The Pennsylvania State University

The Graduate School

Department of Veterinary and Biomedical Sciences

MECHANISM OF GP55 MEDIATED SF-STK ACTIVATION

A Dissertation in

Pathobiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2009

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ABSTRACT

Since Friend disease was first reported in 1957, the acute erythroleukemia induced by the various strains of Friend virus has provided an excellent model to study multistage carcinogenesis. In the first stage, the virus infects erythroid progenitor cells, resulting in the Epo-independent (Epo^{ind}) expansion of erythroid progenitor cells. In the late stage, integration of the virus into the *Spi-1* locus leads to inappropriate expression of Pu.1 and subsequent inactivation of the *p53* locus in erythroid cells, causing a block in their differentiation and eventually acute erythroleukemia.

Friend virus induced erythroleukemia in susceptible mice is initiated by the interaction of the Friend virus encoded glycoprotein, gp55, with the erythropoietin receptor and the product of the host *Fv2* gene, a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). We have previously demonstrated that the activation of Sf-Stk and recruitment of a Grb2/Gab2/Stat3 signaling complex is required for the ability of Friend virus to induce Epo independent colony formation of erythroid progenitor cells. Here we demonstrate that gp55 interacts with Sf-Stk through the cysteine residues Cys^{306}, Cys^{309}, Cys^{337} and Cys^{338} in the ecotropic domain of gp55 and the cysteine residues Cys^{8}, Cys^{19}, Cys^{37} and Cys^{42} in the extracellular domain of Sf-Stk. This interaction does not promote the dimerization of Sf-Stk, but results in the enhanced phosphorylation of pre-formed Sf-Stk dimers. Point mutation of these cysteine residues or deletion of these domains abrogates the interaction between gp55 and Sf-Stk, resulting in the inability of these mutants to promote the Epo independent growth of erythroid progenitor cells.

We further demonstrate that cell surface localization of Sf-Stk is critical for receptor phosphorylation and activation of downstream signaling. We show that the interaction between gp55 and Sf-Stk induces the cell surface translocation of Sf-Stk. We also show that mutation of the cysteines in the extracellular domain of Sf-Stk results in enhanced cell surface localization in the absence of gp55, but not enhanced phosphorylation of Sf-Stk. Alternatively, while a constitutively activating mutation in the kinase domain of Sf-Stk is not sufficient to induce receptor phosphorylation and downstream signaling, mutation of the cysteines in the extracellular domain of Sf-Stk in this context restores full activity of Sf-Stk in the absence of gp55. These results indicate that both kinase activation and cell surface localization play an essential role in the activation of Sf-Stk signaling by gp55.

Finally, we demonstrate that gp55 activation of Sf-Stk results in the recruitment and activation of Gab1 through the C terminal docking sites Tyr429 and Tyr436, and that Gab1 activation by Sf-Stk correlates with its ability to promote the phosphorylation of Erk and the activation of AP1. Further, we show that Src interacts with Sf-Stk in the presence of gp55 and that Src activity is essential for the phosphorylation of Gab1 and Gab2 and the ability of gp55 to promote Sf-Stk activation of the MAPK signaling pathway. We also demonstrate that Src kinase activity is essential for the Epo independent growth of primary erythroblasts in response to Friend virus infection.

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LIST OF ABBREVIATIONS

AGM	Aorta-Gonad-Mesonephros
AML	Acute myeloid leukemia
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP1	Activator protein 1
BFU-E	Burst Forming Unit-Erythroid
CD	Cluster of differentiation
CIS	Cytokine inducible SH2 containing protein
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
CHO	Chinese hamster ovary
CKI	Cytokine dependent kinase inhibitor
CLP	Common Lymphoid Progenitor
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor

EGFP	Enhanced Green Fluorescent Protein
EKLF	Erythroid Kruppel-like factor
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
ER	Endoplasmic reticulum
Erk	Extracellular signal regulated kinase
Fak	Focal adhesion kinase
F-MuLV	Friend Murine Leukemia Virus
FV	Friend Virus
Fv2	Friend virus resistant 2
FVA	Friend Virus Anemia
FVP	Friend Virus Polycythemia
Gab	Grb associated binder
GATA	GATA transcription factor
GDNF	Glial cell derived neurotrophic factor
GMP	Granulocyte-monocyte progenitor
Grb	Growth factor receptor bound protein
HEK	Human embryonic kidney
HIF	Hypoxia inducible factor
HGF	Hepatocyte growth factor
HPRC	Human papillary renal carcinoma
HSC	Hematopoietic Stem Cell

IGF	Insulin-like growth factor
IgM	Immunoglobulin M
IL	Interleukin
IRS	Insulin receptor substrate
JAK	Janus Kinase
JNK	Janus N-terminal linked Kinase
LT-HSC	Long Term Hematopoietic Stem Cell
MAPK	Mitogen Activated Protein Kinase
MBD	Met Binding Domain
MBS	Met Binding Sequence
MCFV	Mink cell focus forming virs
M-CSF	Macrophage-Colony Stimulating Factor
MEL	Murine erythroleukemia cells
MEP	Megakarycyte and erythrocyte progenitor
Met	Mesenchymal-epithelial transition factor
MSCV	Murine stem cell virus
MSP	Macrophage Stimulating Protein
MT-SP	Membrane type serine protease
MuLV	Murine leukemia virus
NFkB	Nuclear factor kB
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PI3K	Phosphatidylinositol-3-Kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
PP1	4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]- pyrimidine
RON	Receptor d'origine Nantais
SCF	Stem cell factor
SFFV	Spleen focus forming virus
SFK	Src family kinase
Sf-Stk	Short form stem cell derived kinase
Spi-1	SFFV proviral integration 1 locus
SH2	Src Homology 2
Shc	SH2 domain containing
Ship	SH2 inositol 5-phosphatase
Shp	SH2 containing tyrosine phosphatase
STAT	Signal transducer and activatior of transcription
Stk	Stem cell derived kinase
ST-HSC	Short Term Hematopoietic Stem Cell
YFP	Yellow fluorescent protein

ACKNOWLEDGEMENTS

This dissertation is completed with contributions of many colleagues and unconditional love of family and friends. I would like to thank all who have helped and inspired me during my doctoral study and who have given me the possibility to complete this dissertation.

I want to thank the Department of Veterinary and Biomedical Sciences and all the staff for having provided me with an excellent and friendly research environment. In addition, your administrative support has made my research smooth and helped me to stay focused.

My utmost gratitude goes to my supervisor, Dr. Pamela Hankey, whose expertise, stimulating suggestions, guidance, patience, encouragement, and kindness have supported me through all the high and low times of research and the writing of this dissertation. She is always accessible, willing to listen and help, and exhaustive in offering her valuable opinion and insight. All her advisees, including me, have been tremendously motivated by and have greatly received from her energy and enthusiasm in research as well as care for people. Dr. Hankey has truly made this dissertation writing a rewarding experience for me.

Dr. Avery August, Dr. Biao He, Dr. Robert Paulson and Dr. Zhi-Chun Lai deserve special thanks as my dissertation committee members and advisors. In particular, I am deeply indebted to Dr. Paulson for his knowledge of my research topic has enlightened me with novel ideas.

This work would not have been possible without the support and encouragement of my colleagues and friends. All my lab buddies have made the lab a convivial place to work in. Especially, I would like to thank Daniel Sharda and Shuang Ni for their generous friendship and help in the past 6 years. All other folks, including former lab members, have inspired me in research and life through our daily interactions during those long hours in the lab. You have made those long hours at lab enjoyable. Thanks.

I cannot end without thanking my family on whose constant encouragement and love I have relied throughout education at home and overseas. I thank my parents who have brought me up to be an honest and responsible person. You are my best role models and I will be a son you can be proud of. My beloved wife, I cannot thank you enough for leaving the home in China, walking through each day gratefully with me, and giving me your hearty support and a sweet home.

Chapter 1

Literature Review

1.1 Preface

Friend virus induces an acute erythroleukemia in susceptible mice initiated by the interaction of the Friend virus encoded glycoprotein, gp55. With the erythropoietin receptor (EpoR) and the product of the host *Fv2* gene, a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). The Friend disease model has provided a powerful platform for biologists of different research interests. Virology and immunology studies have contributed to insightful understanding of not only the Friend virus life cycle, infection and host immunity, but also disease progression induced by many other retroviruses, such as Rous sarcoma virus and human immunodeficiency virus. Oncology, hematology, cell biology and molecular biology approaches to studying Friend erythroleukemia have uncovered the pathologic progression of this multiple stage leukemia. Several critical virus and host genes involved in the disease have been identified and their roles in the disease have been intensely investigated. The lessons learned from the study of Friend disease have enriched our knowledge regarding the molecular mechanisms governing the progression of cancer, the development of the hematopoietic system, and immunity to infection. Given the highly homologous nature of the proteins involved in these responses between the murine and human genomes, these studies will also lead to a better understanding of the events that regulate human physiology and disease.

1.2 Hematopoiesis

1.2.1 Hematopoietic Stem Cells

Hematopoiesis is the continuous development of blood cells which occurs throughout the lifetime of an animal. In the fetus, primitive hematopoiesis first occurs in yolk sac blood islands. Definitive hematopoiesis begins in the aorta-gonad-mesonephros (AGM) region and eventually migrates to the fetal liver. The spleen also contributes to hematopoiesis at this stage. Around birth, the bone marrow becomes the major organ of hematopoiesis. At this time, all axial and appendicular skeletons carry red bone marrow that function to support hematopoietic development. With an increase in age, red bone marrow harboring hematopoietic activity retreats to the axial skeleton and proximal ends of long bones. Fat cells fill the hollow interior of the middle portion of long bones, resulting in the appearance of yellow bone marrow. In addition to the red bone marrow, extramedullary hematopoiesis can still occur in the liver, spleen and yellow bone marrow under conditions when the red bone marrow cannot keep up with the demand of blood cell production, including conditions of severe blood loss and hypoxia.

The changes in the sites of hematopoiesis during ontogeny are reflected by changes in the character of the associated hematopoietic activities. Primitive yolk sac hematopoiesis results in the production of nucleated red blood cells which are approximately six fold bigger than the red blood cells produced by definitive hematopoiesis. Primitive red blood cells derived from the yolk sac express both embryonic and adult hemoglobins while definitive red blood cells express predominantly adult hemoglobins. Yolk sac-derived primitive erythropoiesis can be independent of

erythropoietin, while definitive erythropoiesis is strictly dependent on erythropoietin [1]. However, the yolk sac can also produce definitive BFU-E, which later mature in the fetal liver [2]. It is now believed that the yolk sac provides committed progenitor cells and maturing red blood cells so that the embryo can survive until Aorta-Gonad-Mesonephro(AGM) derived hematopoietic stem cells emerge and differentiate into mature blood cells [3].

All blood cells are derived from hematopoietic stem cells (HSCs). These stem cells represent a small population that retain the ability to self-renew, however this potential is lost in a programmed way as these cells differentiate into more and more committed lineage progenitor cells and eventually become functional mature blood cells. Transplantation of HSCs into lethally irradiated mice can restore the full capacity of hematopoiesis in the recipient animal. It has been reported that even transplantation of a single HSC can regenerate all of the blood cell lineages[4].

Based on their origin, HSCs can be divided into primitive HSCs and definitive HSCs. Primitive HSCs arise from the yolk sac of the embryo, whereas definitive HSCs originate in the AGM and are eventually confined to the adult bone marrow. Though these two populations of HSCs display distinct features, increasing evidence suggests that they are closely related. Isolated yolk sac cells transplanted into myeloablated adult mice fail to engraft and reconstitute hematopoiesis in the recipient animal. However, in sublethally myeloablated newborn mice, yolk sac cells can engraft and repopulate all lineages of the hematopoietic system for up to 12 months. The ability of the yolk sac HSCs to differentiate into all hematopoietic lineages in these recipients strongly suggests

that the local cellular microenvironment plays a prominent role in regulating yolk sac HSCs[5].

Based on their ability to self-renew, HSCs can also be divided into long-term and short-term reconstituting HSCs (LT-HSCs and ST-HSCs, respectively). LT-HSCs have unlimited self-renewal capacity and can sustain lifelong hematopoiesis when transplanted into lethally irradiated mice, whereas ST-HSCs are more defined stem cells that are restricted in self-renewal potential, sustaining hematopoiesis only for a limited time[6, 7]. Using fluorochrome-conjugated antibody binding and flow cytometry, HSCs can be enriched by sorting for the lineage negative/Sca1+/kit+ cell population[8]. Using a similar approach, LT-HSC and ST-HSC can be further separated by other cell surface markers including Thy1.1 low/FLT3-/CD34-/Endoglin+/SP+/Rho low/CD150+ for LT-HSCs and Thy1.1 low/FLT3-/CD34+/CD11b low for ST-HSCs[9].

