-R) in myeloid cells also induces IL-3 independence (177). Although Bcr-Abl expressing myeloid cells are resistant to apoptosis induced by etoposide, growth factor withdrawal, or serum deprivation,

cells expressing Epo-R are resistant only to IL-3 withdrawal suggesting additional mechanisms of survival in Bcr-Abl expressing cells (177). As noted above, erythropoietin may protect cells from apoptosis through activation of Stat5. In Bcr-Abl-expressing cells Stat5 is constitutively tyrosine phosphorylated (178-183). Therefore, Stat5 is a likely candidate for mediating at least a part of Bcr-Abl's transforming function.

This thesis investigates proteins that may be important in both cytokine and Bcr-Abl signaling. CrkL is the major tyrosine phosphorylated protein in Bcr-Abl-expressing cells. CrkL is also tyrosine phosphorylated in hematopoietic cells following stimulation with a variety of cytokines. Thus, CrkL is a clear example of overlap between the two systems. We hypothesize that the CrkL adaptor protein may mediate interactions between Bcr-Abl and tyrosine phosphorylated effector molecules necessary for transformation, such as Stat5. As Bcr-Abl induces IL-3 independence in myeloid cells, indicating an activation of cytokine signaling pathways, it follows that CrkL interactions detected in Bcr-Abl expressing cells may also be observed following cytokine stimulation. In examining signaling pathways mediated by CrkL in cytokine stimulated or Bcr-Abl-expressing cells, we identified that CrkL interacts with Signal Transducer and Activator of Transcription (Stat)5. Similar to CrkL, Stat5 is also tyrosine phosphorylated following cytokine stimulation or in Bcr-Abl-expressing cells. The inducible phosphorylation and association of Stat5 with CrkL suggested a role in signaling, therefore, this interaction was further investigated.

Chapter II

Materials and Methods

Cell culture and preparation of lysates

K562, MO7e, MO7p210, 32D, 32Dp210, Rat-1, Rat-1p185, TF-1, and UT-7 cell lines were grown in RPMI supplemented with 10% fetal bovine serum (UBI), 1% penicillin-streptomycin, and 2% L-glutamine. MO7e, TF-1, and UT-7 cells were supplemented with 5 ng/ml GM-CSF (Immunex Corporation). 32D cells were supplemented with 15% WEHI-CM. COS7 and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 2% L-glutamine. Cells were serum and growth factor starved for 8 to 12 hours prior to lysis. UT-7, TF-1 or 293T cells were stimulated with GM-CSF for 10 minutes or alpha interferon stimulation for 45 minutes, respectively. Cells were lysed at 5 X 10^7 cells/1 mL lysis buffer (1% NP-40, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 10 ug/mL aprotinin, 1 mM sodium vanadate (Na_3VO_4), and 1 mM phenylmethylsulfonylfluoride (PMSF)) (108). Whole cell lysates were clarified by centrifugation at 10,000 rpm for 10 minutes. K562 nuclear lysates were prepared by the Dignam method using 1x10^8 cells (184). Nuclear extracts from starved or IL-3 stimulated TF-1 cells were prepared by hypotonic lysis as described (185).

Immunoblotting

Total protein was quantitated by Bradford assay (BioRad) and 1.5-2 mg was immunoprecipitated with 2 µg CrkL antibody (Santa Cruz), resolved by SDS-PAGE on an 8% gel, then immunoblotted with phosphotyrosine antibody 4G10 (1.5 µg/ml) (108, 186), Stat5a (Zymed, 1 µg/ml), Stat5b (Zymed, 1 µg/ml), Stat5b (Santa Cruz, 0.1 µg/ml), or CrkL (Santa Cruz, 0.1 µg/ml) antibodies. Antibodies

were detected either by alkaline phosphatase conjugated antibody (Promega) and developed with NBT/BCIP (Promega) or by horseradish peroxidase conjugated antibody (Promega) and developed with enhanced chemiluminescence (Pierce). Blots were stripped (625 mM Tris-HCl pH 6.8, 2% SDS, and 0.7% 2-mercaptoethanol) for 30 minutes at 70 degrees or 2 hours at room temperature.

GST Fusion proteins

GST proteins were isolated from bacterial lysates and quantitated by Coomassie staining as previously described (108). 1.5 mg of K562 whole cell lysate was incubated for 2 hours with glutathione sepharose beads, 5 µg purified GST protein, or 5 µg purified GST-CrkL fusion proteins (100). Fusion proteins included GST-CrkL full length, GST-CrkL SH2, GST-CrkL SH3, or a GST-CrkL full length with an arginine to lysine mutation (K39) in the SH2 phosphotyrosine binding pocket (FLVRES). 1 mg of TF-1 or UT-7 lysates were incubated overnight with 2 ug of purified GST alone or GST CrkL fusion proteins. Following incubation with glutathione sepharose beads, reactions were washed twice with 0.5M LiCl and four times with 1X PBS. Complexes were immunoblotted as above.

Far-Western Analysis

Far Western analysis was performed on COS7 expressed pcDNA3 constructs of Bcr-Abl and FLAG tagged Stat5a. COS7 cells were transfected using lipofectAMINE (GIBCO-BRL) according to manufacturer's instructions. Flag immunoprecipitates were performed on 2-3 mg of whole cell lysate, resolved by SDS-PAGE on an 8% gel, and transferred to PVDF. Membranes were blocked overnight in 10% nonfat dried milk in PBST followed by incubation with 2 ug of purified GST, GST-CrkL full length, GST-CrkL SH2, or Bcr-Abl per ml of binding buffer (25mM $NaPO_4$, 150 NaCl, 0.1% Tween, 2.3 mM EDTA, 20 mM NaF, 1 mM

Na_3Vo_4, 5% nonfat dry milk, 1mM DTT, 1 ug/ml aprotinin, and 1ug/ml leupeptin). Immunoblots developed as previously described (108).

Electrophoretic Mobility Shift Assay

K562 EMSA reactions were performed using 5 µl nuclear extract, 0.1 ng ^{32}P-end labeled wild type Stat5 DNA binding sequence, 1 µg poly dI/dC, 1 mM dithiothreitol, 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, and 5% glycerol. The wild type Stat5 binding sequence is 5' agatttctaggaattcaatcc 3' and the mutant sequence is 5' agatttagtttaattcaatcc 3'. Reactions were incubated at room temperature for 15 minutes. EMSA's were performed with end-labeled wild type probe alone or with an additional 10 ng of cold wild type or mutant sequences. Cold sequences were added 15 minutes prior to the labeled probe. For supershifts of the Stat/DNA complex, 1 µl Stat5 (Santa Cruz), Stat1 (Santa Cruz), Grb2 (Santa Cruz), or 2 µl CrkL (Santa Cruz) antisera were added and incubated at room temperature for 15 additional minutes. Antibodies were peptide blocked according to manufacturer's instructions (Santa Cruz). Reactions were run on a 5% nondenaturing Tris/glycine polyacrylamide gel (187).

TF-1 or UT-7 EMSA reactions combined 2 uL nuclear lysate with 20 uL binding buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol, 1 mg/mL bovine serum albumin, and 2 mg/ml poly dI-dC, pH7.5), and 50,000 c.p.m. end-labeled bovine ß-casein promoter oligonucleotide 5' agattctaggaattcaaatc 3'. Reactions were incubated at room temperature for 30 minutes. For supershifts, 1 uL of antibody was added and incubated for 30 additional minutes. Complexes were separated on 5% non-denaturing polyacrylamide gels in 0.25 X TBE and detected by autoradiography.

Immunofluorescence

Rat-1 and Rat-1p185 cells grown on glass cover slips were washed in TBS with 1 mM $CaCl_2$ (TBS/Ca) then fixed for 1 minute in acetone: methanol (1:1) at room temperature. Slides were washed four times with TBS/Ca then incubated with CrkL monoclonal antibody 2-2 (1:2 dilution), courtesy of J. D. Griffin, Dana-Farber Cancer Institute, Santa Cruz CrkL polyclonal antibody C-20 (1:10 dilution), or peptide blocked C-20 (1:10 dilution) for 1 hour at room temperature. Antibodies were peptide blocked according to manufacturers instructions (Santa Cruz). Slides were washed four times in TBS/Ca with 0.1% Saponin then four times in TBS/Ca. CrkL was detected with FITC conjugated antibody (Cappel, 1:200 dilution) and washed as before. DAPI II (Vysis, Inc.) was placed on C-20 slides for fifteen minutes at room temperature and briefly washed with TBS/Ca. Slides were overlaid with Slowfade light antifade reagents (Molecular Probes). Confocal images were acquired at the Oregon Health Sciences University MMI Core Facility with a Leica (L900) confocal laser scanning microscope and analysis system.

293T cells were allowed to attach onto glass coverslips overnight. Cells were serum starved for 8 hours and stimulated with 10,000 u/ml alpha-interferon for 45 minutes. Coverslips were processed as above with a 1:10 dilution of anti-CrkL (C-20) antibody or peptide blocked antibody and a 1:200 dilution of anti-rabbit rhodamine conjugated antibody (Cappel). Nuclei were counterstained green with 100 uM Sytox (Molecular Probes) overlaid on the coverslips followed by SlowFade light antifade reagents (Molecular Probes). Confocal images were acquired at the MMI Core Facility with a Leica (L900) confocal laser scanning microscope and analysis system.

Luciferase Assay

COS7 cells were transfected using lipofectAMINE (GIBCO-BRL) according to manufacturer's instructions with 8XGAS luciferase reporter construct (188) (4 µg), CMV-CAT (2 µg), and pcDNA3 (InVitrogen) constructs (5 µg). The pcDNA3 constructs included CrkL, Grb2, p210Bcr-Abl and a kinase defective Bcr-Abl. Total DNA transfected was equalized with pcDNA3 vector. Cells were harvested 48-72 hours after transfection and luciferase assays were performed as previously described (189). Transfections were performed in triplicate. Luciferase data (luciferase value divided by CAT value) are specified by relative promoter activity as compared to the reporter construct (sample luciferase data divided by the background luciferase data). Values were then averaged and plotted with the error bars representing standard error. Phase extraction CAT assays were performed as previously described (189).

PCR Mutagenesis

The Stat5 tyrosine 694 was mutated to phenylalanine by PCR mutagenesis. An oligonucleotide primer containing the tyrosine mutation (mutation in bold, codon underlined) and a silent mutation incorporating an Acc1 site (mutation in bold), 5' cagt**c**gacggat**tc**gtgaagccacag 3' , was paired with the reverse primer 5' ctttcgcaagtacaggag 3' to amplify the entire Stat5a-flag tagged in pBluescript plasmid. PCR reactions were performed with 10 ng of plamid in 2.5 U Pfu turbo with supplied buffer (Strategene), 5% DMSO, MgCl2 1.5 mM, dNTPs 2.5 mM, and 50 pmol of each primer. The PCR program included a 2' dwell at 95 degrees prior to cycling, with cycles of 95 degrees for 1 minute, 55 degrees for 1 minute, and 72 degrees for 4 minutes repeated 30 times in a Thermolyne Temp-Tronic machine. Products were screened by size and Acc1(New England Biolabs) restriction

analysis. The mutant Stat5a-flag product was sequenced for mutations and subcloned into pcDNA3.

CHAPTER III

Cytokine induction of CrkL and Stat5 Association in Hematopoietic Cells

Keystone, 1998, Rhodes et. al.

and partial data published in Blood, Ozaki et. al.

Volume 92, 1998

Page numbers 4652-62

INTRODUCTION

Hematopoiesis is the development of a pluripotent stem cell through distinct lineages to form mature blood cells (1). The progenitor and resulting lineages are regulated by a broad spectrum of cytokines, soluble factors that mediate cell-cell communication, which collaborate to control the differentiation, proliferation, maturation, and survival of developing cells (7). An integral part of most cytokine receptors are associated cytoplasmic tyrosine kinases that are activated in response to cytokine binding. These activated tyrosine kinases in turn phosphorylate a variety of substrates that perform many of the cytokine stimulated functions. CrkL, an SH2, SH3 domain containing adaptor protein, is one such protein that is tyrosine phosphorylated following cytokine stimulation with IL-2 (97), steel factor (stem cell factor) (98), erythropoietin (99), IL-3 (99), and thrombopoietin (93).

CrkL is a member of the Crk family. Crk was originally identified as the homologue of the v-Crk oncogene from the CT10 retrovirus (85). Crk is known to transform cells by activating intracellular tyrosine kinases and associating with various tyrosine phosphorylated proteins (85, 87). CrkL has previously been shown to function as a cytoplasmic adaptor protein linking activated tyrosine kinases to effector molecules such as Cbl and Paxillin (SH2 domain) and Abl, Sos, and C3G (N-terminal SH3 domain) (95). Overexpression of CrkL in fibroblasts induces transformation and in hematopoietic cells increases adhesion to fibronectin (95). Examination of chronic myelogenous leukemia patient samples for novel tyrosine phosphorylated proteins demonstrated that CrkL is the major tyrosine phosphorylated protein in CML neutrophils as compared to normal neutrophils (90-92). CML cells express the fusion protein Bcr-Abl and, in contrast to normal samples, are IL-3 and GM-CSF independent. As CrkL can be tyrosine phosphorylated following stimulation of

hematopoietic cells and constitutive phosphorylation of CrkL correlates with a leukemic phenotype, we explored the role of CrkL in cytokine signaling.

To determine the role of CrkL in cytokine signaling, we examined tyrosine phosphorylated proteins that associated with CrkL following IL-3 or GM-CSF stimulation. Previously, it has been shown that IL-3 and GM-CSF stimulation produced very low levels of CrkL tyrosine phosphorylation (185) . Although we did not detect CrkL tyrosine phosphorylation, IL-3 or GM-CSF stimulation induced the robust tyrosine phosphorylation of 94/96 kDa proteins that associate with CrkL. In this report we identify the 94/96 kDa protein as Signal Transducer and Activator of Transcription (Stat) 5, characterize the interaction between CrkL and Stat5, and demonstrate that CrkL is present in a Stat5/DNA complex.

RESULTS

To analyze the role of CrkL in cytokine signaling, tyrosine phosphorylated proteins that associate with CrkL following cytokine stimulation were examined. Examination of phosphotyrosine containing proteins in whole cell lysates from GM-CSF stimulated cells indicated an increase in overall tyrosine phosphorylation as compared to unstimulated cells (Fig 1A). To determine whether CrkL is tyrosine phosphorylated or associated with tyrosine phosphorylated proteins after GM-CSF stimulation, CrkL immunoprecipitations followed by phosphotyrosine immunoblots were performed. As seen in Figure 1B, no CrkL tyrosine phosphorylation was observed, however a prominent 94/96 kDa tyrosine phosphorylated protiens is observed in the CrkL immunoprecipitations. The association of CrkL and the 94/96 kDa proteins was observed in two GM-CSF stimulated cell lines, but not in unstimulated cell lysates. These cell lines included UT-7 and TF-1 cells, both derived

from patients with acute myelogenous leukemia. Notably, the 94/96 kDa is the most prominent association with few other phosphoproteins interacting with CrkL.

Two highly homologous Stat5 genes code for the Stat5 proteins. These proteins have molecular weights of 94 kDa (Stat5b) and 96 kDa (Stat5a) and are known to be tyrosine phosphorylated after cytokine stimulation. Therefore, we examined whether the 94/96 kDa bands were Stat5. CrkL immunoprecipitates were immunoblotted with a Stat5 antisera that recognizes both Stat5a and Stat5b confirming the identity of the 94/96 kDa proteins as Stat5 (Fig 2A). The Stat5 band appears as a doublet, which may be a result of CrkL interaction with both Stat5a and Stat5b or may be due to differences in levels of tyrosine phosphorylation. In collaboration with Atsushi Oda, similar results have been obtained following IL-3 stimulation of the UT-7 and TF-1 cell lines (data not shown). The CrkL/Stat5 association was also observed following interferon alpha stimulation of 293T cells (190).

The CrkL/Stat5 interaction was analyzed using GST fusion proteins containing full length CrkL, CrkL SH2 domain, CrkL SH3 domains, and full length CrkL with the K39 mutation (Fig 3). The K39 mutation of arginine to lysine in the conserved sequence Phe-Leu-Val-**Arg**-Glu-Ser (FLVRES) renders the SH2 domain incapable of binding phosphotyrosine residues. GST pull down analysis determined that in stimulated cells full length CrkL and the CrkL SH2 domain interact with Stat5. This interaction was disrupted in the K39 mutant, indicating that SH2 domain function is necessary for this interaction.

As Stat5 funcitons in the nucleus but only cytoplasmic adaptors functions for CrkL have been identified, the possibility that the CrkL/Stat5 complex was present in the

nucleus was examined. As CrkL has not previously been reported to be present in the nucleus, indirect immunofluorescence was used to determine CrkL localization in stimulated and unstimulated cells. Using a polyclonal CrkL antibody, CrkL was observed to be largely cytoplasmic in unstimulated 293T cells, but in alpha-interferon stimulated cells nuclear CrkL was observed largely in punctate nodules (Fig 4). Peptide blocked CrkL antibody or secondary antibody alone produced negligible signal (data not shown). Sytox staining (green) indicates the nucleus for verification of nuclear localization.

