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A NOVEL CELL SIGNALING PATHWAY IN FRIEND VIRUS INDUCED ERYTHROLEUKEMIA

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Abstract

Friend virus-induced erythroleukemia is a model for the study of the multi-stage nature of carcinogenesis. The early stage of the disease is characterized by a polyclonal expansion of infected erythroid precursor cells, and the later stage is marked by the emergence of fully transformed cells in the spleen, blood, bone marrow and liver, leading to the development of erythroleukemia. Several loci have been identified in the early stages of erythroleukemia that are required for the expansion of infected progenitor cells, in particular *W, Sl, f* and *Fv2*. *Fv2* has been shown to encode a naturally occurring, N-terminally truncated form of the Stk receptor tyrosine kinase (Sf-Stk). Previous findings have demonstrated that Sf-Stk provides signals necessary for the polycolonal expansion of infected cells. Here we propose to study the mechanism by which Sf-Stk relays signals for the expansion of Friend virus infected progenitor cells.

Previously, we demonstrated that the ability of Sf-Stk to participate in the transformation of Friend virus-infected cells requires the kinase activity and Grb2 binding site of Sf-Stk. In the first chapter, we show that Grb2 heterozygous mice exhibit decreased susceptibility to Friend erythroleukemia virus *in vivo* and that expansion of erythroid progenitors in response to infection requires the c-terminal SH3 domain of Grb2. Furthermore, Gab2 is expressed in spleens of Friend virus infected mice, co-immunoprecipiates with Sf-Stk and is tyrosine phosphorylated in the presence of Sf-Stk, which suggests that Gab2 is a downstream target of Sf-Stk. Using an *in vitro* colony assay, we find that a fusion protein, in which the docking site tyrosines in Sf-Stk are replaced by the coding sequence of Gab2, supports the growth of progenitors from mice

lacking Sf-Stk, whereas a Sf-Stk/Gab1 fusion protein does not. Finally, mice with a targeted deletion in Gab2 are less susceptible to Friend erythroleukemia and the expansion of erythroid progenitor cells in response to infection can be rescued by expression of Gab2, but not Gab1.

To further investigate the signaling events downstream of the Sf-Stk/Grb2/Gab2 complex in the development of erythroleukemia in response to Friend virus infection, in the second chapter, we demonstrate that the recruitment and activation of Stat3 by Sf-stk is mediated by Gab2. We find that the Sf-Stk/Gab2 fusion protein, but not the Sf-Stk/Gab1 fusion protein, which fails to support hematopoietic transformation in this system, results in enhanced tyrosine phosphorylation of, and interaction with, signal transducer and activator of transcription 3 (Stat3). Our in vivo studies demostrate that Stat3 is tyrosine phosphorylated and interacts with Gab2 in splenocytes following injection of sensitive Balb/CJ mice with Friend Virus. Exogenous expression of dominant negative Stat3 in sensitive erythroblasts significantly inhibits BFU-E and CFU-E colony formation induced by Friend Virus. Furthermore, bone marrow cells from floxed-Stat3 mice fail to form Epo-independent colonies in response to Friend Virus infection following the retroviral introduction of Cre-recombinase into primary erythroblasts. Moreover, the Cre-flox/stat3 mice have decreased susceptibility to Friend erythroleukemia virus *in vivo* and exogenous expression of stat3 can rescue this response. In order to map the domains of Gab2 which are critical for the activation of Stat3 and the Epo-independent growth of Friend Virus infected cells, we utilized a series of Gab1/Gab2 chimeric molecules. Our data suggest that the region between Q^{120} and P^{358} of Gab2 is required for the tyrosine phosphorylation of Stat3. By scanning this region, we identified

a Y^{194}LHQ site, a potential Stat3 binding motif, in Gab2, but not in Gab1. The ability of Sf-Stk /Gab2 to recruit and activate Stat3, and support Epo-independent colony formation of primary erythroblasts in response to Friend Virus infection in vitro was abolished when Y^{194} was mutated to phenylalanine. In addition, mutation of this tyrosine in the context of wide type Gab2 abrogated its ability to rescue the defective response of Gab2-/- bone marrow to Friend Virus.

In the third chapter, we investigate the function of Src kinases in the course of Friend virus infection. Previous research demonstrated that v-Src causes erythroleukemia in mice. Furthermore, growth factors can induce Stat3 in a Src family kinase (SFK)-dependent manner. Here we show that dominant negative c-Src significantly inhibits BFU-E and CFU-E colony formation induced by Friend Virus. In addition, the non-specific SFK inhibitor PP1 also abrogated Epo-independent colony formation. Moreover we found that the inhibition of Src family kinases by PP1 suppresses Stat3 tyrosine phosphorylation downstream of Sf-Stk. Taken together, these data suggest that c-Src is downstream of Sf-Stk in Friend virus infected cells and that the ability of c-Src to promote Stat3 tyrosine phosphorylation in response to Friend virus is critical in the early stages of erythroblast transformation.

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List of Abbreviations

AGM	Aorta-Gonad-Mesonephros
BFU-E	Burst Forming Unit-Erythroid
BSA	Bovine Serum Albumin
c-kit	kit receptor tyrosine kinase
CFU	Colony Forming Units
CFU-E	Colony Forming Unit-Erythroid
CFU-EMB	CFU-Erythroid, Myeloid, Basophilic
CFU-GEMM	Colony Forming Unit-Granulocyte, Esonophil, Megakaryocyte, Monocyte
CLP	Common Lymphoid Progenitor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EGFP	Enhanced Green Fluorescent Protein
Epo	Erythroprotein
Epo-R	Erythroprotein-Receptor
Erk	Extracellular response kinase
FACS	Flourescence Activated Cell Sorter
FBS	Fetal Bovine Serum
F-MuLV	Friend Murine Leukemia Virus
FV	Friend Virus
FVA	Friend Virus Anemia

FVP	Friend Virus Polycythemia
Gab	Grb Associated Binding
gp	glycoprotein
Grb	Growth factor Binding protein
HSC	Hematopoietic Stem Cell
IFN	Interferon
IL	Interleukin
JAK	Janus Family of Kinase
JNK	c-Jun N-terminal Kinase
LT-HSC	Long-Term Hematopoietic Stem Cell
MAPK	Mitogen Activated Protein Kinase
MBD	Met Binding Domain
M-CSF	Macrophage-Colony Stimulating Factor
MSP	Macrophage Stimulating Protein
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-3-Kinase
PMSF	Phenyl-Methylsulfonyl Fluride
PTB	Phosphotyrosine-Binding
RBC	Red Blood Cell
RBD	Raf Binding Domain
RON	Receptor d’origine Nantais
RT	Reverse Transcriptase

RTK	Receptor Tyrosine Kinase
SCF	Stem Cell Factor
SDS	Sodium Dodecyl Sulfate
SEA	Sarcoma, Erythroblastosis, Anemia
SFFV	Spleen Focus Forming Virus
SF-STK	Short Form Stem cell-derived Tyrosine Kinase
SH	Src-Homology
SH-HSC	Short-Term Hematopoietic Stem Cell
SHIP	Src Homology Phosphatase
SOCS	Suppressors of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
STK	Stem cell-derived Tyrosine Kinase

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Chapter 1

General Introduction

1.1 Objective

This research will enrich our knowledge of the signals that govern normal erythroblast proliferation, differentiation and homeostasis, and potentially provide information aimed at identifying pharmacological targets for the regulation of the early events in leukemic transformation.

1.2 Background

1.2.1 Hematopoiesis.

The production of all types of blood cells, generated by a remarkable self-regulated system, is known as hematopoiesis. Prenatally, hematopoiesis occurs in the yolk Sac, then liver, and eventually the bone marrow. In normal adults it occurs in bone marrow and lymphatic tissues.

Hematopoietic stem cells (HSCs), first isolated in 1981, are multipotent cells originally formed during embryogenesis in a complex developmental process which involves several anatomical sites (the yolk sac, the aorta-gonad-mesonephros region, the placenta and the fetal liver), after which HSCs colonize the bone marrow at birth. During postnatal life, a steady state is established in which the HSC pool size is maintained by the regulation of HSC self-renewal and differentiation (80).HSCs cells are distinguished by their capacity to self-renew *in vivo* and differentiate into all of the mature hematopoietic cell lineages (1).

There are at least two classes of HSCs—long-term (LT-HSCs) and short-term reconstitutive cells (ST-HSCs). The long-term subset self-renews for the life of the host, and a single LT-HSC is both necessary and sufficient for life-long sustenance of the entire hematopoietic system. LT-HSC's can be divided into two groups, the adult HSCs which originate in the aorta-gonad-mesonephros (AGM) and the embryonic HSCs which originate in the yolk sac during development (79). On the other hand, the short-term subset of HSCs retains self-renewal capacity only for approximately 8 weeks (56, 102, 133). ST-HSCs are relatively immature cells that are capable of proliferating and differentiating, but have a more limited self-renewal capacity.

Definitive HSCs move from the AGM region to the fetal liver, where the multipotent HSCs finally populate the bone marrow. The bone marrow has a specific three- dimensional structure which facilitates the early commitment of HSCs. Stromal cells within the bone marrow provide a microenvironment conducive to the self-renewal, proliferation and differentiation of HSCs. The microvascular endothelium not only works to control the trafficking of HSCs, but also provides cell-cell contact signals and the secretion of cytokines and growth factors which are critical for steady state hematopoiesis (102). Although HSCs are present in low numbers in the bone marrow, and few of them are actively recycling, they can differentiation into all types of blood cells, depending on both stochastic and environmental conditions.

The multipotent stem cells divide more frequently into either more multipotent cells with self-renewing ability, or committed progenitor cells, which can differentiate into mature blood cells. Commitment is not a jump from the multipotent state to mature blood cells; instead, it is a stepwise process. The first step is commitment to either a

lymphoid cell or common myeloid progenitor cell (Figure 1.1). In the second step, the lymphoid progenitor cell gives rise to T or B lymphocytes responsible for the adaptive immune response, while the common myeloid progenitor cell gives rise to different classes of leukocytes, erythrocytes and megakaryocytes.

Both T and B lymphocytes are involved in the regulation of the immune response. However, they are distinguished by their sites of differentiation: T lymphocytes in the thymus and B lymphocytes in the bone marrow, and by their antigen receptors. Mature lymphocytes circulate in the blood and peripheral lymphoid tissues. When they encounter antigens, both the B and T cells will differentiate into effector cells: B cells into plasma cells, which have antibody secreting function, and T cells into activated CD4+ helper or CD8+ cytotoxic T cells. The third lineage of lymphoid-like cells, the natural killer cells, come from the same progenitor cell but have no antigen-specificity which is a hallmark of the adaptive immune response. Nature killer cells are critical in innate immunity, and also important in the early immune response to virus infection and intracellular pathogens.

Common myeloid progenitor cells develop into granulocyte/macrophage progenitors and erythrocyte/megakaryocyte progenitors in the bone marrow. The leukocytes that derive from the granulocyte/macrophage progenitor include monocytes, dendritic cells, basophils, eosinophils and neutrophils. The latter three have cytoplasmic granules whose characteristic staining gives them a specific appearance (hence the name granulocytes) and they all have irregular shaped nuclei (hence the name polymorphonuclear leukocytes). Basophils and eosinophils are involved in the immune response to parasitic infection by being recruited to sites of allergic inflammation. Immature dendritic cells migrate to peripheral tissues where they become mature

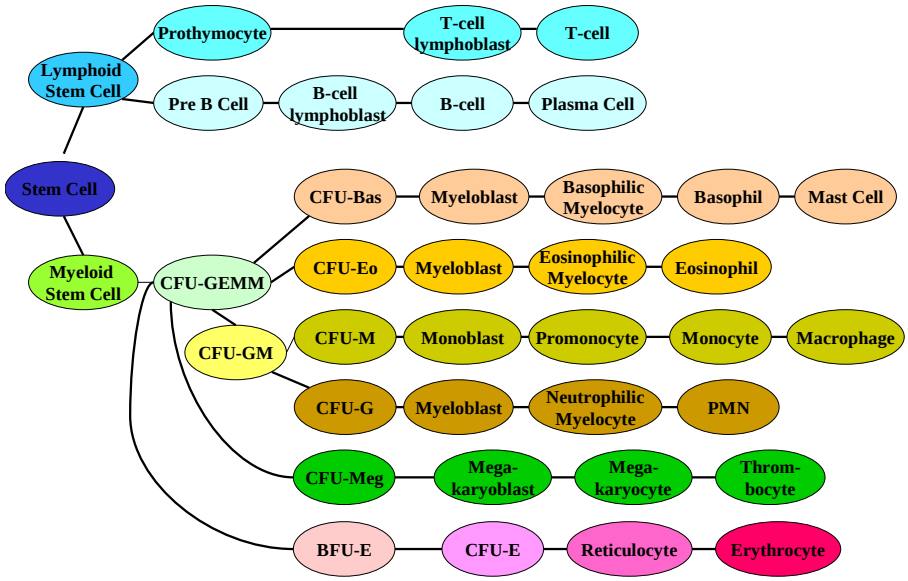

Figure 1.1. Normal Hematopoiesis. The multipotent stem cells committed into either lymphoid stem cells or myeloid progenitor cells. Then the lymphoid progenitor cell gives rise to T or B lymphocytes, while the common myeloid progenitor cell gives rise to different classes of leukocytes, erythrocytes and megakaryocytes. CFU-GEMM: colony forming unit-granulocyte, esonophil, megakaryocyte, monocyte.

dendritic cells. They then migrate to lymphoid tissues to activate antigen specific T lymphocytes following antigen ingestion. Monocytes enter tissues to differentiate into macrophages, which are the main tissue-resident phagocytic cells in the innate immune system. Mast cells complete their differentiation in tissues and are involved in the allergic response. On the other hand, erythrocyte/megakaryocyte progenitors differentiate into megakaryocytes and erythroblasts in the bone marrow. Platelets arise from megakaryocyte progenitors and are critical in blood clotting; while erythrocytes, which develop from erythrocyte progenitors, are responsible for transporting oxygen to tissues and bringing back carbon dioxide (87).

1.2.2 Erythropoiesis.

Erythropoiesis is the process by which mature erythrocytes are produced. In human adults, this usually occurs within the bone marrow. In the early fetus, erythropoiesis takes place in the mesodermal cells of the yolk sac. By the third or fourth month, erythropoiesis moves to the spleen and liver.

Cell differentiation along the erythroid lineage occurs over a two week span in humans. The earliest erythroid progenitor, the burst forming unit-erythroid (BFU-E), is small and without distinguishing histologic characteristics. The BFU-E has been detected in mouse bone marrow, spleen and peripheral blood as well as in human bone marrow and peripheral blood. It takes about eight days for BFU-E to differentiate into mature erythrocytes in methylcellulose media supplied with erythroid specific cytokins (33, 34). Epo receptor expression on BFU-E is low.

The stage of progenitor after the BFU-E is the colony forming unit-erythroid (CFU-E), which is a larger cell and is the stage right before hemoglobin production

begins. CFU-E take two days to differentiate into mature erythrocytes *in vitro* (33). CFU-Es do not contain hemoglobin and depend on Epo for their survival and proliferation.

The CFU-E differentiates further through a series of sequential events from a large basophilic normoblast to a polychromatophilic normoblast, at which stage most cells remain in G0/G1 of the cell cycle, to give rise to mature erythrocytes (Figure 1.2). Reticulocytes are polychromatophilic normoblasts without a nucleus, and they synthesize hemoglobin. Once they enter the peripheral blood, reticulocytes lose their polyribosomes and develop into mature erythrocytes, which are packed with hemoglobin and contain practically none of the usual cell organelles. The erythrocytes can not divide; the only way to make more erythrocytes is by means of erythropoiesis.

The serial events in the maturation of erythrocytes depend on a number of growth factors and cytokins, among which, erythroprotein (Epo) is the most import one. Furthermore, SCF, GM-CSF and IL-3 are all required in the process of proliferation.

1.2.3 Friend virus induced erythroleukemia.

Erythroleukemia is a form of acute myeloid leukemia in which the body produces a large number of abnormal, immature red blood cells. However, the specific signals responsible for the progression of this disease are unknown. Friend virus-induced erythroleukemia is a good model for the study of the multi-stage nature of carcinogenesis, and is characterized by polyclonal proliferation of nonleukemic erythroid progenitor cells followed by a later stage in which there is clonal or oligoclonal expansion of malignant cells (9).

In 1957, Charlotte Friend described a novel retrovirus, which she named Friend virus, derived from passing Ehrlich ascites cells from newborn Swiss mice. Since that

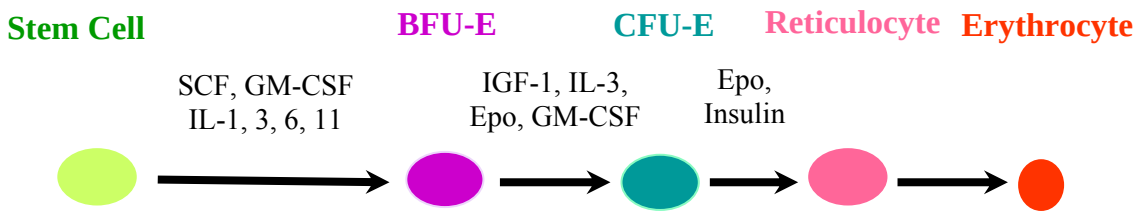

Figure 1.2 Erythrocyte Formation. The development of multipotent Hematopoietic Stem Cell into mature erythrocyte is a stepwise process, which dependent on specific cytokines and growth factors. BFU-E: Erythroid Burst Froming Unit, CFU-E: Erythroid-Colony Forming Unit.

time, the leukemia induced by Friend virus has become an important model of the multistage nature of carcinogenesis and the contribution of the host genotype and stage of differentiation of the target cells to the oncogenic process. The virus causes rapidly progressive disease in susceptible mice, which is characterized by splenic enlargement, erythroleukemia, and death.

Friend virus is a complex of two viruses, spleen focus forming virus (SFFV), a replication defective virus which is responsible for the acute pathogenicity of the complex, and the replication competent helper virus, Friend murine leukemia virus (F-MuLV), which causes no disease itself in adult mice but works as a helper for the replication of the defective SFFV (122, 126). F-MuLV, the nondefective mouse retrovirus, which lacks oncogenes, has a genome that consists of a single-stranded RNA molecule that encodes three polyproteins, *gag, pol,* and *env.* The *gag, pol,* and *env* genes are flanked by long terminal repeats (LTRs), each of which are about 600 nucleotides long and composed of three functional segments, U3, R, and U5. In target cells, the proviral DNA randomly integrates in the vicinity of cellular proto-oncogenes, perturbing the structure or expression of those genes (52). F-MuLV itself does induce splenomegaly, severe anemia, and erythroleukemia when inoculated into newborn BALB/c or NIH/Swiss mice (9). On the other hand, SFFV is responsible for the erythroblastosis and the proliferation of erythroid precursors, characteristic of the first stage of Friend disease. Unlike other replication defective retroviruses that induce cancer, the erythroleukemia caused by the Friend virus complex is distinctive due to the lack of an oncogene encoded by SFFV. Once SFFV was molecularly cloned, it was shown to be a relative of F-MuLV. SFFV leads to not only erythroid hyperplasia, but also transformation of erythroid

precursor cells by integrating into specific cellular genes, resulting in their activation (108). Studies from previous research demonstrated that helper-free preparations of SFFV can cause the initial erythroproliferation and also the second phase of leukemic immortalization of erythroblasts during Friend disease, albeit at a lower efficiency (121).

There are two variant strains of SFFV: SFFV-A (FVA) and SFFV-P (FVP). Mice infected with viral FVP become polycythemic, while mice infected with FVA suffer anemia. However, these mice are not truly anemic, with an increase in erythrocyte production but decreased hematocrits due to the increased plasma volume(122, 126). The difference in the biological phenotypes between FVA and FVP is due to differences at the molecular level: there are nine amino acid differences between the two viruses within the 113bp region in the transmembrane domain, including two additional leucines (due to a six nucleotide duplication) in the hydrophobic membrane anchor region at the C-terminus of the glycoprotein in SFFV-P, not in SFFV-A (108). Erythroid cells from the spleens of mice infected with SFFV-P can both proliferate and differentiate in the absence of Epo. In contrast to SFFV-P, erythroid cells from spleens of mice infected with SFFV-A can not differentiate in the absence of Epo. Withdraw of Epo from FVA infected erythroblasts at the CFU-E stage results in a rapid commitment to undergo apoptosis. The *in vitro* colony assay utilizing day 12.5 fetal liver cells, or erythroid precursor cells from mice that had been treated with phenylhydrazine to increase the number of erythroid precursor cells in the spleen and bone marrow, both have shown that SFFV-P can induce both the proliferation and differentiation of erythroid cells in the absence of Epo, although cells infected with SFFV-A can only form large erythroid bursts without Epo,

and these bursts can not differentiate into mature erythrocytes unless a small amount of Epo is added to the cultures (108).

Friend virus-induced erythroleukemia is characterized by polyclonal proliferation of nonleukemic erythroid progenitor cells followed by a later stage in which there is clonal or oligoclonal expansion of malignant cells (Figure 1.3). The early stage of Friend disease commences virtually immediately after the innoculation of virus into susceptible mice. It is characterized by massive splenic proliferation, due to the polyclonal proliferation of non-tumorigenic erythroid cells with limited self-renewal capability that retain the capacity to terminally differentiate into mature red blood cells. During the first stage of disease, there is a marked expansion of BFU-E and CFU-E. The second stage of Friend disease is characteristized by the emergence of fully transformed cells in the spleen, and finally the blood, bone marrow and liver (88). 3-8 weeks after Friend virus inoculation, malignant clones are capable of transplantation in vivo or growth in semisolid media *in vitro*. The fully transformed cells form spleen colonies in genetically anemic *Sl/Sl* ^{d} mice, and can establish stable cell lines *in vitro*. While some of the mice die during the first stage of disease due to splenic rupture; mice that survive the first stage will develop erythroleukemia. The transformation events involve proviral integration into preferred sites, like PU.1 and Fli-1, resulting in transcriptional activation of Ets family genes, and further the inactivation of the tumor suppressor p53, finally leads to erythroleukemia. Both PU.1 and Fli-1 are members of the *ets* gene family, which contain a conserved 80-90 amino acid domain, the ETS domain, which is involved in specific DNA binding. PU.1 is activated through proviral insertion in 95% of erythroleukemias induced by FVP and FVA, but not in leukemias induced by F-MuLV. Instead, Fli-1 can

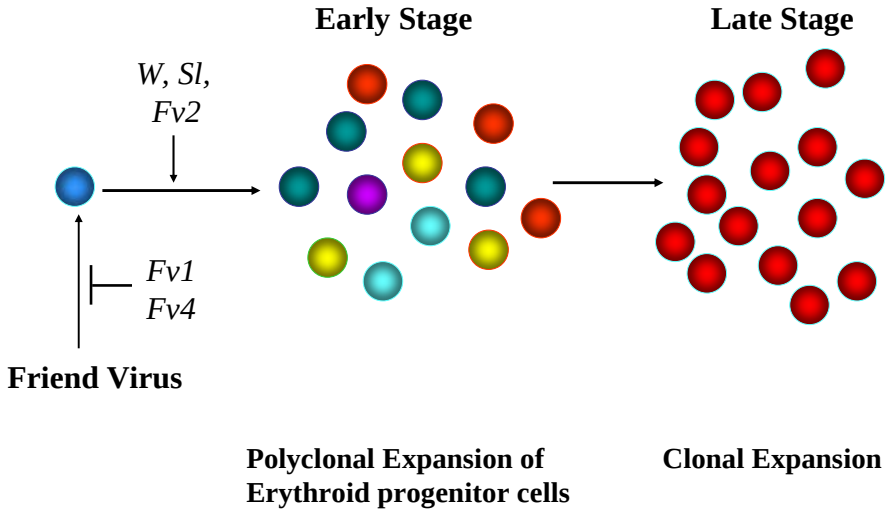

Figure 1.3 The development of Friend erythroleukemia. The early stage of the erythroleukemia is characterized by a polyclonal expansion of infected erythroid precursor cells, and the later stage is marked by the emergence of fully transformed cells in the spleen, blood, bone marrow and liver, leading to the development of erythroleukemia. Several loci have been identified that control susceptibility to Friend virus infection. The genes *Fv1* and *Fv4* affect the ability of Friend virus to infect early erythroid progenitor cells. *W*, *Sl*, *Fv2* are required for the expansion of infected progenitor cells and progression of the early stages of erythroleukemia.

be induced in erythroleukemic clones induced by F-MuLV. The strict specificity for PU.1 and Fli-1 may reflect the differences in the availability of these loci to integration in target cells for SFFV and F-MuLV. P53 is a dominant –acting gene, and about 30% of leukemic clones transformed by Friend virus have an inactivated p53 gene as the result of internal deletions or SFFV proviral insertion in the late stage of Friend disease (9).