HSC self-renewal is tightly controlled both intrinsically and extrinsically. Intrinsically, the self-renewal of HSCs is regulated by anti-apoptotic proteins, transcription factors, and signal transducers including BCL2, STAT5 and Pten[10]. Extrinsically, the endosteal bone marrow niche[11] provides a hypoxic microenvironment that keeps HSCs in a long term dormant state. Synapses between the stromal cells found in the hematopoietic niche and HSCs mediate HSC adhesion, migration, dormancy and self-renewal by providing ligands and influencing HSC signaling through activation of pathways such as Wnt, Notch, Shh, and the Tie2/Ang-1 pathway. Another type of vascular niche is located near blood vessels. The oxygen rich environment of this niche is believed to support the activated and cycling HSCs. After asymmetric cell division, activated daughter cells move out of the niche and initiate differentiation[12-14].

HSC differentiation begins with asymmetric cell division, resulting in one daughter HSC and one multipotent progenitor cell. The detailed molecular mechanism governing asymmetric cell division is not clear, but several lines of evidence suggest that this can arise either from the unequal distribution of intrinsic cell factors in the daughter cells or from differential microenvironmental exposure which can change the phenotype of one of the daughter cells in response to external signals[15]. The cause of lineage restriction is likely to be the result of both intrinsic and extrinsic regulation. The progenitor cells undergo progressive epigenetic changes and permanently silence those genes that may participate in the development of other cell types.

1.2.2 Hematopoiesis

During progenitor cell maturation, various signals are spatiotemporally required to regulate their differentiation, proliferation, survival and apoptosis. The HSCs first differentiate into either common lymphoid progenitor cells (CLPs) or common myeloid progenitor cells (CMPs). The CLPs further differentiate into T or B lymphocytes, which are responsible for the host adaptive immune response, and nature killer T cells, which are important in the innate immune response. Unlike the development of other lineages, T lymphopoiesis primarily occurs in the thymus rather than the bone marrow. The CMPs develop into granulocyte–monocyte progenitors (GMPs) or progenitors for megakarycytes and erythrocytes (MEPs). The final products of GMPs include dendritic cells, macrophages, mast cells, basophils, neutrophils and eosinophils. Megakaryocytes are responsible for the production of platelets, which are necessary for normal blood

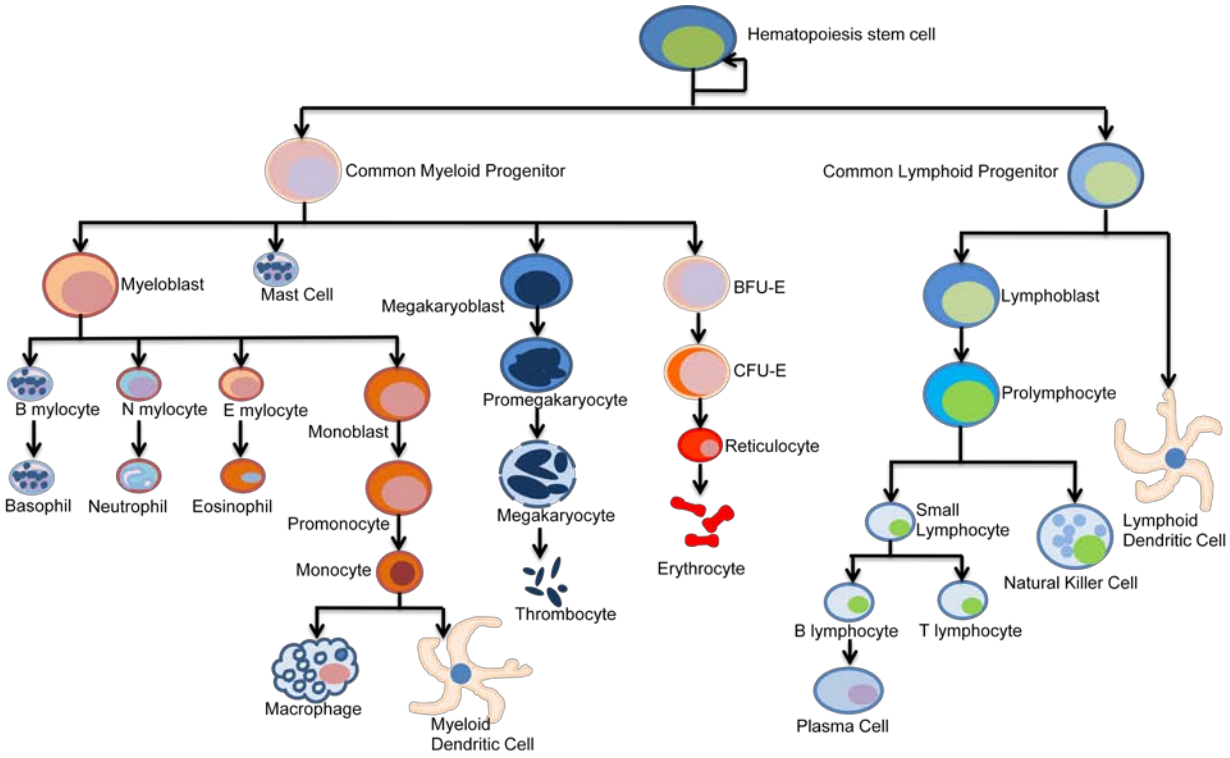

Fig 1.1 Steady State Hematopoiesis

Hematopoietic stem cells (HSCs) first differentiate into either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). CLPs further differentiate into T or B lymphocytes. CMPs develop into dendritic cells, macrophages, mast cells, basophils, neutrophils, eosinophils, megakaryocytes and erythrocytes.

clotting, while erythrocytes are responsible for oxygen transportation from the lung to the tissues.

1.2.3 Erythropoiesis

Erythropoiesis is the process of erythrocyte production. Erythrocytes, also known as red blood cells, deliver oxygen from the lung to the tissues and clear the tissues of carbon dioxide. Erythrocytes are the most abundant blood cells in the human body. They account for approximately 40% of the total blood volume. Erythrocytes are shaped like biconcave discs, which allow the cells to maximize laminar flow and minimize platelet scattering[16]. Red blood cell membranes consist of a lipid bilayer with a cytoskeleton structure underneath to support their shape. Mutation of the genes encoding these cytoskeletal components can cause the loss of the biconcave shape and shorten the usual 120 day lifespan of the red blood cells. Mature erythrocytes contain no nucleus or organelles, but are rather filled with hemoglobins. Hemoglobins are composed of four globin chains, each of which is associated with a heme group containing an iron ion that can bind and release oxygen at the center of the heme ring.

In erythroid development, primitive erythropoiesis originates in the yolk sac, but erythroid cells are released into the blood stream where they complete maturation and enucleation. However, definitive erythropoiesis in the adult occurs entirely in the bone marrow. Adult erythropoiesis can be divided into three distinctive stages. The first stage is characterized by the accruing of erythroid linage committed erythroid progenitor cells. The earliest recognizable erythroid lineage committed progenitor cell is the erythroid-

burst forming unit (BFU-E). It takes eight days and fourteen days for BFU-E to differentiate into mature red blood cells in mice and humans, respectively. From BFU-E, a more mature erythroid committed progenitor cell, the erythroid-colony forming unit (CFU-E) is generated. It take two days and seven days for these late stage erythroid progenitor cells to differentiate into mature red blood cells in mice and humans, respectively[17, 18]. The proliferation and differentiation of the erythroid progenitor cells are regulated, in part, by the coordinated activity of cell surface receptors for a number of cytokines and growth factors[19]. Generally, BFU-E have a strong proliferative potential, and several cytokines such as erythropoietin (Epo), interlukin-3 (IL-3), and stem cell factor (SCF) can enhance the colony formation of BFU-E. In contrast, CFU-E have a very limited proliferative potential and the survival of CFU-E is exclusively dependent on low concentrations of Epo.

The second stage of erythropoiesis is the progression from proerythroblast to basophilic, polychromatophilic, and orthochromatic forms. This stage is characterized by the accumulation of hemoglobins in the cells, expansion of erythroid blast numbers, progressive nuclear pyknosis and loss of the nucleus. In the bone marrow, erythroid progenitor cells mature in association with macrophages in erythroblast islands[20]. Interaction with the stromal microenvironment is critical for normal erythroblast maturation. The culture of human erythroblasts in the absence of macrophage cells results in decreased erythroblast proliferation and enucleation and increased erythroblast apoptosis[21].

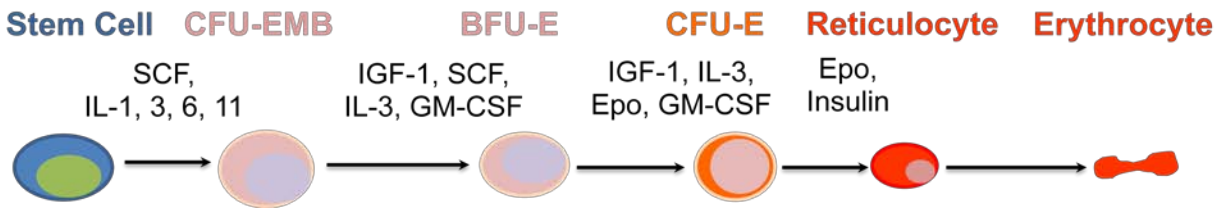

Fig 1.2 Erythropoiesis

The process of erythropoiesis, the development of erythrocytes from hematopoietic stem cells, is defined by several steps. Burst forming unit-erythroid (BFU-E) is the first recognizable erythroid progenitor cell. It takes 8 days for BFU-Es to fully develop into erythrocytes in culture. Cytokines such as Epo and IL-3 are important for the survival and development of erythroid cells.

The third stage of erythropoiesis is the progression of reticulocyte maturation and the release of mature red blood cells into the circulatory system. As reticulocytes terminally differentiate into erythrocytes, an active internal degradation results in the loss of internal organelles such as the endoplasmic reticulum (ER) and mitochondria[22]. Reticulocytes migrate through the sinusoidal endothelium of the bone marrow and enter the bloodstream. In the bloodstream, reticulocytes lose the remaining ribosomes and transit to mature erythrocytes. Normally, human erythrocytes can circulate for about 120 days. Companioned with the aging of circulating red blood cells, a gradual loss of cell membrane, hemoglobin and CD47 expression occurs[23, 24]. Eventually, phosphotidylserine is expressed on the cell surface of senescent erythrocytes. Macrophages recognize the phosphotidylserine and engulf the aged red blood cells[25].

1.3 Erythropoietin and Erythropoietin Receptor Signaling

Erythropoietin (Epo) is a cytokine that is produced primarily in the fetal liver or kidney and released into the circulation. Epo is one of the most important cytokines in erythropoiesis, while primitive erythropoiesis can proceed in the absence of Epo, definitive erythropoiesis is critically dependent on it. Epo gene transcription is regulated by the hypoxia inducible factor1α (HIF1α) through enhancer binding[26]. Under normoxic conditions, high oxygen levels induce hydroxylation of the HIF1α proline residues by hydroxylase. This drives the ubiquitination and degradation of HIF1α[27]. Under hypoxic conditions, HIF1α is not ubiquitinated and can bind the enhancer

sequence in the Epo gene resulting in the upregulation of Epo transcription by seven fold. Thus, the expression of Epo is ultimately regulated by tissue oxygen levels.

Studies in mice show that reducing Epo levels only affects *in vitro* BFU-E colony formation. However, CFU-E numbers are significantly affected by Epo levels both *in vivo* and *in vitro*[28]. This suggests that, during the differentiation of primary erythroblasts from the BFU-E to the CFU-E stage, the progenitor cells acquire Epo dependence. Accordingly, the cell surface expression of the Epo receptor (EpoR) on erythroid progenitor cells increases during erythroid lineage commitment and peaks around the CFU-E stage. After that, EpoR expression declines with the terminal differentiation of the erythroid progenitor cells. Consequently, reticulocytes appear to have lost their Epo dependence[29, 30].