As CrkL appear to be present in the nucleus of stimulated cells, we investigated whether CrkL might be present in a Stat5/DNA complex. In collaboration with Atsushi Oda, EMSA experiments were performed using Stat5 DNA binding sequence derived from the beta-casein promoter. A specific Stat5/DNA complex was apparent in IL-3 stimulated cells (Fig 5) that was not present in unstimulated cells and was competed with excess cold probe. The presence of Stat5 was confirmed by using Stat5 antiserum to supershift the complex, whereas Stat3 antisera produced no supershift. Similarly, the presence of CrkL was detected in the DNA-binding complex by a supershift with CrkL antisera.

DISCUSSION

Cytokine signaling cascades transduce extracellular signals into a nuclear response. Specifically, IL-3, GM-CSF, and alpha-interferon stimulation of appropriate cell types results in the tyrosine phosphorylation and activation of signal transducer and activator of transcription (Stat) 5. Although the precise function of CrkL is unknown, CrkL has previously been described as a cytoplasmic adaptor protein that is activated by cytokine stimulation. In this report we have identified an association of Crkl with tyrosine phosphorylated Stat5 following GM-CSF and IL-3 stimulation.

Using GST-fusion constructs, we detected an inducible association between full length CrkI and the CrkI SH2 domain with Stat5. This association was abolished in a full length CrkI with a mutation in the SH2 phosphotyrosine binding domain. These associations occur in the absence of CrkL tyrosine phosphorylation. Taken together, this suggests that the SH2 domain of CrkL binds to tyrosine phosphorylated Stat5 and is likely responsible for the observed co-immunoprecipitation.

As Stat5 is present in the cytoplasm but translocates to the nucleus after cytokine stimulation, it was necessary to determine whether CrkL is also present in the nucleus. Immunofluorescence studies indicate clear nuclear localization in cytokine stimulated cells. In these cells, CrkL localization is predominantly observed in punctate nodules; however, the functional significance of these nodules is unknown. In nuclear lysates of cytokine stimulated cells, CrkI antiserum supershifts a Stat5-DNA complex in electrophoretic mobility shift assays. These data suggest that CrkI may be a component of the Stat5-DNA binding complex in the nucleus. Experiments are in progress to test this hypothesis. Whether other proteins, such as additional Stats, interact with Crk family members is as yet undetermined. These data suggest a novel function for CrkL, functioning as a nuclear adaptor protein to influence Stat5 transcriptional activation of genes important in cytokine stimulation.

Recent reports have indicated that Stat5/CrkL association is induced by alpha-interferon stimulation (190). Alpha-interferon results in growth inhibition of hematopoietic progenitors whereas IL-3 and GM-CSF result in proliferation of progenitor cells. The apparent discrepancy of the CrkL/Stat5 association induced by each of these pathways may be resolved by the consistent anti-apoptotic effects of each of these cytokines. Further studies are necessary to confirm this hypothesis.

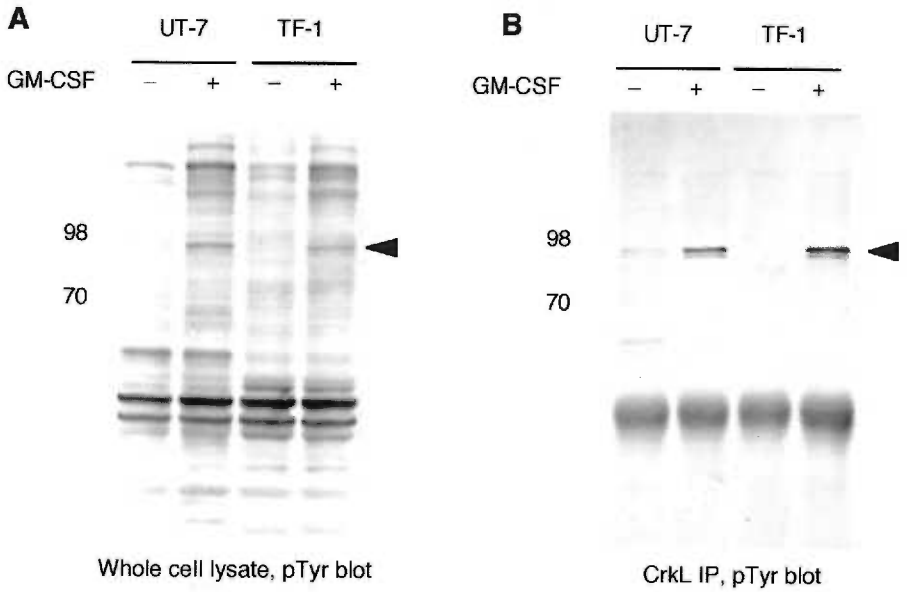


Figure 1. CrkL interacts with 94/96 kDa tyrosine phosphorylated proteins in cytokine stimulated cells. (A) Whole cell lysates from UT-7 and TF-1 cells either starved or stimulated with 100 ng/ml GM-CSF for 10 minutes were analyzed by phosphotyrosine immunoblotting. The + and - signs indicate stimulation with GM-CSF. Note the band of 95 kDa in the presence of stimulation or Bcr-Abl as indicated by an arrow. (B) Lysates from UT-7 and TF-1 cells either starved (-) or GM-CSF stimulated (+) were immunoprecipitated with CrkL antisera and analyzed by antiphosphotyrosine immunoblotting.

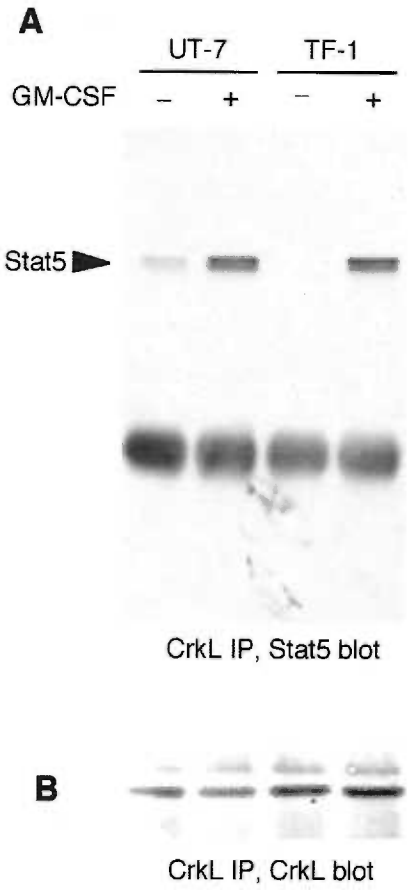
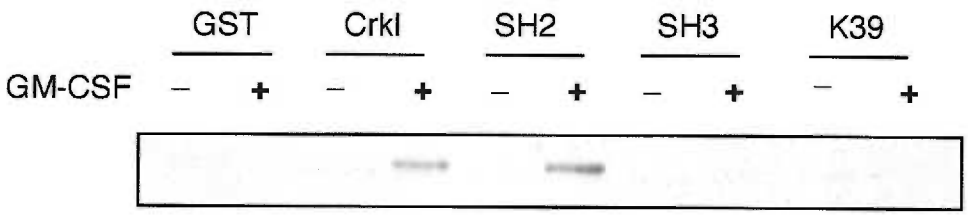


Figure 2. CrkL associates with Stat5 following cytokine stimulation. (A) CrkL immunoprecipitates of lysates from UT-7 and TF-1 starved (-) and GM-CSF stimulated (+) cells were analyzed by immunoblotting with Stat5 antisera. Similar results were obtained following IL-3 stimulation of TF-1 and UT-7 cells (data not shown). (B) Stripped blots were reprobed with CrkL antisera. Similar results were obtained following IL-3 stimulation of TF-1 and UT-7 cells (data not shown).



GST-CrkI Fusion Binding Assay, Stat5 blot

Figure 3. CrkL SH2 domain mediates interaction with Stat5 in cytokine stimulated cells. Lysates of starved or stimulated TF-1 cells were incubated with the following GST fusion proteins: GST alone, GST-full length CrkL, GST-CrkL SH2 domain, GST-N terminal SH3 domain of CrkL, and GST-K39 (full length CrkL with a arginine to lysine substitution at amino acid 39 in the FLVRES, phosphotyrosine binding motif of the CrkL SH2 domain). Proteins bound to CrkL fusions were immunoblotted for Stat5. Similar results were obtained with UT-7 lysates (data not shown).

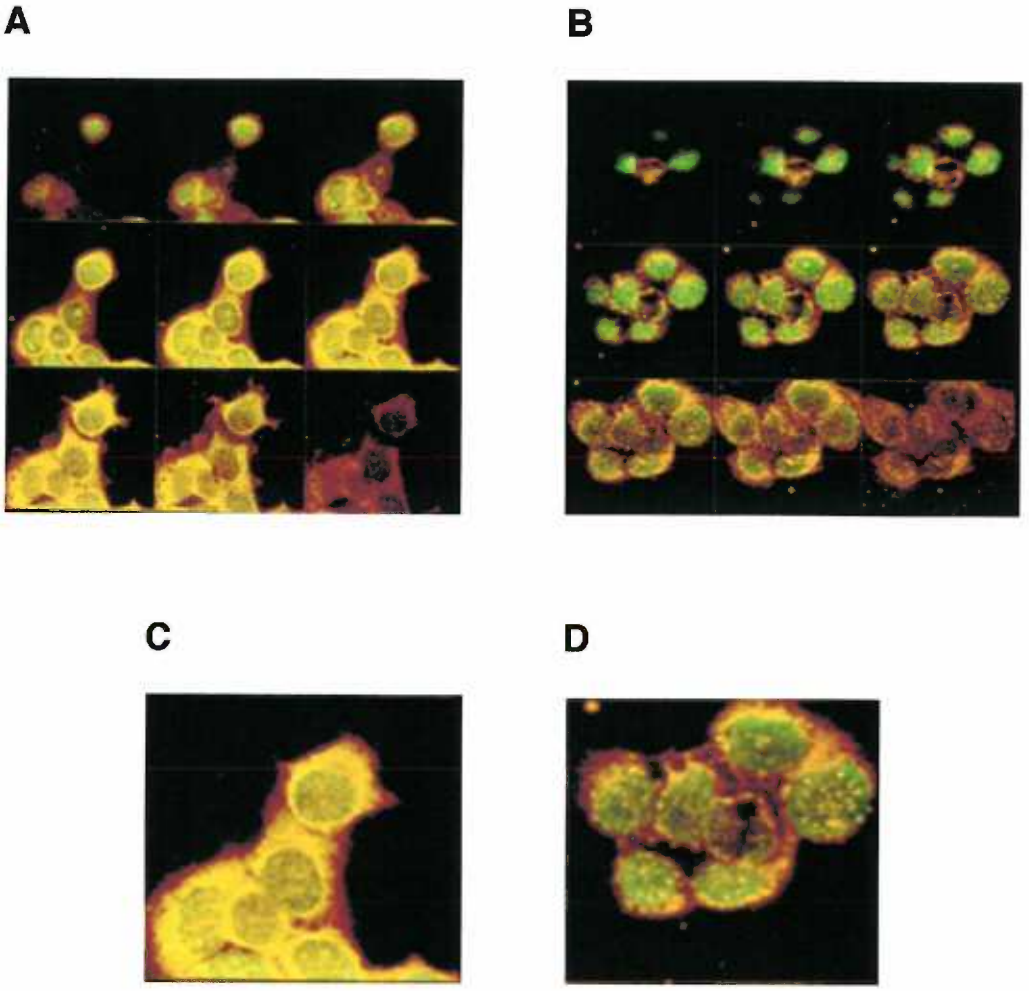


Figure 4. CrkL localization in starved and alpha-interferon stimulated cells. Confocal images of starved (A) and alpha interferon stimulated (B) 293T cells. A and B, images are successive planes from the top of the cell (top left) to the bottom (lower right). Enlarged images of a single plane of starved (C) and stimulated (D) 293T cells. Green Sytox staining indicates the nucleus.

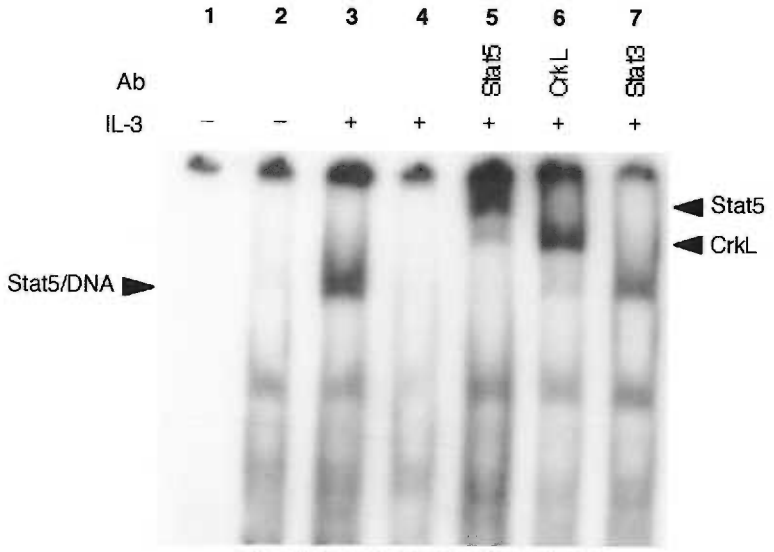


Figure 5. CrkL is present in a Stat/DNA complex. EMSAs were performed using a double stranded oligonucleotide which contains a ß-casein promoter derived Stat5 binding site. End-labeled oligonucleotide was incubated with no lysate (lane 1), nuclear extracts from TF-1 unstimulated cells (lane 2), and nuclear extracts from IL-3 stimulated TF-1 cells in the absence or presence of 50 fold excess cold oligonucleotide (lane 3 and 4, respectively). Lanes 5, 6, and 7 represent IL-3 stimulated nuclear lysates from TF-1 cells incubated with the ß-casein probe in the presence of Stat5, CrkL, or Stat3 antibodies. The left side arrow indicates the Stat5/ DNA complex and the right side arrows indicates Stat5 or CrkL supershifts. Similar data has been obtained from UT-7 cells. This figure was kindly provided by our collaborator Atsushi Oda.

CHAPTER IV

CrkL Functions as a Nuclear Adaptor and Transcriptional Activator in Bcr-Abl-Expressing Cells

Experimental Hematology, Rhodes et. al.

In press, March 2000

INTRODUCTION

Chronic myelogenous leukemia (CML) is a malignancy of a pluripotent hematopoietic stem cell. The majority of CML patients have a specific chromosomal translocation, known as the Philadelphia (Ph) chromosome, that is identifiable throughout the course of the disease (21, 22). The Ph chromosome is a somatic mutation that results from a reciprocal translocation between the long arms of chromosomes 9 and 22 which fuse genetic sequences on chromosome 22 (Bcr) with c-Abl sequences translocated from chromosome 9 (22-24). The Bcr-Abl fusion protein has elevated protein tyrosine kinase activity as compared to c-Abl (34, 35). In bone marrow reconstitution studies using bone marrow infected with Bcr-Abl, transplanted mice develop a CML-like syndrome along with other leukemias (27, 28).

Examination of CML patient samples for novel tyrosine phosphorylated proteins demonstrated that CrkL is the major tyrosine phosphorylated protein in CML neutrophils as compared to normal neutrophils (90-92). CrkL, an SH2, SH3 domain containing adaptor protein, is a member of the Crk family. Crk was originally identified as the homologue of the v-Crk oncogene from the CT10 retrovirus (85). Crk is known to transform cells by associating with and activating intracellular tyrosine kinases (85, 87). Further, CrkL has been shown to bind directly to Bcr-Abl and is known to associate with a variety of tyrosine phosphorylated proteins (87, 100, 101).

Another tyrosine phosphorylated protein in Bcr-Abl-expressing cells is Signal Transducer and Activator of Transcription (Stat)5 (178, 179). When tyrosine phosphorylated, Stat proteins dimerize and translocate to the nucleus to activate transcription (126). In exploring the role of CrkL in Bcr-Abl transformation, we have identified CrkL as a link between Bcr-Abl and Stat5 signaling. Further, we have explored the functional interactions of CrkL and Stat5.

RESULTS

To analyze the role of CrkL in Bcr-Abl transformation, tyrosine phosphorylated proteins that associate with CrkL in Bcr-Abl-expressing cells were examined. In CrkL immunoprecipitates from Bcr-Abl-expressing cells, we observed 94/96 kDa tyrosine phosphorylated proteins (Fig 1A). The association of CrkL and the 94/96 kDa proteins was consistently observed in all Bcr-Abl-expressing cells examined. These included MO7e, a human megakaryoblastic cell line (191), K562 cells, a human Ph (+) cell line (192), 32D cells, a mouse lymphoblastic cell line, and Rat-1 fibroblasts (data not shown). Using specific monoclonal antibodies the 94/96 kDa proteins were identified as Stat5a and Stat5b (Fig 1B). Despite the fact that the Stat5b monoclonal antibody is not reported to cross react with Stat5a (Zymed), the Stat5b associated with CrkL appears to migrate as a doublet. The CrkL/Stat5 interaction was confirmed *in vitro* using GST-CrkL fusion proteins (Fig 2). The association of GST-CrkL with Stat5 was seen in all of the above Bcr-Abl expressing lines, such as K562 (Fig 2A) and Rat-1p185 (Fig 2B) cell lines. This interaction was detected using GST fusion proteins containing full length CrkL as well as the SH2 domain and the amino terminal SH3 domain of CrkL (Fig 2C).