Several host genes have been identified that control susceptibility to Friend virus infection. The genes *Fv1* and *Fv4* affect the ability of Friend virus to infect early erythroid progenitor cells. Another set of genes, *W*, *Sl*, *Rfv*, *Fv3, Fv5* and *Fv2* are required for the expansion of infected progenitor cells and progression of the early stages of erythroleukemia. *Fv1* encodes a gag-related protein that interferes with the retroviral life cycle by an unclear mechanism. *Fv4* encodes an endogenous retroviral envelop protein, blocking ecotropic retroviral receptors. For the other set of genes, *W* controls an intrinsic property of the stem cells that give rise to melanocytes, erythrocytes and germ cells; while *Sl* affects the microenvironment in which these stem cells develop during embryogenesis and function in adult life. *W* encodes the c-Kit receptor tyrosine kinase and *Sl* encodes a ligand, Stem Cell Factor (SCF), for this transmembrane receptor. The SCF/Kit signaling pathway plays a key role in erythroid differentiation by stimulating the proliferation of early erythroid progenitor cells, leading to the development of late erythroid progenitors. In the bone marrow, W/W^v mice have near normal numbers of Friend virus target cells and the initial infection of bone marrow occurs normally *in vivo*. However, spleen cells from W/W^v mice infected both *in vitro* and *in vivo* with Friend virus failed to give rise to erythropoietin-independent colonies following Friend virus infection, indicating that mutation of the Kit receptor specifically affects target cells in

the spleen, rendering the mutant mice resistant to the development of Friend virus-induced erythroleukemia. The Kit-dependent pathogenic targets of Friend virus in the spleen are distinct from the pathogenic targets in bone marrow and this population of spleen target cells is markedly decreased in W/W^v mice (5). *Fv3* affects the susceptibility of mice to Friend virus-induced immunosuppression, while *Fv5* determines whether FVP induces anemia or polycythemia in a strain of mice (97). Two H2 linked loci, Rfv1, Rfv2, and one non-H2 linked locus, Rfv3, affect the recovery from Friend virus infection. All these genes are required for normal erythropoiesis, suggesting the importance of the normal erythropoietic pathway for the development of Friend disease (9, 88, 97).

Fv2 is another host factor that affects the proliferation of SFFV-induced erythroblasts. It does not interfere with retroviral entry into host cells or the retroviral life cycle. Instead, *Fv2* determines the potential of SFFV infected erythroblasts to development in response to gp55, a truncated form of the retroviral envelope protein (88). Previously, we demonstrated that the Friend virus susceptibility gene, *Fv2,* encodes the stem cell-derived tyrosine kinase (Stk) receptor, which confers susceptibility to Friend virus induced erythroleukemia in mice. Mice homozygous for the resistant allele of *Fv2* ($Fv2^{rr}$), such as C57Bl/6, fail to express a naturally occurring, N-terminally truncated form of Stk, called short form-Stk (Sf-Stk), while expression of full-length Stk is unaffected. Mice homozygous for the susceptible allele of *Fv2* ($Fv2^{ss}$), such as Balb/CJ, express both full-length Stk and Sf-Stk. An internal promoter within the Stk locus drives the expression of Sf-Stk, which lacks the N-terminal ligand binding domain, but retains the transmembrane and tyrosine kinase domains. Mice deficient for Stk are resistant to Friend virus-induced expansion of erythroid progenitor cells (98), while enforced

expression of Sf-Stk in C57Bl/6 mice has been shown to be sufficient to confer Friend virus susceptibility to *Fv2*^{rr} mice (26, 97, 98).

Since the genomes of both SFFV-P and SFFV-A do not have oncogenic sequences, the pathogencity is caused by the Friend virus *env* gene which encodes a 55kd fusion glycoprotein (gp55). The N terminus of gp55 is homologous to env sequences present in a family of endogenous murine retroviruses, and the C terminus is related to the ecotropic MuLV gp70 (9). gp55 is required for both the proliferation of infected erythroblasts in Friend erythroleukemia and the differences in Epo-responsiveness associated with anemia- and polycythemia- inducing forms of gp55. Most of the envelope protein remains as a 55kd form within the Rough Endoplasmic Reticulum (RER). Only about 5% of gp55 leaves the RER as a disulfide-bound dimer that translocates to the cell surface, and is required for pathogenicity (9, 97). It has been shown that gp55 binds to the Epo receptor at the cell surface within the transmembrane region of these cell surface proteins (88, 97). Furthere more, gp55 can replace Epo to support the formation of erythroid colonies in vitro, but only in the presence of the Epo receptor (97). Sf-Stk has also been shown to interact with gp55 both covalently and non-covalently in erythroid cells resulting in constitutive activation of Sf-Stk (93), and this activation results in the early stages of transformation induced by Friend virus (93, 107). Interestingly, *c-sea*, the chicken homologue of Stk, is the cellular homologue of the avian retroviral oncoprotein *v-sea*, which causes erythroblastosis and anemia in chickens (47). V-sea (155kD) can be cleaved into two subunits, 85kD and 70kD, which are linked by disulfide bonds. The 85kD *env* subunit mediates the interaction between SEA molecules and results in constitutive activation of the tyrosine kinase domain (120). Therefore, the potential of

viral envelope proteins to induce leukemic transformation through the constitutive activation of this family of receptors is conserved.

1.2.4 Signaling downstream of Stk

Overview of Stk function

Stk (the stem cell derived tyrosine kinase), first isolated from murine hematopoitic stem cells (57), belongs to a super-family of receptors which includes the proto-oncogene Met. The human ortholog of Stk, Ron, was first cloned by screening a cDNA library prepared from human tumors and foreskin keratinocytes (105). These receptors share a high degree of sequence homology with the hepatocyte receptor, Met, and the chicken oncoprotein, c-Sea, which is currently considered the chicken ortholog of Ron. This small Receptor Tyrosine Kinase (RTK) subfamily distinguishes itself from other RTKs through the presence of a conserved two tyrosine docking site motif in the C terminal tail which plays a central role in Stk-mediated signaling. The Stk/Ron receptor is first synthesized as a single precursor chain of 180 KD, which then is proteolytically cleaved into a 160 KD transmembrane β chain and a 40 KD α chain that is linked to the extracellular region of the β chain by a disulfide bridge (68). The Stk/Ron receptor consists of three basic regions: an extracellular domain that recognizes growth factor ligand, a single-span transmembrane sequence that anchors the receptor to the membrane and propagates the free energy produced by ligand-receptor interactions across the membrane, and a cytoplasmic domain which undergoes a conformational change and leads to downstream signaling in the cells following ligand binding.

The ligand for Stk is Macrophage Stimulating Protein (MSP), an 80 KD serum protein secreted mainly from the liver in an in-active form, and then activated by serum

proteases of the intrinsic coagulation cascade at sites of inflammation (73). It is widely accepted that MSP activates Stk/Ron by inducing receptor dimerization to increase local concentration of the receptor and conformational changes in the kinase domain, which are the driving forces for the upregulation of kinase activity.

The Stk/Ron receptor can elicit cell scattering and invasive growth (78, 112). Moreover, MSP also exibits mitogenic and anti-apoptotic effects on various cell types, which is a common feature of growth factor signaling (31, 131). A specific cellular response mediated by Ron is the downregulation of inflammatory mediators produced by macrophages both *in vitro* and *in vivo*. Our lab has shown that MSP/Ron attenuates NO production in response to IFN and LPS by inhibiting the expression of inducible NO synthase (iNOS). Furthermore, we demonstrate that stimulation of murine peritoneal macrophages with MSP results in the RON-dependent up-regulation of arginase, an enzyme that competes with iNOS for the substrate L-arginine. More recently, we found that MSP may actively suppress cell-mediated immune responses through its ability to down-regulate IL-12 production and thus inhibit classical activation of macrophages (4, 25).

Several studies have implicated Stk receptor in the development of erythroleukemia. Enforced expression of short form Stk *ex vivo* confers Friend virus sensitivity to erythroid progenitor cells from resistant strains of mice. Moreover, deletion of Stk causes the resistance of susceptible strains of mice to Friend Virus induced erythroleukemia (97). One proposed mechanism for the role of Sf-Stk in this process is that the gp55 envelop glycoprotein and intra-cellular Sf-Stk can form a full length "receptor-like" protein complex which modulates the activity of the EPO receptor

through a direct interaction (107). This hypothesis is also inspired by the role of v-sea, a truncated chicken version of Ron (Ron) reminiscent of Sf-Stk, in chicken erythroleukemia induced by the S13 avian erythrolastosis virus. In the viral genome, the v-sea is fused in frame with a viral envelope gene and the resultant large membrane protein is responsible for the transformation of chicken erythroblasts (27). Interestingly, an N-terminally truncated Ron receptor, similar to Sf-Stk, has been cloned from human cells, indicating that this tuncated receptor may have an evolutionarily conserved function. A recent study also implicates full length Ron in Epo receptor signaling. Epo mediated Jak2 activation by EpoR leads to phosphorylation of the Ron receptor, that further generates the proliferation signals required for erythroid progenitor expansion (130). We have demonstrated that expression of mouse RON (Stk), but not human RON, in the absence of ligand induces the formation of Epo-independent erythroid colonies (CFU-E and BFU-E) from primary bone marrow cells, while stimulation of both receptors with MSP promoted the growth of Epo-independent colonies. This difference in ligand-independent signaling by Ron in erythroid progenitor cells maps to a deletion in the juxtamembrane domain in Stk. The kinase activity of the receptor is essential for the ability of Stk to promote progenitor cell proliferation, as demonstrated by the failure of kinase-inactive form of Stk to promote this response. Moreover, using tyrosine to phenylalanine mutants, we found that the docking site tyrosines are required for ligand-dependant induction of erythroid colony formation, whereas tyrosines in kinase domain are required for ligand independent colony growth (139, 140).

Signalings downstream of Stk (Figure 1.4)

The tyrosine kinase Met receptor controls multiple cellular events, ranging from cell motility and angiogenesis to morphological differentiation and tissue regeneration. The cytoplasmic C-terminal region of this receptor mediates these activities by serving as a docking site to mediate diverse signaling traffic by multiple protein substrates, including Grb2, Gab1, STAT3, Shc, SHIP-1 and Src. These substrates are characterized by the presence of multiple specific domains, including the PH, PTB, SH2 and SH3 domains, which directly or indirectly interact with the multisubstrate C-terminal region of Met (15).

The most studied signaling cascade of protein-protein interactions downstream of STKs is the Grb2/SOS/Ras/MAPK pathway (Figure 1.4). Previous research demonstrated that a single carboxyl-terminal tyrosine residue, Tyr^{489} of Met, is essential for efficient transformation of 3T3 fibroblasts and forms a multisubstrate binding site for the adaptor protein Grb2 (37). Grb2 is composed of a src-homology-2 (SH2) domain which is flanked by two SH3 domains. The N-terminal SH3 domain, through its ability to interact with SOS, mediates downstream activation of the Ras/MAPK pathway(40). The Ras family of small GTPases acts as molecular switch: it is in the inactive form when bound with GDP and can be activated when GDP is replaced by GTP. The link between receptor phosphorylation and Ras activation is mediated by Sos, a nucleotide exchange factor which catalyzes the dissociation of GDP and subsequent association of GTP with Ras. In quiescent cells, Sos, which is constitutively associated with the Grb2 adaptor protein via a proline-rich C-terminal region, is uncoupled from membrane anchored Ras. Growth factor-induced tyrosine phosphorylation of a receptor results in recruitment of the Grb2/Sos complex to the proximity of the cell membrane through its SH2 domain.

Consequently, sub-cellular co-localization of SOS with Ras triggers the GTP/GDP exchange reaction and concomitant Ras activation (116). Activated Ras undergoes a conformational change which favors the interaction of the Raf binding domain (RBD) of Ras with Raf-1. This leads to the upregulation of Raf-1 serine/threonine kinase activity (106) and the further activation of a kinase cascade including MAPKK, MAPK and other pleiotropic responses essential for cell growth and differentiation.

The C-terminal SH3 domain of Grb2 recruits large adaptor proteins to the complex, including the Grb2 associated binding protein (Gab), which are important factors in activation of the PI3K/Akt pathway which promotes cell survival. Previous experiments demonstrated that Gab1 can bind stably to the C terminal SH3 domain of Grb2. Moreover, these experiments define a 13 amino acid sequence with the unusual consensus motif P-x-x-x-R-x-x-K-P that is required for stable binding of Gab1 to the C terminal SH3 domain of Grb2 (38, 69) Alternatively, Gab1 can be directly recruited to Y1349, the first docking site tyrosine in Met, through its unique Met Binding Domain (MBD). As a scaffolding adapter protein, Gab1 provides a broader binding platform for the next layer of signal transducers including the p85 subunit of PI3 kinase, CrkL and SHP2 (91, 109), which are upstream signals for Akt, Rap1, and Erk, functioning as critical factors for cell survival, proliferation and differention. The functional importance of the Gab1-SHP2 interaction has been extensively studied using Gab mutants that are unable to bind SHP2. In unstimulated cells, SHP2, a protein tyrosine phosphatase, is held in an inactive conformation by inhibitory intramolecular interactions. Phosphorylation of specific tyrosines on Gab1 induced by the active receptor serve as “secondary” docking sites for the SHP2 SH2 domain, leading to the membrane localization of SHP2

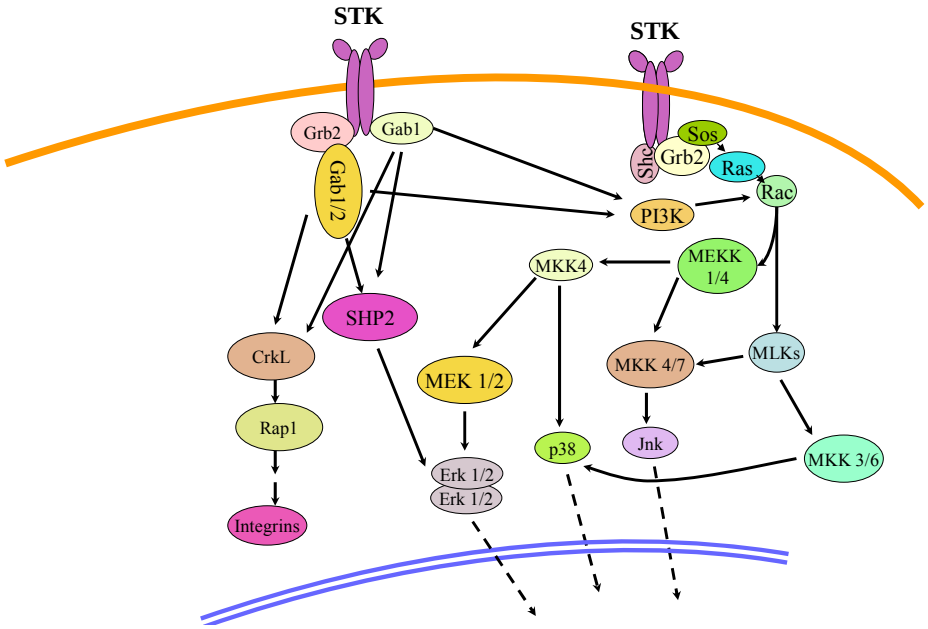

Figure 1.4 A schematic figure of signaling network downstream of STK. Activation of STK leads to recruitment and tyrosine phosphorylation of Gab2/1, which binds constitutively to the Grb2 SH3 domain. Tyrosine phosphorylated Gab2/1 binds to signal relay molecules, including PI3K, which is required for Akt activation, SHP2, which may be important for Erk activation, and CrkL, which is essential for Rap1 activation. On the other hand, Gab1 can be recruited and activated by STK directly through MBD, and are upstream signals for Akt, Rap1 and Erk, functioning as critical factors for cell survival, proliferation and differention.

and the subsequent activation of phosphatase activity. Gab1 mediated activation of PI3K/AKT by Met is responsible for the anti-apoptotic effects of HGF, the ligand for Met, while the SHP2 association with Gab1 has been shown to be crucial for Met promoted tubulogenesis and sustained MAPK/Erk activation (91, 109).

The recruitment of Gab1 to the Met receptor is both Grb2 dependent and Grb2 independent. In contrast, Gab2 recruitment to Met is strictly mediated by Grb2. Genetic comparison of the MBD region of Gab1 and Gab2 revealed a 10-amino acid segment that is lacking in Gab2, which is critical for the Grb2-independent recruitment of Gab1 to the Met receptor (77). However both Gab1 and Gab2 have highly homologous PH domains, which are important for their membrane recruitment, and contain tyrosine residues within a consensus for recruitment of p85, SHP-2 and Crk (77, 91). It has been reported that transformation of cells by chicken sea is mediated by Gab2 and requires the activation of the PI3K and MAPK pathways (3, 55). In previous analysis of Sf-Stk signaling during Friend virus infection, we demonstrated that the activation loop of the kinase domain and docking site tyrosine 436 in the c-terminal tail, which is responsible for the interaction with Grb2, are required for the erythroblast expansion(36). In studies described here, we found that Grb2-mediated recruitment of Gab2, but not Gab1, to Sf-Stk supports the expansion of Friend virus-infected erythroid progenitor cells (127).

1.2.5 Cytokine, Growth Factor Receptors and Receptor Activation

Cell proliferation and differentiation are regulated by the binding of either cytokines or growth factors to their receptors. Cytokine and growth factor receptors can be divided into three categories: Class I, Class II and RTKs. Class I and Class II cytokine receptors lack intrinsic catalytic activity and have similar tertiary structures. Class I

cytokine receptors have a conserved cysteine and a conserved WSXWS (Trp-Ser-X-Trp-Ser) motif in the membrane proximal region of the extracellular domain, which is critical for structural stability and ligand binding, although the exact role of the WSXWS motif is unknown. The cytoplasmic domains of cytokine receptors, contain conserved Box1 and Box2 motifs, which mediate signal transduction. Class II cytokine receptors maintain the conserved cysteine motif, Box1 and Box2, but lack the WSXWS motif which are conserved in Class I cytokine receptors (84). RTKs, on the other hand, have intrinsic kinase activity and diverse extracellular domains which are responsible for ligand binding.

Both Class I and Class II cytokines receptors bind to their specific ligands through the extracellular domain, and induce the formation of either homodimers or multimeric complexes. The Erythropoietin Receptor (EPOR) forms single chain complex of homodimers upon ligand stimulation. EPOR controls the proliferation, differentiation and survival of the erythroid progenitors, opening the way to clinical trials of hematopoietic growth factors for the treatment of anemia after it was cloned in 1985 (65). However, EPOR is not required for the generation of committed erythroid progenitors (BFU-E and CFU-E) (138). The common sequence motif, WSXWS, near the transmembrane domain is highly conserved in this family (145). Mice lacking the EpoR are embryonic lethal due to lack of fetal liver erythropoiesis. The carboxy-terminal tyrosine, Y479, is sufficient for Epo-stimulated proliferation and differentiation of fetal liver progenitors (62).

The mechanisms in signal transduction downstream of cytokine receptors are quite similar, although there is diversity among different classes of cytokines and growth factors. The ligand binding of RTKs induces the dimerization of these receptors, and the subsequent phosphorylation of tyrosines in the activation loop of kinase domain. This

results in movement of the activation loop which occludes the active site in the unphosphorylated form, leading to increased kinase activity. Alternatively, Class I and Class II cytokine receptors utilize the receptor associated tyrosine kinase, Janus Kinase (JAK) to induce downstream signals due to the lack of intrinsic catalytic domains.

There are seven common homology domains in JAKs. The Janus Homology Domain 1 (JH1) harbors the catalytic activity, while JH2 encodes a pseudokinase domain. JH3/4/5/6/7 have no catalytic activity but are important in the regulation of JAK kinase activity (21, 110, 144). Four JAKs have been cloned from mammalian cells, JAK1, JAK2, JAK3 and TYK2. Mice lacking JAK1 die perinatally due to a neurologic defect, whereas JAK2 deficient mice die during embryogenesis due to a failure of definitive erythropoiesis in the fetal liver. JAK3 deficient mice are viable but have defects in T, B and NK cells. However, TYK2 deficient mice have no obvious abnormalities, indicating the function of TYK2 is not essential (39, 128).

1.2.6 JAK/STAT Signaling

Cross phosphorylation of JAKs and subsequent phosphorylation of the cytokine receptor chains results in the recruitment of proteins containing SH2 or phosphotyrosine-binding (PTB) domains. One major class of SH2 containing proteins downstream of JAKs in cytokine signaling is the STAT family of transcription factors. Once STATs are phosphorylated, they dimerize and translocate to nucleus where they bind to DNA and induce the expression of STAT-dependent target genes. STAT proteins were originally discovered in interferon (IFN)-regulated gene transcription in the early 1990's, and have subsequently been shown to play a key role in diverse biological processes, including cell growth, differentiation, apoptosis, transformation, and immunity (10). There are seven

members of STATs that have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Although there are functional differences among members of the STAT protein family, they share common structural features, those are : 1) a central DNA-binding domain, which determines DNA sequence specificity of individual STATs; 2) a Transcriptional Activation Domain (TAD) domain, which is located at the C-terminal end of the molecule and is involved in communication with transcriptional complexes; 3) a critical tyrosine residue that is required for SH2-phosphotyrosine interactions and therefore, STAT activation which is located near the SH2 domain; 4) a coiled-coil domain, which provides potential contacts for transcription factors and other regulatory proteins (10, 15). Activation of the EPOR by ligand binding leads to the cross-phosphorylation of the constitutively associated JAK2 molecules. The activated JAK2 then phosphorylates the tyrosines in the cytoplasmic domain of EPOR, which are docking sites for SH2 containing molecules, and induces the activation (phosphorylation) of STAT5.