Epo is the ligand for the erythropoietin receptor (EpoR). One Epo molecule binds to two EpoR molecules to induce the activation of preformed EpoR dimers. The EpoR is encoded by a single-copy gene which shares about 82% similarity between mice and humans. The murine and human EpoR genes encode 507 amino acid (aa) and 508aa proteins, respectively. From N terminal to C terminal, the EpoR peptide contains a 24aa signal peptide, a 225aa extracellular domain, a 22aa transmembrane domain and a 236aa cytoplasmic domain. The molecular weight of the EpoR is about 55KDa, while glycosylated and phosphorylated EpoR molecules can reach up to 72-85KDa. EpoR is a member of the hemopoietin/cytokine receptor superfamily. Members of this superfamily share some common features. They have four conserved cysteines that form two intramolecular disulfide bonds at relatively similar positions in the N terminal extracellular domain. A conserved 20aa sequence in the extracellular domain near the

transmembrane domain contains the specific amino acid sequence Trp-Ser-X-Trp-Ser (WSXWS). The cytoplasmic region of the EpoR can be divided into two parts. The juxtamembrane domain contains a hydrophobic motif and amino acid residues Leu253, Ile257 and Trp282 in this motif are important for Janus kinase 2 (JAK2) binding[31]. The distant C terminal tail contains eight tyrosine residues (Tyr343, Tyr401, Tyr429, Tyr431, Tyr443, Tyr460, Tyr464 and Tyr479) which serve as docking sites for multiple Src homology 2 (SH2) domain-containing proteins[32, 33]. EpoR ligand independent dimerization is mediated by the transmembrane domain. Epo binding or a point mutation of residue Arg129 to Cys (R129C) induces a change in the intermolecular disulfide bond of the EpoR to promote the active conformation. It is believed that the correct orientation of the JAK2 binding motif between the two EpoR molecules is critical for EpoR activation. Cysteine scanning mutations in the EpoR show that only dimerization of certain residues can activate the EpoR[34, 35]. The EpoR does not have intrinsic enzymatic activity. Epo binding leads to the phosphorylation of the constitutively associated JAK2, which induces EpoR phosphorylation resulting in the recruitment of downstream signals. Mice deficient in either Epo or the EpoR exhibit severe deficiencies in erythroid cell development, resulting in decreased BFU-E/CFU-E formation and embryonic lethality. Similarly, JAK2 knockout mice also exhibit impaired definitive erythropoiesis and $JAK2^{-/-}$ embryos die around day 12.5 of gestation[36, 37]. Interestingly, JAK2 not only serves as a downstream signal of the EpoR, but Huang *et al.* demonstrated that Jak2 also specifically promotes Golgi processing and cell surface expression of the EpoR[38].

One of the signaling molecules recruited following EpoR phosphorylation is signal transducer and activator of transcription 5 (STAT5) [39, 40]. Phosphorylation on Tyr343 on the EpoR results in the recruitment and phosphorylation of STAT5 [41]. Activated STAT5 dimerizes and translocates to nucleus to stimulate the transcription of the Bcl-x(L) gene, which protects primitive and definitive erythroid progenitor cells from apoptosis[42]. Investigations into the role of STAT5 *in vivo* revealed that STAT5a$^{-/-}$b$^{-/-}$ mice are anemic and the erythroid progenitor cells from these animals exhibit higher rates of apoptosis[43-45]. Furthermore, exogenous expression of dominant negative STAT5 suppresses Epo-dependent erythroid differentiation[46].

Lyn, a non-receptor tyrosine kinase of the Src family, also associates with the EpoR. Lyn binds with the phosphorylated EpoR (on Tyr464 and Tyr479) and JAK2 through its Src homology 2 (SH2) domain. The Lyn tyrosine kinase domain also interacts with the unphosphorylated EpoR membrane proximal region and over expression of Lyn is found to induce EpoR and STAT5 phosphorylation[47]. Lyn deficient mice exhibit lower levels of the transcription factors STAT5, GATA-1 and erythroid Kruppel-like factor (EKLF)[48, 49]. Lyn is not required for the susceptibility of Friend virus infection, however, Lyn deficient mice do not develop Polycythemia following FVP infection, suggesting that Lyn-/- infected erythroblasts have a defect in terminal differentiation[50].

Mitogen activated protein kinase (MAPK) extracellular signal regulated kinase (Erk1/2) activation upon Epo stimulation is dependent on JAK2/STAT5 phosphorylation. Further, an EpoR HM mutant, which is truncated with all the C terminal docking sites except the JAK2 binding site, and an EpoR harboring a Tyr343Phe point mutation on the STAT5 binding site, still retain the ability to induce Erk1/2 activation, suggesting that

this signaling pathway is dependent on the direct binding of JAK2, not STAT5[51]. Indirect activation of MAPK by the EpoR can also be mediated by the SH2 inositol 5-phosphatase 1 (Ship1). Phosphorylation of the EpoR on Tyr401 results in the recruitment of Ship1 through its SH2 domain. Subsequent tyrosine phosphorylation of Ship1, promotes its adaptor function and binding of Shc and Grb2, leading to the activation of the Ras/Raf1/MEK/MAP kinase pathway[52, 53].

Phosphorylation on EpoR Tyr 479 results in the direct binding of the p85 subunit of phosphoinositide 3-kinase (PI3K)[54]. A mutant EpoR that contains only tyrosine 479 is sufficient to support CFU-E proliferation and differentiation[55]. PI3K signaling can also be activated indirectly through the Grb2 associated binder1/2 (Gab1/2) binding sites on Tyr343 and Tyr401. Phosphorylation of the insulin receptor substrate 2 (IRS2) following Epo stimulation also promotes PI3K/AKT signaling[56]. The PI3K inhibitor, LY294002, induces apoptosis in erythroid progenitor cells. It has been suggested that PI3K promotes erythroid progenitor cell proliferation by downregulating the cytokine dependent kinase inhibitor (CKI) P27KIP1 via proteasomal degradation[57, 58].

A few negative regulators are also recruited to the phosphorylated EpoR. The SH2 containing tyrosine phosphatase (SHP1) is activated through binding to the phosphorylated Tyr429 site on the EpoR. SHP1 dephosphorylates the EpoR and JAK2, thus inactivating them. An EpoR Y429F mutant that does not bind to SHP1, displays prolonged JAK2 phosphorylation, and is hypersensitive to Epo stimulation[59]. In addition to Gab1 and Gab2, phosphorylated Tyr343 and Tyr401 also recruit the SHP2 phosphatase[60]. Another negative regulator of EpoR signaling is cytokine inducible SH2 containing protein (CIS) which binds to Tyr401 of the EpoR. STAT5 binds to the CIS

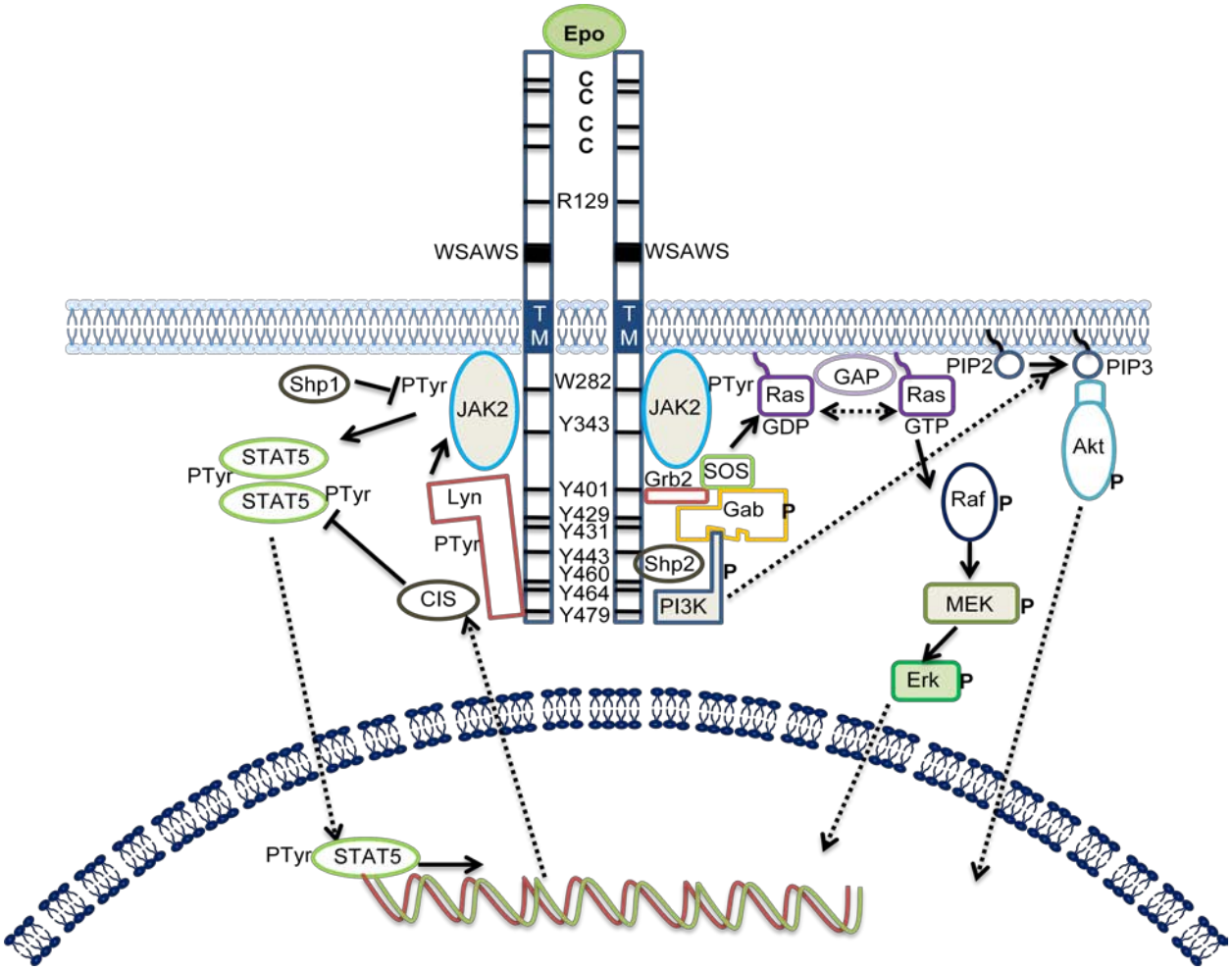

Fig 1.3 Epo/EpoR signaling

Binding of a single Epo molecule to a preformed EpoR dimer results in the JAK2 and STAT5 activation. JAK2 also enhances the phosphorylation of the EpoR which subsequently recruits SH2 domain containing signaling molecules such as Grb2, PI3K, Lyn and Shp, which further amplify downstream signaling pathways.

promoter and activates CIS transcription, while CIS expression suppresses STAT5 phosphorylation. Thus, CIS is a classic negative feedback modulator of STAT5[61]

1.4 Friend erythroleukemia

In normal erythropoiesis, erythroid progenitor cells develop into mature enucleated red blood cells. In Friend virus induced erythroleukemia, mice produce large numbers of abnormal, immature red blood cells. This virally induced disease is named after Dr Charlotte Friend (1921-1987), who first found that a filterable cell free agent obtained from the spleen of a leukemic Swiss mouse caused transmission of the leukemia in adult mice[62].

The target cells of Friend virus infection are Epo responsive erythroid progenitor cells. Friend virus is a complex of two viruses, the spleen focus forming virus (SFFV), which is a replication defective C type retrovirus, and the ecotropic helper Friend murine leukemia virus (F-MuLV). SFFV is responsible for the rapid splenomegaly and acute erythroleukemia induced by Friend virus infection[63-66], while F-MuLV provides the replication-rescue ability and can be substituted for by other murine leukemia viruses[67]. Specifically, the glycoprotein gp55, encoded by the SFFV *env* gene, appears to act as the transforming viral oncoprotein. A retroviral vector containing the isolated gp55 coding sequence can induce a rapid erythroid disease that is indistinguishable from that induced by the entire SFFV genome[64, 68]. The late stage of erythroleukemia in Friend virus infected mice is marked by inactivation of the *p53* locus[69-73] and integration of the virus into the SFFV proviral integration-1(*Spi-1*) locus[74-76] which

leads to inappropriate expression of the transcription factor PU.1 in erythroid cells, causing a block in their differentiation and eventually acute erythroleukemia. These leukemic cells have extensive proliferative capacity, they are transplantable *in vivo*, and can be used to establish permanent cell lines *in vitro*. Permanent cell lines established in culture from these tumors are called murine erythroleukemia (MEL) cells[77]. These lines are known to contain both SFFV and MuLV sequences. Interestingly, treatment of MEL cells with 1% dimethylsulphoxide (DMSO) in *vitro* causes them to undergo erythroid differentiation, resulting in syndissertation of hemoglobin[78]. Similarly, expression of a dominant negative Ras or treatment with Epo and DMSO in FVA erythroleukemia derived SKT6 cells promotes differentiation. Epo induced SKT6 differentiation is inhibited by expression of a constitutive active Ras. Inactivation of Erk is also observed after Epo treatment, suggesting that the Ras/MAPK signaling is a negative regulator of the terminal differentiation.