CrkL has previously been shown to function as a cytoplasmic adaptor protein linking activated tyrosine kinases to small GTP binding proteins. Having shown that CrkL and Stat5 interact in Bcr-Abl-expressing cells, the possibility that the CrkL/Stat5 complex was present in the nucleus was examined. For these experiments, CrkL immunoprecipitates were performed using nuclear lysates from K562 cells. Immunoblotting for CrkL or Stat5 demonstrated the presence of CrkL in the nuclear lysates and an association between CrkL and Stat5 (Fig 1B). As CrkL has not previously been reported to be present in the nucleus, indirect immunofluorescence was used to determine CrkL localization in parental and Bcr-Abl expressing cells.

Using a monoclonal antibody, CrkL was observed to be largely cytoplasmic in Rat-1 cells (Fig 3A), but in Rat-1p185 cells nuclear CrkL was observed (Fig 3B). The confocal images show CrkL throughout the nucleus with punctate nodules of CrkL immunostaining. This result was confirmed with a polyclonal CrkL antibody (Fig 3C), binding of which was blocked by preincubation with immunizing peptide (data not shown). DAPI staining indicates the nucleus for verification of nuclear localization. Results of secondary antibody alone showed negligible background (data not shown).

As CrkL appears to be present in the nucleus of Bcr-Abl-expressing cells, we investigated whether CrkL might be present in a Stat5/DNA complex. Using a Stat5 DNA binding sequence derived from the ß-casein promoter, electrophoretic mobility shift assay (EMSA) experiments were performed. K562 nuclear lysates shifted multiple bands (Fig 4, *lanes 2 and 11*). Two of these shifted bands were competed with an excess of cold wild type sequence, but not with a mutant core binding sequence, indicating specificity of these two complexes (Fig 4, *lanes 3 and 4*). To confirm that Stat5 was present in these complexes, a Stat5 antisera that recognizes both Stat5a and Stat5b was used to supershift the complex (Fig 4, *lane 5*), while peptide blocked antibody (Fig 4, *lane 6*) did not supershift the complex. Multiple supershift bands suggest that Stat5a and Stat5b proteins, which homo- and heterodimerize, may be present in these complexes. Similarly, the presence of CrkL was detected in the DNA binding complex by a supershift with CrkL antisera (Fig 4, *lane 8*), while peptide blocked antibody (Fig 4, *lane 9*) did not supershift the complex. Although faint in comparison to the Stat5 supershifts, the CrkL supershift was reproducible in multiple independent experiments. In contrast, no supershift was seen with Stat1 (Fig 4, *lane 7*) or Grb2 (Fig 4, *lane 10*) antibodies demonstrating the specificity of the Stat5 and CrkL supershifts.

To explore the functional consequences of a CrkL/Stat5 interaction, the effect of Bcr-Abl and CrkL on Stat transcriptional activation was examined. A reporter construct with eight consecutive γ-interferon activated sequences (GAS) linked to a luciferase reporter gene (188) was cotransfected with Bcr-Abl or CrkL into COS7 cells and Stat transcriptional activation was examined (Fig 5A). Data is plotted as the average relative promoter activity over baseline of the reporter construct alone. Transfection of p210 Bcr-Abl resulted in a significant activation over baseline. This is presumably due to endogenous Stat5 proteins in COS7 cells whose presence has been confirmed by immunoblotting of whole cell lysates (data not shown) and is consistent with previous data demonstrating activation of Stat5 in Bcr-Abl-expressing cells (178, 179). Transfection of a kinase defective Bcr-Abl resulted in no Stat transcriptional activation over baseline. Transfection of CrkL alone, also, resulted in no significant activation. However, cotransfection of CrkL with Bcr-Abl caused a two fold increase in activation over Bcr-Abl alone. This two fold increase has been seen in multiple independent experiments. As CrkL alone did not activate Stat transcription, these data suggest that Bcr-Abl activates CrkL's adaptor function resulting in increased Stat activation. To determine whether this effect is specific to CrkL, another SH2, SH3 domain containing protein that also binds directly to Bcr-Abl, Grb2, was examined (Fig 5B). In contrast to CrkL, Grb2 cotransfected with Bcr-Abl slightly inhibited Stat activation. The slight inhibitory effect of Grb2 on Bcr-Abl induced Stat transcriptional activation may be a result of binding site competition for Bcr-Abl, other interacting proteins, or retention of Stat in the cytoplasm.

DISCUSSION

Following identification of Crk family members as SH2, SH3 domain-containing proteins, multiple protein-protein interactions with Crk proteins have been observed. The amino terminal SH3 domain of CrkL interacts with Bcr-Abl, Abl, and

C3G (103). Tyrosine phosphorylated proteins that bind to the CrkL SH2 domain include Cbl, Paxillin, and p130Cas (103). Additional interactions between Crk proteins and the PDGF receptor, IRS-1, and IRS-4 have also been identified (193-195). Although the precise function of CrkL is unknown, these data suggest that Crk proteins function as cytoplasmic adaptors. In examining the role of CrkL in Bcr-Abl transformation, we have identified an interaction between CrkL and Stat5 and have presented data suggesting a novel nuclear function for CrkL.

As Stat5 is present in the cytoplasm, but functions in the nucleus, it was necessary to identify whether CrkL is present in the nucleus. Not only was CrkL present in nuclear lysates of K562 cells, but immunofluorescent studies clearly demonstrated nuclear localization of CrkL in Bcr-Abl expressing cells. In these cells, CrkL localization is predominantly observed in punctate nodules, however, the functional significance of these nodules is unknown.

A nuclear role for CrkL was suggested by demonstrating that CrkL is present in a Stat5/DNA complex and subsequently by showing that in the presence of Bcr-Abl, CrkL increases transcriptional activation from a Stat responsive reporter construct. However, CrkL alone did not increase Stat transcriptional activation. This indicates that CrkL is not sufficient for this effect and that other factors are required for an enhancement of Stat activation to be observed in the presence of CrkL. Whether these factors activate a previously undescribed function of CrkL, allow CrkL to link directly to Stat, or stimulate a pathway leading to Stat activation is unknown. However, data using the kinase defective Bcr-Abl indicates that transcriptional activation in the presence of CrkL is dependent on the kinase activity of Bcr-Abl.

The interaction of tyrosine phosphorylated Stat5 and CrkL is consistently observed in cells expressing Bcr-Abl. An inducible association of Stat5 and CrkL was reported following IL-3 and GM-CSF stimulation of hematopoietic cells (185). In these cells, Stat5 is inducibly tyrosine phosphorylated, but minimal CrkL phosphorylation is observed. This indicates that Stat5, but not CrkL, tyrosine phosphorylation is necessary for this interaction suggesting that the SH2 domain of CrkL interacts with tyrosine phosphorylated Stat5. Our data using GST-fusion proteins demonstrate that both the SH2 and SH3 domains of CrkL are capable of binding to Stat5 from Bcr-Abl expressing lysates. As Bcr-Abl is known to bind directly to the SH3 domain of CrkL and also interacts with Stat5, it is likely that the SH3 domain interaction of CrkL with Stat5 is due to indirect associations. Whether the SH2 domain of CrkL interacts directly with Stat5 is currently under investigation.

Stat5 has previously been demonstrated to be tyrosine phosphorylated and activated in Bcr-Abl-expressing cells (178, 179). Our data demonstrates that CrkL and tyrosine phosphorylated Stat5 form a complex in Bcr-Abl-expressing cells. Our co-transfection data suggest that the interaction of CrkL and Stat5 has functional implications resulting in enhanced Stat5 transcriptional activation. The finding of CrkL association with tyrosine phosphorylated Stat5 may be a more general phenomenon. This interaction may be induced in other instances in which Stat5 is tyrosine phosphorylated, including α-interferon, GM-CSF and IL-3 stimulation of the appropriate cell types (185, 190). Whether other Stat proteins interact with Crk proteins is as yet undetermined. These data suggest a novel function for CrkL, functioning as a nuclear adaptor protein that can increase Stat transcriptional activation.

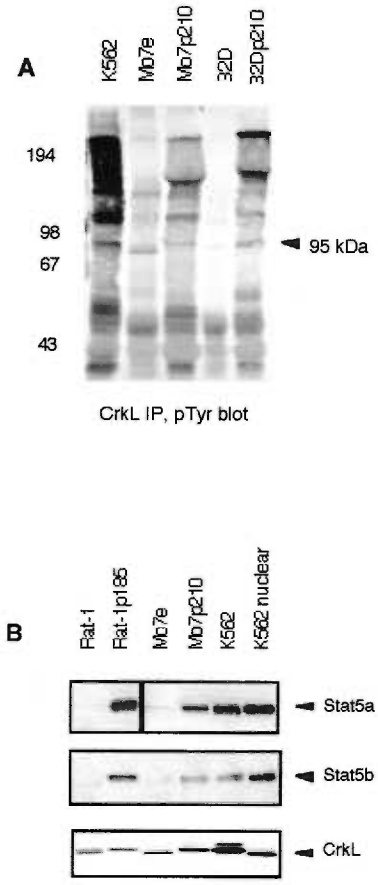


Fig 1. CrkL association with tyrosine phosphorylated Stat5 in Bcr-Abl-expressing cells. (A) MO7e and 32D cell lines expressing the p210 forms of Bcr-Abl have previously been described (196, 197). The CML patient derived K562 cell line expresses p210Bcr-Abl (192). Whole cell lysates from the indicated cell lines were immunoprecipitated with CrkL antisera then immunoblotted with phosphotyrosine antibody 4G10. The 94/96 kDa tyrosine phosphorylated proteins are indicated by an arrow. Molecular size standards are labeled in kDa. (B) CrkL immunoprecipitates from the indicated cell lines were immunoblotted for Stat5a or Stat5b. Immunoblotting for CrkL following CrkL immunoprecipitations demonstrated equal

amounts of CrkL in paired lanes. K562 nuclear indicates nuclear lysate from K562 cells.

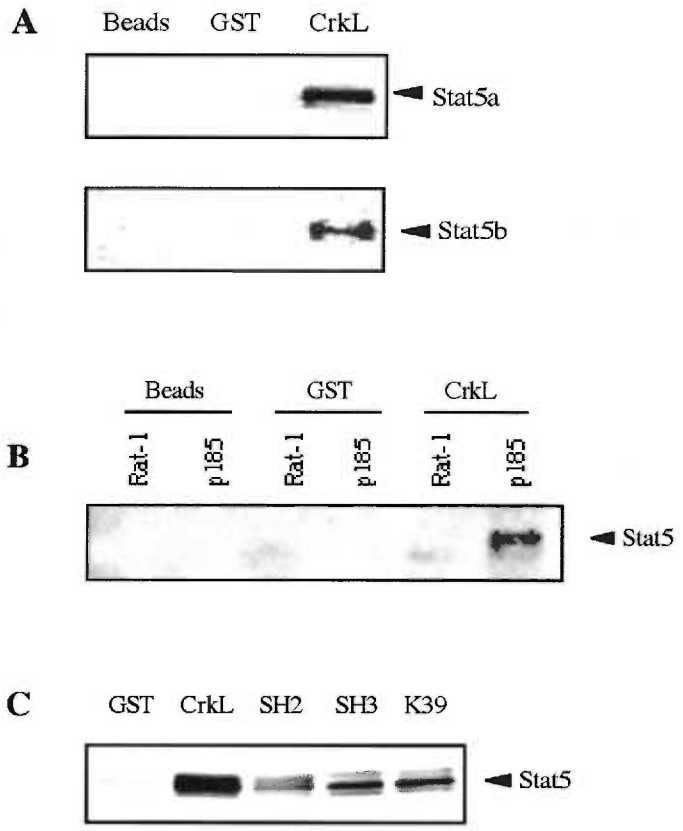


Fig 2. CrkL interaction with Stat5a and Stat5b in vitro. (A) K562 whole cell lysates were incubated with glutathione sepharose beads, purified GST protein, or purified GST-CrkL fusion protein. Complexes were immunoblotted for Stat5a or Stat5b. (B) Rat-1 and Rat-1p185 whole cell lysates were incubated with beads, purified GST protein, or purified GST- CrkL. Complexes were immunoblotted with a polyclonal Stat5 antibody that recognizes both Stat5a and Stat5b. Similar results were obtained using MO7e and MO7p210 lysates (data not shown). K562 whole cell lysates were incubated with GST or GST fusions including CrkL full length (CrkL), CrkL SH2 domain, CrkL SH3 domains, or a full length CrkL with an arginine to lysine mutation in conserved FLVRES sequence of the SH2 phosphotyrosine binding pocket (K39). Proteins bound to the GST proteins were then immunoblotted for Stat5 (polyclonal for 5a and 5b).

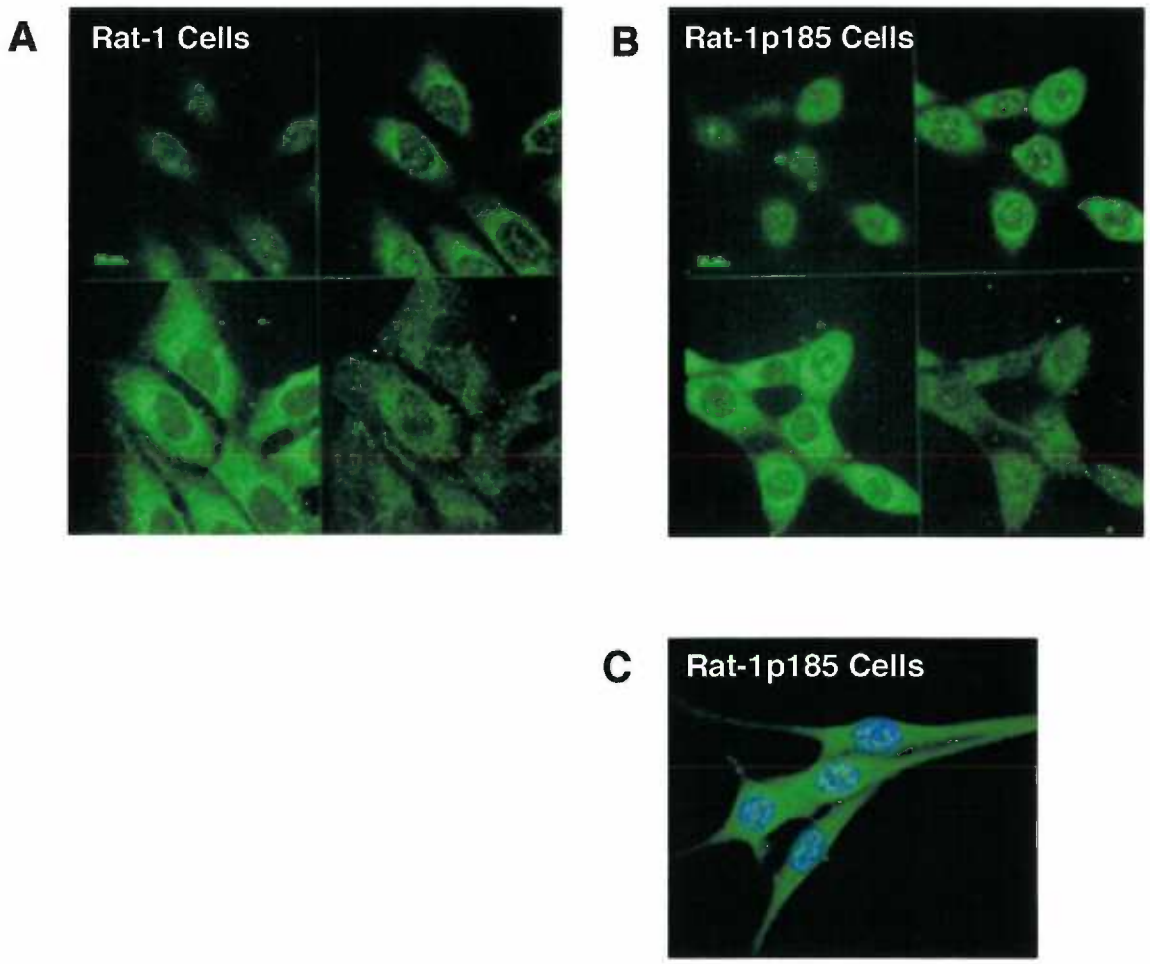


Fig 3. Nuclear localization of CrkL in Bcr-Abl-expressing cells. Indirect immunofluorescence of a monoclonal CrkL antibody was used to detect CrkL (green) in (A) Rat-1 and (B) Rat-1p185 cells. Cells were scanned at 630X magnification into four slices, the upper left corner signifying the top of the cell layering down from left to right and top to bottom of the figure. Relative size is indicated by the 10 μm bar in the upper left panel. (C) Similar results were obtained using polyclonal CrkL antisera. The single section shown of the Rat-1p185 cells is also marked with DAPI (blue) nuclear stain. Negligible signal was detected using secondary antibody alone or the CrkL antisera preincubated with the immunizing peptide (data not shown).

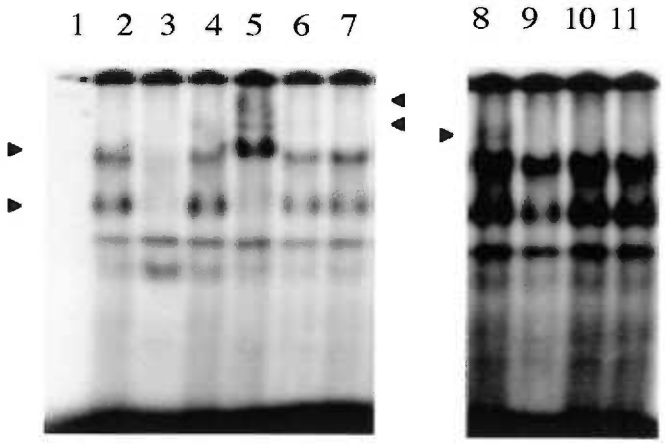


Fig 4. CrkL is present in a Stat5/DNA complex. EMSA's were performed with wild type probe (probe alone, *lane 1*) and K562 nuclear lysate (*lanes 2 and 11*). Competitive binding was examined with an 100X excess cold wild type (*lane 3*) or mutant sequences (*lane 4*). For supershifts, the Stat/DNA complex was incubated with Stat5 (*lane 5*), Stat1 (*lane 7*), CrkL (*lane 8*), or Grb2 (*lane 10*) antisera or peptide blocked antibodies for Stat5 (*lane 6*) or CrkL (*lane 9*). The specific Stat5 bands are indicated by arrows on the left and supershifted complexes are noted by arrows in the center column.