STATs not only function as downstream effectors of cytokine signaling, but also play key roles in mediating growth factor receptor signalling. While ligand dependent activation of the STATs is often associated with the regulation of normal cell growth and differentiation, constitutive activation of STAT3 has been detected in a wide variety of human cancer cell lines and primary tumours, such as multiple myelomas, and head and neck cancers (15, 17). It is reported that the transcription factor Stat3 is a downstream signaling molecule activated by HGF/Sf-Met signaling (152). Furthermore, receptors harboring a Met(p + 1 loop) → Thr point mutation in the kinase domain of Met, lead to the formation of tumours with high metastatic potential, including oral squamous cell

carcinoma. This dramatic event is apparently the result of the constitutive phosphorylation of STAT3 by the mutant Met receptor (15, 152).

A number of positive and negative regulatory molecules in the JAK/STAT pathway exist, including Cytokine-inducible SH2-domain containing protein (CIS) and SHP-1/2. Acting as a negative regulator, CIS is member of Suppressor Of Cytokine Signaling (SOCS) family. It downregulates cytokine receptor signaling by binding to docking sites tyrosines in the cytokine receptor and blocking STAT recruitment. SOCS1 also negatively regulates the activation of JAK1/2 and Tyk2 by acting as a pseudosubstrate (143), whereas activated SOCS3 can maintain Ras in an activated form, inducing the activation of Erk pathway (18). Finally SOCS family members can serve as E3 ubiquitin ligases, targeting cytokine receptors for proteasome-mediated degradation. SHP-1, an SH2-containing protein tyrosine phosphatase, can dephosphorylate and inactivate JAKs, as well as dephosphorylate tyrosines in cytokine receptors critical for downstream signaling events (150). However, SHP-2 plays different roles in the cytokine signaling pathway. On one hand, SHP-2 plays a positive role in early hematopoiesis by working as adaptor protein to induce PI3K and MAPK activation. On the other hand, SHP-2 acts as negative regulator in more differentiated cells with the same mechanism as described for SHP-1 (142).

1.2.7 Src Family Kinases

Some recent studies have indicated that G protein-coupled receptors and growth factor receptors can induce the activation of Stat3 in Src-dependent mechanism (24, 76). Src family kinases have been implicated downstream of the Met receptor tyrosine kinase in the modulation of multiple cellular events via its direct interaction with Met. It binds,

with fast recruiting and dis-recruiting rates, either of the two docking site phosphotyrosines in the sequence Y l 1349-VHVNAT-Y * 1356~VNV, in which both residues can be simultaneously phosphorylated (100). The Src family of protein kinases regulates a diverse array of cellular processes in both normal and transformed cells, including cell proliferation, survival, differentiation, adhesion and motility. There are eight members in the Src family of kinases expressed in mammalian cells, including Hck, Fgr, Lyn, Lck, Blk, Src, Yes, Fyn. The latter three of them are ubiquitously expressed, while the others are limited to specific cell lineages. Src family kinases are members of a family of non-receptor tyrosine kinases defined by a common modular structure. The N-terminal region of Src family members are unique to each family member and provide signals including myristoylation, a modification determined by the first seven amino acids of the molecule that causes association with the plasma membrane. Src family members also contain conserved SH3 and SH2 domains (15, 117). These domains contribute to the negative regulation of Src kinases by promoting intramolecular interactions. Disruption of these interactions is a major mechanism by which Src kinases become activated. The conserved kinase tyrosine residue, Y527, located in the c-terminal tail, is critical for Src regulation due to its ability to interact intramolecularly with the SH2 domain of Src. Interestingly, in some signaling networks, Src appears to work as an adaptor molecule which may function by recruiting or activating other tyrosine kinases (35). Recent studies have demonstrated that phosphorylation of Stat3 and/or Gab2 may occur in a Src family kinase dependent manner following growth factor stimulation (24, 64). In conclusion, Src may play different roles in the context of different signaling systems.

Chapter 2

GRB2-mediated recruitment of GAB2, but not GAB1, to SF-STK supports the expansion of Friend virus-infected erythroid progenitor cells

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2.1 Abstract

Friend virus induces the development of erythroleukemia in mice through the interaction of a viral glycoprotein, gp55, with a truncated form of the Stk receptor tyrosine kinase, short form-Stk (Sf-Stk), and the EpoR. We have shown previously that the ability of Sf-Stk to participate in the transformation of Friend virus-infected cells requires the kinase activity and Grb2-binding site of Sf-Stk. Here we show that Grb2 heterozygous mice exhibit decreased susceptibility to Friend erythroleukemia and that expansion of erythroid progenitors in response to infection requires the C- terminal SH3 domain of Grb2. A fusion protein, in which the Grb2-binding site in Sf-Stk is replaced by Gab2, supports the growth of progenitors from mice lacking Sf-Stk, whereas Sf-Stk/Gab1 fusion protein does not. Gab2 is expressed in spleens from Friend virus-infected mice, co-immunoprecipitates with Sf-Stk and is tyrosine phosphorylated in the presence of Sf-Stk. Mice with a targeted deletion in Gab2 are less susceptible to Friend erythroleukemia and the expansion of erythroid progenitor cells in response to infection can be rescued by

expression of Gab2, but not Gab1. Taken together, these data indicate that a Sf-Stk/Grb2/Gab2 complex mediates the growth of primary erythroid progenitor cells in response to Friend virus.

2.2 Introduction

The development of leukemia proceeds in response to a series of events resulting in transformation, however the signals that govern the progression of disease through these discrete phases are largely unknown. Friend erythroleukemia virus (FV) provides an experimental model with which to study the signaling events that direct leukemia development through progressive stages of transformation *in vivo.* FV is a complex of two viruses, the replication defective spleen focus forming virus (SFFV) which expresses gp55, the pathogenic component, and the replication competent helper virus, Friend murine leukemia virus (F-MuLV) (9). In the early stages of FV infection, erythroid progenitor cells are infected resulting in the polyclonal expansion of the infected cells (53, 71, 82, 125). The late stage of erythroleukemia in FV infected mice is characterized by subsequent mutations in p53 combined with the insertional activation of the *Spi-1* oncogene, resulting in acute erythroleukemia (83, 85).

Several loci have been identified that control susceptibility to FV infection (6). The genes *Fv1* and *Fv4* affect the ability of FV to infect the early erythroid progenitor cells. Another set of genes, *W*, *Sl*, *f* and *Fv2* are required for the expansion of infected progenitor cells and the progression of the early stages of erythroleukemia. The host gene *Fv2* acts in a cell autonomous manner and determines the proliferative response of infected erythroblasts to gp55 (8, 72). Previously, we demonstrated that *Fv2* encodes the Met-related Macrophage Stimulating 1-Receptor (Mst1r) tyrosine kinase, also known as Stem cell-derived tyrosine kinase receptor (Stk) (97). Mice homozygous for the resistant allele of *Fv2* ($Fv2^{rr}$), including C57BL/6 mice, fail to express a naturally occurring, N-terminally truncated form of Stk, called short form-Stk (Sf-Stk). An internal promoter

within the Stk locus drives Sf-Stk expression which lacks the N-terminal ligand-binding domain of the full-length Stk, but retains the transmembrane and tyrosine kinase domains. Enforced expression of Sf-Stk in C57BL/6 mice is sufficient to confer FV susceptibility in *Fv2*^{rr} mice (97).

Previous work has shown that gp55 interacts with the EpoR, and that co-expression of EpoR and gp55 in IL-3-dependent BaF/3 cells results in factor-independent cell growth (29, 70). Sf-Stk has also been shown to co-immunoprecipitate with gp55 in hematopoietic cells resulting in constitutive activation of Sf-Stk (93), and studies from our laboratory clearly demonstrate that Sf-Stk kinase activity is essential for Epo-independent growth of infected cells (36). Interestingly, the chicken homologue of Stk, *c-sea*, is the cellular homologue of the avian retroviral oncoprotein *v-sea*, which causes erythroblastosis and anemia in chickens (48). *v-sea* is a 155 kDa glycoprotein, which is cleaved into 85 and 70 kDa subunits and linked by a disulfide bridge (48). The 85 kDa *env* subunit of *v-sea* mediates interaction between SEA molecules resulting in autophosphorylation and constitutive activation of the tyrosine kinase domain (120). Thus, the ability of viral envelope proteins to induce erythroleukemia through the constitutive activation of this family of RTKs is conserved across species.

In vitro infection of primary bone marrow cells with FV results in Epo-independent erythroid colony formation. In previous analysis of Sf-Stk signaling during FV infection *in vitro,* we demonstrated that tyrosine 1337, which binds Grb2, is required for the formation of Epo-independent erythroid colonies by FV (36). In addition, mutation of the asparagine following this tyrosine (YVNV to YVHV), which eliminates Grb2 binding without affecting the binding of other SH2 domain-containing proteins,

abrogates FV-induced cell transformation *in vitro*, which further underscores the key role of Grb2-dependent signals (36, 37). Here we show that Grb2 haploid insufficiency decreases the susceptibility of mice to Friend erythroleukemia *in vivo*. Furthermore, we demonstrate that primary erythroblasts from Grb2 +/- mice exhibit reduced erythropoietin-independent colony formation in response to Friend virus infection *in vitro*, and that this defect can be rescued by retroviral introduction of wild-type Grb2, but not a Grb2 mutant containing a mutation in the c-terminal SH3 domain of Grb2. This domain binds the Gab family of adaptor proteins and we demonstrate here that Sf-Stk co-immunoprecipitates with Gab2 and Gab2 -/- mice are largely resistant to Friend virus infection *in* vivo. Furthermore, introduction of a Sf-Stk/Gab2 fusion protein into bone marrow lacking Sf-Stk supports Epo-independent colony formation. These data demonstrate that a Sf-Stk/Grb2/Gab2 complex is both necessary and sufficient for the development of erythroleukemia in response to Friend virus infection.

2.3 Experimental Procedures and Preparation

2.3.1 Mice

Grb2 +/- mice, on a FV2-sensitive BALB/c background, were genotyped as previously described (22). Gab2 -/- mice on a mixed genetic background were crossed for 1 generation onto BALB/c. Genotypes of Gab2 knock-out mice (generated by GS Feng, The Burnham Institute, to be described elsewhere) were determined by PCR with the appropriate primers. For preparing genomic DNA, about 5mm of mouse tails were clipped and lysed in lysis buffer (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.1% SDS, 100 μl Proteinase K) at 55 °C overnight. After centrifugation, add 2 volume of ethanol to cleared lysate to precipitate the genomic DNA. The pellet was washed with 75% ethanol; air dried and resuspended in 100 μl of 10 mM Tris-Cl pH 8.0. For the genotype of Gab2, primer pair 5'-GGCTTACAGAGCCTCTGACCCAG-3' (forward) and 5'-CTGCTATACCTCTGCCATGGTGGG-3' (reverse) were used. The PCR parameters are: 95 °C 1 minute followed by 30 cycles of 95 °C 30 seconds, 55 °C 30 seconds and 72 °C 1 minute and 30seconds. The PCR products were loaded onto 1% agarose gel for eletrophoresis and visualized with etherdium bromide staining. The wild type allele amplifies a 1.5 kb fragment and null allele a 0.7 kb fragment. For the Fv2 loci, the primers are 5'-GGTGGGTTTAACGGTTAGGG-3' (forward) and 5'-TCTGGGCTCTGCCTCCTTAT-3' (reverse). The PCR parameters are: 95 °C 1 minute followed by 30 cycles of 95 °C 30 seconds, 55 °C 30 seconds and 72 °C 30 seconds. The PCR products then loaded on 12.5% Acrylamide gel electrophoresis to visualize the DNA fragment by eherdium bromide staining. The sensitive allele amplifies a 53 bp fragment and resistant allele 50 bp. There are three bands for the heterozygous mice.

BALB/c and C57BL/6 mice were obtained from Jackson Laboratories. All research involving the use of mice were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Pennsylvania State University. For *in vivo* infections with Friend virus, wild-type and mutant mice were tail-vein injected with 200 µl of FV. Two weeks post injection, mice were sacrificed and spleens removed. The spleen was weighed with an analytical balance and measured in grams.

2.3.2 Plasmid construction

All the restriction and modification enzymes used here were from New England BioLabs, unless otherwise stated. The Grb2 cDNA was subcloned in-frame with EGFP into the XhoI site of the pEGFP vector (Clontech) and subsequently the 1.4 kb EGFP-tagged Grb2 was subcloned in the HpaI site of the MSCV-neo retroviral vector (provided by A. Henderson, Pennsylvania State University) to produce MSCV-neo-EGFP-Grb2. Grb2 mutants MSCV-neo-EGFP-Grb2W36K, MSCV-neo-EGFP-Grb2W193K and MSCV-neo-EGFP-Grb2W36,193K were produced by site-directed mutagenesis using the Stratagene QuickChange™ kit (Stratagene), following the manufacturer's protocol. Using MSCV-neo-EGFP-Grb2 as template, the mutations were introduced with the following oligos: MSCV-neo-EGFP-Grb2W36K, N-terminal SH3 mutation: sense-5'-CGAAGAATGTGATCAGAACAAGTACAA GGCAGAGCTTAATGG-3'; MSCV-neo-EGFP-Grb2W193K, C-terminal SH3 mutation: sense-5'-GGATAACTCAGACCCCAACAAGTGGAAAGGAGCTTGC-3'. MSCV-neo-myc/Sf-Stk was described previously (36).

The murine Gab2 coding sequence was RT-PCR amplified from mouse liver total RNA (RNAeasy Kit from Qiagen) using the forward primer 5'-

CGGCGGGCTCCAGTTTAGCCG-3' and reverse primer 5'-CCCCTTCATTACAGCTTGGCACCC-3'. The PCR product was cloned into pcDNA3.1 (Invitrogen) at the EcoV site to produce pcDNA3.1-Gab2. To generate the Myc-Sf-Stk and Gab2 fusion, the Myc/Sf-Stk fragment was amplified from MSCV-neo-Myc/SF-Stk (11) using primers 5'-gcaggatcccatcgatttaaagc-3' (forward) and 5'-atgctagcagtgaggccactacctgc-3' (reverse) and the Gab2 fragment was amplified from pcDNA3.1-Gab2 with primers 5'-atgctagcCGGCTTCAATCAGGCTGAAGAG-3' (forward) and vector primer 5'-ctagaaggcacagtcgaggctg-3' (reverse). The fragments were digested with proper enzymes, purified and three-way ligated into pcDNA3.1 vector. The fused gene was then cut out from pcDNA3.1 vector with Pme I and subcloned into MSCV-neo at the Hpa I site to produce MSCV-neo-Myc/SF-Stk-Gab2. The mutations in Myc/Sf-Stk-Gab2Y3F were introduced by site-directed mutagenesis (Stratagene QuickChange ^{TM} Kit) following the manufacturer's protocol. The template was MSCV-neo-Myc/SF-Stk-Gab2 and the primers (sense) were as follows: 5'-ccagctctgatgacaactTcgtgcccatgaacccagg-3' (Y441F); 5'-gacaactcccagagtgtctTcatccccatgagcccagg-3' (Y465F); 5'-ggagacagtgaggagaactTtgtccctatgcaaaaccc-3' (Y574).

2.3.3 Retrovirus production

293T cells were transiently transfected by either $CaPO_4$ coprecipitation (136) or TransIT-293 transfection reagent (Mirus corporation) using 1-2 μg pEco and the appropriate MSCV-neo constructs (1-10 μg). For $CaPO_4$ coprecipitation, cells were transfected for 16 hours at 37 °C, where the media was then aspirated, changed, and cells

were grown for an additional 30 hours at 37 °C prior to harvest of the viral supernatant. For TransIT-293 transfection, cells were transfected for 48-72 hours at 37 °C prior to harvest of the viral supernatant. Protein expression of MSCV-EGFP, MSCV-myc/Sf-Stk-EGFP, MSCV-neo-EGFP-Grb2, MSCV-neo-EGFP-Grb2W36K, and MSCV-neo-EGFP-Grb2W193K was verified by western blot, by probing with antibodies against the EGFP- and myc-tagged fusions (Clontech and Cell Signaling, respectively).

2.3.4 Antibodies, Immunoprecipitation, and Western blotting.

Mouse anti-Myc (1:1000 dilution), mouse anti-phospho-tyrosine (1:1000 dilution) and mouse anti-GFP (1:2000 dilution) antibodies were purchased from Cell Signaling. Rabbit anti-Gab2 antibody (1:200 dilution) was purchased from Upstate Biotechnology. Anti Gab2 was used to detect the Gab2 protein, anti-GFP antibody was used to detect Grb-GFP fusion and mutants, and anti-Myc antibody was used to detect the expression of Myc/sfStk, Myc/sfStk-Gab2 fusion protein and mutants.

293T cells were transiently transfected with plasmids expressing desired protein. Twenty-four hours post transfection, cells were lysed in lysis buffer (1% Digitonin, 150 mM NaCL, 0.4 mM EDTA, 2mM Na_3VO_4, 10 mM NaF, and 2mM PMSF). After 20 minutes centrifugation, the cleared cell lysates were incubated with antibodies and protein A-sepharose at 4°C overnight. The immunoprecipitates were analyzed by reduced SDS-PAGE and transferred to PVDF membrane. The blots were incubated with antibodies and detected with ECL (Amersham).

2.3.5 Colony assay and in vitro infection

For Epo-independent colony analysis, total bone marrow cells from Grb2 +/- and wild-type control mice were harvested and incubated with supernatant from cells expressing polycythemia-inducing FV (derived from FP63 cells, Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada) and DMEM (10 % FBS, P/S, L-glu) on ice for 1 hour. Cells were then plated in methocult M3534 (Stem Cell Technologies) in triplicate with or without 1 U/ml Epo (R&D Systems). Cultures were incubated for 2-8 days in 5 % CO_2 at 37 °C. Erythroid colonies (BFU-E and CFU-E) were visualized by acid-Benzidine staining as previously described (36). For *in vitro* infection of bone marrow, cells were harvested from various strains of mice and washed in PBS. Bone marrow was infected with viral supernatant from the transient transfections for 20 hours, as previously described (36). The cells were then infected with FVP and suspended in methocult 3234 containing IL-3 (2.5 ng/ml) (Peprotech), either in the presence or absence of Epo (1 U/ml).

2.3.6 Reverse transcriptase-PCR

Expression of Gab1, 2 and 3 mRNA in total bone marrow, liver, spleen, and FV infected spleen was determined by reverse transcriptase (RT)-PCR. Total RNA was obtained from the above samples using the RNeasy kit (Qiagen). cDNA was generated by GeneAmp RNA PCR kit components where reverse transcription of RNA was followed by PCR amplification of cDNA (Applied Biosystems). For Gab1 gene the primers are 5'-GGAGGTGTCTCGGGTGAAGAGC-3' (sense) and 5'-CGGCAGAGGCGACGGCATG -3' (anti-sense) which amplify a 0.8 kb fragment, for

Gab2 gene the primers are 5'-ATGTCCCAACCACTCCTCTCTCAGC-3' (sense) and 5'-CCATAGCCAGCAGGGTAGAAGAAC-3' (anti-sense) which amplify a 0.43 kb fragment, and for Gab3 gene the primers are 5'-CCCAGTGCTGAAGACAGCTATGTGC-3' (sense) and 5'-atcagtccatgcctgcttggtgctc-3' (anti-sense) which amplify a 0.6 kb fragment.

Ten percent of the reaction mixture was electrophoresed on a 1 % agarose gel (Fisher Scientific) and stained with ethidium bromide (International Biotechnologies). RT cycling parameters were: 5 min at 70 °C, 40 min at 40 °C, 5 min at 99 °C, then hold at 4 °C. PCR cycling parameters were: 1 min at 95 °C, 20 sec at 94 °C, 30 sec at 62 °C, 1 min at 72 °C, for 40 cycles.

2.4 Results

2.4.1 Grb2 +/- mice exhibit reduced susceptibility to Friend erythroleukemia virus.

Mice with a targeted deletion in the gene encoding Grb2 are embryonic lethal (22), however haplo-insufficiency results in several defects in the adult animal including impaired negative selection of T cells, a block in cardiac hypertrophy and fibrosis in response to pressure overload and resistance to neointima formation following carotid injury (42, 151). In order to determine the susceptibility of Grb2 +/- mice on a sensitive Balb/c background to FV *in vivo*, eight-week old Grb2 +/- and wild-type control mice were infected with FV and spleens were harvested two weeks later. The average spleen size of infected Grb2 +/- animals was significantly reduced when compared to wild-type controls, suggesting that Grb2 may be required for the expansion of FV infected cells in the spleen (Figure 2.1). *In vitro* FV infection of primary bone marrow cells from sensitive mice has previously been shown to result in Epo-independent erythroid colony formation (36). In order to determine whether Grb2 is required for this response, we infected bone marrow cells from Grb2 +/- and wild-type control mice with FV and assessed the ability of these cells to form Epo-independent erythroid colonies. The number of FV induced Epo-independent colonies was then compared to the number of Epo-dependent colonies formed in the absence of FV infection. Bone marrow from wild-type controls gave rise to approximately equal numbers of Epo-dependent and FV-induced Epo-independent colonies; however, bone marrow cells from Grb2 +/- mice yielded an average of about 3-fold fewer Epo-independent colonies compared to the number of Epo-dependent colonies (Figure 2.2C).

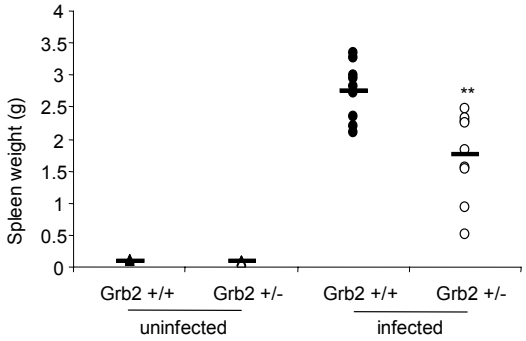

Figure 2.1 Grb2 +/- mice are less susceptible to Friend virus *in vivo*. Eight-week old Grb2 +/- mice on a sensitive Balb/c background and wild-type Balb/c control mice were infected with FV. Spleens were weighed two-weeks post-infection. **p<0.01. Figure was provided by Hami E. Teal.

2.4.2 The C-terminal SH3 subdomain of Grb2 is critical for FV-induced erythroid colony formation.

Previously we demonstrated that exogenous expression of Sf-Stk in C57BL/6 bone marrow cells, which fail to express endogenous Sf-Stk, could rescue the ability of these cells to form Epo-independent colonies following FV infection *in vitro* (36). However, a mutant form of the receptor which lacked the ability to bind Grb2 could not rescue this response. Similarly, to determine whether the defect in the Grb2 +/- mice can be rescued by wild-type Grb2, we transduced bone marrow cells from Grb2 +/- mice with an MSCV-based retroviral vector expressing wild-type and mutant forms of Grb2 (Figure 2.2 A) fused to GFP. Equivalent protein expression in the packaging cells was confirmed by Western blot analysis with anti-GFP (Figure 2.2 B), and infection equivalent infection efficiency was confirmed by flow analysis for GFP of infected bone marrow cells (Figure 2.2 C). While control infections resulted in a reduced percentage of FV induced Epo-independent colonies/Epo-dependent colonies as expected, bone marrow from Grb2 +/- mice expressing wild-type Grb2 supported Epo-independent BFU-E colony formation in response to FV infection at levels comparable to the number of Epo-dependent colonies (Figure 2.2 D). These data support our previous conclusion that Grb2 binding to Sf-Stk is required for FV mediated erythroid colony formation.