The disease induced by Friend virus is driven, in part, by the interaction of the EpoR and gp55. Researchers have demonstrated the significance of the Epo activated EpoR signaling pathway in normal erythropoiesis, however, the role of Epo independent EpoR activation in response to Friend virus infection remains unclear. It is suggested that Epo/EpoR signaling is distinct from gp55/EpoR signal transduction. It is well known that Epo/EpoR interactions transiently activate JAK2 and STAT5[39, 79, 80]. However, in Friend disease, constitutive tyrosine phosphorylation of both JAK1 and JAK2 in gp55 infected primary erythroid cells was reported[81-83]. In addition, the role of STAT5 in EpoR/gp55 signaling is not clear. Yamamura *et al* demonstrated STAT5 tyrosine phosphorylation in Friend virus derived erythroleukemia cell lines, however Epo is

required for this STAT5 to translocate to the nucleus and bind to DNA[82]. In the erythroleukemia cell line HB60-5, Zochodne *et al*. reported that gp55 expression overrides Epo induced STAT5b phosphorylation and blocks cell differentiation[84]. In contrast to this, Moucadel *et al.* reported that Epo stimulates both STAT5a and STAT5b, while only STAT5b is activated in a gp55 transformed cell line, and this STAT5b binds both N3 high affinity and N4 GAS low affinity sites on chromatin[85]. Recently, by using Friend virus infected STAT5a^{-}/b^{-} mice, Zhang *et al.* demonstrated that STAT5a/b is not required for gp55 induced erythroid proliferation, but is required for the development of polycythemia[83]. Also, Zhang *et al* reported that JAK2 and STAT5a/b are constitutively activated by Friend virus, in the presence or absence of Epo.

The 409aa gp55 protein is a naturally occurring fusion envelope protein of gp70 and p15E [86-89]. However, gp55 is not processed in the assembly of the viral particle and remains in the infected cell. The majority of the gp55 protein in the infected cells is retained in the rough endoplasmic reticulum (ER) while about 3-5% of the gp55 is further processed, glycosylated and translocated to the cell surface[90]. Comparative analysis of amino acid sequences suggests that the SFFV *env* gene could have been derived from mink cell focus forming virus (MCFV, aa1-aa331) and F-MuLV(aa279-aa398). A 585bp deletion removed the gp70-p15E cleavage site, while a single nucleotide insertion in the p15E coding region resulted in a frame shift and the loss of 33aa in the cytoplasmic domain. This truncation mutation is essential for gp55 pathogenesis, as a gp55 mutant in which this sequence is replaced is non leukemogenic[91, 92]. Protein co-immunoprecipitation and electrophoresis under non-denaturing conditions has revealed that gp55 must be correctly folded and form a homodimer in order to be translocated to

the cell membrane[93, 94]. Though the bulk of gp55 and EpoR were found to co-immunoprecipitate in the ER[95], a productive interaction between EpoR and gp55 requires localization on the cell membrane[96, 97].

From amino terminal to carboxyl terminal, the gp55 protein is composed of a dualtropic domain, a hypervariable proline rich linker (aa235-280), an ecotropic domain and a hydrophobic membrane anchor. Since gp55 has only 2 amino acids in its cytoplasmic tail, it is unlikely that the tail contributes to the interaction of gp55 with the EpoR. Consistent with this hypodissertation, a mutant in which the cytoplasmic domain of the EpoR is truncated retains its ability to co-immunoprecipitate with gp55[32].

Previous studies suggested that the transmembrane domain is important for the gp55/EpoR interaction. There are 2 variant strains of SFFV: SFFV-Anemia (FVA) and SFFV-Polycythemia (FVP). SFFV-A induces erythroleukemia and anemia, due to hemodilution, whereas SFFV-P induces erythroleukemia and polycythemia (a massive increase in the number of mature red cells). In the early stages of FVA infection, Epo is required for the polyclonal expansion of infected erythroid progenitor cells[98, 99]. On the other hand, in the early stages of FVP infection, Epo is not required for the polyclonal expansion of the infected cells. The critical difference between SFFV-A and SVVF-P lies within the transmembrane domain of gp55-A and gp55-P [100-102]. Point mutagenesis in the transmembrane domain of gp55-P demonstrated that two continuous Leu residues within this region directly mediate gp55 and EpoR binding. Deletion of these Leu residues (dLL) in gp55-A is responsible for the decreased affinity of gp55-A for the EpoR. Another methionine to isoleucine point mutation (M390I), also found in gp55-A, hampers the full mitogenic activation of the EpoR though has no effect on EpoR

binding[103]. Human EpoR is not responsive to gp55 activation, but a point mutation, L238S, in the human EpoR transmembrane domain results in its activation by gp55-P. Conversely, a S238L point mutant in the murine EpoR abolishes its activation by gp55-P[104]. Furthermore, a chimeric protein in which the non responsive insulin receptor is fused to the EpoR TM domain, can colocalize with gp55 in a fluorescence assay, demonstrating that gp55 interacts with the EpoR through the transmembrane domain[105]. Other evidence also suggested that the gp55 dualtropic domain is important for its ability to interact with the EpoR. This area is highly homologous to other mink cell focus-forming murine leukemia virus (MCFV) envelope proteins and Li *et al* found that the gp70 envelope protein of MCFV can coprecipitate with EpoR and induce the growth of Ba/F3 cells expressing the EpoR in the absence of growth factor, while gp70 from ecotropic MuLV cannot[106]. Also, an EpoR/IL-3R chimeric protein that contains only the extracellular region of the EpoR through the conserved WSXWS motif was found to co-immunoprecipitate with gp55[107].

Several loci in the mouse genome have been identified that control Friend virus susceptibility. *Fv1* and *Fv4* affect the ability of Friend virus to infect early erythroid progenitor cells. The *Fv1* gene product inhibits Friend virus infection by interacting with its capsid protein[108], and the *Fv4* gene product affects Friend virus binding by competing with receptors on the cell membrane[108]. Another set of genes, *W*, *Sl*, *f* and *Fv2* are required for the development or expansion of infected progenitor cells. The dominant white spotting, *W*, and Steel, *Sl*, loci encode the Kit receptor tyrosine kinase and its ligand, stem cell factor, respectively. Subramanian *et al* demonstrated that Friend virus has distinct target cells in the bone marrow and spleen, and that *W* mutant mice lack

the spleen target cells [109]. In 1999, we demonstrated that the Friend virus susceptibility gene, *Fv2*, encodes the stem cell-derived tyrosine kinase (Stk) receptor[110]. A naturally occurring N-terminally truncated form of Stk, short form Stk (Sf-Stk), is required for Friend virus susceptibility. $Fv2^{r/r}$ mice, including C57BL/6 mice, lack expression of Sf-Stk and are resistant to Friend virus infection, while full-length Stk expression is unaffected. An internal promoter within the Stk locus drives Sf-Stk expression, and $Fv2^{r/r}$ mice harbor mutations in the internal promoter. Sf-Stk lacks the N-terminal ligand binding domain of full-length Stk, but retains the transmembrane and tyrosine kinase domains. *In vitro* and *in vivo* expression of Sf-Stk in C57BL/6 bone marrow cells has been shown to confer Friend virus susceptibility to $Fv2^{r/r}$ mice[111]. Furthermore, Sf-Stk has been shown to covalently interact with gp55, resulting in constitutive activation of Sf-Stk[112]. However, the mechanism by which this activation occurs is currently unknown. It has also been reported that when Sf-Stk is present, SFFV can induce the transformation of rodent fibroblast 3T3 cells, which express no EpoR[113]. Zhang J et al reported that the distal region of the EpoR is not necessary for Sf-Stk induced erythroid progenitor cell proliferation and that mouse EpoR can be substituted for by the human EpoR, which cannot interact with gp55, to support Friend virus induced erythroblastosis[83], suggesting that EpoR is not required for gp55/Sf-Stk mediated transformation.

The chicken c-sea[114] and human Ron receptor tyrosine kinases have been generally accepted as the orthologs of murine full length Stk. In chickens, erythroleukemia is induced by the S13 avian erythroblastosis virus harboring the viral sea

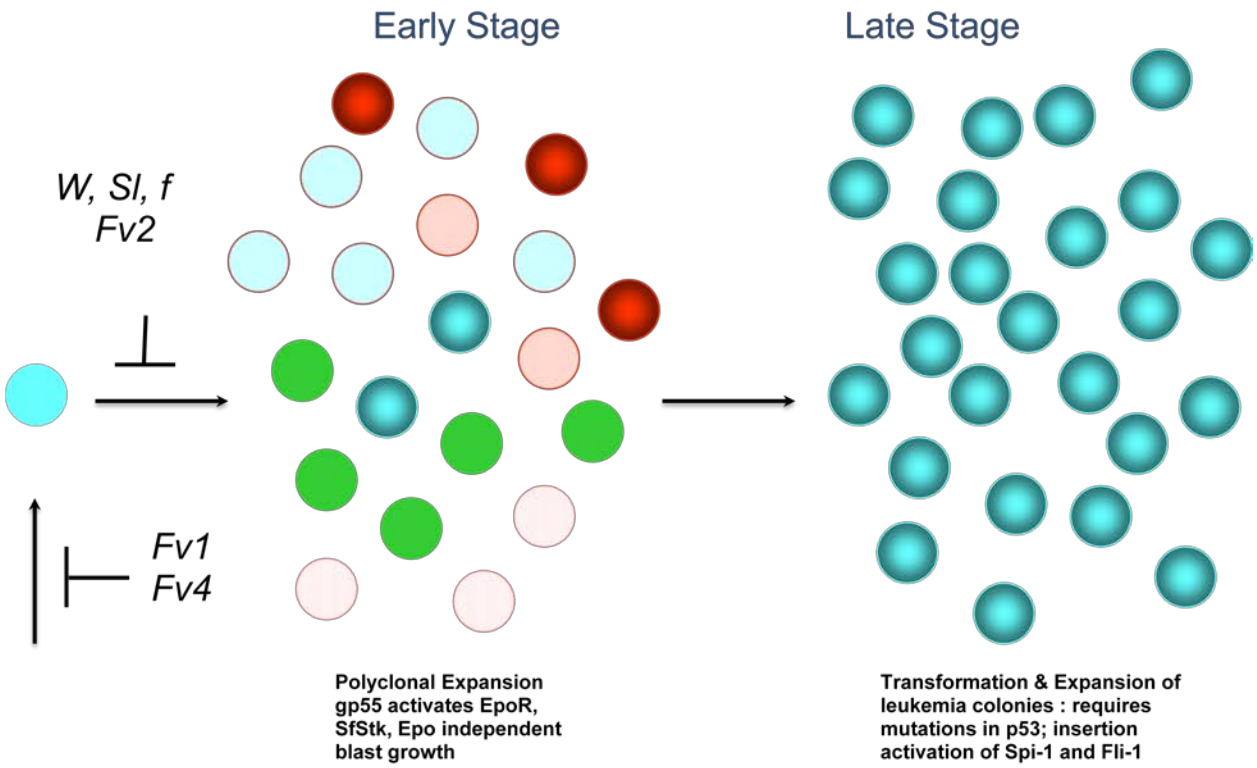

Fig 1.4 Friend erythroleukemia

Friend virus induces erythroleukemia in susceptible mice. In the early stage of the disease, Friend virus infection results in the uncontrolled polyclonal expansion of erythroid progenitor cells in the spleen. In the late stage of the disease, integration of the virus into Spi-1 locus blocks differentiation and results in leukemic transformation.

(v-sea) which is derived from its cellular counterpart, c-sea. In *v-sea*, c-sea is fused in frame to the 3' end of the viral envelop gene from the S13 viral genome, and is expressed as a fusion protein $gp155^{env\text{-}sea}$. Hence this fusion protein retains the extracellular and transmembrane domains of the viral envelope and the intracellular tyrosine kinase domain of *sea*[115]. By using a temperature sensitive mutant of S13, Knight, J *et al* demonstrated that the tyrosine kinase activity of v-sea is required for the acute transforming ability of S13[116].

1.5 Stk/Ron Signaling

Ron, the human homologue of the Stk receptor tyrosine kinase, belongs to the scatter factor receptor tyrosine kinase family. Ron and mesenchymal-epithelial transition factor (Met), are the only two members of this family expressed in humans. Met is the receptor for hepatocyte growth factor (HGF), and Ron is the receptor for macrophage stimulating protein (MSP)[117, 118]. Ron is expressed in epithelial tissues, neurons, macrophages and hematopoietic cells[119, 120] Ron regulated biological functions include the ability to promote adhesion and motility, as well as cell growth and survival. Up to 20 fold over expression of Ron was first observed in primary breast cancer samples. In the breast carcinoma derived cell line, ZR75.1, Ron activation results in cell proliferation, migration and invasion[121]. Ron overexpression and constitutive activation has also been demonstrated in human colon cancer cell lines[122], and immunohistochemical staining demonstrated Ron overexpression in significant numbers

of cancers including breast (56%), colon (51%), lung (48%), thyroid (42%), skin (37%), bladder (36%), and pancreas (33%)[123].

The Ron cDNA was first cloned in 1993 from cDNA libraries prepared from human tumors and from human foreskin keratinocytes[124]. The Stk cDNA was first cloned in 1994 from highly purified murine hematopoietic stem cells[125]. The gene encoding Ron is located on chromosome 3p21.3[126]. It has 20 exons and 19 introns encoding a total of 1400aa including an N terminal signal peptide. Ron is first expressed as a single chain precursor which is then cleaved at a specific site (K305-R-R-R-R-309) into a 40kDa α chain and a 150kD β chain linked by a disulfide bond, forming the mature heterodimeric Ron receptor. The α chain contains 284aa and is completely extracellular, while the β chain is composed of 1096aa containing an extracellular domain, a single transmembrane domain and a cytoplasmic domain. Important structural features of the extracellular domain of the β chain include a Sema domain at the N terminus followed by a plexin semaphorin-integrin (PSI) motif and four immunoglobulin, plexin, and transcription factor (IPT) domains[126]. The cytoplasmic region of Ron can be divided into the juxtamembrane domain, the tyrosine kinase domain and the non-catalytic c-terminal tail.