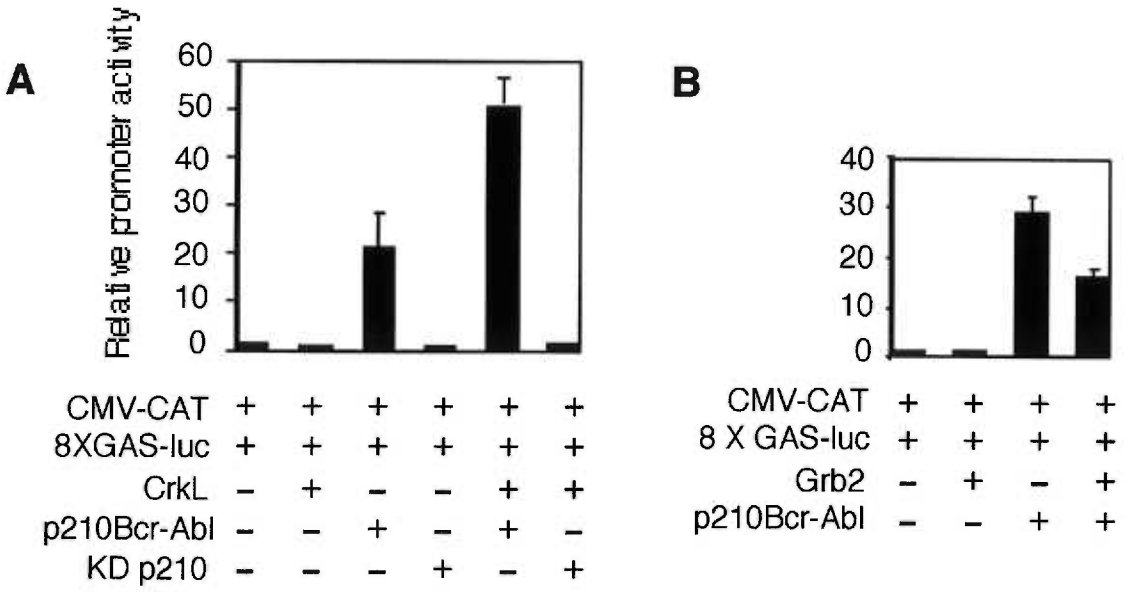


Fig 5. CrkL increases Stat transcriptional activation. Transient transfections of COS7 cells were used to determine the combinatorial effects of the indicated constructs on a Stat responsive luciferase reporter gene. Transfections were performed in triplicate and representative data from one transfection set is shown as relative promoter activity compared to the reporter construct. Error bars represent the standard error. Phase extraction CAT assays demonstrated comparable levels of transfection efficiency (data not shown).

CHAPTER V

Discussion

Bcr-Abl and Cytokine Signaling

This thesis has analyzed the activation and associations of specific proteins mutual to cytokine and Bcr-Abl signaling. Two such proteins are CrkL and Stat5. In examining signaling pathways activated in cytokine stimulated or Bcr-Abl-expressing cells, we demonstrated that CrkL interacts with Signal Transducer and Activator of Transcription (Stat)5. We have identified a novel role for CrkL in which CrkL is present in a Stat5/DNA complex, has nuclear localization, and increases Stat mediated transcriptional activation. This discusion will explore the implications of the CrkL/Stat5 interaction including the mechanism of interaction of Stat5 with CrkL, the role of nuclear CrkL and CrkL transcriptional activation, the kinases responsible for Stat5 phosphorylation, and possible pathways activated by the CrkL/Stat5 interaction.

Interaction of Stat5 and CrkL

In lysates from cytokine stimulated cells, Stat5 interacted with full length CrkL or the CrkL SH2 domain expressed as GST fusion proteins. No interaction was observed using lysates from unstimulated cells. This interaction was abolished using a GST fusion protein that expressed the CrkL K39 mutant, which has an arginine to lysine mutation in the SH2 binding pocket that interfers with phosphotyrosine binding. This suggests that the SH2 domain of CrkL targets a phosphotyrosine on Stat5 in cytokine stimulated cells. In lysates from Bcr-Abl-expressing cells, GST pull down experiments demonstrate that both the SH2 and SH3 domains of CrkL are capable of binding to Stat5. As Bcr-Abl is known to bind directly to the SH3 domain of CrkL, this would allow an indirect interaction of the SH3 domain of CrkL with Stat5. Specifically, the SH3 domain of CrkL binds Bcr-Abl directly, with Stat5 binding to

another site on Bcr-Abl such that it is present in the CrkL-Bcr-Abl complex. To determine whether the SH2 domain interaction of CrkL with Stat5 is direct, interactions were explored by gel overlay analysis. Initial reports, using these lysate of cells stimulated with GM-CSF, showed that the SH2 domain of CrkL directly interacts with Stat5 (198). However, we have been unable to confirm this data in Bcr-Abl expressing cells (data not shown). Thus, we have been unable to confirm a direct interaction between CrkL and Stat5.

Interactions between Stat5 and CrkL and Bcr-Abl may be further defined by mutants in Bcr-Abl and Stat5 that disrupt the interactions. As noted above, it appears that tyrosine phosphorylation of Stat5 is required for the interaction with CrkL. Stat5 contains tyrosines at amino acid residues 694, 686, and 668, that are consistent with the preferred binding sequence for the CrkL SH2 domain. Ota et al. performed experiments following cytokine stimulation in which phosphopeptides containing these tyrosines were analyzed for their ability to disrupt the CrkL/Stat5 interaction (198). These studies suggested that the activating tyrosine, residue 694, is the main tyrosine that interacts with CrkL (198). Tryptic mapping of Stat1 co-expressed in insect cells with a kinase (Jak1) indicated that only the activating tyrosine is phosphorylated. Using PCR mutagenesis, a Stat5 mutant was generated with a tyrosine (694) to phenylalanine mutation (Fig 1). The mutant Stat5 (Y694F) is still tyrosine phosphorylated when co-expressed with Bcr-Abl (Fig 2), suggesting that more than one tyrosine can be phosphorylated on Stat5. Experiments to directly compare equal amounts of wild type versus mutant protein phosphorylation levels are ongoing, however, the amount of tyrosine phosphorylation of the mutant is similar to wild type Stat5 in equal amounts of whole cell lysate. Whether Stat5Y694F retains the ability to interact with CrkL or Bcr-Abl is currently under investigation. Additional Stat5 tyrosines are being mutated to

examine the phosphorylation and interaction of CrkL and Bcr-Abl with these mutants.

Kinases Responsible for Stat5 Phosphorylation

Stat5 tyrosine phosphorylation is necessary for transcriptional activation and interaction with CrkL suggesting an important role for a tyrosine kinase in Stat5 signal transduction. Constitutive tyrosine phosphorylation of Stat5 is observed in Bcr-Abl-expressing cells. Likewise, Stat5 is inducibly tyrosine phosphorylated following IL-3 and GM-CSF stimulation of hematopoietic cells (185). In cytokine signaling, ligand binding induces activation of Janus kinases which then phosphorylate Stat proteins. Stat5 is constitutively phosphorylated in Bcr-Abl-expressing cell, however Janus kinases are not consistently activated. This suggests that another kinase is involved in Stat5 tyrosine phosphorylation. Putative candidate kinases include Abl or Src family members. As additional Stat transcriptional activity has been reported upon serine phosphorylation of Stat proteins by ERK kinases, the potential role of these kinases will also be discussed.

Stat5 contains a consensus site for the Abl tyrosine kinase, but it is not known whether Stat5 is a substrate of the Abl kinase. In vitro kinase assays using purified proteins would determine whether Stat5 is an Abl substrate. Other kinases that may be responsible for phosphorylation of Stat5 include the Src kinase family members Src, Yes, Fgr, Fyn, Lck, Lyn, Hck, and Blk. Src tyrosine kinase is a potential activator of Stat5 activation following epidermal growth factor stimulation (203). Prolactin and Src activate nuclear translocation of Stat5a/b, however a dominant negative Jak2 inhibited only prolactin mediated Stat5 activation (205). Src family kinases Lyn and Hck associate with Bcr-Abl in 32Dp210 cells and are activated in K562 cells (206, 207). Lyn induces activation of Stat5 when co-expressed in Cos7 cells and

phosphorylates Stat5 in vitro (208). Whether Lyn or other Src kinases activate Stat5 in Bcr-Abl expressing cells has yet to be determined. Another tyrosine kinase, Fes, is activated in Bcr-Abl expressing cells and has been shown to activate Stat3 DNA binding when expressed in 293T cells (209). However, it is not known whether Fes activates Stat5. Each of these kinases could be tested for their ability to phosphorylate Stat5 with in vitro kinase assays using purified proteins. The function of the phosphorylation site may be confirmed with trancriptional reporter assays in cells null for the appropriate kinase in which lack of a specific kinase may lead to the lack of Stat activation. Subsequently, the phosphorylated sites on Stat5 can be mapped by phosphotryptic analysis, mutated, and tested for CrkL interaction.

Bcr-Abl expression activates Ras, ERK, and JNK proteins. (211). The Stat5 transactivation domain must be present for transcriptional activation and serine phosphorylation of this domain increases the activation of Stat5 but is not necessary for basic function (204). Erks co-immunoprecipitate and phosphorylate Stat5 on serine 780, mutation of which inhibits phosphorylation by Erk (212). In general, serine 780 phosphorylation leads to an increase in Stat5 transcriptional activation, however, serine phosphorylation can occur independently of Map kinase pathway, suggesting that alternate unidentified kinases may be involved (213). To examine the pathway from Bcr-Abl to the activated Stat5 we examined transcriptional activation of a Stat responsive reporter construct in the presence of dominant negative Erks, dominant negative map kinase, and map kinase phosphatase. In this expression system, inhibitors of the Map kinase pathway did not consistently affect transcriptional activation of Stat5/CrkL in the presence of Bcr-Abl indicating alternate pathways of Stat5 activation (data not shown).

Nuclear CrkL

Stat5 functions in the nucleus, however, CrkL has not previously been identified as having nuclear localization or function. To examine whether CrkL is present in the nucleus, CrkL immunoprecipitations were performed on K562 nuclear lysates followed by immunoblotting for CrkL. Not only was CrkL present in the nuclear lysates, but immunblotting for Stat5 demonstrated that the Stat5/CrkL complex was also present. Indirect immunofluorescence of CrkL clearly demonstrated nuclear localization of CrkL in Bcr-Abl expressing and cytokine stimulated cells. Nuclear CrkL is observed predominantly in punctate nodules, however, the functional significance of these nodules is unknown. Stat5 is localized to both the cytoplasm and the nucleus with Stat5 evenly dispersed throughout the nucleus. Simultaneous detection of CrkL and Stat5 by immunofluorescence did not definitively co-localize Stat5 and CrkL (data not shown). This may be due to CrkL complex formations in the nucleus that inhibit antibody binding to Stat5 or that Stat5 may not exclusively interact with or may be more mobile than CrkL in the nucleus. This latter hypothesis is supported by the finding that a polyclonal Stat5 antibody did not produce a punctate nuclear pattern (data not shown).

A nuclear role for CrkL was suggested by demonstrating that CrkL is present in a Stat5/DNA complex. Although immunofluorescence did not clearly co-localize CrkL and Stat5, electrophoretic mobility shift assays indicated a specific CrkL/Stat5/ DNA complex in nuclear lysates from cytokine stimulated or expressing Bcr-Abl cells. In cytokine stimulated cells, one major Stat5/DNA complex is present, whereas in Bcr-Abl-expressing cells there appears to be at least two specific Stat5 supershifted bands. This indicates that there may be additional Stat5 complexes present in Bcr-Abl-expressing cells as compared to cytokine stimulated cells. The Stat5/DNA complexes were determined to contain CrkL by supershift analysis. The single CrkL

supershift in cytokine stimulated cells is robust whereas the CrkL supershift in Bcr-Abl-expressing cells is minimal, although reproducible. This may be a result of multiple Stat5 complexes in Bcr-Abl expressing cells which may not all contain CrkL. Whether other proteins, such as additional Stats, interact with Crk family members is as yet undetermined. Further supershift analysis may determine the presence of additional proteins in the CrkL/DNA complexes. As Stat1, Stat3, or Grb2 antibodies did not produce supershifts these proteins are not likely to be present in the Stat5/DNA complexes.

CrkL Transcriptional Activation

As CrkL is present in a Stat5/DNA complex, we explored the function of CrkL in Stat mediated transciptional activation. In the presence of Bcr-Abl, CrkL increases transcriptional activation from a Stat responsive reporter construct, however, CrkL without Bcr-Abl did not increase Stat transcriptional activation. This indicates that CrkL is not sufficient for this effect and that other factors are required for an enhancement of Stat activation to be observed in the presence of CrkL. Whether these factors activate a previously undescribed function of CrkL, allow CrkL to link directly to Stat, or stimulate a pathway leading to Stat activation is unknown. However, data using the kinase defective Bcr-Abl indicates that transcriptional activation in the presence of CrkL is dependent on the kinase activity of Bcr-Abl. Howeever, our data suggest a novel function for CrkL, functioning as a nuclear adaptor protein that may increase Stat dependent transcriptional activation of genes required for Bcr-Abl transformation or cytokine signaling.

To determine whether CrkL trancriptional activation was dependent on Stat5, the above described Stat5 Tyr694Phe mutant was co-expressed with Bcr-Abl and CrkL. However, the transcriptional activation induced by Bcr-Abl and CrkL was

unchanged (data not shown). A corresponding mutant of Stat3 was reported to function as a dominant negative by failing to be released from receptor complexes and therefore blocking wild type Stat3 activation (166). As Bcr-Abl is a non-receptor tyrosine kinase, the mechanism of activation of Stat5 may differ to account for the lack of inhibition when the mutant was present. A carboxy truncated dominant negative might be more useful for confirming the dependence of transcriptional activation on Stat5 and determining the pathways in which Stat5 functions.

Examination of Bcr-Abl transformation in CrkL null or Stat5 null cells would determine whether CrkL or Stat5 are necessary in Bcr-Abl induced transcriptional activation and transformation. Similarly, cytokine stimulation in CrkL null or Stat5 null cells would determine the necessity of these proteins in cytokine signaling. Since CrkL is ubiquitously expressed, examination of Stat5 transcriptional activation in the complete absence of CrkL has not been possible. The activation of Stat5 in CrkL null cells would be informative for the dependence of Stat5 on CrkL for transcriptional activation. Likewise, Stat5 null cells would be informative for the dependence of CrkL on Stat5 for Bcr-Abl mediated transformation. Additionally, mouse knockouts may be used to determine the pathways involved in Stat5 activation and Bcr-Abl transformation, such as Stat5 activation in Ras null cells or Bcr-Abl anti-apoptotic effects in Bcl-X deficient mice.

Pathways Activated by Stat5 in Bcr-Abl Transformation

Stat5 activation by cytokine stimulation has been well documented (see Appendix I). Although the pathway of Stat5 activation following cytokine stimulation is well defined (see appendix I), the cytokines that stimulate the CrkL/Stat interaction have not been fully identified. Further work is necessary to identify the circumstances that induce the Stat5/CrkL association. Following cytokine stimulation, Stat 5 has been

shown to mediate cell proliferation, differentiation, and survival. Bcr-Abl transformation has overlaping effects with Stat5 including cellular proliferation and cell survival suggesting a possible role for Stat5 in these pathways. Examination of genes activated by Stat5 or proteins associated with Stat5 following cytokine stimulation may be useful for determining the role of Stat5 in Bcr-Abl transformation.

Cytokine induced genes activated by Stat5 continue to be identified, however, the previously identified genes may be useful in determining the role of Stat5 in Bcr-Abl transformation. Previously identified Stat5 activated genes include B cell lymphoma protein (Bcl-x), FBJ osteosarcoma (Fos), Oncostatin M (OSM), Cytokine Inducible SH2 protein (CIS), c-Myc, Pim.1, Serine Protease Inhibitor (Spi 2.1), Interferon Regulatory Factor (IRF-1), IL-2 Receptor alpha, Fc gamma Receptor 1, p21 WAF/Cip1, Whey Acidic Protein (WAP), Beta-lactoglobulin, and Beta Casein. As CrkL has not previously been identifed as a nuclear factor, the involvement of CrkL in Stat5 activation of genes, modification of specificity or localization, or additional functions is not known. Our data suggests that CrkL functions to increase trancriptional activation of Stat responsive genes, however, EMSA or reporter assays with endogenous sequences would better define the involvement of CrkL in activation of known targets of Stat5.