Grb2 is an adapter protein composed of a central Src homology 2 (SH2) domain and two flanking SH3 domains. The SH2 domain is responsible for recruitment of Grb2 to tyrosine phosphorylated receptors. The N-terminal SH3 domain recognizes a P-x-x-P-x-R binding motif, which is found in a number of signaling proteins, including SOS and c-Cbl, while the consensus-binding motif for the C-terminal SH3 domain is P-x-x-x-R-x-

Fig 2

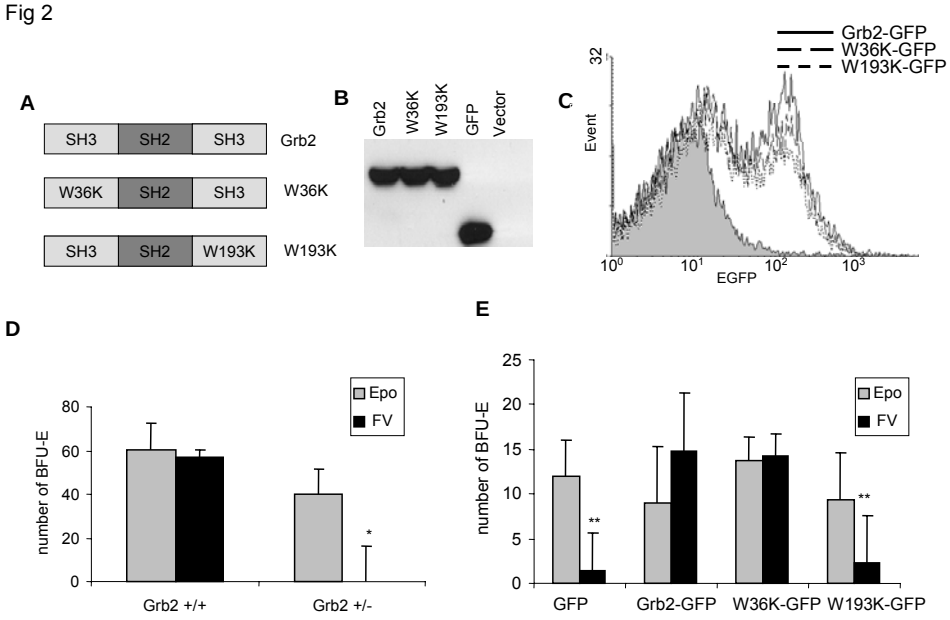

Figure 2.2 The C-terminal SH3 subdomain of Grb2 is required for FV-induced colony formation. (*A*) Schematic of wild-type and mutant Grb2 proteins used in this study. (*B*) Expression of wild-type and mutant Grb2 in 293T cells. C) Total bone marrow from Grb2 +/- and wild-type control mice were infected with vector (shaded area), Grb2-GFP, W36K-GFP and W193K-GFP. Infection efficiency was determined by flow cytometry for GFP. D) Wild-type and Grb2+/- bone marrow was plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of either FV or Epo (1 U/ml). E)Total bone marrow from Grb2 +/- mice was transduced with vector or MSCV-neo-EGFP-Grb2 with or without SH3 domain mutations and plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E were stained with acid-benzidine and scored on day 5. Bars denote the mean ± S.D. of three independent experiments performed in replicates of three and reflect normalized values where the number of Epo-dependent colonies was set at 100%. *$p<0.05$, ***$p<0.001$. Figure (A) (C) (D) were provided by Hami E. Teal.

x-K-P (63, 69, 77). In order to map the subdomains of Grb2 that are required for FV induced Epo-independent growth of erythroid progenitor cells, we mutated a conserved tryptophan in either the N- or C-terminal SH3 domain to lysine, which abrogates the ability of the SH3 domains to bind ligand. Retroviral-mediated expression of the Grb2 N-terminal SH3 subdomain mutant, W36K, in bone marrow from Grb2 +/- animals was fully capable of rescuing the ability of these cells to form BFU-E in response to FV infection. Conversely, the expression of the Grb2 C-terminal SH3 subdomain mutant, W193K, in Grb2 +/- bone marrow failed to support Epo-independent erythroid colony formation in response to FV infection (Figure 2.2 E). These results suggest that the C-terminal SH3 domain of Grb2 is essential for the ability of FV to drive the Epo-independent growth of these cells.

2.4.3 Gab2 is a downstream target of Sf-Stk signaling

Previous work has demonstrated that the adapter proteins, Gab and SLP76, interact with the C-terminal SH3 subdomain of Grb2 (69). The Gab family of adapter proteins consists of three members in mammalian cells, Gab1, Gab2 and Gab3. All three Gab proteins are composed of an N-terminal PH domain, two proline-rich regions and numerous tyrosines that, upon phosphorylation, serve as docking sites for signaling proteins including p85 and SHP-2 (51, 75). These adapter proteins are thought to play a role in the amplification of signals initiated by RTKs. To determine whether the Gab family of proteins could be a target of Grb2 in FV-infected erythroblasts, we first examined the expression of Gab1, Gab2 and Gab3 in FV infected spleens of BALB/c mice by RT-PCR. All three Gab genes were found to be expressed in FV infected

spleens and, to a lesser extent, in uninfected liver and spleen. These data suggest that Gab family members are expressed in the cells that repopulate the spleen in the course of FV infection and could therefore be targets for the C-terminal SH3 domain of Grb2 in FV-infected cells.

Both Gab1 and Gab2 have been demonstrated to signal downstream of the MET family of receptor tyrosine kinases. While Gab1 binds directly to the MET and RON receptors through the Met binding domain (MBD), Gab2, which lacks a MBD, is indirectly recruited to RTKs through its interaction with Grb2. Overexpression of the MBD from Gab1, which blocks recruitment of Gab1 to MET and RON, in primary erythroid progenitor cells failed to inhibit colony formation in response to Friend virus infection (data not shown). Conversely, Gab2 has been shown to play a critical role in the transformation of cells by the chicken homologue of STK, c-Sea. Therefore, we analyzed the ability of Gab2 to co-immunoprecipitate with Sf-Stk. 293T cells were transfected with plasmids expressing myc-Sf-Stk and mGab2 and cell lysates were immunoprecipitated with anti-myc or anti-Gab2 and probed with anti-phosphotyrosine, anti-myc or anti-Gab2 (Figure 2.3 A). Data from these studies clearly indicate that Gab2 co-immunoprecipitates with Sf-Stk in these cells and that Gab2 becomes tyrosine phosphorylated in the presence of Sf-Stk. We failed to detect a similar interaction when Sf-Stk was co-transfected with Gab1 in this system (Figure 2.3 C). The interaction of Gab2 and Myc/Sf-Stk in primary infected cells was confirmed using spleens from FV-infected animals (Figure 2.3 D).

2.4.4 Recruitment of Gab2, but not Gab1, by Sf-Stk is sufficient to support the growth of primary erythroblasts in response to Friend virus infection

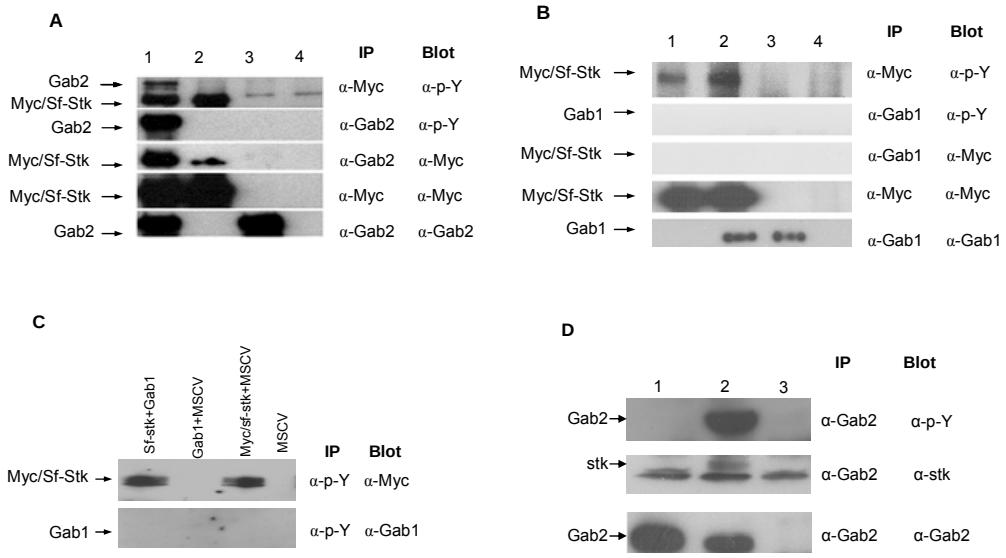

Figure 2.3 Gab2 is present in spleens from FV-infected mice and co-immunoprecipitates with Sf-Stk. (A) 293T cells were transiently transfected with plasmids expressing (1) Myc-Sf-Stk and Gab2, (2) Myc-Sf-Stk, (3) Gab2 and (4) vector control. Cell lysates were immunoprecipitated with anti-Myc or anti-Gab2 antibodies and subsequently analysed by SDS–PAGE and Western blotting with antiphosphotyrosine, anti-Gab2 or anti-Myc antibody. (B) 293T cells were transiently transfected with plasmids expressing (1) Myc-Sf-Stk and Gab2, (2) Myc-Sf-Stk, (3) Gab2 and (4) vector control. Cell lysates were immunoprecipitated with anti-Myc or anti-Gab1, and subsequently analysed by SDS–PAGE and Western blotting with antiphosphotyrosine, anti-Gab1 and anti-Myc. (C) 293 cells were transiently transfected with (1) Sf-Stk and Gab1, (2) Gab1, (3) Sf-Stk and (4) vector control. Cell lysates were immunoprecipitated with antiphosphotyrosine, resolved by SDS–PAGE and probed with anti-Myc or anti-Gab1. (D) Splenocytes from day14 FVP-infected mice were immunoprecipiated with anti-Gab2 and blotted with antiphosphotyrosine, anti-Stk or anti-Gab2 as indicated. Lane 1, uninfected wild-type mice; Lane 2, FVP-infected wild-type mice; Lane 3, FVP-infected Gab2-/- mice. Figure (A) was provided by Jie Xu.

In order to determine whether the recruitment of Gab proteins by Sf-Stk is sufficient to support Friend virus induced growth, we generated Sf-Stk/Gab fusions in which the C-terminal docking site tyrosines Y429 and Y436 of Sf-Stk were replaced with the coding sequence of Gab1 or Gab2 (Figure 2.4 A). We have shown that Y436 is essential for the growth of primary erythroblasts in response to FV infection in vitro. Therefore, the absence of this docking site tyrosine in the context of the fusion proteins would prevent recruitment of other signaling molecules to this site. We removed the PH domain of Gab1 and Gab2 in these constructs because this domain is generally involved in recruiting these scaffolding proteins to the membrane and this should be accomplished by fusion with Sf-Stk. Retroviral-mediated expression of the Sf-Stk/Gab2 fusion in cells from C57Bl/6 mice lacking endogenous Sf-Stk, rescued the ability of these cells to form Epo-independent colonies in response to Friend virus infection. However, expression of the analogous Sf-Stk/Gab1 fusion protein in these cells repeatedly failed to support the growth of FV infected erythroblasts (Figure 2.4 C). Both of these fusion proteins were expressed at similar levels and efficiently induced the phosphorylation of Akt and Erk in transiently transfected 293T cells (Figure 2.4 B). The kinase activity of Sf-Stk is required for the ability of Sf-Stk/Gab2 to support Epo-independent colony formation as demonstrated using a kinase-dead fusion protein (Figure 2.4 D), suggesting that Sf-Stk kinase activity is required for more than the recruitment of Gab2 to the docking site tyrosines.

We and others have shown previously that activation of the Erk and PI3K signaling pathways by this family of receptors is required for cellular transformation. Both Gab1 and Gab2 contain three p85 binding sites, resulting in PI3K activation, and

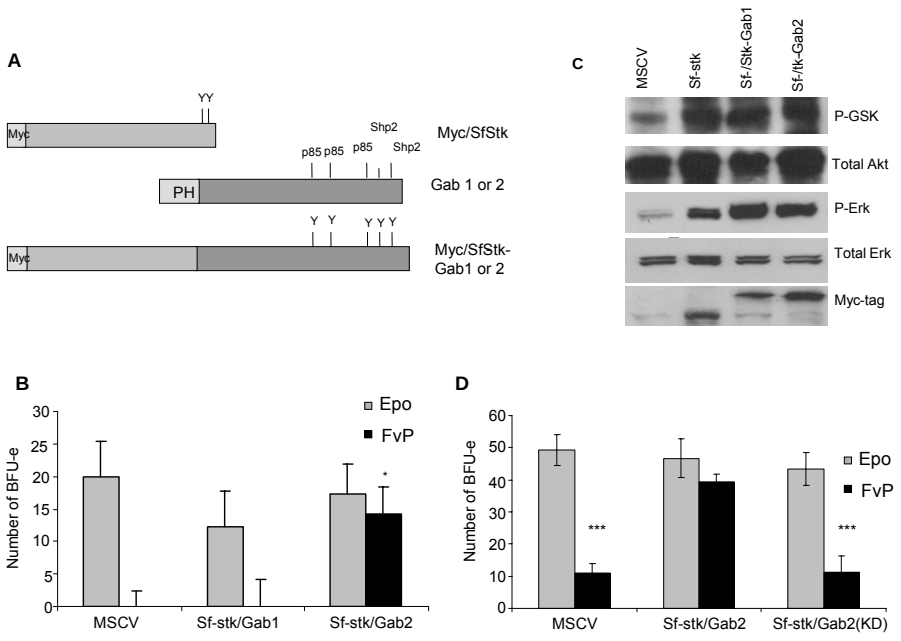

Figure 2.4 Retroviral expression of a Sf-Stk-Gab2 fusion protein supports FV-induced colony formation in C57BL/6 mice. (A) Schematic of WT and fusion Sf-Stk and Gab proteins (B) Total bone marrow from C57Bl/6 mice was transduced with MSCVvector, MSCV-myc-Sf-Stk/Gab1 and MSCV-myc-Sf-Stk-Gab2. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence/absence of FV or Epo (1 U/ml). BFU-E were stained with acid-benzidine and scored on day 5. The data is representative of three independent experiments and the s.e. bars denote the mean $\pm$s.d. of three replicates. (C) 293T cells were transiently transfected with empty vector, myc-Sf-Stk, myc-Sf-Stk/Gab1 or myc-Sf-Stk/Gab2. Cell lysates were probed with antiphospho-Akt, anti-Akt, antiphospho-Erk, anti-Erk and anti-Myc. (D) Total bone marrow from C57Bl/6 mice was transduced with Sf-Stk/Gab2 or KDSf-Stk/Gab2. Cells were plated and BFU-E were analysed as described in (B). *P<0.05, ***P<0.001.

two Shp2 binding sites that lead to the activation of Erk through an as yet unidentified mechanism. Therefore, we mutated either the p85 or Shp2 docking sites in Gab2 in the context of the Sf-Stk/Gab2 fusion protein and tested the ability of these mutants to support Friend virus-induced colony formation. Mutations in the three p85 binding sites (Figure 2.5A) or two Shp2 binding sites (Figure 2.5B) resulted in reduced colony number, but not colony size (data not shown), in response to FV infection, indicating that these two signaling pathways play a key role in the transformation of erythroid progenitor cells. Protein expression of the mutant proteins was confirmed in the packaging cells with anti-Myc (Figure 2.5C and D), and equal infection efficiency in primary bone marrow cells was confirmed by flow cytometry with anti-Myc following cell permeabilization (Figure 2.5E). Interestingly, mutation of either the p85 or Shp2 binding sites in Gab2 resulted in both reduced Erk phosphorylation and Akt activity in 293T cells (Figure 2.5F).

2.4.5 Gab2 is required for the efficient expansion of Friend virus infected cells in vitro and in vivo

To further study the potential role of Gab2 in the growth of Friend virus infected cells, we examined the effect of a targeted mutation in Gab2 on the progression of Friend disease. Mice homozygous for a deletion in Gab2 on a mixed genetic background (Figure 2.6A) were crossed to the FV2 sensitive Balb/c strain of mice and resultant heterozygous mice were bred to obtain progeny with varying alleles of Gab2 and Fv2 (Figure 2.6B). The mice were injected with FV i.v. and spleen size was determined two weeks later. Data from these studies indicate that, while the Gab2 mutation had no effect on the response of FV2-resistant strains of mice to Friend virus, Gab2 -/- animals on a

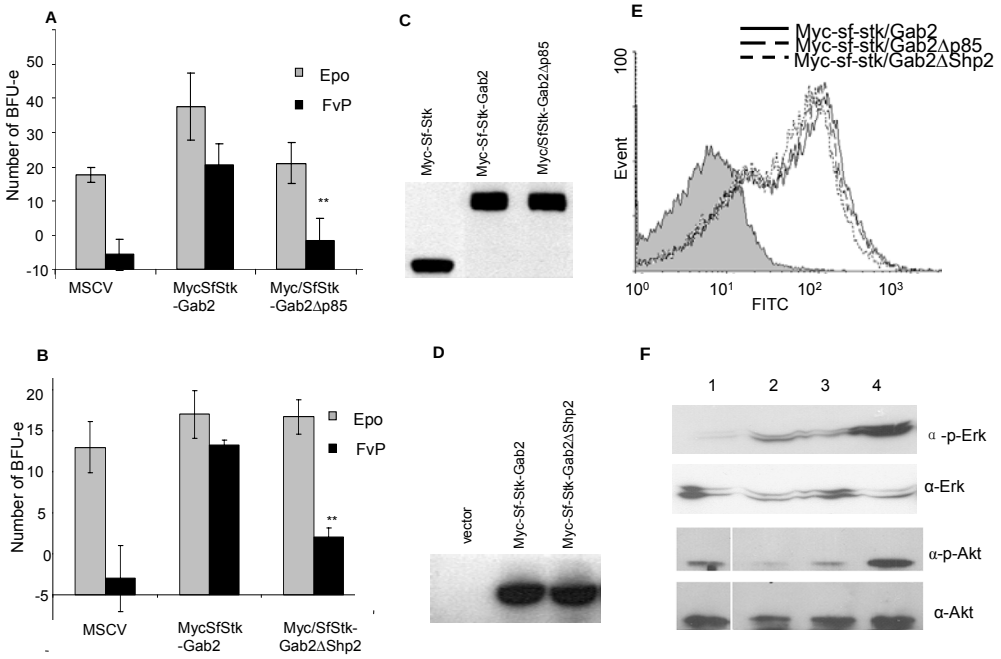

Figure 2.5 Efficient colony formation of Friend virus-infected cells in the presence of Sf-Stk-Gab2 requires the p85- and Shp2-binding sites of Gab2. (A) Total bone marrow from C57BL/6 mice was transduced with empty vector, Myc-Sf-Stk/Gab2 or Myc-Sf-Stk/Gab2-harboring Y to F mutations at the three p85-binding sites (Myc-Sf-Stk/Gab2Dp85). Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence/absence of FV or Epo (1U/ml). (B) Total bone marrow from C57BL/6 mice was transduced with empty vector, Myc-Sf-Stk/Gab2 or Myc-Sf-Stk/Gab2 harboring Y to F mutations at the two Shp2-binding sites (Myc-Sf-Stk/Gab2DShp2). Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence/absence of FV or Epo (1 U/ml). BFU-E were stained with acid-benzidine and scored on day 5. The data shown is representative of three independent experiments and s.e. bars denote the mean7s.d. of three replicates. **P<0.01. (C) Expression of Myc-Sf-Stk, Myc-Sf-Stk/Gab2 and Myc-Sf-Stk/Gab2Δp85 in 293T cells. (D) Expression of Myc-Sf-Stk/Gab2 and Myc-Sf-Stk/Gab2ΔShp2 in 293T cells. (E) Total bone marrow from C57BL/6 mice was transduced with vector (shaded area), Myc-sf-stk/Gab2, Myc-sf-stk/Gab2Δp85 or Myc-sf-Stk/Gab2ΔShp2. Transduced cells were permeabilized and stained with anti-Myc antibody, followed by an FITC-conjugated secondary antibody. (F) 293T cells were transiently transfected with (1) vector control, (2) Myc-Sf-Stk/Gab2Δp85, (3) Myc-Sf-Stk/Gab2ΔShp2 or (4) Myc-Sf-Stk/Gab2. Erk phosphorylation was examined using antiphospho-Erk. Blots were stripped and reprobed for total Erk. Akt was immunoprecipiated with anti-Akt, and resulting lysates were blotted with antiphospho-Akt. Blots were stripped and reprobed for Akt. Figure (A) (B) (C) (D) were provided by Jie Xu.

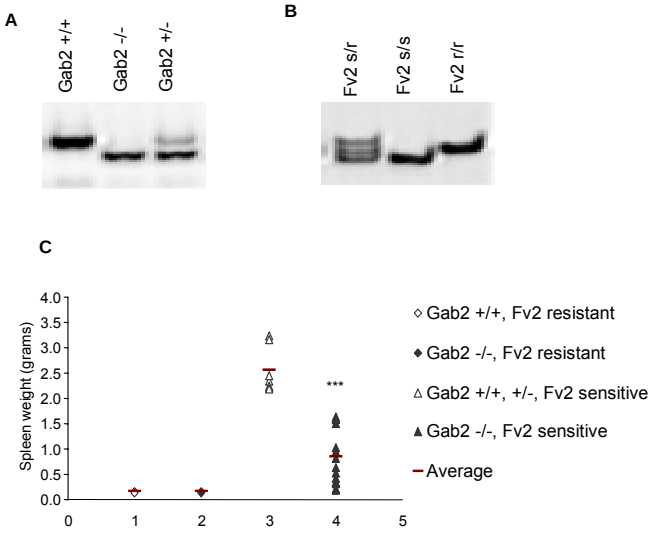

Figure 2.6 Gab2-/- mice exhibit reduced susceptibility to Friend virus in vivo. Gab2-/- mice on a mixed genetic background were crossed with sensitive BALB/c mice and the Fv2 locus was genotyped. Mice (8–10 weeks old) of Gab2-/-, and WT genotype (A) on Fv2-sensitive and resistant backgrounds (B) were injected with 200 ml FVP virus supernatant collected from FP63 cells. Spleens were weighed 2 weeks postinjection (C). Lanes: (1) Gab2 +/+, Fv2 resistant, (2) Gab2-/-, Fv2 resistant, (3) Gab2 +/+, +/-, Fv2 sensitive, (4) Gab2-/-, Fv2 sensitive. Figure was provided by Jie Xu.