In the human gastric cancer cell line, KATO-III, mutants of Ron harboring an in frame deletion of 49aa in the proline rich motif in the extracellular domain of the β chain (RonΔ165) have been isolated[127]. This deletion is due to variant splicing of exon 11, and the cleavage of RonΔ165 into the α and β chains does not occur. Thus RonΔ165 is retained in the cytoplasm, and forms constitutively active homodimers through the generation of intermolecular disulfide bonds. While RonΔ165 does not contain cell

transforming ability, it can induce cell motility when transfected into NIH 3T3 cells. Another Ron variant containing a 109aa in frame deletion generated by aberrant splicing of exons 5 and 6 (RonΔ160) was cloned from colon cancer cell lines HT29 and SW837[122, 128]. RonΔ160 can be cleaved into the two chain form and translocate to the cell surface. The 109aa deletion in the cysteine rich Sema domain results in an unbalanced number of cysteines that form abnormal disulfide bonds resulting in the constitutive activation of RonΔ160. A third variant of Ron found in two primary colorectal adenocarcinoma samples contains both the 49aa and 109aa in frame deletions (RonΔ155)[129]. RonΔ155 is retained in the cytoplasm in a monomeric form and is constitutively activated.

The Ron gene contains an internal promoter in intron 10 and translation can be initiated from an ATG codon in exon 11 to produce Sf-Ron. Sf-Ron, like Sf-Stk, lacks most of the extracellular domain of the receptor but retains intact transmembrane and cytoplasmic domains. This truncated transcript has long been observed in northern blots[118]. Recently, the expression of Sf-Ron was found in normal and cancer tissues including those from ovarian, pancreatic and gastrointestinal cancers, as well as in leukemic cells[130]. Proliferation assays indicate that cells expressing Sf-Ron grow faster than control cells. Sf-Ron is found to be constitutively tyrosine phosphorylated in transfected epithelial cells, but so far the signaling pathways activated downstream of Sf-Ron have not been reported. Sf-Ron and Sf-Stk share about 88% sequence similarity[125]. Given the fact that Sf-Stk and v-sea play essential roles in acute leukemic disease, it is reasonable to speculate that Sf-Ron may mediate similar transforming potential in human cells.

In addition to the extracellular domain variants of Ron, a splice variant of Ron harboring a deletion of exon 19 (RonΔ170) has also been reported. Exon 19 encodes 46aa of the catalytic kinase domain and this deletion causes a frame shift and the introduction of a new stop codon, resulting in the truncation of 84aa in the C terminus of Ron, including the multi-functional docking site. RonΔ170 is a naturally occurring dominant negative form of Ron. Co-expression of RonΔ170 with Ron or RonΔ160 inhibits MSP induced MAPK and PI3K signaling in colorectal cancer cells HT-29, HCT115 and SW620. RonΔ170 expression also significantly reduces in vivo tumor growth mediated by Sw620 cells[131].

So far, MSP is the only identified specific ligand for Ron. The gene encoding MSP is also located on chromosome 3p21[132]. MSP is constitutively expressed in liver cells[133] and circulates in the blood as an inactive pro-MSP precursor. Membrane type serine proteases (MT-SP) conduct cleavage of pro-MSP into a disulfide bond linked heterodimer, which is required for MSP/Ron binding and activation[133, 134]. MSP binding results in the tyrosine phosphorylation of Tyr1238 and Tyr1239 in the activation loop of the kinase domain catalytic site, the phosphorylation of which are required for ligand-induced kinase activation, and of two docking site tyrosines in the c-terminal tail, which are responsible for recruiting downstream signaling molecules to the receptor complex. In a murine breast cancer model, expression of MSP promotes breast tumor growth and metastasis to several organs, including bone, which is prominent in human breast cancer. Moreover, statistical analysis demonstrated that coordinated overexpression of MT-SP, MSP and Ron is correlated with accelerated metastasis and poor prognosis in human breast cancer patients[135], .

Transforming mutations in the kinase domain have been described for Met and Ron (MetM1268T, RonM1254T), which result in constitutive receptor activity in the absence of ligand stimulation[136-138]. The MetM1268T mutant was originally identified in patients with human papillary renal carcinoma (HPRC), a kidney cancer that is either inherited or sporadic[139]. While a naturally occurring RonM1254T mutant has not been identified, injection of RonM1254T expressing 3T3 cells results in the development of large tumors in the lungs of nude mice[138]. Interestingly, MetM1268T requires only the phosphorylation of Tyr1235 in the activation loop of the kinase domain, and mutation of Tyr1234 in this context does not affect the biological activity of the receptor [140]. In addition, this mutation overcomes the requirement for the docking site tyrosines in Ron, accompanied by a shift in substrate specificity and receptor autophosphorylation[141]. Generation and expression of the M1231T mutation in Stk (StkM1231T) is associated with enhanced phosphorylation and metastasis compared with wild type Stk[142].

Previous studies indicated that overexpression of the full-length murine Stk receptor, but not the human Ron receptor, is transforming in an NIH3T3 assay[143]. In comparing the activity of Stk and Ron, we found that Stk exhibits a high degree of ligand-independent activity compared with Ron. As observed for Ron M1254T, the ligand-independent activity of Stk is independent of the docking site tyrosines. Chimeric analysis indicated that this enhanced activity mapped to the juxtamembrane domain of Stk[144]. Comparison of the coding sequence of Stk and Ron revealed that exon 13, which encodes part of the juxtamembrane domain of Ron, is not included in the mature Stk transcript. While the splice donor and splice acceptor sites surrounding this exon are

conserved in Stk, a premature stop codon exists in exon 13 of Stk arising from a 1 base-pair frameshift in this pseudo-exon. Deletion of the sequences encoded by exon 13 in Ron, resulted in a similar increase in ligand-independent activity, transforming capacity and changes in substrate specificity. A splice variant in the same juxtamembrane domain region of Met, in which exon 14 is deleted, has also been isolated from murine tissues[145]. Deletion of exon 14 in Met has been observed in lung cancer and is associated with transforming potential in several experimental models. Furthermore, point mutations found in the juxtamembrane domain of Met have been observed in thyroid carcinoma, lung cancer, large B cell lymphoma, AML, and malignant pleural mesothelioma. Finally, deletion of this region is required for the transforming activity of the Tpr/Met fusion protein.

Following kinase activation, downstream signals are recruited to the Stk/Ron receptor through the multifunctional docking site in the c-terminal tail, resulting in activation of the MAPK and PI3K signaling pathways. It has been suggested that activation of the MAPK/Erk pathway by overexpression or constitutive activation of Ron is necessary and sufficient to promote cell differentiation, proliferation and transformation in multiple cell lines [138, 146-148]. Activation of Ron was also found to increase cell growth and migration via the MAPK and PI3K/AKT pathways. Silencing RON expression significantly prevented cell growth and increased cell apoptotic death[123]. Pull down experiments demonstrated that deletion of the c-terminal tail in Ron results in a receptor that fails to dimerize following stimulation with an agonist antibody. Both MSP dependent and independent phosphorylation of Ron were blocked,

and this deletion was sufficient to abolish Ron mediated cell proliferation, shape change and migration[149].

The multifunctional docking site of Stk is composed of Tyr1330 and Tyr1337, and is a conserved feature of the Hepatocyte Growth Factor/Scatter Factor (HGF/SF) receptor family[150]. Upon phosphorylation, the specific sequences of the two docking sites in Stk/Ron are recognized by a number of SH2 domain containing proteins including phospholipase C-γ (PLC- γ), p85, Shc and Grb2. Mutation of both docking site tyrosines to phenylalanine results in the inability of Ron to mediate epithelial cell scattering and migration[151]. Detailed studies using point mutations at these two sites in Stk demonstrated that binding of the p85 subunit of PI3K is more severely affected by a Y1337F than a Y1330F mutant, while Grb2 binding is abrogated by the Y1337F mutant. PLC- γ and Shc association are moderately reduced in these mutants[146]. Similar studies in Met show that the second docking site tyrosine, Tyr1356, is responsible for Grb2, p85, PLC- γ and Shp2 association[152]. Interestingly, p85 does not interact with c-Met in the presence of Grb2, Gab1 or SHIP-1, suggesting there is a preference for binding of these signaling molecules over p85.

In addition to the two tyrosines in the docking site, Ron can also be phosphorylated on Ser1394 in the c-terminal tail by the protein serine/threonine kinase B (Akt or PKB), and this site can be dephosporylated by protein phosphatase 1 (PP1) *in vitro*. Akt phosphorylation of Ron on Ser1394 provides a docking site for 14-3-3, which, in turn, serves as a docking protein for the $\alpha_6\beta_4$ integrin. This Ron-$\alpha_6\beta_4$ complex removes $\alpha_6\beta_4$ integrin from cell-adhesion structures and relocalizes it at lamellipodia. The $\alpha_6\beta_4$ integrin is phosphorylated in this complex, resulting in the activation of p38 MAPK and

nuclear factor kB (NFkB) signaling, which are required for transcriptional upregulation and are normally induced during keratinocyte wound healing[153, 154].

1.6 Src tyrosine kinase

Src is a non-receptor protein tyrosine kinase which was originally found encoded by the avian oncogene of Rous sarcoma virus (v-Src). The cellular homologue of Src (c-Src) is a proto-oncogene that plays key roles in cell proliferation, differentiation and survival. In humans, eleven members of the Src protein tyrosine kinase family have been identified; Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm and Yes[155]. Src, Fyn and Yes are expressed ubiquitously while other Src family kinases (SFKs) are limited to specific cell lineages[156]. From N to C terminus, Src contains an N-terminal 14-carbon myrsitoyl group, a unique segment, an SH3 domain, an SH2 domain, a protein tyrosine kinase domain and a C-terminal regulatory tail[157]. During biosyndissertation, the N-terminal glycine is myristoylated and this myristoylation facilitates the attachment of Src to membranes, including endosomes, secretory vesicles and the cytoplasmic face of the plasma membrane where it can interact with a variety of growth factor and integrin receptors[158]. The SH3 domain of Src interacts with the linker region between the SH2 domain and the tyrosine kinase domain, and this intramolecular interaction helps maintain Src in an inactive conformation. The SH2 domain of Fgr, Fyn, Lck and Src preferentially binds to pYEEI sequences[159], however the Src SH2 domain can also interact with a variety of other docking sites, and amino acids beyond the minimal recognition sequence can also contribute to binding affinity. Under basal conditions, the

majority of Src molecules are phosphorylated on Tyr527 in the C-terminal tail[160] and this YQPG sequence interacts with the SH2 domain of Src, thus further promoting its inactive conformation. These c-terminal amino acids are missing in v-Src, resulting in constitutive activation of v-Src. However, the intramolecular interaction inhibition is weak enough to allow activation, and mutation of this sequence to YEEI results in the abolishment of Hck activation by exogenous SH2 ligands[161]. The Src kinase domain consists of the typical bilobed protein kinase structure. Residues 267-337 make up the small N-lobe and residues 341-520 make up the large C-lobe. In the dormant state, the catalytic center is displaced and the enzyme is inactive. Binding of SH3 and SH2 domains with exogenous ligands changes the conformation of the kinase domain into its active form. Phosphorylation of Tyr416 in the C-lobe of the kinase domain then stabilizes this active conformation. Thus Src has two important regulatory phosphorylation sites: Tyr416 is stimulatory, while Tyr527 is inhibitory.

Extracellular matrix (ECM) induced integrin aggregation was found to lead to Ron oligomerization and transphosphorylation, resulting in an increase in Ron kinase activity in the absence of MSP. Integrin mediated focal adhesion kinase (FAK) activation triggers the activation of c-Src, which targets phosphorylated Ron and results in the phosphorylation of additional Ron tyrosines[162]. Recently, MSP independent Ron activation was reported in MCF-10A breast epithelial cells. PP2, a c-Src inhibitor, reduced both MSP dependent and independent Ron phosphorylation in these cells, and co-expression of a dominant negative c-Src blocked MSP dependent and independent Ron phosphorylation[163]. Our laboratory further demonstrated that Src interacts with Stk in a ligand-independent manner in 293 cells, and that Src activity is required for the

ligand-independent signaling of Stk in these cells [164]. Taken together, these studies suggest that Src can promote Stk/Ron signaling by mediating, directly or indirectly, the phosphorylation of the receptor and/or its downstream targets.