CrkL and Stat5 are present in a novel complex, however, the components of this complex have not been fully examined. By EMSA analysis, Stat1, Stat3, and Grb2 are not present in the Stat5/DNA complex. Further EMSA analysis may define other proteins present in the Stat5/DNA complex that have yet to be identified. As CrkL is an adaptor proteins, a hypothesis for the increase in Stat5 transcriptional activation due to CrkL may be that CrkL is recruiting additional transcription co-factors. Stat1 proteins have been identified to interact with

transcription factors CBP/p300, p48, and Sp1, however, only a prolactin induced association of CBP/p300 with Stat5 has been identified (215-218). The glucocorticoid receptor can act as a co-activator to increase Stat5 mediated transcriptional activation (146). The role of glucocorticoids in hematopoiesis is not fully defined, although glucocorticoids are required for the proliferation of erythroid progenitor cells and lead to apoptosis of B-cell lymphocytes (219, 220). Additional proteins, including c-Abl or proteins activated in Bcr-Abl-expressing cells such as Retinoblastoma (Rb), NFkappaB, c-Fos/Jun, or c-Myc, have yet to be examined for interaction with CrkL or Stat5 in a DNA complex. Stat5 transcriptional activation is increased in by CrkL in Bcr-Abl expressing cells, suggesting the involvement of a transcriptional activator. A possible role for CrkL may be as a nuclear adaptor which functions by recruiting activating molecules to the Stat5/DNA complex and, therefore, increasing Stat5 mediated gene transcription.

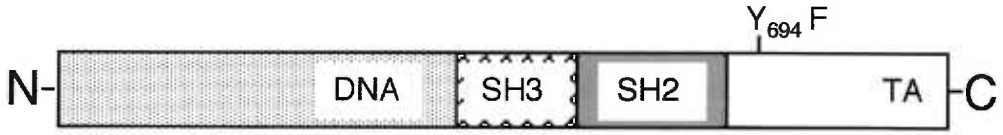


Figure 1. Diagram of Stat5 Tyrosine 694 to phenylalanine mutation. Features Stat5 domains as previously described and activating tyrosine 694 mutated to phenylalanine, $Y_{694}F$.

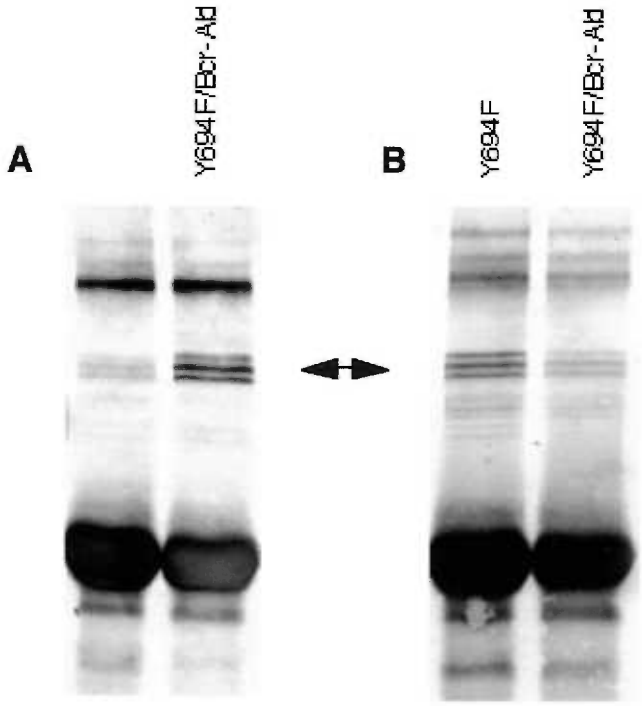


Figure 2. Stat5 $Y_{694}F$ mutant tyrosine phosphorylation. Flag tagged mutant Stat5 was transfected alone or co-expressed with Bcr-Abl in COS7 cells. Flag immunoprecipitations were performed followed by immunoblotting for (A) phosphotyrosine or (B) flag.

CHAPTER VI

Conclusions

Bcr-Abl expression results in proliferation, prevention of apoptosis, and adhesion defects in myeloid cells. Transformation of myeloid cells is dependent on the Bcr-Abl kinase activity suggesting that the molecular mechanism depends on the tyrosine phosphorylation of proteins. Therefore, Oda et. al. looked for novel tyrosine phosphorylated proteins in CML patient neutrophils in comparison to normal neutrophils (91). This initial study identified CrkL as being a novel constitutively tyrosine phosphorylated protein in CML patient neutrophils and suggests that CrkL may have a role in Bcr-Abl transformation. In this thesis the proteins associated with CrkL in Bcr-Abl expressing cells have been explored. In Bcr-Abl-expressing cells, we observed a constitutive interaction between CrkL and tyrosine phosphorylated Stat5. Similar to CrkL, Stat5 is constitutively tyrosine phosphorylated in Bcr-Abl expressing cells.

Stat5 has been implicated in proliferation, differentation, and cell survival of myeloid cells. The Stat5 pathway is regulated in normal cells by cytokine induced tyrosine phosphorylation and subsequent activation of Stat5. One of the goals of this thesis was to determine if Bcr-Abl and cytokines utilize common pathways to induce proliferation or protection from apoptosis. Thus, we explored whether the Stat5/CrkL interaction was present following cytokine stimulation. We observed an inducible assocation between CrkL and tyrosine phosphorylated Stat5. This association was mediated by the CrkL SH2 domain, whereas the constitutive association observed in Bcr-Abl expressing cells was regulated by both the CrkL SH2 and SH3 domains.

In the course of these experiments we have determined that CrkL is found in a Stat5/DNA complex and increases transcriptional activation from a Stat responsive reporter construct. As CrkL has only been described as having cytoplasmic adaptor functions, this suggests a novel role for CrkL acting as a nuclear protein in a Stat5/DNA complex.

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Appendix I

JAK-Stat Signal Transduction

by Jennifer Rhodes and Brian J. Druker

Chapter in Developmental Hematopoiesis

edited by Len Zon

Jak-STAT Signal Transduction

Introduction

Essential to an understanding of cytokine biology are studies of the molecular mechanism of action of cytokines. Although cytokine receptors lack kinase domains, the induction of protein tyrosine phosphorylation has been identified as critical to their function. Numerous studies have demonstrated an essential role for activation of Janus kinases (Jaks) and Signal Transducers and Activators of Transcription (STAT) in cytokine signal transduction. This pathway is activated by virtually all cytokines and allows for direct signaling from receptors to the nucleus. More recent data from animals lacking specific Jak or STAT proteins has confirmed the necessity of these proteins in cytokine signaling.

Janus Kinases (Jaks)

The Janus kinase (Jak) family members (Jak 1, 2, 3 and Tyk2) were cloned using two different strategies. The kinase domains of Jak1 and Jak2 were cloned by PCR using degenerate oligonucleotides complementary to highly conserved regions of tyrosine kinase domains (1). Subsequently, the kinase domain sequences were used as probes to clone the full length cDNAs (2). Jak3 was similarly cloned using a PCR based strategy (3, 4). Tyk2 was cloned by low stringency hybridization from a T-cell cDNA library using the sequences of the c-fms tyrosine kinase domain (5, 6). By sequence homology, Jak1 and Jak2 had two potential kinase domains, thus, were named Janus kinases after Janus, the Roman God with two faces (2). Subsequent work has demonstrated that only one of these domains has tyrosine kinase activity.

The role of Jaks in hematopoietic cytokine signaling was originally described in two separate sets of experiments. The first of these was a genetic analysis of interferon

signaling. The interferon responsive 2fTGH cell line, derived from a human fibrosarcoma, was mutagenized and cell lines were obtained that were unresponsive to interferons (7-9). Genomic DNA libraries were transfected into these lines and clones that rescued interferon (IFN) responsiveness were identified. The sequences of clones that rescued interferon alpha responsiveness were identical to the Tyk2 tyrosine kinase (10). Another cell line (U4A) that is unresponsive to interferon alpha and gamma was found to be deficient in Jak1. The interferon responsiveness of this cell line was restored by transfection of Jak1 (11). A third cell line (gamma1A), unresponsive to interferon gamma, was rescued by expression of Jak2 (12). These experiments are summarized in Table 1. In a set of separate experiments, it was shown that stimulation of certain cells with growth hormone or erythropoietin results in Jak2 activation (13, 14). In the case of the erythropoietin receptor, a constitutive association between Jak2 and a membrane proximal region of the receptor was identified (14). Subsequent work has identified Janus kinase associations with a variety of cytokine receptors (see below).

Characteristics of Jaks

Janus kinases contain approximately 1150 amino acids with apparent molecular masses between 120-140 kDa. Jak1, Jak2, and Tyk2 are expressed ubiquitously, whereas Jak3 expression originally appeared to be restricted to hematopoietic cells (15). Additional characterization has shown that Jak3 expression can be induced by cytokine treatment in a variety of non-hematopoietic tissues, such as vascular endothelial and vascular smooth muscle cells (16). Splice variants of Jak3 have been identified that differ in the their carboxy termini, one of which lacks tyrosine kinase activity (17), however, the function of these splice variants is unknown. Chromosomal locations of the Janus kinases are summarized in Table 2.

Jak proteins have been divided into seven domains, JH1 through JH7, based on sequence homology between the Jak kinases (2, 18) (Figure 1). With the exception of the kinase domain, JH1, the precise functions of the other domains are not known, nor is it clear that these are modular domains. The carboxyl terminus of Jaks contain the kinase domain, JH1, and the JH2 region, a pseudokinase domain that lacks kinase activity. The kinase domains do not confer cell-type or pathway specificity. Specifically, the kinase domains can be interchanged between the Janus kinases without an apparent change in function. For example, the Jak2 kinase and pseudokinase domains can replace those of Jak1 in interferon alpha and interferon gamma pathways (19) and other Jak kinase domains are able to substitute for Jak2 in interferon gamma signaling (20). A yeast two-hybrid screen detected direct binding of the JH2 region of Jak1, 2, and 3 to STAT5a and STAT5b, known substrates of the Janus kinases (21). This suggests a role for the pseudokinase domain in substrate binding. Whereas the kinase domains of the Janus kinases appear to be interchangeable, the domains JH3-JH7 are implicated in specific receptor interactions (19, 22-25). For example, IL-2 gamma common receptor binding and signaling can be transfered from Jak 3 to a chimeric Jak2 that contains the amino terminal domains of Jak3 (23).

Mechanism of Activation

The Janus kinases have been found to associate with a variety of cytokine receptors. This includes the interleukin and interferon receptors that lack intrinsic kinase activity, growth factor receptor tyrosine kinases, as well as GTP-coupled receptors. The intracellular portions of the cytokine receptors mediate binding to specific Jaks. Following ligand binding, receptors form homo- or hetero-oligomers allowing the associated Janus kinases to cross-phosphorylate and become activated. Tyrosine

phosphorylation of the receptors and the Janus kinases creates the necessary docking sites for downstream signaling proteins.

In general, tyrosine phosphorylation of Jaks has correlated with activation, suggesting an important role for tyrosine phosphorylation in regulation of Janus kinase activity. However, there are significant differences in the regulation of catalytic activity between the Jaks. Mutation of tyrosine 1007 to phenylalanine in Jak2 abolished kinase activity and the ability to transmit erythropoietin receptor signals, whereas mutation of tyrosine 1008 had no effect (26). Mutations of tyrosine residue 980 of Jak3 that corresponds to tyrosine 1007 of Jak2, inhibits kinase activity while mutation of tyrosine 981 augments activity (27). In contrast, mutation of the corresponding tyrosine residues in Tyk2, amino acids 1054 and 1055, prevented ligand-dependent activation of Tyk2 without abolishing its catalytic activity (28). This mutant was capable of transmitting an interferon alpha response, albeit at reduced levels (28). Additionally, a K930R mutation in the ATP binding site of Tyk2 generated a kinase-deficient protein, which was phosphorylated upon interferon-alpha treatment (28). This data demonstrates that Janus kinases can be phosphorylated without activation. It also demonstrates that Jaks can be phosphorylated by other kinases, including other Janus kinase family members.

The pseudokinase domain, JH2, has also been shown to regulate Janus kinase activity. Most notable is a gain of function mutation in the JH2 domain in a Drosophila homologue of Jak2 (29). Generation of the corresponding glutamic acid to lysine mutation at amino acid 695 in murine Jak2 resulted in increased autophosphorylation and increased activation of STAT5 in Cos cells (29). However, removal of the JH2 domain from Tyk2 abolished kinase activity and the ability to transmit interferon alpha/beta signals (30). Further, JH2 domain mutations that inactivate Jak3 have

been identified as the cause of severe combined immunodeficiency (SCID) in several patients (31).

Consistent with a role for tyrosine phosphorylation in the regulation of Janus kinase activity, tyrosine phosphatases have been shown to regulate Jaks. One of the best examples is the murine tyrosine phosphatase encoded by the murine moth-eaten locus. The moth-eaten phenotype results from a deletion or mutation in the tyrosine phosphatase SHPTP1 (SH2 domain-containing protein tyrosine phosphatase), previously called PTP1, PTP1c, HCP, or SHP (32, 33). Homozygous moth-eaten mice die at three weeks of age with an accumulation of macrophages and granulocytes in the lungs (33). There is an excess proliferation of both lymphoid and myeloid progenitors with hypersensitivity of erythroid progenitors to erythropoietin (34, 35). SHPTP-1 has been shown to bind to a distinct tyrosine residue in the IL-3 receptor beta chain and erythropoietin receptor through its amino terminal SH2 domain (36, 37). This interaction results in dephosphorylation and inactivation of Jak2 (36, 38). Interestingly, an erythropoietin receptor mutant lacking this tyrosine phosphorylation site is the molecular basis of an autosomal dominant benign erythrocytosis (39). In macrophages from moth-eaten mice Jak1 also had sustained and increased activation in response to IFN alpha/beta, whereas Tyk2 activity was largely unaffected (40). In contrast to SHPTP-1, SHPTP-2 appears to positively regulate Jak signaling. Overexpression of SHPTP-2, also called SHP-2, SYP, and PTP1D, enhances prolactin and IL-2 signaling, whereas a dominant negative mutant inhibits signaling (41, 42). However, there may be receptor type specificity in that mutation of the SHPTP-2 binding site of gp130 enhanced signaling from a G-CSF/gp130 chimera (43).

As might be expected for any signal transduction cascade, negative regulators of the Jak/STAT signaling pathway have recently been identified. These include a family of proteins named SOCS, suppressor of cytokine signaling. SOCS proteins are also termed SSI-1, STAT inducible STAT inhibitor, and JAB, Jak binding protein. Included in this family is CIS, a cytokine inducible SH2 protein that was characterized as an immediate early cytokine responsive gene with structural homology to the SOCS proteins (44, 45). SOCS proteins have been cloned by a variety of methods. Starr, et al., infected a murine myeloid leukemia cell line with a cDNA library and cloned SOCS-1 by virtue of it's ability to inhibit IL-6 induced macrophage differentiation (46). The same protein was also cloned by a yeast two hybrid screen with the Jak2 JH1 domain and called JAB (47). Lastly, Naka, et. al., screened a murine thymus cDNA expression library with an antibody recognizing the SH2 domain of STAT3 and cloned SSI-1, which is homologous to JAB and SOCS-1 (48). Search of expressed sequence tagged databases resulted in the cloning of SOCS-2 and SOCS-3 (46). There are now at least eight members of this family.

Structurally the SOCS proteins have an amino terminal region of variable length, a central SH2 domain, and a conserved carboxy terminal motif called the SOCS box (49). The SOCS proteins are part of a classical negative feedback loop that regulates cytokine signal transduction. Transcription of the SOCS genes occurs in response to various cytokines and inhibits cytokine signaling. SOCS-1 inhibits signal transduction by binding to and inhibiting the kinase activity of Jak family members while CIS appears to act by competing with signaling molecules, such as STAT proteins, for binding to phosphorylated intracellular receptor domains (44, 46-48).

Associations

Janus kinases have been shown to associate with all cytokine receptors and each of the cytokine receptors associate with and activate distinct patterns of Jaks as summarized in Table 3. In some cases, more than one Janus kinase is associated with a specific cytokine receptor. Additionally, there are examples of interdependence of Janus kinases for activation of cytokine induced signaling events.

As previously noted, a role for Jak family members in cytokine signaling was initially identified through genetic analysis of interferon signaling. This data demonstrated that Jak2 is required for an interferon gamma response and Tyk2 is required for an interferon alpha/beta response. However, a cell line generated in those experiments that was unable to respond to interferon alpha/beta or gamma was found to express a truncated Jak1 transcript (11). Responsiveness to either interferon can be restored by expression of Jak1 (11). Thus, interferon alpha requires both Jak1 and Tyk2 while interferon gamma requires Jak1 and Jak2. In wild type cells, interferon gamma stimulation results in tyrosine phosphorylation of Jak1 and Jak2. However, in cell lines lacking either Jak1 or Jak2, phosphorylation of the remaining Jak kinase is not observed. Restoring the appropriate Jak results in the tyrosine phosphorylation of both Jak 1 and 2 in response to interferon gamma (11). Thus, both kinases must be present for signaling activation to occur.

A slightly different situation exists in IL-6 signaling. Following IL-6 stimulation Jak1, Jak2, and Tyk2 are activated (50, 51). However, the absence of any single Jak does not interfere with activation of the others (52). Although Jak2 and Tyk2 are tyrosine phosphorylated in the absence of Jak1, IL-6 induced signaling events, including phosphorylation of gp130 and activation of STAT1 and STAT3, are

greatly reduced (52). Thus, Jak1 appears to be the critical Janus kinase required for IL-6 signaling.