FV2-sensitive background were unable to support the efficient growth of infected cells in the spleen when compared to wild-type and Gab2 +/- mice (Figure 2.6 C). These data support a role for Gab2 in the progression of Friend virus-induced erythroleukemia *in vivo.*

To determine whether Gab2 is required for the expansion of infected erythroid progenitor cells *in vitro*, we infected bone marrow cells from wild-type and Gab2 -/- mice with Friend virus and assessed colony formation in the presence or absence of Epo. While bone marrow cells from wild-type controls formed Epo-independent colonies in response to FV infection as expected, the virus failed to induce colony formation following infection of bone marrow cells from Gab2 -/- mice (Figure 2.7A). The defect in Gab2 -/- erythroblasts could be rescued by exogenous expression of wild-type Gab2, however Gab1 in these cells did not rescue this defect (Figure 2.7B). Both Gab1 and Gab2 were efficiently expressed in 293T packaging cells (Figure 2.7C). Mutation of either the p85 or Shp2 binding sites in wild-type Gab2 resulted in reduced colony formation (Figure 2.7D), reflecting the previous results using the chimeric proteins. Taken together, these data demonstrate that recruitment of Gab2, but not Gab1, to Sf-Stk is sufficient to support the growth of primary erythroblasts in response to Friend erythroleukemia virus.

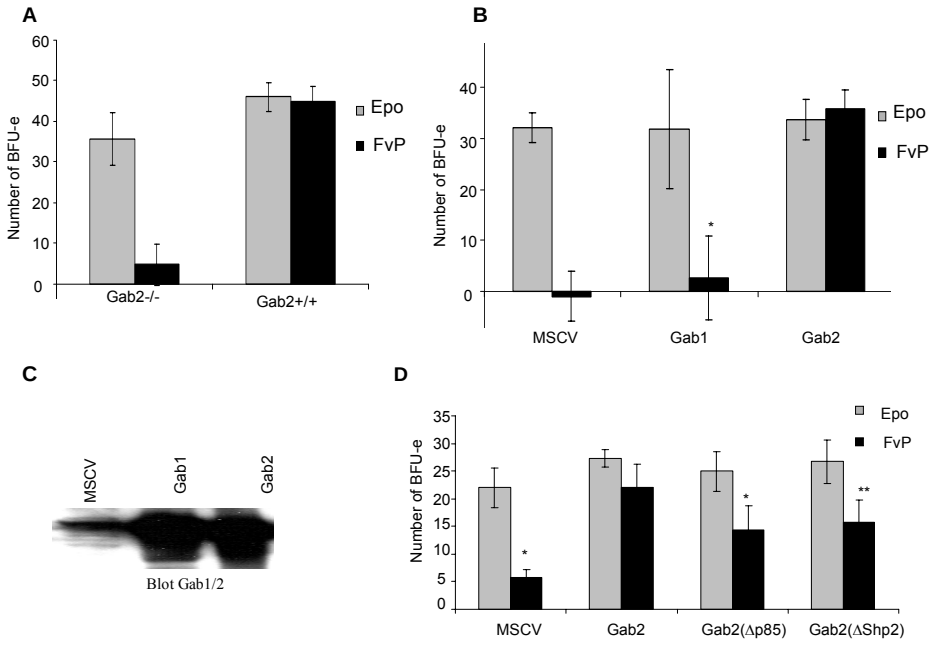

Figure 2.7 Gab2-/- erythroblasts fail to form colonies in response to FV in vitro. (A) Total bone marrow from wild-type and Gab2-/- mice was incubated with or without FVP and plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence or absence of Epo. (B) Total bone marrow from Gab2-/- mice was transduced with empty vector, Gab1 or Gab2 followed by infection with FVP and plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence or absence of Epo. BFU-E were stained with acid-benzidine and scored on day 8. The data shown are representative of three independent experiments and s.e. bars denote the mean7s.d. of three replicates. (C) Expression of Gab1 and Gab2 in transiently transfected 293 cells. (D) Total bone marrow from Gab2-/- mice was transduced with empty vector, Gab2, Gab2Δp85 or Gab2ΔShp2. Cells were plated and BFU-E were assessed as described in (B). *P<0.05, **P<0.01.

2.5 Discussion

Grb2 has emerged as a central adapter molecule in RTK signaling in both invertebrates and mammals. Grb2 is recruited to phosphotyrosine motifs through a central SH2 domain, leading to the activation of the MAPK and PI3K pathways via binding of flanking SH3 domains to SOS and a number of adapter proteins, including the Gab family. While gene-targeting experiments demonstrated a requirement for Grb2 in endoderm differentiation at day 4.0, this defect could be rescued by a Grb2/SOS fusion protein indicating that the primary role of Grb2 in early development may be to activate the ras/MAPK pathway (22). Subsequent studies have revealed gene dosage-dependent functions for Grb2. A hypomorphic mutation, analogous to one characterized in the *C.elegans* Grb2 ortholog *Sem-5*, which reduces binding of the SH2 domain to phosphotyrosine, results in perinatal lethality, and fibroblasts from these mice showed a defect in Erk activation and Gab1 tyrosine phosphorylation (114, 123). In this report, we show that Grb2 +/- animals exhibit decreased susceptibility to Friend erythroleukemia, adding to the growing list of defects observed in the adult animal due to Grb2 haplo-insufficiency. Futhermore, our data indicate that the primary role of Grb2 in this process is to recruit Gab2 to the Sf-Stk receptor.

The Gab (Grb2-associated binder) family of adapter proteins are the mammalian homologues of *Drosophila* DOS (daughter of sevenless) and *C.elegans* Soc1, which are critical for signaling downstream of RTKs and thus development of the fly and nematode, respectively (49, 101, 118). There are three mammalian Gabs and the critical nature of these proteins in RTK signaling in mammals was highlighted by the phenotype of the Gab1 knockout mice. These mice are embryonic lethal, exhibiting developmental defects

similar to those observed in mice homozygous mutant for the MET RTK, with which Gab1 interacts directly through its Met binding domain (MBD) (13, 109, 129). While Gab2 and Gab3 lack a MBD, all of the Gab proteins share a proline-rich domain that serves as a consensus Grb2 binding site (44). Gab1 recruitment to the EGF receptor is dependent upon the Grb2 binding site, and cytokine receptors that utilize the β_c chain recruit Gab2 through a SHC-Grb2-Gab2 interaction (43, 77, 104). Our data indicate that Sf-Stk recruits Gab2 through an indirect interaction with Grb2, and that the ability of Sf-Stk/Grb2 to recruit Gab2 is critical for the progression of Friend disease. However, the Gab2 -/- mice are not completely resistant to FV induced disease as demonstrated by the enhanced spleen size when compared with FV2 resistant controls. The residual growth of infected cells in the Gab2 -/- animals could be due to compensation by other adaptor proteins, or the retained ability of Grb2 to recruit SOS. Interestingly, transformation by BCR/Abl has also recently been shown to depend on the recruitment of a Grb2/Gab2 complex, suggesting the potential of a broader role for Grb2/Gab2 in the development of myeloid leukemias (113).

All Gab family members contain multiple p85 binding sites that become phosphorylated upon receptor activation (75), and we demonstrate here that the p85 binding sites in Gab2 are required for efficient cytokine-independent growth of Friend virus infected erythroblasts. The ability of Gab proteins to amplify Sf-Stk signaling through the activation of PI3K is consistent with previous findings from our laboratory that demonstrate a requirement for PI3K activation in the transformation of cells by FV (36, 95). Our data also extend previous studies demonstrating constitutive tyrosine phosphorylation of Gab1 and Gab2 and their association with p85 in FV-infected cells

grown in the absence of Epo (66, 92, 134). In these cells, PI3K is constitutively active, and PI3K activity, but not EpoR phosphorylation, is required for the proliferation of these cells in the absence of Epo (92). Furthermore, *v-sea*, which induces erythroleukemia in chickens, preferentially interacts with Gab2 and is essential for cellular transformation induced by *v-sea* (55), and mutagenesis studies revealed a critical role in *v-sea* induced transformation for PI3K activation (95). EpoR stimulation has also been shown to result in the phosphorylation of Gab1 and Gab2 in HCD57 and UT7 cells, however there was no significant impairment of the Gab2 -/- erythroblasts to respond to Epo stimulation, and gp55-mediated growth of primary erythroblasts was more sensitive to inhibition of the p85-PI3K pathway than Epo-induced growth. Therefore, we conclude that the Grb2/Gab2/p85 signaling pathway downstream of Sf-Stk is specifically required for the transformation of erythroid progenitor cells by Friend virus.

In addition to the Grb2 binding site, all Gab proteins contain an N-terminal PH domain with sequence similarity to the Btk PH domain which recognizes primarily PIP3, the lipid products of PI3K (54). Therefore, the requirement for PI3K signaling in the transformation of cells by FV could also reflect a role for this signaling pathway in the recruitment of Gab2. A PH domain deletion mutant of Gab1 which fails to support Met-induced morphogenesis, fails to translocate in a PIP3-dependent manner, and a Met/Gab1 fusion protein lacking the PH domain fully supports this process (77). Conversely, while mutations in the PH domain of DOS inhibits its ability to function in rescue experiments, this mutation does not affect its ability to localize to the membrane suggesting that the PH domain of DOS may also play a role in propagating downstream signals (7, 32, 118). While overexpression of a PH domain from Gab1 blocked the ability of FV-infected bone

marrow to form Epo-independent colonies (unpublished observation), deletion of the PH domain of Gab2 in our fusion proteins did not appear to affect the ability of Sf-Stk/Gab2 to support the Epo-independent growth of infected cells. It is therefore likely that the PH domain is required for the recruitment of Gab2 to the signaling complex, but not for generating further signaling events.

The activation of MAPK is also required for the transformation of cells by FV, and previous studies have demonstrated that while Gab2-mediated transformation of cells in response to *v-sea* requires PI3K activation, efficient transformation also requires binding of Gab2 to bind the SH2-containing protein phosphatase, SHP-2 (55). In addition, constitutively active forms of SHP-2 have been implicated in the progression of some human leukemias (44). All Gab proteins contain two tandem tyrosines in the C-terminal portion of the protein which, upon phosphorylation, result in the recruitment of SHP-2. In our studies, mutation of these two tyrosines in the context of the Sf-Stk/Gab2 fusion protein, reduced the ability of this fusion to support the growth of primary erythroblasts in response to Friend virus. Mutation of these tyrosines, but not the p85 binding sites, in DOS and Soc1 also failed to rescue loss-of-function alleles, suggesting a critical role for the recruitment of SHP-2 by Gab (50, 118). Furthermore, SHP-2 binding sites in Gab are critical for the induction of branching morphogenesis in epithelial cells by Met (77, 109). SHP-2 contains two tandem SH2 domains and the N-terminal SH2 domain serves an auto-inhibitory function in the absence of binding to phosphorylated tyrosines on adjacent proteins (153). Recruitment of SHP-2 by Gab most likely has the dual function to activate the PTP activity of SHP-2 and to bring SHP-2 in proximity to its substrates at the membrane. Interestingly, like the Grb2-SOS-Ras-MAPK pathway,

recent studies have suggested that the Grb2-Gab1-SHP-2 pathway results in the activation of Erk, and may play a role in the enhanced or sustained activation of this signal (28).

The three mammalian Gab proteins, despite their high degree of homology, appear to play largely non-overlapping roles *in vivo* (137). Mice with targeted deletions in Gab2 and Gab3, unlike Gab1, are viable. Gab2-deficient mice display defects in allergic responses due to defective IgE receptor signaling in bone marrow-derived mast cells, whereas Gab3-deficient mice appear normal to date (90, 137). Furthermore, while both Gab1 and Gab2 have similar effects on the activation of Erk by growth factor stimulation, they have reciprocal effects in mediating Elk induction (17). In this study, the resistance of Gab2 -/- mice to Friend virus was not absolute, supporting the presence of other compensating signals, perhaps provided by other Gab family members. However, the exogenous expression of Gab1 failed to rescue the Epo-independent growth of Friend virus-infected erythroblasts from Gab2 -/- mice *in vitro*. It will therefore be of future interest to take advantage of this system in order to examine the functional similarities and/or differences in signaling through the Gab family of adaptor proteins and to map the domains of Gab2 required for the transformation of primary hematopoietic cells.

2.6 Acknowledgements

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Chapter 3

A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/Ron receptor tyrosine kinase in response to Friend virus infection.

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3.1 Abstract

Friend erythroleukemia virus has long served as a paradigm for the study of the multistage progression of leukemia. Friend virus infects erythroid progenitor cells, followed by an initial polyclonal expansion of infected cells, which is driven by the activation of a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). Subsequently, the accumulation of additional mutations in p53 and the activation of PU.1 result in full leukemic transformation. The early stages of transformation induced by Friend virus are characterized in vitro by the Epo-independent growth of infected erythroblasts. We have shown previously that this transforming event requires the kinase activity and Grb2 binding site of Sf-Stk and the recruitment of a Grb2/Gab2 complex to Sf-Stk. Here, we demonstrate that Stat3 is required for the Epo-independent growth of Friend virus-infected cells and that the activation of Stat3 by Sf-Stk is mediated by a

novel Stat3 binding site in Gab2. These results underscore a central role for Stat3 in hematopoietic transformation and describe a previously unidentified role for Gab2 in the recruitment and activation of Stat3 in response to transforming signals generated by tyrosine kinases.

3.2 Introduction

Friend virus induces in mice acute erythroleukemia characterized by two distinct stages, an initial polyclonal expansion of infected erythroid progenitor cells mediated by the viral glycoprotein gp55, followed by the acquisition of additional genetic alterations, including the mutation of p53 and retroviral insertion into the PU.1 locus, resulting in the expansion of leukemic clones (88). Genetic analysis has led to the identification of several signaling pathways required for the early stage of progenitor cell expansion in response to viral infection. The *W*, *Sl*, *Fv2*, and *f* loci have been demonstrated to regulate the expansion of infected progenitor cells in vivo. The *W* locus, encoding the Kit receptor tyrosine kinase, is required for the normal development of target cells for the virus in the spleen (124), whereas an analysis of the *f* locus, encoding Madh5 (67), has suggested a role for the BMP4/Smad5 signaling pathway in the acquisition of additional target cells in the spleen following infection (A. Subramanian, submitted for publication).

The Friend virus susceptibility locus 2, *Fv2*, encodes a naturally occurring, N-terminally truncated form of the Stk receptor tyrosine kinase, the murine homologue of the human Ron receptor, called Sf-Stk (97). The expression of Sf-Stk in erythroid progenitor cells is driven by an internal promoter, and the translation of this transcript generates a protein that lacks the extracellular ligand binding domain of full-length Stk, while retaining the transmembrane and kinase domains. C57BL/6 mice harbor mutations in the internal promoter in the gene encoding Stk, resulting in reduced Sf-Stk expression and Friend virus resistance. Sf-Stk is required for Epo-independent colony formation induced by Friend virus infection. Exogenous expression of Sf-Stk rescues the Epo-

independent growth of Friend virus-infected erythroid progenitor cells in vitro (36) and renders C57BL/6 mice susceptible to Friend erythroleukemia in vivo (97).

The viral glycoprotein gp55, from the spleen focus forming virus, interacts with the erythropoietin receptor (EpoR) (70) and Sf-Stk (93), and this signaling complex drives the Epo-independent expansion of Friend virus-infected cells. We have shown previously that the kinase domain and Grb2 binding site of Sf-Stk are required for this response to Friend virus infection (36) and that a Grb2/Gab2 complex downstream of Sf-Stk mediates the growth of primary erythroid progenitor cells infected in vitro with Friend virus (127). Recent studies have demonstrated that mice harboring a human EpoR knocked in to the murine locus (which fails to interact with gp55) develop leukemia, but not the characteristic polycythemia, in response to Friend virus infection (149). Taken together, these data suggest that signaling through Sf-Stk plays a central role in the polyclonal expansion of infected progenitor cells, whereas EpoR regulates the differentiation of these cells.

The activation of Stat5 by EpoR has been extensively studied and plays a key role in the differentiation of erythroid progenitor cells in response to Epo. However, while Epo and gp55 both lead to the activation of Stat5 in erythroid cells, nuclear translocation and DNA binding of Stat5 are not observed in response to gp55, suggesting that Stat5 may not play an essential role in the transmission of the cell growth signals in gp55-induced erythroleukemia cells (141). Furthermore, mice with a targeted deletion in Stat5 develop erythroleukemia in response to the polycythemia-inducing strain of Friend virus (FVP), but without the characteristic development of polycythemia (149), indicating that

like EpoR, Stat5 is required for the differentiation, but not the expansion, of Friend virus target cells (149).

Stat1 and Stat3 are also activated by EpoR and have been suggested to play distinct roles in erythropoiesis (60, 61), although the mechanism of tyrosine phosphorylation of Stat1 and Stat3 by EpoR is distinct from that of Stat5 (46, 59). Mice harboring a targeted deletion in Stat1 exhibit overall reductions in erythroid progenitor cells (45); however, the role of Stat3 in erythropoiesis in vivo has not been elucidated. Constitutive activation of Stat1 and Stat3 has been demonstrated in primary erythroleukemic cells (58) and cell lines infected with spleen focus forming virus (94). Taken together, these data suggest potential roles for Stat1 and Stat3 in regulating the aberrant growth of erythroid progenitor cells in Friend disease.

Here, we set out to determine the role of Stat3 in the progression of Friend erythroleukemia. We demonstrate that Stat3 is required for the Epo-independent growth of Friend virus-infected erythroid progenitor cells. Furthermore, we identified a novel Stat3 binding site in Gab2 which is required for this process, suggesting that the activation of Stat3 in Friend virus-infected cells is mediated by an Sf-Stk/Grb2/Gab2 signaling pathway. These studies highlight a critical role for Stat3 activation in the progression of Friend erythroleukemia and identify a novel mechanism for the activation of Stat3 downstream of tyrosine kinases. Interestingly, Gab2 is also required for the transformation of hematopoietic cells by BCR/Abl (113), which suggests that the observations described here could have broader implications for the function of Gab2 in various hematopoietic malignancies.

3.3 Experimental Procedures and Preparation

3.3.1 Mice.

Gab2-/- mice, on an FV2-sensitive BALB/c background, were genotyped as previously described (127). Floxed stat3 mice (111) on a C57BL/6 background were obtained from Charles Drake (John Hopkins University) and were crossed for two generations onto the BALB/c background. Genotypes of Fv2 loci were determined by PCR with appropriate primers. For preparing genomic DNA, about 5 mm of mouse tails was clipped and lysed in lysis buffer (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 100µl proteinase K) at 55°C overnight. After centrifugation, 2 volumes of ethanol were added to cleared lysates to precipitate the genomic DNA. The pellet was washed with 75% ethanol, air dried, and resuspended in 100 µl of 10 mMTris-Cl, pH 8.0. For the genotype of the floxed Stat3 allele, the primer pair 5'-CCTGAAGACCAAGTTCATCTGTGTGAC-3' (forward) and 5'-ACACAAGCCATCAAACTCTGGTCTCC-3' (reverse) was used. The PCR parameters were 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min and 30 s. The PCR products were loaded onto a 1.5% agarose gel for electrophoresis and visualized with ethidium bromide staining. The wild-type allele amplifies a 200-bp fragment, and the floxed allele amplifies a 250-bp fragment. For the Fv2 loci, the primers were 5'-GGTGGGTTTAACGGTTAGGG-3' (forward) and 5'-TCTGGGCTCTGCCTCCTTAT-3' (reverse). The PCR parameters were 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were then loaded on a 12.5% acrylamide gel for electrophoresis to visualize the DNA fragments by ethidium bromide staining. The sensitive allele amplifies a 53-bp

fragment, and the resistant allele amplifies a 50-bp fragment. All research involving the use of mice was performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Pennsylvania State University.

For polyI/polyC injection, 8 week-old Cre-flox/Stat3 mice were injected with interferon-inducible polyI/polyC at 20µg/g of body weight in 200µg PBS. IP injections will be performed every other day for 6 days, for a total of 3 injections. Two weeks following pI/pC administration, the same mice will be injected with FVP.

For in vivo infection with Friend Virus, wild type mice were tail-vein injected with 200µl of FVP. Mice were sacrificed and spleens removed on different time point after injection. The spleen was weighted with an analytical balance and measured in grams.

3.3.2 DNA constructs.

Gab1/2 chimeric molecules were obtained by three-way ligation. The primers used were 1S1, 5'-CGGGGTACCATGTATGATGTTCCTGATTATGCTAGCCTCAGCGGTGGTGAAGTGGTCTG-3'; 1S2, 5'-CCAACAGAAGAAGATCCTGTG-3'; 1S3, 5'-CATCCAGCTCATGACCGATC-3'; 1S4, 5'-AACCCAAACCTGTCCAGTGAAG-3'; 1AS1, 5'-CCGGAATTCTCATTTCACACTCTTCGCTGGCG-3'; 1AS2, 5'-ATTAAACCCACAGATGTCACAAATAC-3'; 1AS3, 5'-TGGTTTCGGTGGCCGAGGTG-3'; 1AS4, 5'-CATGGGAACATAATTCTCTTCAC-3'; 2S1, 5'-CGGGGTACCATGTATGATGTTCCTGATTATGCTAGCCTCAGCGGCGGCGGCGACGTGG-3'; 2S2, 5'-CAGGCTGAGGAGAGCACAG-3'; 2S3, 5'-AGTCAGGCAGAAACACCTCG-3'; 2S4, 5'-CAAAACCCAGTGTCTGCATCTC-3';

2AS1, 5'-GCGGATCCTCTCATCACAGCTTG-3'; 2AS2, 5'-ATTGAAGCCACAGATCTGGCAG-3'; 2AS3, 5'-TGGCTTGGGGGGGCGGG-3'; and 2AS4, 5'-CATAGGGACATAGTTCT CTTCG-3'. Partial cDNA fragments of Gab1 and Gab2 were synthesized independently by PCRs with the appropriate primer pairs, digested with restriction enzymes, and inserted into the pcDNA3 vector. For each fragment, primer pairs were as follows: for CM1, Gab12-592 (1S1 and 1AS4) and Gab2588-676 (2S4 and 2AS1); for CM2, Gab12-348 (1S1 and 1AS3) and Gab2359-676 (2S3 and 2AS1); for CM3, Gab22-118 (1S1 and 1AS2) and Gab2120-676 (2S2 and 2AS1); for CM4, Gab22-119 (2S1 and 2AS2) and Gab1119-694 (1S2 and 1AS1); for CM5, Gab22-358 (2S1 and 2AS3) and Gab1349-694 (1S3 and 1AS1); and for CM6, Gab22-587 (2S1 and 2AS4) and Gab1593-694 (1S4 and 1AS1). For the purpose of ligation reactions, phosphate groups were added to the 5' end of the primers prior to PCRs. These oligonucleotides (250 pmol) were incubated with 10 U T4 polynucleotide kinase (Biolabs) and 1 mM ATP in a 25µl reaction volume for 30 min at 37°C. The enzyme was deactivated by 10 min of incubation at 70°C. MSCV-neo-myc/sf-stk, MSCV-neo-myc/sf-stk(Y429F), and MSCV-neo-myc/sf-stk(Y436F) were described previously (36).

MSCV-neo-myc/sf-stk(KD) was produced by site-directed mutagenesis using the Stratagene QuikChange kit (Stratagene), following the manufacturer's protocol. Using MSCV-neo-myc/sfstk as a template, the mutation was introduced with the oligonucleotide (sense) 5'-GCGTCCTAGACAAGGAATTCTTCAGTGTTCGCCAGCATC-3'.