1.7 Gab1/2 signaling

Gab proteins belong to a family of large scaffolding proteins that are conserved from worms to mammals. These signaling molecules do not harbor catalytic activity but bind to multiple signaling molecules and serve as signal amplifiers. In humans, Gab1 and Gab2 are ubiquitously expressed while Gab3 is highly expressed in lymphoid tissues.

Gab proteins share a common structural organization. They contain an N-terminal pleckstrin-homology (PH) domain, several proline rich motifs and multiple tyrosine, serine or threonine phosphorylation sites. Each Gab protein also has unique structural motifs which are responsible for their activation downstream of specific receptors[165]. The PH domain targets Gab to the cell membrane through binding with lipid products of PI3K, particularly phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The proline rich motifs can interact with SH3 domain containing proteins including Grb2 and Src, and the tyrosines serve as docking sites for the recruitment of downstream signals.

Generally, receptor association of Gab is mediated indirectly through Grb2. Grb2 contains two SH3 domains at the N and C terminus flanking a central SH2 domain. While the Grb2 SH2 domain interacts with consensus YXNX binding sites[166] on activated receptors, the c-terminal SH3 domain of Grb2 interacts with a proline-rich motif in Gab, and recruits Gab proteins to the signaling complex. While Gab2 can only interact with

receptors indirectly through Grb2, Gab1 can also interact with c-Met Tyr1349 directly via the Met binding domain (MBD) of Gab1. A 13aa Met binding sequence (MBS) in the MBD is sufficient for direct binding between Gab1 and c-Met, suggesting this motif does not form a structured SH2 domain. Also, unlike the binding of Grb2 to Met, residues upstream of Tyr1249 in Met are required for MBD binding while Tyr1249 can be substituted for by a negatively charged residue and still retains MBD binding ability to recruit and activate Gab1[167].

Gab proteins contain multiple tyrosine residues and, upon phosphorylation, these sites can recruit and activate the SH2 domain containing signaling molecules p85/PI3K and Shp2. All Gab proteins contain at least one YxxM binding motif which mediates the binding of the SH2 domain of the p85 subunit of PI3K. Upon activation, Gab provides a mechanism for the activation of the PI3K signaling pathway by those receptors, such as the EGFR, that lack this binding motif. In addition, activation of PI3K leads to generation of PIP3, which promotes the recruitment of Gab proteins to the surrounding membrane and sustains receptor signaling. Gab proteins also contain a Shp2 binding motif, which is critical for sustained Ras-Erk activation downstream of cMet [168, 169]. Gab1 in which the Shp2 binding motif is mutated fails to activate Erk or a downstream transcriptional reporter [170].Further, activated Erk can bind Gab1 through the MBD and phosphorylate Gab1, suggesting the potential for positive feedback regulation[171]. In addition to the activation of Erk, Shp2 also attenuates EGF dependent PI3K kinase activation by dephosphorylating the p85 binding sites in Gab1. Also, Akt and 14-3-3 are found to bind with specific sites of phosphorylated Gab2. Mutation of these sites results in amplified receptor activation, suggesting a role for these sites in the negative regulation of Gab

signaling [172-174]. While PI3K and Shp2 have been the most intensively studied signaling pathways downstream of Gab proteins, other signaling molecules such as Src, PLC-γ and Crk can also interact with Gab family members.

1.8 Signals involved in the progress of Friend disease

Previous studies by our laboratory have elucidated the downstream signaling pathways by which gp55/Sf-Stk mediates the early stages of Friend disease. By introducing a mutation in the Grb2 binding site on Sf-Stk docking site tyrosines, we demonstrated that Grb2 is required downstream of gp55/Sf-Stk for the Epo independent expansion of Friend virus infected progenitor cells[111]. Grb2 heterozygous mice show decreased sensitivity to Friend virus infection, which is rescued by exogenous expression of Grb2. Mutational analysis of Grb2 demonstrated that the Grb2 C-terminal SH3 domain is essential for the formation of FVP derived Epo independent BFU-E[175]. This SH3 domain binds to a number of large adaptor proteins including the Gab family of adaptors, and we have shown that $Gab2^{-/-}$ mice exhibit significantly smaller spleen sizes than wild type mice following friend virus infection. Exogenous expression of Gab2 rescues Friend virus derived Epo independent BFU-E formation, suggesting that Gab2 activation is also required for the development of Friend erythroleukemia. A Sf-Stk/Gab2 fusion protein containing no Grb2 binding site transfected into erythroid progenitor cells from $Fv2^{r/r}$ mice successfully supported the growth of Friend virus derived Epo independent BFU-E[175], suggesting that Gab2 recruitment and activation downstream of Sf-Stk is both necessary and sufficient to promote Epo^{ind} colony growth in response to Friend virus

infection. Further, we showed that Gab2 recruits STAT3 through a YXXQ binding motif, which is required for Epo independent colony formation following Friend virus infection. This work suggests that gp55 mediates the Epo independent growth of erythroid progenitor cells through a Sf-Stk/Grb2/Gab2/STAT3 signaling pathway. In addition to STAT3, STAT1 and STAT5 activation have been reported in Friend virus infected erythroid cells[176], however, phosphorylation of STAT1 and STAT5 was not observed in gp55 and Sf-Stk expressing 3T3 cells, suggesting that STAT1 and STAT5 activation is regulated by signals other than Sf-Stk.

Both Gab1 and Gab2 phosphorylation have been reported in Sf-Stk expressing 3T3 cells. The activation of Erk, JNK and PI3K/Akt signaling downstream of Sf-Stk has also been reported. Application of the MEK inhibitor, PD98059, and the PI3K inhibitor, wortmannin, blocked Erk and PI3K activation in Sf-Stk expressing 3T3 cells and inhibited Epo^{ind} colony formation. Co-expression of Sf-Stk with a dominant negative p85 in transduced bone marrow cells from $Fv2^{r/r}$ mice failed to rescue the response of these cells to Friend virus infection. Inhibition of JNK by SP600125 also blocked the proliferation of 3T3 cells and SFFV infected HCD-57 cells, suggesting that Sf-Stk, Erk, JNK and PI3K/Akt signaling may play important roles in Friend virus induced erythroblast growth[111, 175, 177-180].

SHIP, Cbl, Shc, IRS and PKC activation have also been detected in Friend virus infected erythroid cells or cell lines derived from Friend erythroleukemia[112, 181-183]. EpoR and Sf-Stk are both expressed in these cells, thus it remains unclear whether the activation of these signals is mediated by Sf-Stk or EpoR. Activation of EpoR or Sf-Stk by gp55 triggers both distinct and overlapping downstream signaling pathways. An

interaction between Sf-Stk and EpoR has also been reported[112], however, little is known regarding the physiological consequences of this interaction. How signaling pathways mediated by Sf-Stk or EpoR might be altered in the presence of the gp55/EpoR/Sf-Stk complex remains an open question.

Chapter 2

Cysteines in the extracellular domain of gp55 mediate the activation of Sf-Stk

2.1 Abstract

Friend virus induces an erythroleukemia in susceptible mice initiated by the interaction of the Friend virus encoded glycoprotein, gp55, with the erythropoietin receptor and the product of the host *Fv2* gene, a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). We have previously demonstrated that the activation of Sf-Stk and recruitment of a Grb2/Gab2/Stat3 signaling complex is required for the ability of Friend virus to induce Epo independent colony formation of erythroid progenitor cells. Here we demonstrate that gp55 interacts with Sf-Stk through the cysteine residues Cys^{306}, Cys^{309}, Cys^{337} and Cys^{338} in the ecotropic domain of gp55 and the cysteine residues Cys^{8}, Cys^{19}, Cys^{37} and Cys^{42} in the extracellular domain of Sf-Stk. This interaction results in enhanced phosphorylation of Sf-Stk. Point mutation of these cysteine residues or deletion of these domains abrogates the interaction between gp55 and Sf-Stk, resulting in the inability of these mutants to promote the Epo independent growth of erythroid progenitor cells.

2.2 Introduction

Since Friend disease was first reported in 1957[62], the acute erythroleukemia induced by the various strains of Friend virus have provided an excellent model to study multistage carcinogenesis[184]. In the first stage, the virus infects erythroid progenitor cells and a viral glycoprotein, gp55, interacts with both the EpoR and a naturally occurring truncated form of the stem cell derived tyrosine kinase, Sf-Stk, resulting in the Epo-independent (Epo^{ind}) expansion of erythroid progenitor cells. The late stage of erythroleukemia in Friend disease is marked by inactivation of the *p53*[69-73] locus and integration of the virus into the *Spi-1* locus[74-76] which leads to inappropriate expression of Pu.1 in erythroid cells, causing a block in their differentiation and eventually acute erythroleukemia.

Friend virus is a complex of two viruses, the spleen focus forming virus (SFFV), which is a replication defective C type retrovirus; and the ecotropic Friend murine leukemia virus (F-MuLV). SFFV is responsible for the rapid splenomegaly and acute erythroleukemia induced by Friend virus infection[63-66], while F-MuLV provides helper function and can be substituted for by other murine leukemia viruses[67]. Helper free preparations of SFFV can cause not only the initial erythroproliferative stage of Friend disease, but also the second stage of erythroblast immortalization. Specifically, the glycoprotein, gp55, encoded by the SFFV *env* gene, acts as the transforming viral oncoprotein[64, 68].

Several loci in the mouse genome have been identified that control Friend virus susceptibility. *Fv1* and *Fv4* affect the ability of Friend virus to infect early erythroid

progenitor cells. The *Fv1* gene product inhibits FV infection by interacting with its capsid protein[185], and the *Fv4* gene product affects FV binding by competing with receptors on the cell membrane[186]. Another set of genes, *W, Sl, f* and *Fv2* are required for the development or expansion of infected progenitor cells. The dominant white spotting locus, *W,* and the Steel locus, *Sl,* encode the Kit receptor tyrosine kinase and its ligand, stem cell factor, respectively. The Friend virus susceptibility gene, *Fv2,* encodes the stem cell-derived tyrosine kinase (Stk) receptor[110]. A naturally occurring N-terminally truncated form of Stk, Sf-Stk, is required for Friend virus susceptibility. $Fv2^{r/r}$ mice, including C57BL/6, lack expression of Sf-Stk and are resistant to FV infection, while full-length Stk expression is unaffected. An internal promoter within the Stk locus drives Sf-Stk expression, and $Fv2^{r/r}$ mice harbor mutations in the internal promoter. Sf-Stk lacks the N-terminal ligand binding domain of full-length Stk, but retains the transmembrane and tyrosine kinase domains. *In vitro* and *in vivo* expression of Sf-Stk in C57BL/6 bone marrow cells has been shown to confer Friend virus susceptibility to $Fv2^{r/r}$ mice[111].

Sf-Stk has been shown to covalently interact with gp55, resulting in constitutive activation of Sf-Stk[112]. Immunoprecipitation results show molecular complexes containing EpoR, gp55 and Sf-Stk, suggesting the EpoR and Sf-Stk binding sites on gp55 are distinct[112]. Furthermore, a deletion in the extracellular domain of Sf-Stk cannot induce transformation of 3T3 fibroblasts when gp55 is co-expressed [113], indicating that this area of Sf-Stk is critical for the activation of Sf-Stk by gp55. However, the mechanism by which this occurs is currently unknown. Here, we identify cysteines in the extracellular domain of Sf-Stk and gp55 that mediate this interaction. Furthermore, we demonstrate that, while the association with gp55 is not required for the dimerization of

Sf-Stk, the interaction of gp55 with Sf-Stk promotes phosphorylation of Sf-Stk. This increase in Sf-Stk activation induced by gp55 mediates the ability of Sf-Stk to induce gene expression and promote the Epo^{ind} growth of primary erythroblasts.

2.3 Materials and Methods

2.3.1 Antibodies and cell culture reagents

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The Mirus-293 transfection reagents were purchased from Mirus Bio, LLC (Madison, WI). The Dual-Luciferase Reporter Assay System was purchased from Promega Corporation (Madison, WI). Antibodies against the Myc tag, HA tag, phospho-tyrosine, phospho-Erk1/2, Erk1/2 and HRP linked anti-rat IgG were purchased from Cell Signaling (Danvers, MA). Antibody against actin and HRP linked anti-mouse IgG were purchased from Sigma-Aldrich, Inc (St.Louis, Mo). Mouse True Blot ULTRA HRP anti-mouse IgG was purchase from eBiosciences (San Diego, CA). HRP linked anti-rabbit IgG was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rat anti-serum against gp55 was kindly provided by Dr. Sandra Ruscetti (National Cancer Institute). Murine interleukin-3 was purchased from Peprotech (Rocky Hill, NJ). Erythropoietin (Epo) was purchased from R&D systems (Minneapolis, MN). Methocult Medium M3234 was purchased from Stem cell technologies (Vancouver, BC). All PCR Primers were ordered from Operon Biotechnologies, Inc (Huntsville, ALlocation). Restriction enzymes and Protein G magnetic beads were

purchased from New England Biolab (Ipswich, MA). PfuTurbo DNA polymerase was purchased from Stratagene (La Jolla, CA). ECL plus Western blotting detection reagents were purchased from GE Healthcare (Piscataway, NJ). Pierce Cell Surface Protein Isolation Kit was purchased from Thermo Fisher Scientific Inc (Rockfold, IL).