Mechanism of Association Between Jaks and Cytokine Receptors

Several general statements can be made about the interactions of Janus kinases with cytokine receptors. Jaks are frequently constitutively associated with receptors, but in several receptor systems, ligand binding may recruit Jaks to the receptor. Typically, the JH6 and 7 domains of the Jaks are responsible for mediating an interaction with the receptors. The JH6 and 7 domains bind to conserved membrane proximal cytokine receptor sequences that include box 1 and box 2 domains. However, in receptors that lack these domains, distal receptor sequences interact with the Jaks. Some of these distal sequences contain proline rich motifs resembling box 1 sequences.

As an example of the above statement, cytokine receptors that homodimerize (GH, PRL, EPO, and TPO) and cytokine receptors that use the beta common chain (IL-3, IL-5, and GM-CSF) have been shown to interact with and activate Jak2 (53). Amino acids 1-294 of Jak2, which includes the JH6 and 7 domains, mediate binding to the membrane proximal regions of the beta common chain and growth hormone receptors (24, 25). The interleukin-2 receptor family (IL-2, IL-4, IL-7, IL-9, and IL-15) are composed of ligand specific alpha and/or beta chains and a gamma common signaling chain. Jak3 has been shown to specifically associate with the box 1 gamma common chain, while Jak1 associates with carboxy terminus serine rich sequences of the alpha or beta subunits (54, 55). The IL-6 family receptors (IL-6, IL-11, ciliary neurotrophic factor (CNTF), oncostatin M (OSM), and leukemia inhibitory factor (LIF)) that signal through gp130 also utilize the membrane proximal box 1-box 2 region for Jak binding (56, 57).

In the interferon family, Jak1 binds to the interferon gamma receptor R1 chain while Jak2 binds to the R2 chain (58). Residues 266-268 (LPKS) of the R1 chain are critical for constitutive association and activation of Jak1 (59). R2 chain sequences mediating binding to Jak2 include a proline rich set of amino acids, 263-267 (PPSIP), and additional sequences at residues 270-274 (IEEYL) (60). These sequences are reminiscent of box 1 and box 2 domains. In the case of interferon alpha, Jak1 binds to the R2 chain and Tyk2 associates with the R1 chain (61). The R2 subunit of the alpha interferon receptor binds to Jak1 through its carboxyl termini cytoplasmic domain, although a specific binding sequence has not been identified (62). Tyk2 binding to the R1 chain is mediated by amino acids 479-511 and an upstream proline containing region similar to box1 sequences (63). Between residues 479 and 511 are a number of hydrophobic and acidic residues that resemble box 2 sequences and are required for Tyk2 binding (63).

Jak Knockouts

The mutant cell lines described above demonstrated an essential role for Janus kinases in interferon signaling. Subsequent studies demonstrated that multiple cytokines activate the various Jaks. More recent studies have characterized the necessity of the Jaks in these signaling pathways using gene targeting studies or naturally occurring mutations. The phenotypes of the Janus kinase null mice are summarized in Table 4.

Jak1

Jak1 null embryos develop normally, however, newborns weigh 40% less than wild type, fail to nurse, and die within 24 hours of birth (64). The only apparent pathologic finding is an atrophic thymus with a deficiency in cell numbers, but no clear defect in the types of cells produced. Thus, Jak 1 deficiency results in a defect in thymocyte

production rather than a block in thymocyte maturation. However, Jak1 deficiency leads to a developmental blockade of B lymphocyte differentiation at the pro-B to pre-B transition. No alteration of development of other hematopoietic lineages was observed (64).

Analysis of the Jak1 deficient mice showed that Jak 1 has a critical role in mediating biological responses to three major cytokine receptor subfamilies: class II cytokine receptors (IFN alpha, beta, gamma and IL-10), cytokine receptors that utilize the gamma common chain receptor subunit (IL-2, IL-4, and IL-7), and receptors that utilize the gp130 subunit (IL-6, CNTF, OSM, CT-1, and LIF) (64). Since IL-7 is known to act early and specifically in lymphocyte development to promote lymphocyte survival (65), the lymphocyte deficiencies in the Jak null mice were linked to the absence of IL-7 receptor function. Proliferation in response to IL-2 and IL-4, whose receptors also utilize the gamma common chain, was defective in Jak null thymocytes.

Similar to data observed in mutant cell lines, Jak1 was required for both signaling and biologic responses to interferon alpha and gamma (64). Specifically, embryonic fibroblasts from Jak 1 null mice did not enhance MHC class 1 expression after exposure to interferon alpha or gamma nor did interferon treatment of Jak1 deficient fibroblasts result in protection from viral infection. These defects were complemented by the addition of Jak 1 to the deficient cells. Additionally, macrophage responses to the third member of the class II cytokine receptor family, IL-10, were defective in Jak1 null cells.

Both biological and signaling responses to cytokines that signal through the common chain gp130, such as IL-6 or LIF, were significantly reduced in Jak1 null cells (64).

However, distinct from Jak 1 null mice, gp130 deficient mice have a malformation of the ventricular walls of the heart (66) This data suggests that Jak1 is not required for all gp130 functions. In contrast, similarities between Jak1 null mice and mice lacking the LIF receptor beta subunit (67, 68), which also signals through gp130, suggest that Jak1 is the major signaling component required by LIFR-beta. As expected, Jak1 deficient macrophages showed normal responses following cellular stimulation with cytokines whose receptors specifically activate Jak2, namely EPO, GM-CSF, or IL-3. Although cell line data had suggested that G-CSF receptor function was lost in the absence of Jak1, colony forming assays from Jak1 null mice demonstrated that Jak1 is not required for responsiveness to G-CSF (64).

Jak2

Homozygous Jak2 deficiency results in embryonic lethality at approximately day 12.5 with mice lacking definitive erythropoiesis (69, 70). Analysis of fetal liver myeloid progenitors showed that Jak2 is essential for response to cytokine receptors that use the beta common chain (GM-CSF, IL-5, and IL-3) along with erythropoietin and thrombopoietin receptors. Responses to receptor tyrosine kinases such as CSF-1 and c-kit receptor were present but reduced.

Jak 2 deficient fibroblasts were unable to respond to IFN gamma as assessed by tyrosine phosphorylation of Jak1 or STAT1, induction of IRF-1 or SOCS-1, or by protecting against cytopathic effects of encephalomyocarditis virus (69, 70). However, fibroblasts were able to respond to interferon alpha and beta. Jak2 deficient cells responded normally to IL-6 and LIF, cytokines that utilize gp130 (69, 70). Although cell line data indicated activation of Jak2 in response to G-CSF, this was not confirmed in Jak2 deficient fetal liver cells (70).

Progenitors for the erythroid lineage are present in Jak2 deficient mice as indicated by the expression of lineage committed gene expression and by the ability to partially rescue erythroid lineage cells by infection with a retrovirus expressing Jak2 (69, 70). Therefore, Jak2 is not required for erythroid progenitor development, but is required for definitive erythropoiesis. Although the Jak2 deficient mice are comparable to erythropoietin and erythropoietin receptor null mice (71), Jak2 null embryos contain fewer circulating primitive erythrocytes and almost no benzidine positive erythroid colonies in BFU-E and CFU-E assays. This indicates that erythropoietin receptor signaling is not the only defect in Jak2 deficient mice and may indicate a contribution from the thrombopoietin or stem cell factor receptor in erythrocyte development (69, 70).

Jak3

Given the relatively ubiquitous expression of Jak 1 and 2 and their associations with numerous cytokines receptors, the severity of the defects in null mice was not entirely unanticipated. In contrast, the expression of Jak 3 is more restricted and Jak3 is only known to associate with the gamma common chain. Mutation of the gamma common chain is the molecular defect in X-linked severe combined immunodeficiency, thus prompting a search for Jak3 mutations in autosomal forms of severe combined immunodeficiency (SCID). There have now been several reports of Jak3 mutations in patients with SCID, making Jak3 the first Janus kinase associated with a human disease (31, 72, 73). Most of the described mutations in Jak3 lead to a truncated protein that lacks the tyrosine kinase domain or are internal deletions and missense mutations that abolish Jak3 activation in response to IL-2 or IL-4.

Jak 3 null mice have been generated and are immunodeficient, similar to the Jak 3 mutants in humans. However, the immunodeficiency in humans and mice are somewhat different. In humans with SCID due to Jak3 or gamma common chain deficiency, there is a marked decrease in circulating mature T cells and NK cells, normal to elevated numbers of nonfunctional B cells, and marked hypoplasia of lymphoid tissues. In contrast, mice lacking Jak3 or gamma common chain have severe defects in both T and B cell development. This difference has been attributed to an essential role for IL-7 signaling through gamma common chain and Jak-3 for B cell development in the mice, but not humans.

In Jak3 null mice, T-cells mature normally in the thymus, but are markedly decreased in number with a slight increase in the number of CD4+ cells. In contrast, there are normal numbers of CD4+ T-cells in the spleens of Jak 3 deficient mice. To evaluate whether the T cell defect in Jak3 deficient mice was due to aberrant T cell development in the thymus or acquired in the periphery, two groups expressed Jak 3 under the control of the thymus specific lck promoter in Jak3 deficient animals (74, 75). These experiments demonstrated that Jak3 expression corrected virtually all defects in Jak3 null thymocytes. However, peripheral T cells in these mice acquire all of the defects of Jak3 null T cells, suggesting that continuous expression of Jak 3 is required to maintain T cell function (74, 75).

Tyk2

Tyk2 null animals have not yet been reported.

Drosophila melanogaster, Jaks, and Leukemogenesis

Jak proteins are evolutionarily conserved and Jak family members have been identified in a wide variety of species, including Drosophila melanogaster and

zebrafish. In Drosophila, a single Jak homolog has been identified, known as hopscotch (76). Loss of hopscotch function results in larval/pupal lethality, underproliferation of diploid tissues, and embryonic defects in segmentation (77). Interestingly, mutations at the hopscotch locus known as Tum-1 (tumorous-lethal) causes formation of plasmocytic tumors and overproliferation of larval blood cells reminiscent of leukemia (78-80). Hop^{t42} and $Hop^{Tum\text{-}1}$ have mutations in the JH2 and JH1 domains, respectively, resulting in a hyperactive kinase (29). Further support for constitutive activation of Janus kinases in leukemogenesis includes recent reports of TEL-JAK2 fusion proteins with constitutive Janus kinase activity as a result of a chromosomal translocation in patients with acute lymphoblastic leukemia (81, 82).

Signal Transducers and Activators of Transcription (STAT)

The STAT proteins were originally identified through studies of transcriptional activation induced by interferon. Identification of interferon inducible genes allowed the characterization of two interferon response elements, termed interferon alpha response element (ISRE) and interferon gamma activation site (GAS) (83-85). Subsequently, proteins binding to these sites were sought. Through these studies it was determined that interferon alpha induces the formation of a transcription complex termed interferon stimulated gene factor 3 (ISGF3) that binds ISRE sequences (83, 86-88). Similarly, gamma activated factor (GAF) was identified as the complex binding to GAS sequences following interferon gamma stimulation (83, 89). The transcription complex ISGF3 was analyzed and found to contain four polypeptides with relative molecular weights of 48, 84, 91, and 113 kDa (90, 91). Microsequencing of tryptic peptides allowed preparation of degenerate oligonucleotides and cloning of cDNAs for all of these proteins. p91 and p84 are alternatively spliced forms of STAT1, now called STAT1α and STAT1β (90-93). The sequence of p113 was distinct but highly homologous to STAT1, indicating the

existence of a family of STAT proteins (94). Thus, p113 was named STAT2. Following the cloning of STAT1, it was determined that GAF is a STAT1 homodimer (95) while ISGF3 is a STAT1/STAT2 heterodimer that also contains a non-STAT protein, p48 (94).

Since the original identification of STAT1 and 2, a variety of approaches were used to clone other STAT proteins, which now number six. STAT3 was cloned by purifying a protein termed acute phase reactive protein (APRF) (96). Following IL-6 stimulation, this protein is tyrosine phosphorylated and co-immunoprecipitates with gp130 and Jak1 (50). STAT3 was also cloned by low stringency hybridization with a STAT1 probe (97, 98). Similarly, STAT4 was cloned using low stringency hybridization as well as a PCR based approach (99, 100).

Studies of lactation in sheep identified a prolactin inducible DNA binding activity termed mammmary growth factor (MGF) (101). MGF was purified from mammary gland tissue of lactating sheep through it's ability to bind to a beta casein promoter (101). MGF was highly homologous to STAT family proteins and was named STAT5a. The sheep cDNA for STAT5a was used to screen a murine MC/9 and a mouse mammary tissue library (102, 103). Two highly homologous genes were identified in these studies, termed STAT5a and STAT5b (102, 103). STAT5b was independently cloned by purifying proteins that bind to the IL-6 responsive 2-macroglobulin promoter (104).

STAT6 was initially identified through efforts to identify GAS binding proteins induced by IL-4. An affinity column containing a GAS element was used to purify proteins, with subsequent microsequencing of tryptic peptides which ultimately led

to the cloning of STAT6 (105). STAT6 was independently cloned by homology searches of a database of expressed sequences (106).

In this chapter, splice variants of STAT proteins are designated as alpha and beta forms, e.g., STAT1α and STAT1β, while distinct genes are indicated as a and b, e.g., STAT5a and STAT5b. STAT proteins are activated following stimulation of cells with a variety of cytokines as summarized in Table 5.

Characteristics of STAT Proteins

The STAT proteins are approximately 750 to 850 amino acids in length with apparent molecular weights between 83 and 113 kDa. The principal size differences result from variable lengths of the carboxy termini of the STAT proteins. STAT1 and STAT4 map to the proximal region of mouse chromosome 1 (human 2q homology), STAT2 and STAT6 are located on mouse chromosome 10, and STAT3, STAT5a, and STAT5b are on mouse chromosome 11 (99, 107, 108). STAT5a and STAT5b are closely positioned on human chromosome 17 (109). The chromosomal locations and relative molecular weights of the STAT proteins are summarized in Table 6.

STAT1, STAT2, and STAT3 are ubiquitously expressed whereas STAT4, STAT5, and STAT6 have a more restricted pattern of expression (107). STAT4 is expressed in several tissues including spleen, heart, brain, peripheral blood cells, and testis (100). The highest expression of STAT5 is in the mammary gland, but transcripts can also be detected in ovary, thymus, lung, adrenal gland, kidney, spleen, muscle, liver, and myeloid cells (101, 110). Although STAT6 is expressed in most tissues, it cannot be detected in muscle and brain (105, 106). Expression of the STAT proteins can also be regulated by stimulation with various growth factors

and cytokines and may change with cellular differentiation. For example, increased levels of STAT1 and STAT2 are observed in cells following treatment with interferon alpha or gamma, with induction occuring at the transcriptional level (93, 94, 111). In contrast, STAT4 levels decrease during differentiation of myeloid and erythroid cells (99).

Structure/Function of STAT Proteins

The STAT proteins are known to be inducibly tyrosine phosphorylated following cytokine stimulation. They then form dimers and translocate to the nucleus, where they bind DNA and activate specific gene transcription. The structural features of the STAT proteins enable them to fulfill these functions (Figure 2).

The amino terminus of the STAT proteins functions as a protein-protein interaction domain. For example, amino terminal mediated interactions have been observed between STAT dimer pairs allowing for oligomerization (112-114). The amino terminus is also likely to be important for interactions with other intracellular proteins. In STAT1, amino acids 150-250 serve as the p48 binding domain (115). In particular, lysine 161 of STAT1, has been implicated in the interaction with p48 (115).

The DNA binding domain of STAT proteins is located at amino acids 300-500 (116). This domain was delineated by comparing chimeric STAT proteins with differing DNA binding specificities (116, 117). The function of this domain has been confirmed by mutation of a number of conserved residues in this region that inactivate the DNA binding activity of STAT proteins without affecting tyrosine phosphorylation or dimerization (116-118). Crystal structure of STAT proteins also confirms this regions as the DNA binding domain and demonstrates that they utilize an immunoglobulin-like fold to bind DNA as a dimer much like NF-κB (119, 120).

The most highly conserved motif of STAT proteins is the SH2 domain between amino acids 570-670. The SH2 domain mediates interactions with cytokine receptor complexes and is critical for STAT dimerization. Crystal structure of STAT1 and STAT3 dimers bound to DNA have confirmed highly specific and reciprocal interactions between the SH2 domain of one monomer and the phosphorylated carboxy terminal tyrosine of the other (119, 120). Consistent with this, mutations in the phosphotyrosine binding pocket of the SH2 domain or mutation of the carboxy terminal conserved tyrosine phosphorylation site prevent STAT dimerization (121).

The SH2 domains of the STAT proteins differ in their recognition of tyrosine phosphorylated motifs, allowing for specificity in binding to and activation by different cytokine receptor complexes. For example, interferon alpha induces the phosphorylation of STAT1 and STAT2 whereas interferon gamma induces phosphorylation of only STAT1 (95, 121, 122). The specificity for STAT activation by interferon gamma resides in the STAT SH2 domain. For example, swapping the SH2 domain from STAT1 into STAT2 allowed activation of the recombinant protein by interferon gamma while insertion of the SH2 domain of STAT2 into STAT1 prevented activation by interferon gamma (123). Similarly, exchanging the SH2 domain of STAT1 with SH2 domains of either STAT3 or STAT6 results in a STAT protein that it is unable to be activated by interferon gamma but is activated by IL-6 (STAT3 chimera) or IL-4 (STAT6 chimera) (117, 124).