The murine MSCV-neo-Gab2 and MSCV-neo-myc/sf-stk/Gab2 cDNAs were

described previously (34). The QuikChange mutagenesis kit was used to mutate MSCV-neo-Gab2 and MSCV-neo-myc/sf-stk/Gab2 to MSCV-neo-Gab2(Y/F) and MSCV-neo-myc/sf-stk/Gab2(Y/F), respectively, by using the oligonucleotide (sense) 5'-CACCTCAGGAGTATCTCTTCTTGCACCAGTGCATAAGC-3'. The MSCV-neo-myc/sf-stk(KD)/Gab2 cDNA was introduced by site-directed mutagenesis (Stratagene QuikChange kit) following the manufacturer's protocol. The template was MSCV-neo-myc/sf-stk/Gab2, and the primer was (sense) 5'-CCACTGTGCCATCATGTCTCTGAGTCGG-3'.

The dominant negatives pRc CMV-Stat3(Y705F) and pRc CMV-Stat3(ZZ/VVV) (kind gifts of C. Horvath, Northwestern University) were excised from the pRc cytomegalovirus vector with NotI and ApaI and subcloned into MSCV-neo at the HpaI site to produce MSCV-neo-Stat3(Y705F) and MSCV-neo-Stat3(ZZ/VVV). Wild-type MSCV-neo-Stat3 was generated by site-directed mutagenesis (Stratagene QuikChange kit) following the manufacturer's protocol. The template was MSCV-neo-Stat3(Y705F) and the primer was (sense) 5'-ACCTTCCTACTGCGCTTCAG-3'.

3.3.3 Transient transfection.

The 293T cell line was acquired from A Henderson (Pennsylvania State University) and grown in DMEM containing 10% FCS, penicillin/Streptomycin, and L-Glytamine. Cells were transiently transfected by TransIT-293 transfection reagent (Mirus Corporation) using 1-2 μg pEco and the appropriate MSCV-neo constructs (1-10 μg) for 48-72 hours at 37 °C prior to harvest of the viral supernatant. Protein expressions of plasmids were verified by western blot, by probing with antibodies.

3.3.4 In vitro infection and colony assay

For *in vitro* infection of bone marrow, cells were harvested from femurs and tibias of >6 week old BALB/c, C57BL/6 or Gab2-/- mice, and washed in PBS. Bone marrow was infected with viral supernatant from the transient transfections for 20 hours, as previously described (36). For Epo-independent colony analysis, total bone marrow cells from different strains of mice were harvested and incubated with supernatant from cells expressing polycythemia-inducing FV (derived from FP63 cells, Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada) or DMEM (10 % FBS, P/S, L-glu) on ice for 1 hour, as previously described(Teal et al, 2005). Cells were added to methocult media M3234 (Stem Cell Technologies) along with 2.5ng IL-3(Peprotech) in triplicate with or without 1 U/ml Epo (R&D Systems). Cultures were incubated for 2-8 days in 5 % CO_2 at 37 °C. Erythroid colonies (BFU-E and CFU-E) were visualized by acid-Benzidine staining as previously described (36).

3.3.5 Antibodies, Immunoprecipitation, and Western blotting.

Mouse anti-Myc (1:1000), rabbit anti-stat3 (1:1000), rabbit anti-phospho-stat3(Y705)(1:1000) antibodies were purchased from Cell Signaling. Mouse anti-Gab1 was purchased from Santa Cruz. Rabbit anti-Gab2, rabbit anti-phospho-Stat3 (S727) were purchased from Upstate. Rabbit-anti-β-actin was purchased from Sigma. Anti-myc was used to detect Myc/Sf-Stk, Myc/Sf-Stk-Gab2 fusion and mutants. Anti-Stat3 was used for immunoprecipitation and to detect Stat3. Anti-Gab2 was used for immunoprecipitation and to detect Gab2 and mutant. Anti-Gab1 and anti-Gab2 were used to detect Gab1/2 chimeric plasmids.

293T cells were transiently transfected with plasmids expressing desired protein. Twenty-four hours after transfection, cells were lysed in lysis buffer (1% NP-40, 150 mM NaCL,

1mM EDTA, 0.25% Deoxycholate, 2mM Na_3VO_4, 10 mM NaF, and 2mM PMSF). After 15 minutes centrifugation, the cleared cell lysates were incubated with antibodies and protein A (or G) -sepharose at 4°C overnight. The immunoprecipitates were analyzed by reduced SDS-PAGE and transferred to PVDF membrane. The blots were incubated with antibodies and detected with ECL (Amersham).

3.3.6 Flow cytometry analysis

Bone marrow cells were collected after in vitro infection, fixed with 2% paraformaldehyde for 10 minutes on ice and incubated with saponin buffer. Cells were stained with mouse-anti-myc antibody or rabbit anti-Gab2 following FITC-conjugated anti-mouse IgG2a (BD Pharmingen) or FITC-conjugated anti-rabbit IgG (kindly provided by Dr. Biao He). Stained cells were analyzes by flow cytometry.

3.4 Results

3.4.1 The region between Q120 and P358 of Gab2 is required for hematopoietic transformation and activation of Stat3 downstream of the Stk receptor.

Previous studies from our laboratory demonstrated a requirement for Gab2 in the transformation of primary hematopoietic cells by Friend erythroleukemia virus both in vitro and in vivo. Furthermore, we found that exogenous expression of Gab2, but not the closely related Gab1, rescued the ability of Gab2-/- hematopoietic progenitor cells to form cytokine-independent colonies in response to Friend virus infection, suggesting a unique role played by Gab2, but not Gab1, in hematopoietic transformation. Here, we set out to map the domains of Gab2 required for this response. A series of six Gab1/Gab2 chimeric proteins (Fig. 3.1A) were generated and tested for their abilities to rescue the Epo-independent colony formation of Gab2-/- hematopoietic cells in response to Friend virus infection. As shown in Fig. 3.1B, while Gab2-/- hematopoietic cells infected with empty vector (MSCV) failed to form Epo-independent colonies following Friend virus infection, the transduction of these cells with MSCV harboring wild-type Gab2 completely rescued this defect. Exogenous expression of the Gab1/Gab2 fusion proteins demonstrated that the region between amino acids Q^{120} and P^{358} in Gab2 is essential to support the transformation of Friend virus-infected erythroblasts.

The transformation of primary erythroblasts by Friend virus requires the activation of a truncated form of the Stk receptor tyrosine kinase (Sf-Stk). Previously, we demonstrated that Y^{436} in the C-terminal tail of Sf-Stk is required for its ability to mediate the transformation of infected cells. Further, we found that a fusion protein in which the C-terminal tail of Sf-Stk, including Y436, was replaced with Gab2 (Sf-Stk/Gab2) was

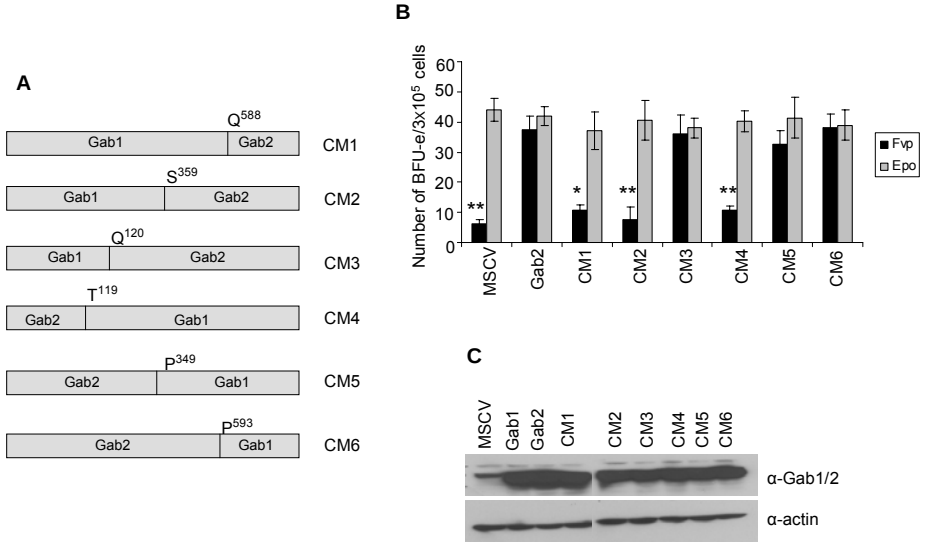


FIG. 3.1. The region between Q120 and P358 of Gab2 is essential to support the transformation of Friend virus-infected erythroblasts. (A) Schematic diagram of the chimeric Gab1/2 constructs used in this study. Gray bars, sequence from Gab1; black bars, sequence from Gab2. (B) Total bone marrow from Gab2-/- mice was transduced with empty vector (MSCV), Gab2, and the indicated Gab1/2 chimeras (CM1 to CM6). Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E cells were stained with acid-benzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means _ SD of one of three independent experiments performed in replicates of three and reflect normalized values. *, $P < 0.05$; **, $P < 0.01$. (C) Expression of Gab1, Gab2, and Gab1/2 chimeric proteins in 293T cells, determined by Western blot analysis using a mixture of anti-Gab1 and anti-Gab2 antibodies.

fully able to support Epo-independent growth induced by Friend virus, suggesting that the primary role of Y436 was to recruit Gab2 to the signaling complex. However, a similar fusion with Gab1 (Sf-Stk/Gab1) failed to support this response, even though both fusion proteins induced strong activation of Erk and Akt in 293 cells. Because Stat3 is often activated downstream of receptor tyrosine kinases, it is phosphorylated in Friend virus-infected cells and has been implicated in the progression of a number of malignancies; we set out to test whether the differences in the abilities of Sf-Stk/Gab1 and Sf-Stk/Gab2 fusions to support the Epo-independent growth of Friend virus-infected cells could be due to differences in activation of Stat3.

3.4.2 The Y194LHQ motif in Gab2 interacts with Stat3 and mediates tyrosine phosphorylation of Stat3 downstream of Stk.

To determine whether Stat3 is downstream of Sf-Stk, we coexpressed Sf-Stk and Stat3 in 293 cells. Here, we demonstrate that Sf-Stk induces tyrosine phosphorylation of Stat3 in 293 cells and that this phosphorylation is dependent on Y436 (Fig. 3.2A). In addition, the expression of the Sf-Stk/Gab2 fusion, but not Sf-Stk/Gab1, enhanced tyrosine phosphorylation of Stat3 in these cells (Fig. 3.2B). Therefore, we tested the abilities of the Gab1/Gab2 fusion proteins to mediate Stat3 activation when coexpressed with Sf-Stk. Interestingly, the ability of Gab2 to enhance tyrosine phosphorylation of Stat3 in the presence of Sf-Stk mapped to the same region of Stat3 that was required for hematopoietic transformation in response to Friend virus infection (Fig. 3.2C).

An analysis of the amino acid sequence in this region of Gab2 revealed a YxxQ motif (a potential Stat3 binding site) at position 194 (Fig. 3.3A), which is conserved in

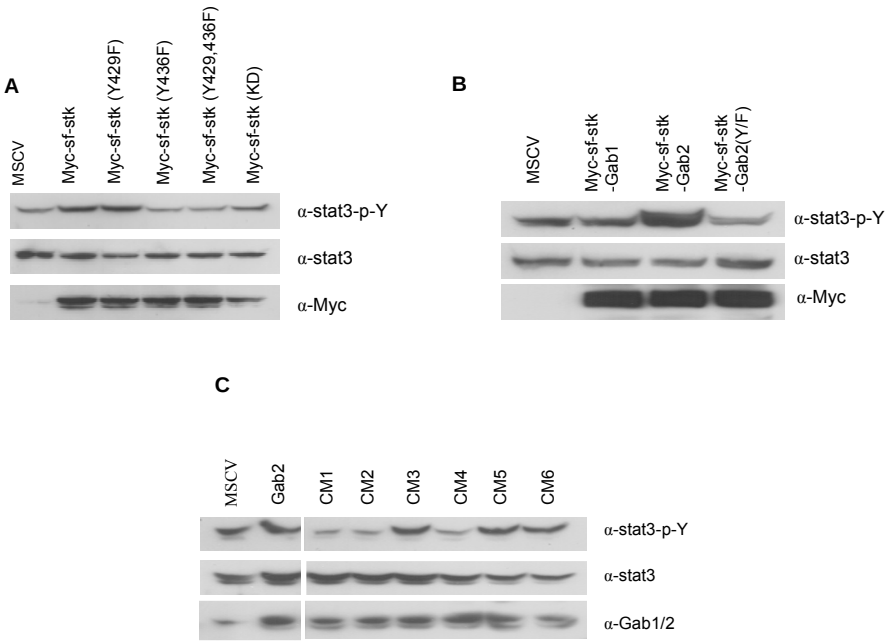


FIG. 3.2. The region between Q^{120} and P^{358} of Gab2 is required to mediate Stat3 activation when coexpressed with Sf-Stk. 293T cells were transiently cotransfected with Stat3 and plasmids expressing (A) wildtype or mutant forms of Sf-Stk or (B) wild-type or mutant forms of Sf-Stk/Gab fusions as indicated. Cell lysates were probed with antiphospho-Stat3, anti-Stat3, and anti-Myc. (C) 293T cells were transiently cotransfected with Myc-Sf-Stk, Stat3, and plasmids expressing the various Gab1/2 fusions as indicated. Cell lysates were probed with anti-phospho-Stat3, anti-Stat3, and anti-Gab1/2.

the human sequence. Therefore, we mutated this sequence to FxxQ in the context of both the Sf-Stk/Gab2 [Sf-Stk/Gab2(Y/F)] fusion protein and wild-type Gab2 [Gab2(Y/F)]. Figure 3.3B demonstrates that Sf-Stk/Gab2, but not Sf-Stk/Gab2(Y/F), immunoprecipitates with Stat3 in 293 cells. Further, the expression of wild-type Sf-Stk with Gab2 in 293 cells resulted in the coimmunoprecipitation of Sf-Stk with Stat3. However, while Sf-Stk retained the ability to recruit Gab2(Y/F) when coexpressed in 293 cells, Stat3 did not coimmunoprecipitate with Sf-Stk under these conditions (Fig. 3.3C). The expression of Sf-Stk/Gab2(Y/F) in 293 cells also failed to enhance tyrosine phosphorylation of Stat3 in these cells (Fig. 3.2B), indicating that the recruitment of Stat3 to this motif is required for the tyrosine phosphorylation of Stat3 downstream of Sf-Stk. We failed to detect the interaction of Sf-Stk with Stat1 or Stat5 in the presence of Gab2 in 293 cells, suggesting that this interaction is specific to Stat3 (data not shown).

3.4.3 Stat3 binding to Y194 is required for Friend virus-induced Epo-independent colony formation.

To determine whether the interaction of Stat3 with Gab2 downstream of Sf-Stk is required for the hematopoietic transformation induced by Friend virus, Sf-Stk/Gab2 and Sf-Stk/Gab2(Y/F) were exogenously expressed in primary hematopoietic progenitor cells from resistant C57BL/6 mice lacking endogenous Sf-Stk. Figure 3.4A shows that while Sf-Stk/Gab2 supported the Epo-independent growth of Friend virus-infected erythroblasts, Sf-Stk/ Gab2(Y/F) failed to support this response. Similarly, exogenous expression of Gab2 in hematopoietic cells from Gab2-/- mice rescued the Epo-independent growth of these cells in response to Friend virus infection, as shown previously, while the

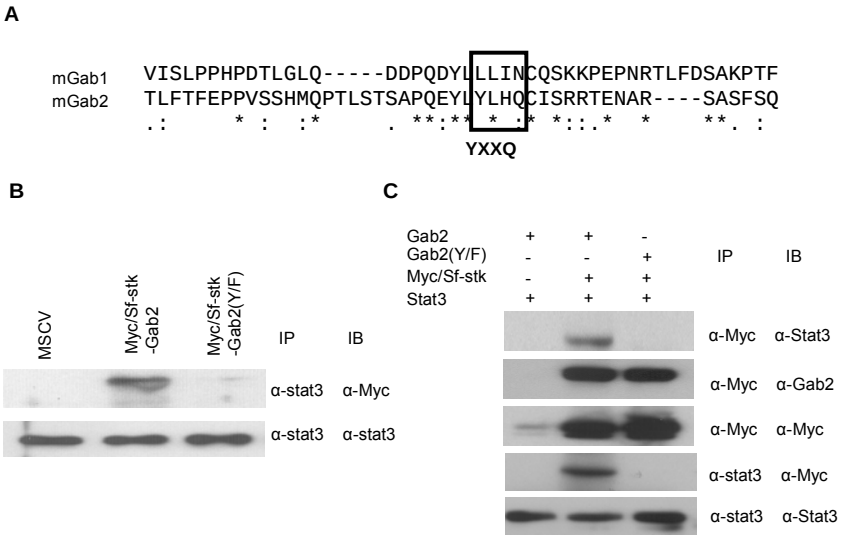


FIG.3.3. The ability of Gab2 to recruit and activate Stat3 is dependent on the Y194LHQ site in Gab2. (A) Sequence alignment of mGab1 and mGab2 proteins. The potential Stat3 binding site is shown in the box. (B) 293T cells were transiently cotransfected with Stat3 and plasmids expressing wild-type (MSCV) or mutant Sf-Stk/Gab2 fusions as indicated. Cell lysates were immunoprecipitated (IP) with anti-Stat3 antibodies and subsequently analyzed by SDS-PAGE and Western immunoblotting (IB) with anti-Myc and anti-Stat3 antibodies. (C) 293T cells were transiently cotransfected with Stat3, Sf-Stk, and wild-type or mutant forms of Gab2. Cell lysates were immunoprecipitated with anti-Stat3 and blotted with anti-Gab2, anti-Myc, and anti-Stat3 antibodies. -, absence of; +, presence of.

expression of Gab2(Y/F) did not rescue the defect in the Gab2-/- cells (Fig. 3.4C). An equivalent expression of the fusion proteins in 293 packaging cells is demonstrated by Western blot analysis (Fig. 3.4B and D), and equal infection efficiencies of primary bone marrow cells by these vectors were verified by flow cytometry (data not shown). Taken together, these data indicate that the YxxQ motif in Gab2, which is not present in Gab1, mediates the interaction of Gab2 with Stat3 downstream of Sf-Stk and that this interaction is critical in the ability of Gab2 to support hematopoietic transformation in response to Friend erythroleukemia virus.

To determine whether the Stat3 binding site in Gab2 is phosphorylated directly by Sf-Stk or indirectly by another kinase, we generated a kinase-dead form of the Sf-Stk/Gab2 fusion protein. We have shown previously that kinase-dead Sf-Stk does not support the Epo-independent growth of Friend virus-infected cells. However, we found that the kinase-dead Sf-Stk/Gab2 fusion at least partially supports the cytokineindependent growth of primary hematopoietic cells following infection with Friend virus (Fig. 3.5A). Furthermore, the kinasedead Sf-Stk/Gab2 fusion protein induces Stat3 tyrosine phosphorylation in 293 cells (Fig. 3.5B). Taken together, these data suggest that the kinase activity of Sf-Stk is required for receptor autophosphorylation but may not be required for the phosphorylation of Gab2 or Stat3 following the recruitment of Gab2 to the receptor complex.

3.4.4 Stat3 is tyrosine phosphorylated and interacts with Gab2 in spleens from Friend virus-infected mice.

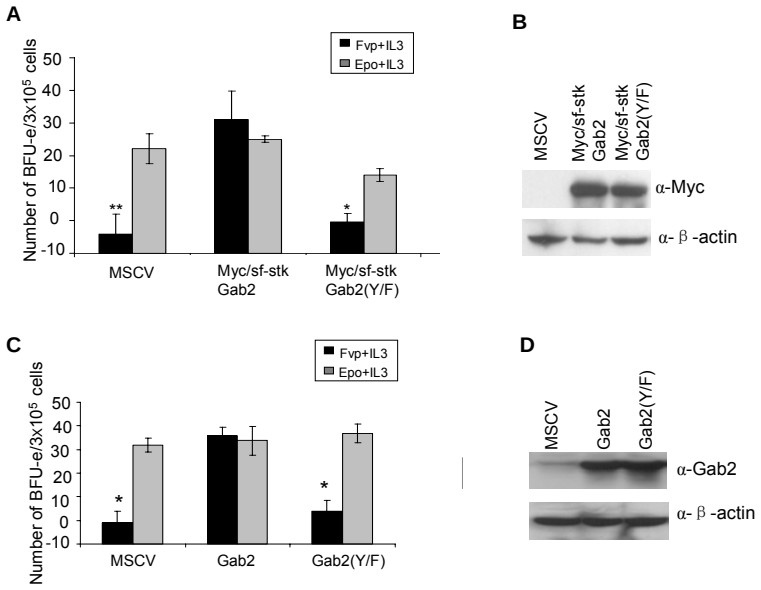


FIG 3.4 The Y^{194}LHQ site in Gab2 is critical to support Epo-independent colony formation of primary erythroblasts in response to Friend virus infection. (A) Total bone marrow from C57BL/6 mice was transduced with empty vector (MSCV), Myc-Sf-Stk/Gab2, or Myc-Sf-Stk/Gab2 harboring the Y194-to-F mutations. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence or absence of FV or Epo (1 U/ml). BFU-E cells were stained with acidbenzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means $\pm$ SD of one of three independent experiments performed in replicates of three and reflect normalized values. (B) Expression of Myc-Sf-Stk/Gab2 and Myc-Sf-Stk/Gab2(Y/F) in 293T packaging cells. (C) Total bone marrow from Gab2-/- mice was transduced with empty vector, Gab2 or Gab2 (Y^{194}/F). Colony assays were performed as described in the legend for panel A. (D) Expression of Gab2 and Gab2(Y/F) in 293T packaging cells. *, $P < 0.05$; **, $P < 0.01$.

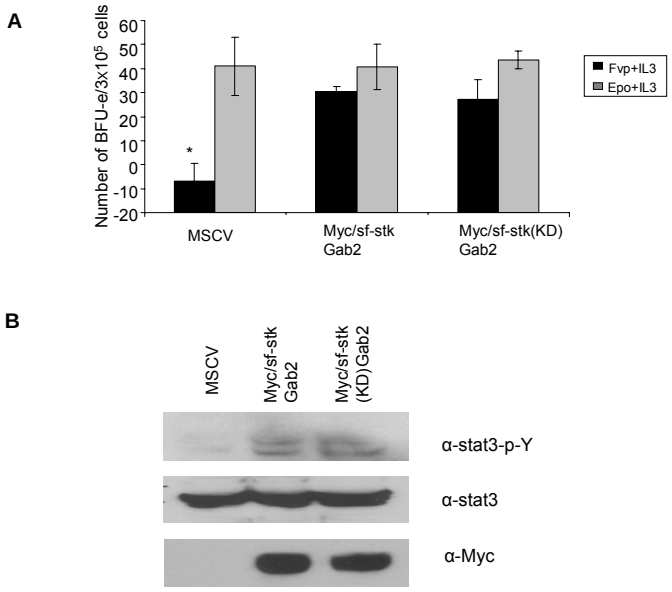


FIG. 3.5. The kinase activity of Sf-Stk is not required for the phosphorylation of Stat3 or Epo-independent colony formation of primary erythroblasts in response to Friend virus infection. (A) Total bone marrow from Gab2-/- C57BL/6 mice was transduced with empty vector (MSCV), Myc-Sf-Stk/Gab2, or Myc-Sf-Stk(KD)/Gab2. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence or absence of FV or Epo (1 U/ml). BFU-E cells were stained with acid-benzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means $\pm$ SD of one of three independent experiments performed in replicates of three and reflect normalized values. *, $P < 0.05$. (B) 293T cells were transiently cotransfected with Stat3 and plasmids indicated above. Cell lysates were probed with anti-phospho-Stat3, anti-Stat3, and anti-Myc.