2.3.2 Gene construction and mutagenesis

MSCV-myc-Sf-Stk C8A mutagenesis was generated from MSCV-myc-Sf-Stk by using the primers 5'-GTGGGTGGTGAGGTCGCCCAACATGAGCTCCG-3' and 5'-CGGAGCTCATGTTGGGCGACCTCACCACCCAC-3'. MSCV-myc-Sf-Stk C19A and MSCV-myc-Sf-Stk C8,19A mutagenesis were generated from MSCV-myc-Sf-Stk and MSCV-myc-Sf-Stk C8 respectively by using the primers 5'-GGGGATGTGGTGATCGCCCCCCTGCCCCCTTC-3' and 5'-GAAGGGGGCAGGGGGGCGATCACCACATCCCC-3'. MSCV-myc-Sf-Stk C37A mutagenesis was generated by using the primers 5'-GTCCCATTGCAGGTCGCTGTAGACGGTGGGTG-3' and 5'-CACCCACCGTCTACAGCGACCTGCAATGGGAC-3'. MSCV-myc-Sf-Stk C42A mutagenesis was generated by using the primers 5'-CTGTGTAGACGGTGGGGCTCACATCCTGAGCC-3' and 5'-GGCTCAGGATGTGAGCCCCACCGTCTACACG-3'. MSCV-myc-Sf-Stk C37,42A mutagenesis was generated from Sf-Stk C37A by using the primers 5'-CGCTGTAGACGGTGGGGCTCACATCCTGAGCC-3' and 5'-GGCTCAGGATGTGAGCCCCACCGTCTACAGCG-3'. MSCV-myc-Sf-Stk C4A

mutagenesis was generated from Sf-Stk C37,42A by using the primers for Sf-Stk C8A and Sf-Stk C19A mutations.

To construct MSCV-myc-Sf-StkΔ19, the Sf-StkΔ19 fragment was PCR amplified form MSCV-myc-Sf-Stk by using the primers 5'-GGGAATTCCCCCCTGCCCCCTTCCCTG-3' and 5'-GAGACGTGCTACTTCCATTTGTC-3'. The Sf-StkΔ19 PCR fragment was then digested with *EcoR* I and cloned into *EcoR* I digested MSCV-myc-Sf-Stk vector. To construct MSCV-myc-Sf-StkΔ42, the Sf-StkΔ42 fragment was PCR amplified form MSCV-myc-Sf-Stk by using the primers 5'-GGGAATTCTCACATCCTGAGCCAAGTGC-3' and 5'-GAGACGTGCTACTTCCATTTGTC-3'. The Sf-StkΔ42 PCR fragment was then digested with *EcoR* I and cloned into *EcoR* I digested MSCV-myc-Sf-Stk vector. To construct MSCV-myc-Sf-StkΔE, the Sf-StkΔE fragment was PCR amplified form MSCV-myc-Sf-Stk by using the primers 5'-GGGAATTCGATACTCCTTATTGCTCTTCTGG-3' and 5'-GAGACGTGCTACTTCCATTTGTC-3'. The Sf-StkΔE PCR fragment was then digested with *EcoR* I and cloned into *EcoR* I digested MSCV-myc-Sf-Stk vector.

To construct MSCV-myc-Sf-StkΔETM, the Sf-StkΔETM fragment was PCR amplified form MSCV-myc-Sf-Stk by using the primers 5'-CGGAATTCTAACTCCCGAAGACGGAAAAAGC-3' and 5'-GAGACGTGCTACTTCCATTTGTC-3'. The Sf-StkΔETM PCR fragment was then digested with *EcoR* I and cloned into *EcoR* I digested MSCV-myc-Sf-Stk vector. To construct MSCV-Sf-Stk-HA, the Sf-Stk fragment was PCR amplified from MSCV-myc-

Sf-Stk by using the primers 5'-GGCAGATCTTGTGACTGTGAACATG-3' and 5'-AGTGGGCAGGGGTGGCTCTG. The PCR SfStk fragment was then purified and inserted into the *EcoR* V site of pcDNA3.1-HAc. The Sf-Stk-HA fragment was cut out using *Bgl* II / *Xho* I and ligated into *Bgl* II / *Xho* I digested MSCV-neo vector.

MSCV-gp55 C306A mutagenesis was generated by using the primers 5'-CCCTGATAAAATTCAAGAGGCCTGGTTATGCCTAGTGTCTGG 3' and 5'-CCAGACACTAGGCATAACCAGGCCTCTTGAATTTTATCAGGG-3'. MSCV-gp55 C309A mutagenesis was generated by using the primers 5'-CAAGAGTGCTGGTTAGCCCTAGTGTCTGGACCCCCC-3' and 5'-GGGGGGTCCAGACACTAGGGCTAACCAGCACTCTTG-3'. MSCV-gp55 C306, 309A mutagenesis was generated by using the primers 5'-CAAGAGGCCTGGTTAGCCCTAGTGTCTGGACCCCCC-3' and 5'-GGGGGGTCCAGACACTAGGGCTAACCAGGCCTCTTG-3'. MSCV-gp55 C337A mutagenesis was generated by using the primers 5'-GCCCTAAAAGAAAAAGCTTGTTTCTATGCTGACCATACAGGCC-3' and 5'-GGCCTGTATGGTCAGCATAGAAACAAGCTTTTTCTTTTAGGGC-3'. MSCV-gp55 C338A mutagenesis was generated by using the primers 5'-GCCCTAAAAGAAAAATGTGCTTTCTATGCTGACCATACAGGCC-3' and 5'-GGCCTGTATGGTCAGCATAGAAAGCACATTTTTCTTTTAGGGC-3'. MSCV-gp55 C337, 338A mutagenesis was generated by using the primers 5'-GCCCTAAAAGAAAAAGCTGCTTTCTATGCTGACCATACAGGCC-3' and 5'-GGCCTGTATGGTCAGCATAGAAAGCAGCTTTTTCTTTTAGGGC-3'. MSCV-gp55 C4A mutagenesis was generated from MSCV-gp55 C306,309A by using the same

primers for MSCV-gp55 C337, 338A mutagenesis. MSCV-gp55M390I mutagenesis was generated by using the primers 5'-CGTTGATATCCGCCATCATCATCGGGTCTCTCATTATACTCC-3' and 5'-GGAGTATAATGAGAGACCCGATGATGGCGGATATCAACG-3'. MSCV-gp55M390IdLL mutagenesis was generated from MSCV-gp55M390I by using the primers 5'-GTCTCTCATTATACTCCTACTCATTCTGCTTATTTGGACCCTG-3' and 5'-CAGGGTCCAAATAAGCAGAATGAGTAGGAGTATAATGAGAGAC-3'. To construct pCDNA-YFP1-gp55, pCDNA-YFP2-gp55, pCDNA-YFP1-gp55C4A and pCDNA-YFP2-gp55C4A the gp55 and gp55C4A fragments were PCR amplified from MSCV-gp55-HA and MSCV-gp55C4A-HA by using the primers 5'-GTTCCGGAATGGAAGGTCCAGCGTCCTC-3' and 5'-GCTCTAGACTGCCTTGGGAAAAGCGCCTC-3'. The PCR amplified gp55 and gp55C4A fragments and pCDNA-YFP1 and pCDNA-YFP2 plasmids were double digested by *BspE* I and *Xba* I and ligated.

2.3.3 Cell Transfection and Luciferase Assay

$5x10^4$ HEK 293 cells were plated into 24-well plates. 20hrs later, a designated mixture of 20ng Sf-Stk or gp55, 20 ng of AP1 luciferase reporter plasmid and 0.5ng renilla reporter plasmid were used for transient transfection in each well with the Mirus-293 transfection reagent according to the manufacturer's protocol. For control vector, 20ng MSCV-neo plasmid was used in the transfection of each well. 48hrs after

transfection, luciferase assays were performed according to the manufacturer's instructions.

2.3.4 Immunoprecipitation and Western Blot Analysis

$3x10^5$ HEK 293 cells/well were plated into 6-well plates. 20 hours later, the cells were transiently transfected with a 300 ng mixture of the designated plasmids per well and 2 wells were transfected for every set of samples. 40-48hrs post transfection, cells were suspended in 500ul ice cold cell lysis buffer containing 150mM NaCl, 20mM Tris·HCl(pH7.5), 5mM EDTA, 1% NP-40, 1mM phehylmethylsulfonyl fluoride, 1mM Na_3VO_4, 10mM NaF. Cell lysates were centrifuged at 1500 rpm for 20 minutes. Following centrifugation, the supernatant lysates were transferred to prechilled tubes. 40 ul cell lysates were mixed with 4X denaturing SDS loading buffer and heated to 100 degrees for 5 minutes. 800 ul of lysates were used for immunoprecipitation as follows: Cell lysates were incubated with the appropriate amount of primary antibody at 4 degrees for 30 minutes, incubated with protein G magnetic beads for 2 hrs and washed by ice cold PBS buffer three times. Beads were resuspended in 40 ul 1X SDS denaturing loading buffer and heated to 100 degrees for 5 minutes. Western blot and immunoprecipitation samples were separated by SDS-PAGE gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% non fat milk TBST buffer for 1 hr and probed with primary antibody at 4 degrees overnight. Membranes were washed 3 times in TBST and incubated with horseradish peroxidase-conjugated IgG for another hour. Membranes were washed 3 times in TBST then applied

to ECL plus western blotting detection reagents for visualization. For reprobing, membranes were stripped with 62.5mM Tris·HCl (pH6.8), 2% SDS and 0.7% β-mercaptoethanol at 55 degrees for 30 minutes.

2.3.5 Retrovirus generation and *in vitro* infection of Primary Murine Bone Marrow Cells

To generate MSCV retroviral supernatants, $3x10^5$ cells/well 293T cells were plated into 6-well plates. 20hrs later, the cells were transiently transfected with 300ng pEco and 700ng MSCV plasmids for each well. 40-48 hours post transfection, viral supernatants were collected and filtered through 0.45um sterile syringe filters (Pall Life Sciences, Ann Arbor, MI) before use. For FVP supernatant generation, FP63 cells (a kind gift from Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada.) were cultured in DMEM medium with 10% FBS. The FVP supernatants were collected and filtered through 0.45um sterile syringe filters, aliquoted into and stored at -80 degrees. For *in vitro* infection of primary bone marrow, cells were harvested from femurs of 8 weeks old mice and resuspended in DMEM medium with 40% FBS. The bone marrow cells were infected by incubation with retroviral supernatants for 20hrs along with 10ng/ml interleukin-3. For Epo-independent BFU-E colony assays, the bone marrow cells were resuspended with FVP supernatants and incubated on ice for 1hr. The cells were then added to Methocult Medium M3234, along with 2.5ng/ml IL-3, in triplicate with or without 1U/ml Epo. Cultures were incubated for 5 days in 5% CO_2 at 37 degrees. Erythroid colonies were visualized by acid-benzidine staining.

2.4 RESULTS

2.4.1 Gp55 oligomerization is regulated by cysteines in the ecotropic domain

Homo-oligomerization is believed to be an intrinsic feature of the gp55 protein[94]. Detailed sequence studies have revealed 12 cysteine residues in the gp55 peptide: eight in the dualtropic N-terminal domain and four in the ecotropic C-terminal domain[86-88]. The eight cysteine residues within the gp55 dualtropic domain are similar to those in the envelope glycoprotein gp70 of F-MCFV and gp71 of F-MuLV, which are highly homologous to gp55 in the dualtropic domain and share the same evolutionary origin[187]. The cysteine residues in gp70 and gp71 are responsible for intrachain disulfide bond formation[188, 189]. By replacing the 3' region of gp70 with that of the gp55, the recombined F-MCFV exhibited pathogenic activity similar to F-SFFV [89]. This finding suggests that cysteine residues in the gp55 dualtropic domain similarly form intrachain disulfide bonds and are not available for interchain disulfide bond formation. This leaves the possibility that gp55 dimerization is mediated by interchain disulfide bond formation between cysteines at positions 306, 309, 337 and 338 in the ecotropic C-terminal domain of gp55. In order to investigate this hypodissertation, we generated cysteine to alanine mutations in the ecotropic domain of gp55. These mutants were transiently transfected into 293 cells and their expression was examined by western blot analysis under both reducing and non-reducing conditions (Fig 2.1.A). Wild type gp55 and gp55 harboring cysteine to alanine substitutions at two of the four cysteines (gp55C306,309A and gp55C337,338A) were detected both as 55Kd monomers and 95Kd dimers under non-reducing western blot conditions. However, gp55 harboring cysteine to

alanine substitions at all four cysteines in the ecotropic domain (gp55C4A) was only detected as a monomeric band. These data confirm the hypodissertation that all the four cysteines in the ecotropic domain are involved in the dimerization of gp55.