The carboxy termini of all STAT proteins, distal to the SH2 domain, contain a conserved tyrosine that is a site of phosphorylation (121, 122). As noted above, phosphorylation of this tyrosine is critical for STAT dimerization. Both homo- and heterodimerization can occur depending on the binding specificity of the SH2 domain and the sequences surrounding the phosphotyrosine (53, 107, 125).

Following dimerization, STAT proteins translocate to the nucleus, although a nuclear localization sequence has not been well defined.

Distal to the conserved tyrosine phosphorylation site of STAT proteins is a non-conserved carboxy terminal transactivation domain. The variability of sequences in this transactivation domain may contribute to the specificity of STAT transcriptional activation. Deletions in the carboxy terminus abolish transcriptional activity but do not prevent tyrosine phosphorylation, dimerization, or DNA binding of STAT proteins (118, 126-129). In addition, several of the STAT proteins (STAT1α, 3, 4, and 5) also have a serine residue in this carboxy terminal domain, whose phosphorylation is important for full transcriptional activity (130-134).

Dominant Negative and Constitutively Active STAT Mutants

Several mutations have resulted in dominant negative STAT proteins. As expected, deletion of the carboxy terminal transactivation domain accomplishes this (128). Interestingly, there are several naturally occurring STAT isoforms that contain deletions of this carboxy terminal region, suggesting that these proteins may serve to regulate STAT activity (135, 136). Mutation of the carboxy terminal tyrosine phosphorylation site of STAT proteins also results in a dominant negative STAT protein (137). This mutant inhibits tyrosine phosphorylation of the endogenous STAT proteins suggesting that this mutant may fail to dissociate from activated receptors or kinases thus preventing endogenous STAT proteins from binding and becoming tyrosine phosphorylated.

A constitutively activated STAT5 mutant was identified by Onishi et al. using PCR driven random mutagenesis and a retrovirus mediated expression screening system

(138). This mutant has two amino acid substitutions, one upstream of the DNA binding domain (H299R) and the other in the transactivation domain (S711F).

STAT DNA Binding Sequences and Target Genes

All STAT proteins have been shown to recognize highly related DNA sequences. These sequences have been identified by mutagenesis of response or promoter elements, oligonucleotide competition studies, or isolation of STAT binding sites from random oligonucleotide pools (107, 139). In particular, these experiments have shown that STAT proteins bind to a semipalindromic sequence TTNNNNNAA, known as a GAS sequence. However, the affinities of STAT proteins for different GAS elements varies depending on the nucleotides in or around the GAS sequence. Thus, $TTC(N)_3GAA$ is the optimal binding site for STAT1, 3, 4, and 5 while STAT6 binds to the sequence $TTC(N)_4GAA$ with an affinity one order of magnitude higher than its affinity for $TTC(N)_3GAA$ (117, 118, 140, 141). A variant of the GAS sequence present in the c-fos promoter, termed sis inducible element (SIE), can be bound by STAT1 or STAT3 (97, 142). STAT binding sequences and their presence in a variety of target genes are summarized in Table 7.

STAT homo- or heterodimers can interact directly with non-STAT transcription factors that can result in alteration of the preferred DNA binding sequence. For example, following interferon stimulation, a STAT1/STAT2 heterodimer interacts with p48, a DNA binding protein, leading to the formation of the ISGF3 complex (115). Interaction of STAT1 and STAT2 with p48 changes the recognition sequence of the complex to an ISRE motif AGTTTCNNTTTCNC/T. Depending on the level of the p48 transcription factor, the ISGF3 complex will predominate over STAT dimers redirecting STAT complexes from their intrinsic recognition of GAS elements to bind ISRE sequences (143).

Association of STAT Proteins with Other Transcription Factors

STAT proteins have been shown to interact with other transcription factors in addition to p48. For example, several groups have shown an association between STAT1 or STAT2 and the cyclic AMP response element binding protein (CBP)/p300 following interferon stimulation (144, 145). The interaction between p300 and STAT1 is mediated by two domains of each protein. Thus, an amino terminal region of STAT1 associates with the CREB binding domain (residues 571-687) of p300 and the carboxyl terminus of STAT1 binds the E1A interaction domain of p300 (residues 1680-1891) (145).

In interferon gamma signaling, full activation of the intercellular adhesion molecule-1 gene (ICAM-1) requires binding of both STAT1 and Sp1 to adjacent promoter elements (146). STAT1 and Sp1 can be co-immunoprecipitated suggesting that these two proteins may synergistically activate the ICAM-1 gene (146). Other described interactions include an association between c-jun with a carboxy terminal truncated STAT3 (STAT3β) in a yeast two hybrid screen (147). Lastly, the glucocorticoid receptor can act as a transcriptional co-activator for STAT5 and enhance STAT5-dependent transcription (148).

Association of STAT Proteins with Receptors and Jaks

As noted above, STAT proteins require tyrosine phosphorylation and dimerization for activation. The current model of STAT activation indicates that STAT proteins are recruited through their SH2 domains to ligand activated receptor complexes where the STAT proteins become tyrosine phosphorylated. Consequently, they undergo dimerization and translocation to the nucleus where they activate transcription (Figure 3). Sequences within the intracellular domains of cytokine receptors recruit specific

STAT proteins, via the STAT SH2 domains, to the activated, tyrosine phosphorylated receptor complexes. Specific tyrosine phosphorylated motifs that mediate this interaction have been identified in a variety of cytokine receptors. For example, tyrosine 440 of the interferon gamma receptor alpha binds to STAT1 (59). Other tyrosine phosphorylated motifs that bind to STAT proteins are summarized in Table 8.

Receptors, such as those for growth hormone, G-CSF, and cytokines that utilize gamma common (IL-3, IL-5, GM-CSF), can activate STAT proteins in the absence of tyrosine residues in the receptor's intracellular domains (127, 149, 150). In these cases, STAT proteins may be recruited to the receptor complex through docking to a tyrosine phosphorylated receptor associated protein, such as a Janus kinase. As previously noted, direct binding of the JH2 domain of Jak1, 2, and 3 to STAT5a and STAT5b has been reported (21). Other proteins that mediate the interaction between receptor complexes and STAT proteins include Src family tyrosine kinases. For example, c-src and STAT proteins co-immunoprecipitate after EGF receptor stimulation (151). Further, c-src was found to be required for STAT activation and mitogenesis induced by the CSF-1 receptor (151). Lastly, STAT proteins may be recruited to receptor complexes by binding to a receptor associated STAT protein. This has been demonstrated in the case of the interferon alpha receptor alpha chain which recruits STAT2, resulting in its tyrosine phosphorylation, allowing recruitment of STAT1 to the complex (126, 152-154).

The kinases responsible for phosphorylating STAT proteins include the Janus kinases as well as the receptor tyrosine kinases (139, 155, 156). In most cases, the phosphorylation of STAT proteins by Jaks appears to be nonspecific. Rather, the

specificity lies in the ability of a given receptor complex to recruit a specific STAT protein.

STAT Knockouts

As noted, STAT proteins are activated following stimulation with a variety of cytokines. To characterize the necessity of STAT proteins in these signaling pathways, gene targeting studies were performed. The phenotypes of the STAT knockout mice are summarized in Table 9.

STAT1

STAT1 has been shown to be activated by a wide variety of receptor systems including interferon alpha (94, 122) and gamma (95, 122), IL-6 (50, 98), leukemia inhibitory factor (50, 157), oncostatin M (50, 158), growth hormone (157, 159), IL-10 (160, 161), prolactin (162), angiotensin II (163, 164), and transmembrane protein tyrosine kinase receptors for epidermal growth factor (142, 165, 166), platelet derived growth factor (167), and colony stimulating factor-1 (168). Despite this activation by a variety of cytokine receptors, STAT1 deficient mice show a relatively specific defect in responsiveness to interferon alpha and gamma (169, 170).

Expected Mendelian frequencies of STAT1 deficient mice were obtained and no gross abnormalities were observed (169, 170). In comparison to the normal phenotype of the homozygous wild type or heterozygous mice, homozygous STAT1 null mice were runted and died within 48 hours of weaning (169, 170). Autopsy findings were consistent with infection with mouse hepatitis virus (MHV), which was present in the heterozygous mothers, but is usually only significant in immunocompromised animals (171).

STAT1 null animals that were bred in MHV negative hosts and a sterile environment appeared normal, but were extremely sensitive to viral infection (169, 170). These mice were highly susceptible to infections with vesicular stomatitis virus (VSV) and Listeria monocytogenes despite normal numbers of B and T lymphocytes, monocytes, and granulocytes (169, 170). This phenotype is similar to mice lacking interferon gamma or interferon gamma receptor alpha chain that die when infected with a dose of Listeria that is sublethal in normal mice (172, 173). Although mice lacking functionally active interferon gamma receptors are able to resist VSV infection, mice lacking interferon alpha receptors are highly susceptible (174), similar to mice lacking STAT1 (169).

This general state of unresponsiveness to interferon in the STAT1 deficient animals was confirmed in a variety of additional experiments. For example, STAT1 deficient macrophages failed to induce transcription of interferon regulatory factor 1 (IRF-1), guanylate-binding protein 1 (GBP-1), and MHC class II transactivating protein (CIITA) in response to IFN alpha or gamma. In addition, STAT1 deficient macrophages did not produce nitrite in response to lipopolysaccaride plus IFN gamma or IFN alpha (169, 170).

In contrast, STAT1 deficient mice have normal responses to a variety of other cytokines tested including growth hormone, IL-10, EGF, CSF-1, and IL-6 (169, 170). Phenotypically, STAT1 null mice do not display features consistent with loss of IL-10, gp130, PDGF, EGF, or CSF-1 (66, 175-181). This indicates that STAT1 activation is irrelevant or redundant in these pathways. Functional redundancy could be provided by STAT3 which is activated by many of the same cytokines that activate STAT1. However, as STAT3 cannot restore interferon responses, this demonstrates that STAT3 alone cannot fully substitute for STAT1 (169, 170).

STAT1 deficient mice have some of the defects observed in IRF-1 deficient mice (182, 183) and CIITA deficient humans (184). Expression of these transcription factors by interferon requires STAT1 with neither gene induced by interferon treatment of cells from STAT1 deficient mice. However, IRF-1 and CIITA expression can be induced by other cytokines, such as IL-6 and deficiencies of these transcription factors results in additional abnormalities not noted in STAT1 deficient mice (182-184). This data places STAT1 upstream of these two transcription factors and demonstrates that other pathways activated by these transcription factors are also deficient in the IRF-1 and CIITA null mice.

STAT2

STAT2 null animals have not yet been reported.

STAT3

As with STAT1, STAT3 is activated in response to a variety of cytokines including the IL-6 family (IL-6, IL-11, LIF, CNTF, OSM) (50, 96, 97, 185, 186), IL-2 family members (IL-2, IL-4, IL-7, IL-15) (187, 188), interferon family members (98, 189), granulocyte-colony stimulating factor (G-CSF) (190-192), epidermal growth factor (EGF) (98, 189), and leptin (185, 193). STAT3 heterozygote deficient mice are phenotypically normal and fertile, however, no STAT3 deficient mice were obtained from crosses of heterozygotes (194). Analysis of embryos revealed relatively normal development through embryonic day 6. However, by day 6.5, STAT3 null embryos began to degrade. At day 7.0, there was no evidence of mesoderm formation and embryos were completely reabsorbed by embryonic day 7.5 (194).

When compared to the phenotype of mice deficient for gp130, LIFR, CNTFR, or EGFR, all of which are lethal, STAT3 null mice die much earlier. LIFR and CNTFR

mice die shortly after birth (67, 68, 195). gp130 mice die around embryonic day 15.5-18.5 as a result of cardiac, placental, and hematopoietic abnormalities (66). EGFR null mice die after embryonic day 11.5 in a similar genetic background as the STAT3 null mice (176, 177). Therefore, the early embryonic lethality of the STAT3 deficient mice may be due to combined loss of two or more of these receptor mediated signals or an unidentified signaling molecule that requires STAT3 activity.

To assess the role of STAT3 deficiency in T cells, Takeda et al. generated mice in which STAT3 is specifically absent from T cells (196). T cell development and numbers were normal in the thymus, spleen, and lymph nodes of these animals. However, T cells from these mice displayed severely impaired proliferative responses to IL-6. IL-6 stimulation resulted in induction of bcl-2 but did not prevent apoptosis of T cells. Thus, STAT3 appears to be required for IL-6 mediated prevention of apoptotic responses.

STAT4

STAT4 is widely expressed, with highest levels in the spleen and testis. However, STAT4 has specifically been demonstrated to be tyrosine phosphorylated after stimulation of T cells with IL-12 (197, 198). IL-12 is required for T cell independent induction of interferon gamma and development of a Th1 response (199, 200). STAT4 null mice are viable and fertile with no detectable defects in hematopoiesis (201, 202). However, all IL-12 functions tested were disrupted including the induction of interferon gamma, IL-12 induced mitogenesis, enhancement of NK cytolytic function, and Th1 differentiation (201, 202). There were no detectable changes in the composition of lymphoid populations as determined by cell surface markers. STAT4 not only seems to be required for promoting Th1 development,

but may also inhibit Th2 differentiation as STAT4 null lymphocytes have a tendency towards Th2 development even under conditions that favor Th1 differentiation.

STAT5

Like STAT1 and STAT3, STAT5 is activated in response to a variety of cytokines. Despite this, STAT5 knockouts show remarkable specificity in their phenotype.

STAT5a

STAT5a null mice appeared normal, but postpartum females were unable to produce and secrete milk (203). Mammary tissue from STAT5a deficient mice indicated incomplete mammopoiesis in which lobuloalveolar tissue was underdeveloped and did not have a secretory phenotype (203). Thus, STAT5a is mandatory for mammopoiesis and lactogenesis and implicates STAT5a as the primary target of prolactin. Interestingly, despite expression of STAT5b in mammary tissues, STAT5b was unable to compensate for the loss of STAT5a. However, the extent of STAT5b expression and phosphorylation was reduced in STAT5a deficient mammary tissue (203).

In examining a potential role for STAT5a in responses to other cytokines, bone marrow macrophages from STAT5a deficient mice had decreased proliferation and gene expression in response to granulocyte/macrophage-colony stimulating factor (GM-CSF) (204). However, the colony forming ability of hematopoietic progenitors from STAT5a deficient mice treated with GM-CSF was normal (205). Further, the number of neutrophils and monocytes in STAT5a knockout mice were normal (205). STAT5a null splenocytes exhibited markedly decreased proliferation in response to IL-2 that could be attributed to defective IL-2-induced expression of the IL-2 receptor alpha chain (IL-2R alpha) (206). This defect can be explained by the

presence of an IL-2 response element in the IL-2R alpha gene that is dependent on STAT5 proteins (207-209).

STAT5b

Normally, male mice have a significantly higher growth rate than females after 3 weeks of age. However, in two separate lines of STAT5b null mice, males grew at a rate similar to female mice (210). The STAT5b deficient male mice had liver specific gene expression that was decreased as compared to wild type male mice but comparable to wild type female levels. Further, female predominant liver gene products are increased in STAT5b deficient males (210). These findings are similar to those observed in growth hormone deficient mice and suggest that STAT5b is the major STAT protein that mediates growth hormone effects in the liver and perhaps other target organs (211, 212). Other phenotypes observed in the STAT5b deficient animals included defects in lactation, decreased ability to carry fetuses to term, decreased fat deposition, and sparse hair with decreased hair regrowth (210). The loss of fat deposition could also be an effect of functional growth hormone deficiency as growth hormone induces the differentiation of preadipocytes to adipocytes and can activate STAT5 in murine preadipocytes (213, 214). Whether these other phenotypes are due to insensitivity to growth hormone or reflect loss of responsiveness to other cytokines is unknown.

STAT5b deficient mice have a modest decrease in both thymic and splenic cellularity (215). Similar to STAT5a deficient T cells, STAT5b null T lymphocytes were deficient in a proliferative response to IL-2 (215). In contrast to STAT5a deficient mice which only have diminished IL-2R alpha expression, STAT5b null mice had a decrease in mRNA for both IL-2R alpha and beta (206). Although the IL-2R alpha gene has a STAT5 response element, no such element is present in

the IL-2R beta gene. NK responses to IL-2 and IL-15 were similarly defective, consistent with the decrease in IL-2R beta expression (215).

STAT5a/STAT5b

The STAT5a/b deficient mice were derived by double targeting of embryonic stem cells, since their genetic colocalization would not allow a purely genetic approach (205). Double homozygous deficient mice were obtained at normal Mendelian frequencies, however, approximately one-third of the mice died within 48 hours of birth (205). The reason for this has yet to be determined.

Although STAT5a and STAT5b deficient mice are fertile, female STAT5a/b null mice are infertile despite normal ovulation. Histologic examination of the ovaries show few or no large corpora lutea in comparison to wild type mice (204). This defect is likely due to an even more severe prolactin defect in the STAT5a/b null mice as compared to the STAT5a deficient animals (216, 217). In addition, the double knockouts were even smaller than STAT5b null mice (204), consistent with a complete lack of growth hormone function (218). These data suggest some redundancy of function for STAT5a and STAT5b in the prolactin and growth hormone pathways.

Despite activation of STAT5 proteins by EPO and TPO, there were no observed differences between the wild type and various STAT5 mutant mice in the red cell numbers, hemoglobin levels, hematocrits, and platelet count. However, STAT5a/b null mice had a decrease in white cell numbers (205). Colony forming assays of bone marrow cells from STAT5a/b null mice demonstrated a decreased response to IL-3, IL-5 and GM-CSF with fewer colonies and smaller colony size. Peripheral T cells from double knockout mice were unable to proliferate in response to T cell

receptor engagement and IL-2. Presumably, this relates to the IL-2 receptor abnormalities noted in the STAT5a or STATb deficient mice (206, 215).