The spleen is the primary site of polyclonal expansion of infected erythroid progenitor cells in vivo. We have shown previously that Gab2 is tyrosine phosphorylated in the spleens of infected mice. Here, we set out to investigate whether the Gab2/Stat3 signaling pathway is active in these cells. Unfractionated spleen cells from mice infected with Friend virus for various amounts of time were collected and Stat3 tyrosine phosphorylation was analyzed. We observed that Stat3 is tyrosine phosphorylated at days 4, 7, and 10 following infection, the time which correlates with the most rapid expansion of erythroblasts in the spleen (Fig. 3.6A). The immunoprecipitation of these lysates with Gab2 and immunoblotting with Stat3 demonstrated that there is enhanced interaction of Gab2 and Stat3 at days 7 and 10 following infection (Fig. 3.6B). Taken together, these data indicate that the interaction of Gab2 with Stat3 and the tyrosine phosphorylation of Stat3 are induced in spleens of Friend virus-infected mice during the expansion phase of infected erythroblasts.

3.4.5 Stat3 is required downstream of Stk for the transformation of primary hematopoietic cells by Friend erythroleukemia virus.

Recent studies have shown that Stat5 is required for Friend virus-induced polycythemia but not for the development of Friend leukemia. However, the potential role of Stat3 in this process has not been addressed. Therefore, we infected primary bone marrow cells from Friend virus-sensitive BALB/c mice with retroviral vectors harboring a dominant negative form of Stat3, followed by infection with Friend leukemia virus and assessment of Epo-independent colony growth. As shown in Fig.3.7, the expression of the dimerization mutant Stat3 (Y705F) (which acts as a dominant negative Stat3)

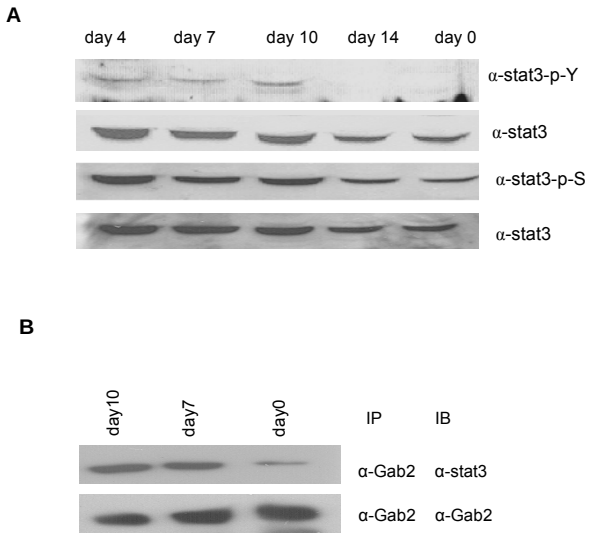


FIG. 3.6. Interaction of Gab2 with Stat3 and tyrosine phosphorylation of Stat3 in spleens of Friend virus-infected mice. (A) Ten-week old BALB/cJ mice were injected via their tail veins with FVP at different time points (day 14, day 10, day 7, and day 4). Spleen protein was harvested and subsequently analyzed by SDS-PAGE and Western blotting with anti-phospho-Stat3(Y705), anti-Stat3, and anti-phospho-Stat3(S727). (B) Ten-week-old BALB/cJ mice were injected via tail vein with FVP at different time points (day 10 and day 7). Spleen protein was immunoprecipitated with anti-Gab2 antibodies and subsequently analyzed by SDS-PAGE and Western blotting with anti-Stat3 and anti-Gab2.

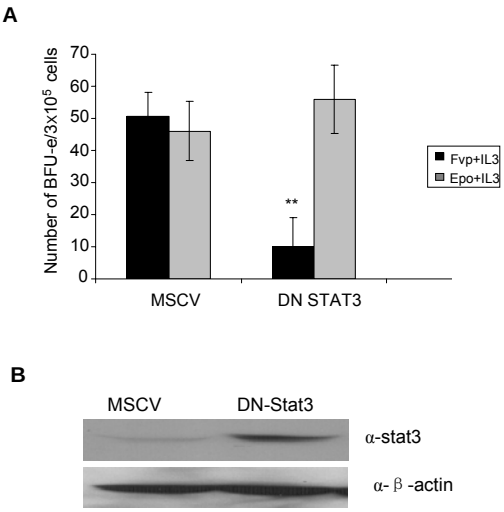


FIG. 3.7. Expression of dominant negative Stat3 inhibits colony formation in response to Friend virus in vitro. (A) Total bone marrow from BALB/c mice was transduced with empty vector (MSCV) or a dominant negative Stat3 [DNStat3(Y705F)]. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E cells were stained with acid-benzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means $\pm$ SD of one of three independent experiments performed in replicates of three and reflect normalized values. **, $P < 0.01$. (B) Expression of dominant negative Stat3 in 293T packaging cells.

inhibited Epo-independent colony formation in response to Friend virus infection but had little effect on the Epo-dependent growth of these cells. This experiment was also performed with the DNA binding mutant Stat3 (ZZ/VVV), and similar results were obtained (data not shown). To confirm a role for Stat3 in the transformation of primary erythroblasts in response to Friend erythroleukemia virus, we harvested bone marrow from floxed Stat3 mice and infected these cells with a retroviral vector expressing Cre recombinase fused to GFP. Figure 3.8A shows that the expression of Cre resulted in a reduction in Epo-independent colony formation in response to Friend virus infection, as observed with the dominant negative Stat3. The expression of Cre in 293 packaging cells was verified by fluorescence (Fig. 3.8B), and that in primary bone marrow cells was verified by flow cytometry (Fig. 3.8C). Reduced levels of Stat3 in bone marrow cells expressing Cre were verified by Western blot analysis (Fig. 3.8D). To further identify the mechanism of Stat3 in the erythroleukemia, we injected Cre-flox/Stat3 (on Friend Virus sensitive background) mice with polyI/poly C prior to Friend Virus infection, followed by the measurement of spleen size to check the susceptibility. As shown in Fig 3.9A, Cre-flox/Stat3 mice are resistant to Friend Virus. The deletion of Stat3 in spleen cells was confirmed on both day7 and day14 by western blot analysis (data not shown). In order to determine whether exogenous expression of stat3 can rescue this response, we utilized retroviral transfer to introduce both wild-type and dominant negative Stat3 (Y705/F) into bone marrow cells from mice previously treated with pI/pC. Fig 3.9B shows that exogenous expression of wild type Stat3 in hematopoietic cells from pI/pC treated mice rescued the Epo-independent growth of these cells in response to Friend Virus infection, while the expression of dominant negative Stat3 fail to rescue this defect. The reduced

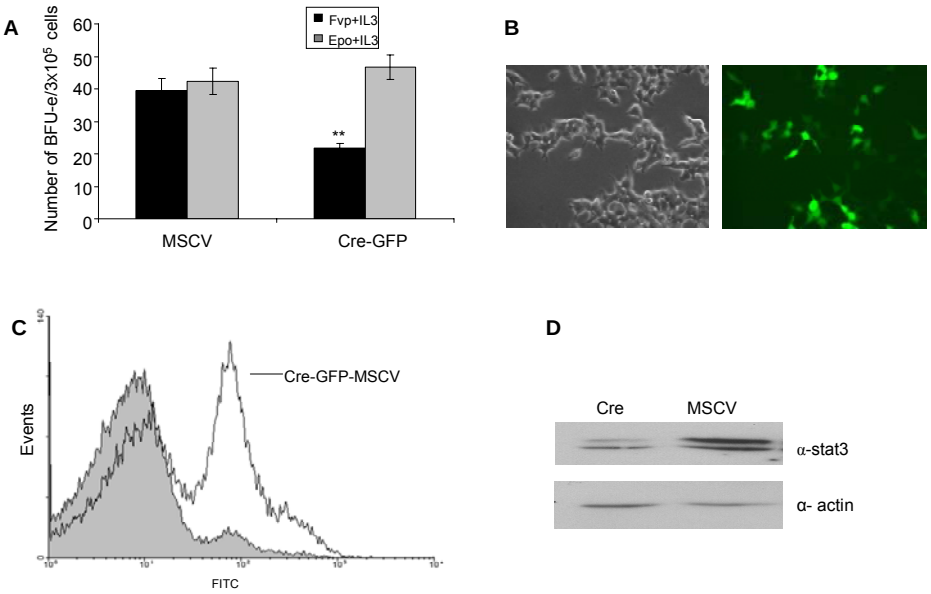
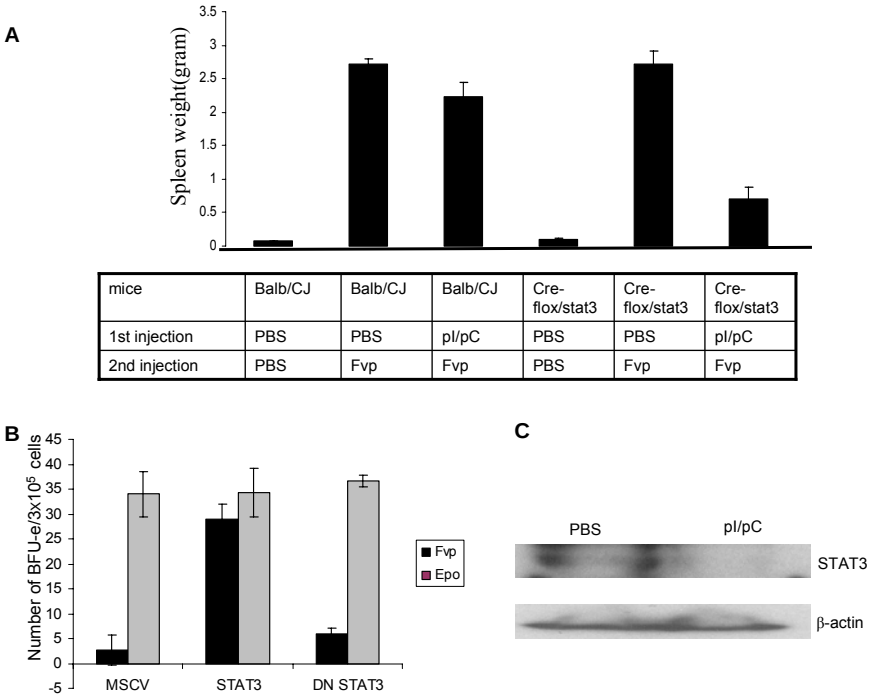


FIG. 3.8. Expression of Cre recombinase inhibits colony formation by bone marrow cells from floxed Stat3 mice in response to Friend virus infection. (A) Total bone marrow from floxed Stat3 BALB/c mice was transduced with empty vector or a Cre-GFP fusion plasmid. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E cells were stained with acid-benzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means $\pm$ SD of one of three independent experiments performed in replicates of three and reflect normalized values. ** $P < 0.01$. (B) Expression of Cre-GFP in 293T packaging cells by immunofluorescence. (C) Total bone marrow from floxed Stat3 mice was infected with vector (shaded area) or Cre-GFP (solid line). Infection efficiency was determined by flow cytometry for GFP. (D) Expression of Stat3 in total bone marrow from floxed Stat3 mice infected with empty vector or Cre-GFP.

expression level of Stat3 in bone marrow cells were verified by western blot analysis (Fig 3.9C). These data support the conclusion that the activation of Stat3 is required for the early stages of transformation of primary erythroblasts by Friend virus.



mice	Balb/CJ	Balb/CJ	Balb/CJ	Cre-flox/stat3	Cre-flox/stat3	Cre-flox/stat3
1st injection	PBS	PBS	pI/pC	PBS	PBS	pI/pC
2nd injection	PBS	Fvp	Fvp	PBS	Fvp	Fvp

Figure 3.9. Conditional deletion of Stat3 inhibits colony formation by bone marrow cells from Cre-floxed Stat3 mice in response to Friend virus infection. Six weeks old Cre-floxed Stat3 mice were injected with PolyI/PloyC or PBS control for three times every other day, (A) the mice were infected with FV or PBS for control. Spleens were weighed 2 weeks postinfection. (B) Total bone marrow from the mice was transduced with empty vector, wild type Stat3 or a DN (Dominant Negative) Stat3. Transduced cells were plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E cells were stained with acid-benzidine and scored on day 5. Black columns, Friend virus; gray columns, Epo. Bars denote the means $\pm$ SD of one of three independent experiments performed in replicates of three and reflect normalized values. (C) Expression of Stat3 in total bone marrow from Cre-floxed Stat3 mice infected with PolyI/Ploy C or PBS control.

3.5 Discussion

Following the initial discoveries that Stat3 is required for cellular transformation by v-*src* (16) and that a constitutiveactive Stat3 molecule itself can lead to cellular transformation (17), evidence for a critical role for Stat3 in transformation has steadily accumulated. Stat3 is persistently activated in several human cancers, including several hematological malignancies, such as multiple myelomas, leukemias, and lymphomas, in which activated kinases appear to promote its constitutive activity. Recently, Chiarle et al. verified a requirement for Stat3 in the development of B-cell lymphomas by the anaplastic lymphoma kinase in vivo following targeted disruption of Stat3 in the B- and T-cell lineages (23). Here, we have identified an essential role for Stat3 in the early stages of transformation of primary erythroblasts in response to Friend erythroleukemia virus. Therefore, our data further support Stat3 as a potential molecular target in cancer therapy. However, while the critical nature of Stat3 in transformation is becoming increasing clear, the mechanism by which Stat3 is activated in transformed cells has, in many cases, remained elusive.

In v-Eyk, the mutation of the receptor tyrosine kinase c-Eyk results in the presence of a YXXQ motif, a canonical Stat3 binding site (135), leading to the enhanced activation of Stat3 and cellular transformation (12). In addition, enhanced tyrosine phosphorylation of Stat3 has been associated with the mutation of the aspartic acid in the kinase domain of the Kit receptor tyrosine kinase; the activation of this pathway is required for its transforming ability (90), and the juxtamembrane mutations in the Kit receptor found in gastrointestinal stromal tumors have propensities similar to those of activate Stat proteins (20). However, while most tyrosine kinases activate the Stat3

signaling pathway, few contain a canonical Stat3 binding motif. Here we demonstrate, for the first time, a functional Stat3 binding motif in Gab2. The ability of receptor tyrosine kinases to recruit Gab2 through a Grb2-dependent mechanism suggests the possibility that a wide range of receptor tyrosine kinases could activate the Stat3 signaling pathway via a Grb2-/Gab2-dependent mechanism.

There is increasing evidence that the Grb2-/Gab2-dependent signaling pathway may also play a central role in transformation. Gab2 maps to 11q13-14, a region that is commonly amplified in breast cancer, that is found to be overexpressed in human breast cancer, and that potentiates breast carcinogenesis in mice induced by ErbB2 (11, 30). In addition, a Grb2/Gab2 signaling pathway is critical for leukemic transformation by BCR/Abl (115) and the inhibition of Gab2 with RNA interference inhibits colony formation by primary chronic myeloid leukemia cells (115). A Grb2 binding site also contributes to leukemogenesis induced by the Tel/Abl tyrosine kinase (81), and Gab2 mediates mast cell proliferation in response to the Kit receptor tyrosine kinase (148). Taken together, these observations indicate that Gab2 could be activated downstream of tyrosine kinases in hematopoietic cells and propagate signals required for the transforming activity of those kinases.

Gab2 is a large adaptor protein (related to the insulin receptor substrate family of adaptors) which transmits signals from a number of receptor tyrosine kinases and is recruited to active signaling complexes through two mechanisms: a polyproline-rich region that binds the N-terminal SH3 domain of Grb2 and a pleckstrin homology domain that is recruited to the membrane by phospholipids generated by PI3 kinase (44, 74). Two Shp-2 binding motifs and three p85 binding sites conserved in all Gab family members

(Gab1, Gab2, and Gab3) have been shown to be critical for the activation of the Erk and PI3 kinase pathways, respectively. Accordingly, we found that the mutation of the Shp2 or p85 binding sites in Gab2 in the context of an Sf-Stk/Gab2 fusion protein reduced cellular transformation of primary erythroblasts in response to Friend virus (127). However, these signaling pathways were retained in an Sf-Stk/Gab1 fusion protein that failed to support the transformation of Friend virus-infected bone marrow cells, indicating that Gab2 must harbor unique functions which are not associated with the activation of Gab1 that are also required for transformation. Our data indicate that the recruitment and activation of Stat3 by Gab2 cooperate with signals generated by Shp2 and p85 and are required for the ability of Gab2 to mediate the transformation of primary hematopoietic cells downstream of the Stk receptor tyrosine kinase.

While the mechanism by which most receptor tyrosine kinases lead to the activation of the Stat3 signaling pathway is unclear, many studies have implicated Src family kinases in this process (41, 103, 132). Recent studies have demonstrated that (i) the hematopoietic cell kinase mediates the phosphorylation of Gab1 and Gab2 in multiple myeloma cells (99), (ii) Lyn and Syk are required for Gab2 tyrosine phosphorylation downstream of FcεR1 (147), and (iii) granulocyte colony-stimulating factor-induced tyrosine phosphorylation of Gab2 is Lyn kinase dependent (154). We have shown here that the kinase activity is not required for the activation of Stat3 and the Epo-independent growth of Friend virus-infected cells in the context of an Sf-Stk/Gab2 fusion protein. This raises the intriguing possibility that the activation of Stat3 downstream of receptor tyrosine kinases, including Stk, could be mediated by the Src-dependent phosphorylation of Gab2, resulting in the recruitment and activation of Stat3. Indeed, our unpublished

observations demonstrate a requirement for Src family kinases in the transformation of primary erythroblasts by Friend leukemia virus in vitro. However, our studies with the Lyn knockout mice did not reveal a critical role for the Lyn tyrosine kinase in the early stages of transformation in vivo (124).

In this study, we have identified a novel receptor tyrosine kinase/Grb2/Gab2/Stat3 signaling pathway required for the transformation of primary hematopoietic cells by using Friend erythroleukemia virus as a model system. Although the targeting of tyrosine kinases has become the first line of therapy in treating leukemia and other malignancies, the occurrence of drug resistance has highlighted the necessity for identifying multiple complementary targets for the successful treatment of these diseases. The identification of specific downstream signaling events that mediate transformation will aid in the elucidation of a new generation of targets for drug therapy. Our data indicate that blocking the ability of Gab2 to recruit and activate Stat3 may provide a novel target in the treatment of leukemia and possibly a wider range of human cancers.

3.6 Acknowledgements

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Chapter 4

Src Family Kinases Play a Role in the Transformation of Primary Erythroblasts by Friend Leukemia Virus

4.1 Introduction

Friend virus-induced erythroleukemia has long served as a paradigm for the study of the multi-stage nature of carcinogenesis, and is characterized by polyclonal proliferation of nonleukemic erythroid progenitor cells followed by a later stage in which there is clonal or oligoclonal expansion of malignant cells (9). Friend virus is a complex of two viruses, spleen focus forming virus (SFFV), a replication defective virus which is responsible for the acute pathogenicity of the complex, and the replication competent helper virus, Friend murine leukemia virus (F-MuLV), which causes no disease itself in adult mice but works as a helper for the replication of the defective SFFV. In the early stages of FVP infection, erythroid cells from the spleens of mice infected with FVP can both proliferate and differentiate in the absence of Epo. The late stage of erythroleukemia in FVP infected mice is marked by the emergence of fully transformed cells in the spleen, and eventually the blood, bone marrow, and liver. Mice surviving the early stages will develop erythroleukemia. Subsequent mutations in p53 combined with transcriptional activation of the *ets* genes, *Fli-1* and *PU.1* are characteristics of this stage (83, 85).

Previously, we demonstrated that the Friend virus susceptibility gene, Fv2, encodes the stem cell-derived tyrosine kinase (Stk) receptor, which confers susceptibility to Friend virus induced erythroleukemia in mice. Mice homozygous for the resistant allele of *Fv2* (*Fv2*^{rr}), such as C57Bl/6, fail to express a naturally occurring, N-terminally

truncated form of Stk, called short form-Stk (Sf-Stk), while expression of full-length Stk is unaffected. Mice homozygous for the susceptible allele of *Fv2* (*Fv2*^{ss}), such as Balb/CJ, express both full-length Stk and Sf-Stk. An internal promoter within the Stk locus drives the expression of Sf-Stk, which lacks the N-terminal ligand binding domain, but retains the transmembrane and tyrosine kinase domains. Mice deficient for Stk are resistant to Friend virus-induced expansion of erythroid progenitor cells (98), while enforced expression of Sf-Stk in C57Bl/6 mice has been shown to be sufficient to confer Friend virus susceptibility to *Fv2*^{rr} mice (98).

In previous analysis of Sf-Stk signaling during Friend virus infection, we found that signaling through Sf-Stk plays a central role in the polyclonal expansion of infected progenitor cells. We have also shown that the kinase domain and Grb2 binding site of Sf-Stk are required for this response to Friend virus infection (36) and that a Grb2/Gab2 complex downstream of Sf-Stk mediates the growth of primary erythroid progenitor cells infected in vitro with Friend virus (127). Our recent study identified a novel Stat3 binding site in Gab2 which is required for Friend virus induced transformation, suggesting that the activation of Stat3 in Friend virus infected cells is mediated by the Sf-stk/Grb2/Gab2 signaling pathway (89). However, while a kinase dead Sf-Stk does not support Epo-independent growth of Friend Virus infected erythroblasts, we have shown previously that a kinase dead Sf-stk/Gab2 fusion protein is functional in this assay. These data suggest that the kinase activity of Sf-Stk is required for receptor autophosphorylation and the subsequent recruitment of Gab2, but may not be required for the phosphorylation of Gab2 or Stat3 following the recruitment of Gab2 to the receptor complex. This raises the

intriguing possibility that the activation of Stat3 downstream of Sf-Stk could be mediated by the other kinases, like Src.

Src family kinases are activated by the Met receptor kinase to modulate multiple cellular events, via the direct interaction of Src with the docking site tyrosines in Met. Previous data in our lab showed that the ability of full-length Stk to active MAP kinase pathway in the absence of ligand is dependent on c-Src activity, and that c-Src co-immunoprecipitates with Stk (140). The Src family of protein kinases regulates a diverse array of cellular processes in both normal and transformed cells, including cell proliferation, survival, differentiation, adhesion and mobility. There are eight members of the Src family of kinases expressed in mammalian cells, including Hck, Fgr, Lyn, Lck, Blk, Src, Yes, Fyn. The latter three of them are ubiquitously expressed, while the others are limited to specific cell lineages (15, 117). Src family kinases are members of a family of non-receptor tyrosine kinases defined by a common modular structure, and consisting of consecutive SH3, SH2, and tyrosine kinase domains. The SH2 and SH3 domains contribute to the negative regulation of the Src kinases by promoting intra-molecular interactions. Disruption of these interactions is a major mechanism to mediate Src activation. All family members also contain a membrane-targeting region at their N-terminus, which is unique to each family member, always myristoylated and sometimes palmitoylated. The membrane-targeting region is followed by a domain of 50–70 residues, which is different among family members (14).