STAT6

STAT6 is activated in response to a limited number of cytokines with the largest number of reports demonstrating activation in response to IL-4 and IL-13. Not surprisingly, STAT6 knockout mice were deficient in responses to these cytokines (219-222). Homozygous STAT6 deficient mice were viable with no gross abnormalities and normal numbers of T and B lymphocytes (219, 221, 222). The proliferation of T and B cells in response to IL-4 was diminished. However, more striking was the inability of IL-4 to induce Th2 differentiation, increase the expression of CD23 and MHCII, and induce Ig class switching of B cells to IgG1 and IgE (219, 221, 222). This is similar to the phenotype of IL-4 deficient mice (223). Consistent with a lack of induction of a Th2 response and class switching to IgE in the STAT6 deficient mice, STAT6 null mice were resistant to allergen induced airway inflammation (224, 225). In addition, responses to IL-13, including augmentation of MHC class II expression and decreases in nitric oxide production by activated macrophages, were not observed in STAT6 deficient mice (220).

Conclusion

The discovery of the Jak/STAT pathway has allowed an improved understanding of the signaling mechanisms used by cytokine receptors. Cytokine receptors associate with distinct Janus kinases that couple ligand binding to intracellular phosphorylation events. These phosphorylation events allow the recruitment and activation of specific STAT proteins that results in transcriptional activation. The critical role of Jak and STAT proteins in cytokine signaling has been confirmed through studies of mice deficient in these proteins. Despite activation of STAT proteins by multiple

cytokines, STAT deficient animals display specific defects in cytokine signaling. This implies redundancy in STAT signaling. In contrast, Jak deficient mice display defects consistent with non-redundant roles for Jaks in cytokine signaling. Additional studies will be required to more precisely define the regulation of the Jak/STAT pathway, the specific target genes of STAT proteins, and the role of this pathway in human disease.

Table 1. Jak/STAT Deficient Cell Lines

Cell Line	Deficient Protein	Defective Interferon Response	Reference
U4A	Jak1	alpha and gamma	(11)
gamma1A	Jak2	gamma	(12)
U1D	Tyk2	alpha	(10)
U3A	STAT1	alpha and gamma	(129)
U6A	STAT2	alpha	(153)

Table 2. Chromosomal Location and Molecular Weight of Jaks

Jak	Location	Molecular Weight
Jak 1	human 1p31	135 kDa
Jak 2	human 10p23-24	130 Kda
Jak 3	human 4q31	120 kDa
Tyk2	human 19p13	140 kDa

Table 3. Activation of Jaks by Hematopoietic Ligands

	Jaks			
Ligands	Jak1	Jak2	Jak3	Tyk2
Interferon family				
IFN α/β	+			+
IFN γ	+	+		
IL-10	+			+
γ Common Chain family				
IL-2	+		+	
IL-4	+	+	+	+
IL-7	+		+	
IL-9	+		+	+
IL-13	+	+		+
IL-15	+	+	+	
gp130 family				
IL-6	+	+		+
IL-11	+	+		
IL-12		+		+
CNTF	+	+		+
G-CSF	+	+		+
LIF	+	+		
OSM	+	+		+

gp140 family			
IL-3	+	+	
IL-5	+	+	
GM-CSF	+	+	
Growth Hormone family			
EPO		+	
GH		+	
PRL		+	
TPO		+	+
Receptor Tyrosine Kinases family			
CSF-1	+	+	+
EGF	+	+	
PDGF	+	+	+
SCF		+	
G-protein Coupled Receptors family			
Angiotensin II		+	+

Table 4. Jak Knockout Mice Phenotypes

Jak Null	Defective cytokine response	Phenotype
Jak1	IL-2 family (IL-2, IL-4, and IL-7), IFN family (alpha/beta, gamma, and IL-10), and gp130 family (IL-6, CNTF, OSM, CT-1, and LIF)	decreased thymocyte cell number, blocked B lymphocyte differentiation, sensitive to viral infection
Jak2	gp140 family (GM-CSF, IL-5, and IL-3), EPO, TPO, and IFN gamma	embryonic lethal, no definitive erythropoiesis
Jak3	IL-2 family (IL-2 and IL-7)	immunodeficient, defects in T and B cell development
Tyk2	not yet reported	

Table 5. Activation of STAT Proteins by Hematopoietic Ligands

	STAT					
Ligands	1	2	3	4	5	6
Interferon family						
IFN alpha/beta	+	+	+	+	+	+
IFN gamma	+				+	
IL-10	+		+		+	
Gamma Common family						
Interleukin-2 (IL-2)	+		+	+	+	
IL-4	+		+		+	+
IL-7	+		+		+	
IL-9	+		+		+	
IL-13						+
IL-15	+		+		+	
gp130 family						
IL-6	+		+		+	
IL-11	+		+			
IL-12	+		+	+		+
CNTF	+		+			
G-CSF	+		+		+	
LIF	+		+		+	
OSM	+		+			

gp140 family					
IL-3	+		+	+	+
IL-5	+		+	+	
GM-CSF	+		+	+	+
Growth Hormone family					
EPO	+		+	+	
GH	+		+	+	
PRL	+		+	+	
TPO	+		+	+	
Receptor Tyrosine Kinase family					
CSF-1	+		+		
EGF	+		+	+	
PDGF	+		+	+	+
SCF	+			+	
G-protein Coupled Receptor family					
Angiotensin II	+	+	+	+	+

Table 6. Chromosomal Location and Molecular Weight of STAT Proteins

STAT	Chromosomal Location	Molecular Weight
STAT 1α	mouse 1 (human 2q)	91 kDa
STAT 1β	mouse 1 (human 2q)	84 kDa
STAT 2	mouse 10	113 kDa
STAT 3	mouse 11	83 kDa
STAT 4	mouse 1 (human 2q)	86 kDa
STAT 5a	mouse 11 (human 17)	96 kDa
STAT 5b	mouse 11 (human 17)	94 kDa
STAT 6	mouse 10	100 kDa

Table 7. STAT Response Elements*

Gene	Response Element	STAT
	ISRE	
ISG54	AGTTTCACTTTCCC	STAT 1, 2
6-16	AGTTTCATTTTCCC	STAT 1, 2
GBP	ACTTCAGTTTCAT	STAT 1, 2
consensus	AGTTTCNNTTTCNC/T	
	Gas-like	
alpha 1 acidic glycoprotein	TTCCCAGAA	STAT 3
beta-casein	TTCTAGGAA	STAT 5
CD23	TACCTGAGAAA	STAT 6, 1
CIS	TTCCTGGAA	STAT 5
	TTCTTGGAA	
	TTCTAGGAA	
	TTCCGGGAA	
CRP	TTCCCGAA	STAT 3
FcγR1 (GRR)	TTCCCAGAA	STAT 1, 3, 5
cFos-SIE	TTCCCGTCA	STAT 1, 3
GBP	TTACTCTAA	STAT 1
ICAM-1	TTCCCGGAA	STAT 1, 3
ICSBP	TTCTCGGAA	STAT 1
IFP53	TTCTCAGAA	STAT 1
IL-2Rα	TTCTGAGAA	STAT 5
IL-4	TTCACAGGAA	STAT 6
IL-4 Receptor	TTCATCTGAA	STAT 6

IRF-1	TTCCCCGAA	STAT 1, 3, 5, 6
junB	GTCAGGAA	STAT 1, 3
Ly-6A/E	TTCCTGTAA	STAT 1
M67-SIE	TTCCCGTAA	STAT 1
alpha 2-Macroglobulin	TTCCCGTAA	STAT 3
MIG	TTACTATAA	STAT 1
Spi2.1	TTCTACTAA	STAT 5
	TTCTGAGAA	
consensus	TTNNNNNAA	

*This is not a comprehensive list.

Table 8. STAT binding of human receptor phosphotyrosine motifs.*

Receptor	STAT	Motif
IFN gammaR1	STAT 1	TSFGY$_{440}$DKPHVLV
IFN alphaR1	STAT 2	RCINY$_{466}$VFFPSLKPSS
gp130 (IL-6)	STAT 1, 3	DEGMPKSY$_{905}$LPQT and
		PQTVTQGGY$_{915}$MPQ
gp130 (IL-6)	STAT 3	TVVGSGY$_{767}$RHQVPSV and
		ILPRQQY$_{814}$FKQNCSQ
IL-2R	STAT 5	EDDAY$_{392}$CTFPSR and
		NTDAY$_{510}$LSLQ
IL-4R	STAT 6	GPPGEAGY$_{578}$KAFSSLL and
		ASSGEEGY$_{606}$KPFQDLI
IL-9R	STAT 1, 3, 5	QTLAY$_{116}$LPQE
EPOR	STAT 5	AQDTY$_{343}$LVLD and
		SFEY$_{401}$TILD
PRLR	STAT1, 3, 5	GGLDY$_{580}$LDPAC (residue 382 in short form)

*This is not a comprehensive list. Additionally, the amino acid assigment corresponds to the published tyrosine, which may not reflect genbank numbering.

Table 9. STAT knockout mice phenotypes.

STAT	Cytokines	Phenotype
STAT1	IFN alpha and gamma	sensitive to viral infection
STAT2	not yet reported	
STAT3	IL-6 in targetted T cells	embryonic lethal, no mesoderm formation
STAT4	IL-12	defective Th1 differentiation, lack of IFN gamma induction
STAT5a	PRL	defective mammopoiesis and lactogenesis
STAT5b	GH	males have female levels in sexually dimorphic traits (growth rate and liver proteins)
STAT5a/b	gp140 family (IL-3, IL-5, and GM-CSF), PRL, and GH	decreased number of lymphocytes, increased percentage of neutrophils, smaller size than STAT5b null mice, and no large corpea lutea
STAT6	IL-4 and IL-13	defective Th2 differentiation, B cell class switching, reduced nitric oxide production by macrophages, and lack of IL-13 induced MHC class II expression

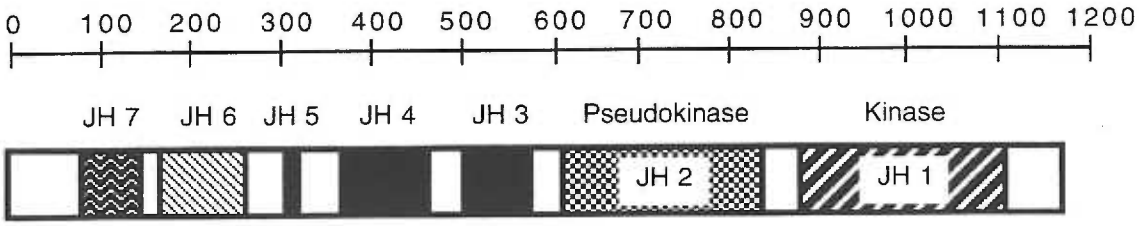

Figure 1. Domains of Jaks. As described in the text, Janus kinases consist of seven conserved Jak homology domains categorized JH 1 through JH 7. The carboxy terminus JH 1 domain is the kinase domain whereas JH 2 is a pseudokinase domain. Amino terminal domains JH 6 and JH 7 are involved in receptor interactions. Domains JH 3 through 5 do not have well defined functions. Amino acid numbering is indicated on the top of the figure.

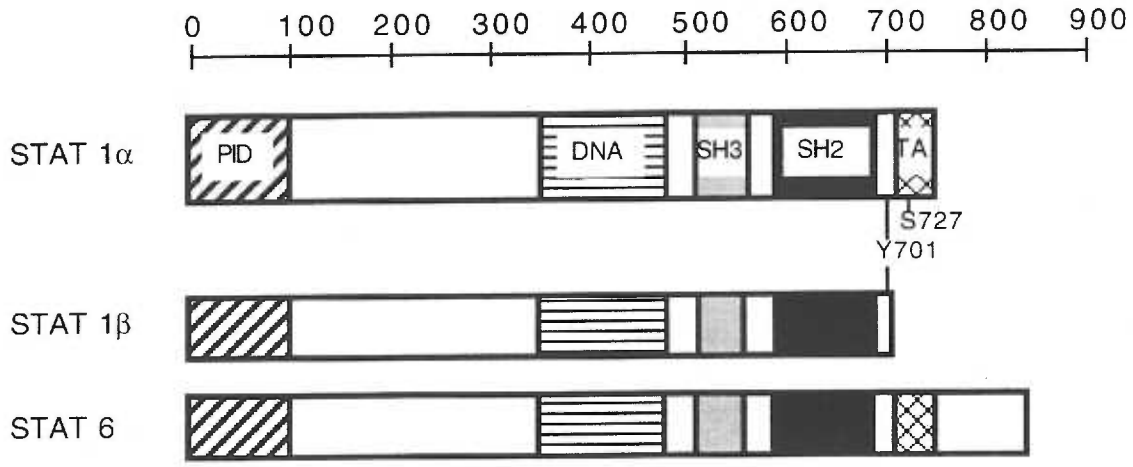

Figure 2. Domains of STAT proteins. The SH2 domain (SH2), the putative SH3 domain (SH3), DNA binding domain (DNA), and a protein interaction domain (PID) are labeled. The carboxy terminal transcriptional activation domain (TA) includes an activating serine at position 727 (S727) in various STAT proteins (STAT1α, 3, 4, and 5). The tyrosine phosphorylation site required for dimerization is at amino acid 701 (Y701). STATs 1a, 2, 3, 5a, and 5b have the same domain configuration. In comparison, STAT 4 and STAT 6 have a longer carboxyl termini. Additionally, the alternately spliced STAT variants, such as STAT1β, are truncated in their carboxy terminus. Amino acid numbering is indicated on the top of the figure.

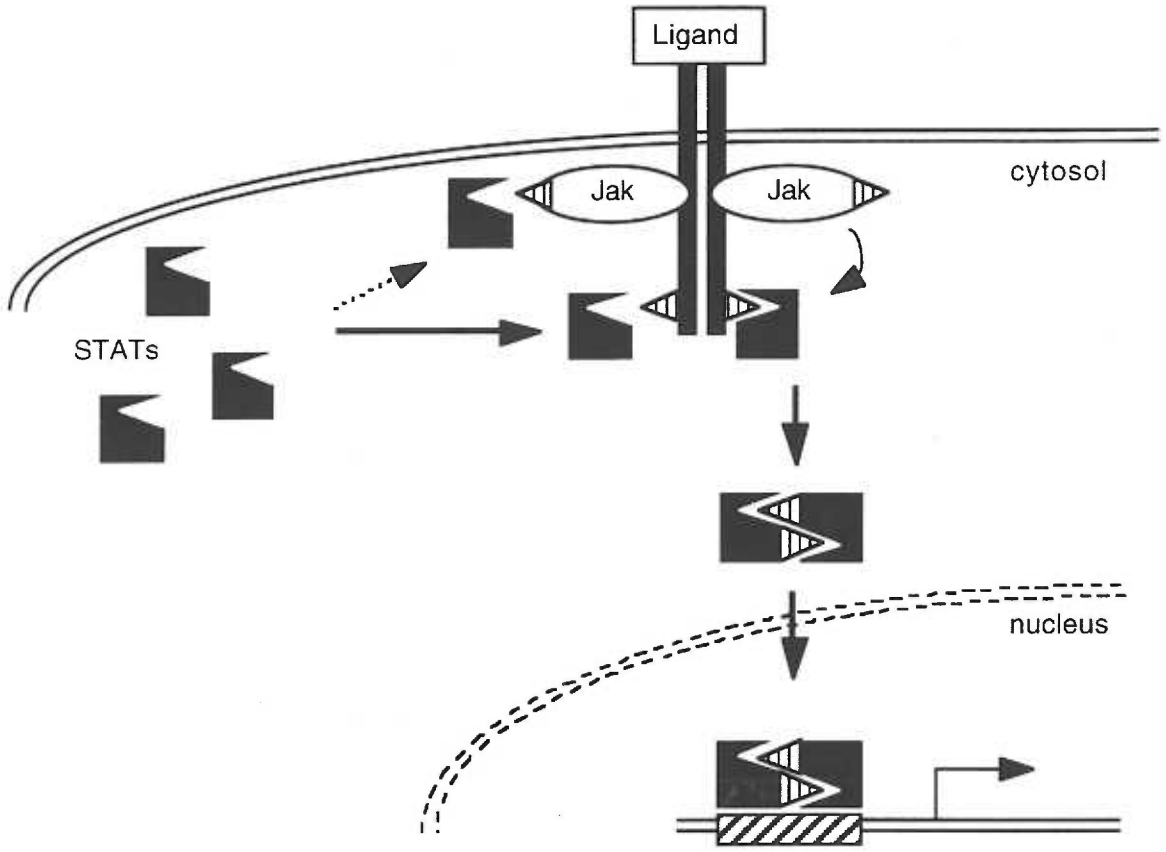

Figure 3. Diagram of Jak/STAT activation. Following ligand binding, receptors dimerize, resulting in Jak activation. Activated Jaks phosphorylate specific sites on the cytoplasmic domains of the receptors. STAT proteins are recruited to phosphotyrosines (striped triangles) on the receptor or receptor associated proteins. Activated Jaks phosphorylate the STAT proteins, which allows the STAT proteins to dimerize and translocate to the nucleus, where they bind specific promoter sequences to activate gene transcription.

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