Previous studies have implicated Src family kinases in the activation of Stat3 and Gab2 following growth factor stimulation (24, 64). Initial evidence that Src family kinases could be involved in STAT activation came from models of v- Src transformation,

demonstrating that the DNA-binding activity of STAT3 was enhanced in cells transformed by the v-Src oncoprotein (146). In some cell models, it was shown that there was an association between STAT3 and v-Src. Furthermore, v-Src was shown to activate not only the tyrosine phosphorylation of STAT3 but also DNA binding, and transcriptional activation (19). A series of in vitro studies demostrated that src, hck, lyn, fyn, and fgr are all capable of phosphorylating STAT3 on tyrosine (117). Experiments by Ren and Schaefer have demonstrated a role for endogenous c-Src in the activation of STAT3 (103, 119). Other studies demonstrate that Src family kinases promote EGF-induced PI3-kinase activation by the tyrosine phosphorylation of Gab2. Moreover, they found that the proline-rich domains of Gab2 are essential for constitutive Src association with and tyrosine phosphorylation of Gab2 (64). Other papers suggested that G-CSF stimulation leads to Lyn-mediated tyrosine phosphorylation of Gab2, which may serve as an important intermediate of enhanced Akt activity (154). We have shown that the kinase activity of Sf-Stk is not required for the activation of Stat3 and the Epo-independent growth of Friend virus-infected cells in the context of a Sf-Stk/Gab2 fusion protein. This raises the possibility that the activation of Stat3 downstream of Sf-Stk, could be mediated by the Src-dependent phosphorylation of Gab2, resulting in the recruitment and activation of Stat3, and/or in the direct phosphorylation of Stat3. However, studies with the Lyn knockout mice did not reveal a critical role for the Lyn tyrosine kinase in the early stages of transformation in vivo (124).

Here, we set out to determine whether Src family kinases play a role in the progression of Friend disease and determine the molecular basis for its interaction with the Sf-Stk/Grb2/Gab2/Stat3 signaling pathway in the transformation of primary

hematopoietic cells. Using the non-specific inhibitor of Src kinases, PP1, and a dominant negative c-Src, we found that Src family kinases mediate the response of progenitor cells to Friend virus downstream of Sf-Stk in the early stages of transformation. Furthermore, we demonstrated that Src family kinases lie upstream of the activation of Stat3 by Sf-Stk. This study highlights the critical role of Src family kinases in promoting the Epo-independent growth of Friend virus infected erythroblasts.

4.2 Experimental Procedures and Preparation

4.2.1 Mice

BALB/c and C57BL/6 mice were obtained from Jackson Laboratories. All research involving the use of mice were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Pennsylvania State University.

4.2.2 DNA Constructs

MSCV-neo-myc/sf-stk was described previously (36). DN-c-Src was ordered from Invitrogen.

4.2.3 Cell Culture and Reagent

The 293T cell line was acquired for A Henderson (Pennsylvania State University) and grown in DMEM containing 10% FCS, penicillin/Streptomycin, and L-glytamine. PP1 was kindly provided by Dr. Avery August (The Pennsylvania State University).

4.2.4 Transient transfection

Cells were transiently transfected by TransIT-293 transfection reagent (Mirus corporation) using 1-2 μg pEco and the appropriate MSCV-neo constructs (1-10 μg) for 48-72 hours at 37 °C prior to harvest of the viral supernatant. Protein expressions of plasmids were verified by western blot, by probing with antibodies.

4.2.5 In vitro infection and colony assay

For *in vitro* infection of bone marrow, cells were harvested from femurs and tibias of >6 week old BALB/c mice, and washed in PBS. For Epo-independent colony analysis, total bone marrow cells from different strains of mice were harvested and incubated with supernatant from cells expressing polycythemia-inducing FV (derived from FP63 cells,

Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada) or DMEM (10 % FBS, P/S, L-glu) on ice for 1 hour, as previously described (127). Cells were added to methocult media M3234 (Stem Cell Technologies) along with 2.5ng IL-3(Peprotech) and different concentrations of PP1 (20nM, 10nM, 5nM, 1nM) and DMSO (for control) in triplicate. Cultures were incubated for 2-8 days in 5 % CO_2 at 37 °C. Erythroid colonies (BFU-E and CFU-E) were visualized by acid-Benzidine staining as previously described (36).

4.2.6 Antibodies, Immunoprecipitation, and Western blotting.

Rabbit anti-Stat3(1:1000), rabbit anti-phospho-Stat3(Y705) (1:1000) antibodies were purchased from Cell Signaling. Rabbit-anti-β-actin was purchased from Sigma.

293T cells were transiently transfected with plasmids expressing desired protein. Twenty-four hours after transfection, cells were lysed in lysis buffer (1% NP-40, 150 mM NaCL, 1mM EDTA, 0.25%Deoxycholate, 2mM Na_3VO_4, 10 mM NaF, and 2mM PMSF). After 15 minutes centrifugation, the cleared cell lysates were incubated with antibodies and protein A (or G) -sepharose at 4°C overnight. The immunoprecipitates were analyzed by reduced SDS-PAGE and transferred to PVDF membrane. The blots were incubated with antibodies and detected with ECL (Amersham).

4.3 Results

4.3.1 Src kinases are downstream of Sf-Stk in the early stages of transformation by FVP

In order to determine whether Src family kinases play a role in the progression of Friend disease, we infected primary bone marrow cells from Friend virus sensitive Balb/ CJ mice with FVP and incubated the cells in methylcellulous media with different concentrations of PP1, which is a non-specific inhibitor of the Src kinase family. In the presence of PP1, infected bone marrow cells failed to form Epo-independent colonies in response to Friend virus infection. Furthermore, Epo-independent colony numbers decreased with increasing concentrations of PP1 (Fig 4.1A). Because PP1 is a non-specific inhibitor of Src kinases, we set out to confirm the role of Src family kniases by retrovirally expressing a dominant negative c-Src. Bone marrow cells from Friend virus sensitive mice failed to form Epo-independent colonies following FVP infection in the presence of the DN Src. (Fig 4.1B). These data suggest that Src family kinases mediate the response of progenitor cells to Friend virus downstream of Sf-Stk in the early stages of transformation.

4.3.2 Src family kinases are upstream of the activation of Stat3 by Sf-Stk.

To determine the role of Src kinases in Friend disease, we tested whether the requirement of Src lies upstream or downstream of stat3. We examined the phosphorylation of Stat3 in 293T cells in which Stat3 and Sf-Stk (or full length Stk) were co-transfected in the presence/absence of PP1. These experiments indicate that Src family kinases lie upstream of the activation of Stat3 by Sf-Stk (Fig 4.2).

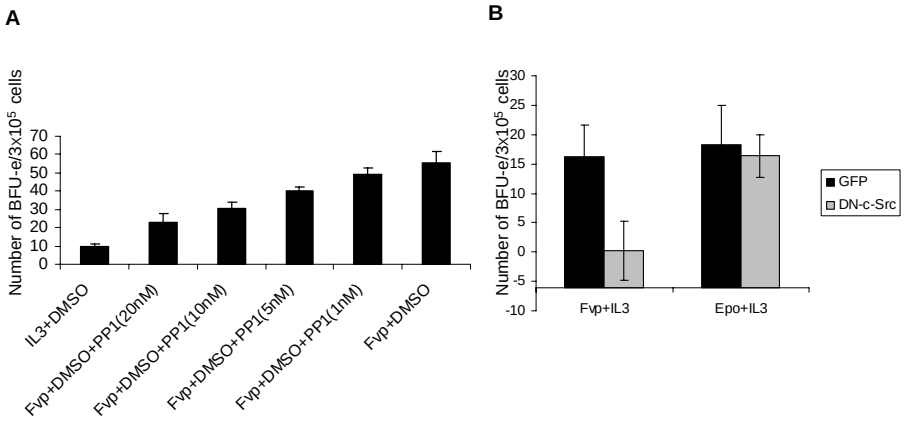

Figure 4.1 Src kinases are downstream of Sf-Stk in the early stages of transformation by FVP. (A) Eight week old Balb/CJ mice bone marrow was plated in methylcellulose containing DMSO, IL-3 (2.5 ng/ml) and dIfferent concentrations of PP1 in the presence of Fvp. (B) Total bone marrow from Balb/CJ mice was transduced with MSCV-neo-EGFP or MSCV-neo-DN-c-Src and plated in methylcellulose containing IL-3 (2.5 ng/ml) in the presence of FV or Epo (1 U/ml). BFU-E were stained with acid-benzidine and scored on day 5. Data are representative of three independent experiments and s.e. bars denote the mean $\pm$ s.d. of three replicates.

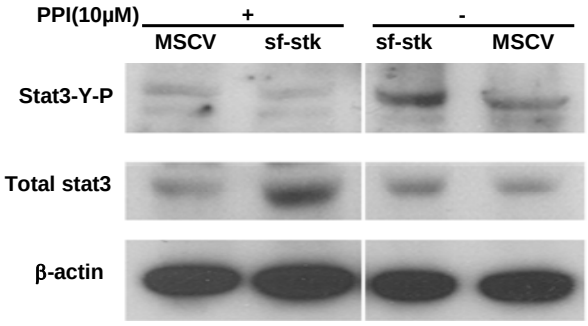

Figure 4.2 Src family kinases are upstream of the activation of Stat3 by Sf-Stk. 293T cells were transiently co-transfected with Stat3 and empty vector or myc-Sf-Stk, and incubated with or without PP1 (10μM). Cell lysates were probed with antiphospho-Stat3, anti-Stat3, anti-β-actin.

4.4 Future Experiments

The data presented here demonstrate that Src kinases mediate the response of progenitor cells to Friend virus, are downstream of Sf-Stk, and lie upstream of the activation of Stat3 by Sf-Stk. Previous work has shown v-Src causes erythroleukemia in mice, and our data indicates the importance of c-Src in the response of erythroid progenitor cells to Friend Virus infection. Therefore, we will focus our studies on c-Src, although multiple Src family members may compensate for the lack of anyone of them.

To determine whether c-Src is required for Friend disease, we will examine the susceptibility of bone marrow cells from Src-deficient mice to FVP infection by analyzing colony formation. In vivo, the spleen size of Src deficient mice will be measured after FVP injection and the expression of c-Src, as well as other Src family members, will be examined by RT-PCR and western blot using splenocytes from infected mice. If we observe a reduction in colony formation in bone marrow from Src-deficient mice in response to FVP, different Src members will be re-introduced into the Src-deficient bone marrow cells by retroviral infection, and Epo-independent colonies will be assessed to clarify which Src family members are capable of mediating the signals downstream of Sf-Stk required for the early stages of Friend disease. Alternatively, siRNA techniques will be used to inhibit various Src kinases in primary erythroblasts prior to Friend virus infection and Epo-independent colony formation will be examined.

We have shown that Gab2 is tyrosine phosphorylated in 293 cells when co-expressed with Sf-Stk. In order to know whether c-Src is responsible for the activation of Gab2, we will transfect 293T cells with Sf-stk and Sf-stk kinase dead (KD), together with Gab2, incubate cells with/without PP1, and determine the phosphorylation level of Gab2.

Using SYF cells (Src, Yes, Fyn deficient cells), the same experiment will be done by transfecting Sf-stk and Sf-stk (KD). Moreover, we will infect bone marrow cells from Src-deficient mice with the Sf-Stk/Gab2 protein and determine the ability of sf-stk/Gab2 to support the growth of Friend virus infected cells in the absence of Src.

To determine the relationship between Src and Stat3, experiments similar to those described above will be performed, and the phosphorylation of Stat3 will be assessed. Moreover, constitutively activated stat3 will be introduced into Src-/- cells, and the response of these cells to Friend virus will be investigated. Taken together, results from these studies will further clarify the role of Src kinases in the progression of leukemia induced by Friend virus, and place Src family kinases in the Sf-Stk/Grb2/Gab2/Stat3 signaling pathway.

Chapter 5

Conclusion and discussion

In this study, we characterize the molecular mechanisms of cell signaling that govern abnormal erythroblast proliferation and differentiation. Cell signaling is part of a complex system of communication that governs basic cellular activities and coordinates cell actions. Errors in cellular information communication can lead to diseases such as cancer and autoimmunity. Here we use Friend Virus induced acute erythroleukemia as an experimental system to study the signaling events involved in the pathogenesis of hematologic neoplasias.

Previous studies from our lab have shown that the ability of Sf-Stk to participate in the transformation of Friend virus-infected cells requires the kinase activity and Grb2-binding site of Sf-Stk. Further, we have demonstrated that Grb2 heterozygous mice exhibit decreased susceptibility to Friend erythroleukemia and expansion of erythroid progenitors in response to infection requires the C- terminal SH3 domain of Grb2. The C-terminal SH3 domain of Grb2 recruits large adaptor proteins to the complex, including the Grb2 associated binding protein (Gab) (93). Gab1 can either bind stably to the C terminal SH3 domain of Grb2, or be directly recruited to the first docking site tyrosine in Met. In contrast, Gab2 recruitment to Met is strictly mediated by Grb2 (77). As a family of scaffolding adapter proteins, Gab provide a broader binding platform for the next layer of signal transducers including the p85 subunit of PI3 kinase, CrkL and SHP2 (91, 109), resulting in the activation of Akt, Rap1, and Erk, respectively. It has been reported that v-sea, which induces erythroleukemia in chickens, preferentially interacts with Gab2 and

is essential for cellular transformation. Furthermore, this transformation requires the activation of the PI3K and MAPK pathways (3, 55). Here we demonstrate that Gab2-/- mice are partially resistant to Friend erythroleukemia in vivo and Gab2-/- erythroblasts fail to form Epo-independent colonies in response to Friend Virus infection. This defect can be rescued by exogenous expression of wild-type Gab2, but not Gab1. All Gab family members contain three p85-binding sites that become phosphorylated upon receptor activation (74, 93), and we demonstrated that the p85-binding sites in Gab2 are required for efficient Epo-independent growth of Friend virus-infected erythroblasts. In addition, constitutively active forms of SHP-2 have been shown to contribute to the progression of some human leukemias (86). Previous data in our lab indicated the activation of MAPK is also required for the transformation of cells by FV, and some studies have demonstrated that efficient transformation in response to v-sea requires binding of Gab2 to the SHP-2 (55). Here we mutated the two tandem tyrosines (binding sites of SHP-2) in the C-terminal in the context of the Sf-Stk/Gab2 fusion protein, which resulted in reduced the ability of the fusion protein to support the growth of primary erythroblasts in response to Friend virus. Therefore, one likely role of Gab2 in this system is to propagate and ampify signals downstream of Sf-Stk, via the activation PI3K and MAPK, through recruitment of p85 and SHP-2, respectively.

The three mammalian Gab proteins exhibit a high degree of homology in structure, although they appear to play largely nonoverlapping roles in vivo (137). A previous study indicated that both Gab1 and Gab2 have similar effects on the activation of Erk in response to growth factor stimulation (153). In addition, recent studies have suggested that the Grb2-Gab1/2-SHP-2 pathway results in the activation of Erk, and may play a role

in the enhanced or sustained activation of this signal (113, 153). Interestingly, in this study, we found that recruitment of Gab2, but not Gab1, by Sf-Stk is required to support the growth of primary erythroblasts in response to Friend virus infection. This suggests that Gab2 must harbor unique functions, not associated with the activation of Gab1, that are also required for transformation. This difference between Gab1 and Gab2 lead us to map the domains of Gab2 responsible for the transformation of primary hematopoietic cells. By the usage of a series of six Gab1/Gab2 chimeric proteins, we found that the Y^{194}LHQ motif in the region between Q^{120} and P^{358} of Gab2, which is missing in Gab1, is required for hematopoietic transformation.

The YxxQ sequence is reported to be a consensus binding motif for Stat3 in various proteins including SHP2, gp130 and leptin (2, 96, 135). Furthermore, in v-Eyk, the mutation of the receptor tyrosine kinase c-Eyk results in the generation of a YxxQ motif, leading to the enhanced activation of Stat3 and cellular transformation (12). Our data suggest that mutations of both the p85- and Shp2-binding sites in wild-type Gab2 only partially block the Epo-independent colony formation (our unpublished data). This raises the intriguing possibility that other proteins, like Stat3, can be activated by Gab2 in the transformation of primary hematopoietic cells. A number of studies provided evidence for a critical role for Stat3 in transformation. Mustafa, et al show that the activation of Stat3 contributes to the development of cancer multiple cancers including breast cancer, head and neck cancer, acute myeloid leukemia, acute promyelocytic leukemia and chronic myeloid leukmia (10). Furthermore, Stat3 is constitutively activated in primary erythroleukemic cells, and a constitutively active variant of Stat3 induces oncogenic transformation and tumorigenesis (17, 58). Here, we have identified

an essential role for Stat3 in the early stages of transformation of primary erythroblasts in response to Friend erythroleukemia virus. In addition, we demonstrated, for the first time, a functional Stat3 binding motif in Gab2. The ability of receptor tyrosine kinases to recruit Gab2 through a Grb2-dependent mechanism suggests the possibility that a wide range of receptor tyrosine kinases could activate the Stat3 signaling pathway via a Grb2-/Gab2-dependent mechanism.

Our previous data have shown that kinase-dead Sf-Stk does not support the Epo-independent growth of Friend virus-infected cells. However, we found that the kinase-dead Sf-Stk/Gab2 fusion at least partially supports the Epo-independent growth of primary hematopoietic cells following Friend virus infection. Furthermore, the kinase dead Sf-Stk/Gab2 fusion protein induces Stat3 tyrosine phosphorylation, at levels comparable with wild type Sf-Stk/Gab2. Taken together, this raises the possibility that the activation of Stat3 downstream of receptor tyrosine kinases, including Stk, could be mediated by an additional kinase which phosphorylates Gab2, resulting in the recruitment and activation of Stat3 later. Ren and Schaefer have demonstrated a role for endogenous c-Src in the activation of STAT3 (103, 119), and several studies have shown that activation of Stat3 and Gab2 is mediated by a Src family kinase dependent mechanism following growth factor stimulation (24, 64). Initial evidence that Src family kinases could be involved in STAT activation came from models of v-Src transformation, demonstrating that the DNA-binding activity of STAT3 was enhanced in cells transformed by the v-Src oncoprotein (146). Furthermore, v-Src was shown to activate not only the tyrosine phosphorylation of STAT3 but also DNA binding, and transcriptional activation (19). A series of in vitro studies demostrated that Src, Hck, Lyn,

Fyn, and Fgr are all capable of phosphorylating STAT3 on tyrosine (117). Other studies indicated that Src family kinases promote EGF-induced PI3-kinase activation by the tyrosine phosphorylation of Gab2, and that the proline-rich domains of Gab2 are essential for constitutive Src association with and tyrosine phosphorylation of Gab2 (64). Furthermore, G-CSF stimulation leads to Lyn-mediated tyrosine phosphorylation of Gab2, which may serve as an important intermediate of enhanced Akt activity (154).Using a non-specific inhibitor of Src kinases, PP1, and a dominant negative c-Src, we found that Src family kinases mediate the response of progenitor cells to Friend virus downstream of sf-stk in the early stages of transformation. Furthermore, we demonstrated that Src family kinases lie upstream of the activation of Stat3 by Sf-Stk. This study highlights the critical role of Src family kinases in Friend virus induced hematopoietic transformation. Studies with the Lyn knockout mice did not reveal a critical role for the Lyn tyrosine kinase in the early stages of transformation in vivo. However, they suggested Lyn plays a role in later events, including the terminal differentiation of infected cells and the expansion of transformed cells (124).

In conclusion, our data define a novel receptor tyrosine kinase/Grb2/Gab2/Stat3 signaling pathway required for the transformation of primary hematopoietic cells, using Friend erythroleukemia virus as a model system (Figure 5.1). Although the targeting of tyrosine kinases has become the first line of therapy in treating leukemia and other malignancies, the occurrence of drug resistance has highlighted the necessity for identifying multiple complementary targets for the successful treatment of these diseases. The identification of specific downstream signaling events that mediate transformation will aid in the elucidation of a new generation of targets for drug therapy. Our data

indicate that blocking the ability of Gab2 to recruit and activate Stat3 may provide a novel target in the treatment of leukemia and possibly a wider range of human cancers.

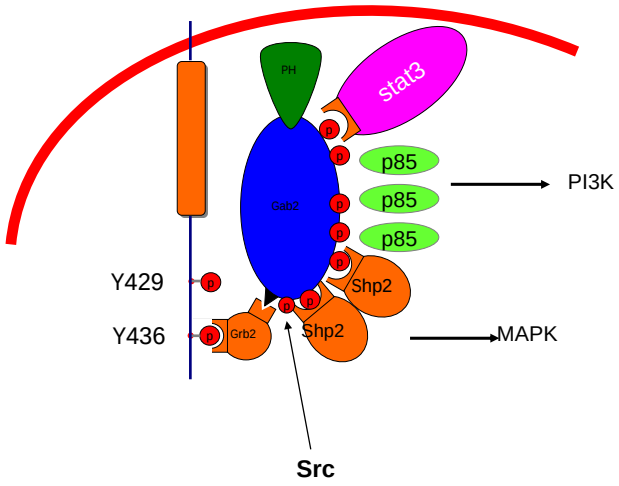

Figure 5.1 Conclusion of signaling pathways in Friend erythroleukemia. During Friend virus infection, the activation loop of the Sf-Stk kinase domain and docking site tyrosine 436 in the c-terminal tail, which is responsible for interaction with Grb2, are required for the erythroblast expansion. This leads to recruitment and tyrosine phosphorylation of Gab2, which binds constitutively to the Grb2 SH3 domain. Tyrosine phosphorylated Gab2 is platform of many downstream molecules, including p85 and Shp2, which are important for PI3K and MAPK activation separately. Meantime, Gab2 is required for the activation of Stat3 by Y^{194}LHQ motif in this system. Src kinase works downstream of Sf-Stk, and the activation of Stat3 could be mediated by Src dependent Gab2 activation.

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Publications

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- **Shuang Ni**, C. Zhao, G. S. Feng, R. F. Paulson, and P. H. Correll. A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/Ron receptor tyrosine kinase in response to Friend virus infection. *Mol Cell Biol. 27(10), 3708-15(2007).*
- **Shuang Ni***, Hami E. Teal*, Pamela H. Correll. Grb2-mediated Recruitment of Gab2, but not Gab1, to Sf-stk supports the expansion of Friend virus infected erythroid progenitor cells. (* co-first auther) *Oncogene. 25, 2433-43 (2006).*
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- **Shuang Ni,** Jie Wang. The Psychotherapy and Analysis to Emergent Patients. *Journal of Chinese Emergent Medical Science. 165-168 (1998).*

Presentations/Abstracts

- 48th annual American Society of Hematology Meeting, Orlando. (**Oral Presentation,**2006).
- 46th annual American Society of Hematology Meeting, San Diego, CA. (**Oral presentation,** 2004)
- 23rd Summer Symposium in Molecular Biology, State College, PA. (Abstract, 2004)