A yellow fluorescent protein (YFP) fragment complementation assay was also conducted to assess the dimerization of gp55. Plasmids expressing the fusion proteins YFP1-gp55, YFP2-gp55, YFP1-gp55C4A and YFP2-gp55C4A were constructed and transfected into 293 cells. 48 hours later, cells were collected and applied to flow cytometry for YFP fluorescence detection (Fig 2.1.B). Using this approach, we demonstrated the presence of dimers of wild-type gp55, but not of gp55C4A. These results are consistent with our previous non-denaturing western blot data, and further support the hypodissertation that the four cysteines in the gp55 ecotropic domain are essential for g55 homodimerization.

2.4.2 Sf-Stk oligomerization is independent of gp55 and regulated by sequences in the cytoplasmic domain

Previous work from our laboratory demonstrated that Sf-Stk kinase activity is required for Friend virus-induced Epo^{ind} erythroid colony formation[111]. A widely accepted model of receptor tyrosine kinase activation is one in which ligand binding to the receptor promotes the dimerization of the receptor resulting in phosphorylation of the activation loop in the tyrosine kinase motif of the receptor[190]. In order to determine whether gp55 promotes Sf-Stk oligomerization, plasmids encoding Myc or HA tagged Sf-Stk (Myc-Sf-Stk and Sf-Stk-HA), were constructed and co-transfected into 293 cells

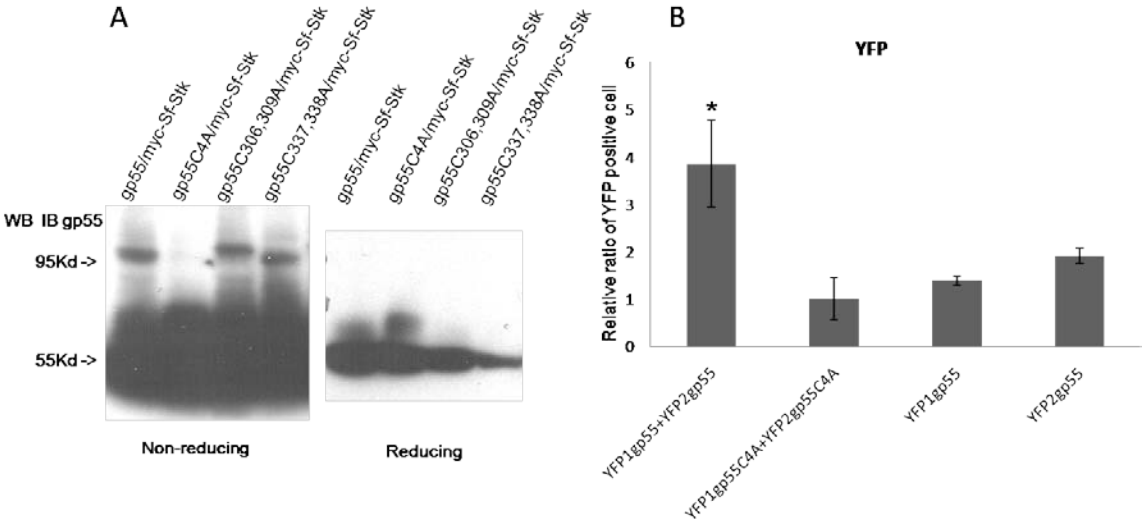

Fig 2.1 Gp55 homodimerizes through the cysteine residues in the ecotropic domain.

A) 293 cells were transfected with wild type gp55 or the indicated gp55 mutants. 48 hours later, cell lysates were separated by SDS-PAGE electrophoresis under non-denaturing and denaturing condition. Membranes were stained with anti-gp55 serum. B) 293 cells were co-transfected with plasmids expressing the fusion proteins YFP1-gp55 and YFP2-gp55, orYFP1-gp55C4A and YFP2-gp55C4A. Plasmids expressing YFP1-gp55 and YFP2-gp55 were also transfected independently as controls. 48 hours later, the transfected cell were collected and sorted by flow cytometry for YFP fluorescence. * $P <$ 0.05

with or without gp55. 48 hours later, cells were lysed and oligomerization was assessed by co- immunoprecipitation of the Myc and HA tags (Fig 2.2.B).

To our surprise, myc tagged Sf-Stk co-immunoprecipitated with HA tagged Sf-Stk in the absence of gp55, and this interaction was not enhanced in the presence of gp55, suggesting that Sf-Stk oligomerization is independent of gp55. In order to map the region of Sf-Stk responsible for promoting oligomerization, we generated N-terminally truncated forms of Sf-Stk lacking the extracellular domain sequences (Sf-StkΔE) or the extracellular and transmembrane domains (Sf-StkΔETM) (Fig 2.2.A). As shown in figure 2.2.B, myc and HA tagged versions of both the Sf-StkΔE and Sf-StkΔETM deletions were also found to co-immunoprecipitate. These data demonstrate that the Sf-Stk extracelluar domain and transmembrane domain are not required for Sf-Stk homo-oligomerization.

2.4.3 Gp55 enhances Sf-Stk tyrosine phosphorylation in a dose-dependent manner

The fact that Sf-Stk oligomerization is independent of gp55 prompted us to further investigate whether gp55 independent Sf-Stk oligomerization is sufficient to promote Sf-Stk tyrosine phosphorylation. Myc-Sf-Stk and Sf-Stk-HA were transfected into 293 cells in the presence of concentrations of gp55-containing plasmid ranging from 0 to 300ng. Sf-Stk tyrosine phosphorylation and oligomerization were assessed by immunoprecipitation and western blot analysis (Fig 2.3). Consistent with our previous results, gp55 did not enhance the co-immunoprecipitation of myc-Sf-Stk and Sf-Stk-HA. However, contrary to the Sf-Stk oligomerization results, gp55 expression strongly

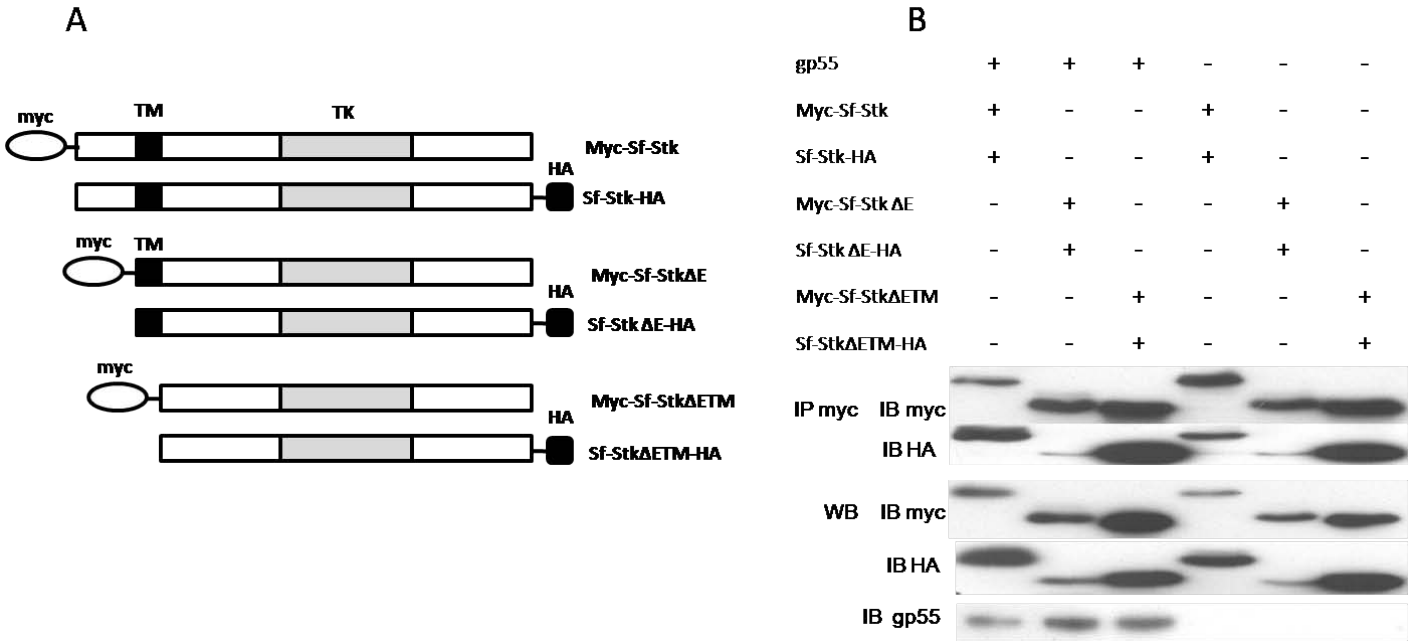

Figure 2.2 Sf-Stk oligomerization is independent of gp55 and mediated by the cytoplasmic domain

A) Schematic representation of myc or HA tagged Sf-Stk, Sf-StkΔE and Sf-StkΔETM structures used in this experiment. TM: transmembrane domain; TK: tyrosine kinase domain. B) 293 cells were transfected with the indicated wild type or deletion mutants of Sf-Stk with gp55 or empty vector. Cell lysates were immunoprecipitated with anti-myc antibody and blotted with anti-HA antibody.

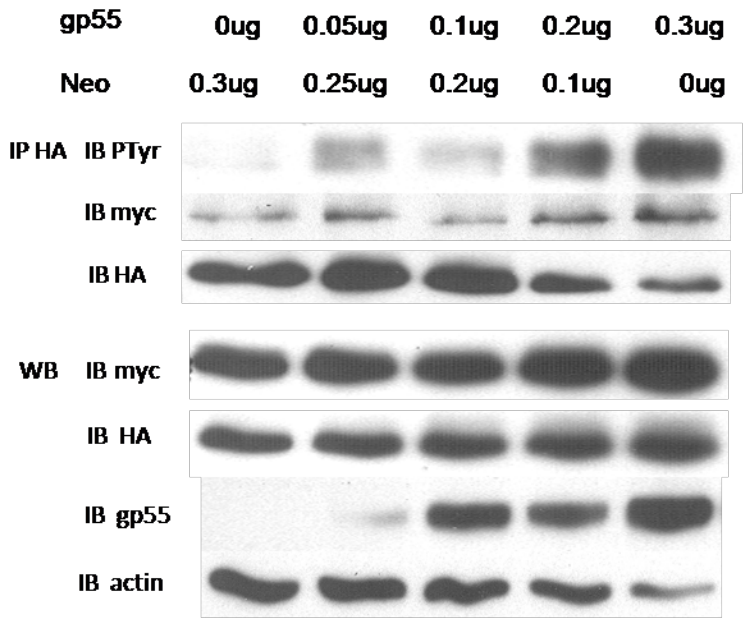

Fig 2.3 Gp55 promotes Sf-Stk tyrosine phosphorylation in a dose dependent manner

293 cells were co-transfected with myc-Sf-Stk and Sf-Stk-HA in the presence of the indicated quantity of gp55 or empty vector. Cell lysates were immunoprecipitated with anti-HA and blotted with anti-phosphotyrosine or anti-myc antibodies.

induced the phosphorylation of Sf-Stk in a dose-dependent manner, suggesting that gp55 is required for efficient Sf-Stk tyrosine phosphorylation. These results suggest that, rather than promoting dimerization of Sf-Stk, gp55 likely induces a conformational change in Sf-Stk oligomers resulting in enhanced tyrosine kinase activity and receptor autophosphorylation.

2.4.4 gp55 interacts with Sf-Stk and this interaction is regulated by the cysteines in the extracellular domain of Sf-Stk

Two forms of Friend virus exist, FVP which promotes erytholeukemia and polycythemia, and FVA which also promotes erythroleukemia, but the mice are anemic. The transmembrane domain of $gp55^{P}$ mediates the interaction of gp55 with the murine EpoR [104, 105] and is essential for promoting Epo^{ind} signaling downstream of the EpoR. In contrast, $gp55^{A}$ cannot effectively activate the EpoR due to sequence alterations in the transmembrane domain. Conversion of sequences in the transmembrane domain of $gp55^{P}$ to reflect that of $gp55^{A}$ shows that two continuous Leu residues directly mediate $gp55^{P}$ and EpoR binding. Deletion of these Leu residues (dLL) is responsible for the reduced affinity of $gp55^{A}$ for the EpoR. Another methionine to isoleucine point mutation, M390I, hampers the full mitogenic activation of EpoR but has no effect on EpoR binding [103] (Fig.2.4.A). We introduced these changes into $gp55^{P}$ (gp55M390IdLL) to test whether these sequences in the transmembrane domain similarly regulate the interaction of gp55 with Sf-Stk. Co-immunoprecipitation experiments using this mutant demonstrated mutation of these residues in the transmembrane domain of gp55 does not affect its ability to associate with Sf-Stk (Fig 2.4.B).

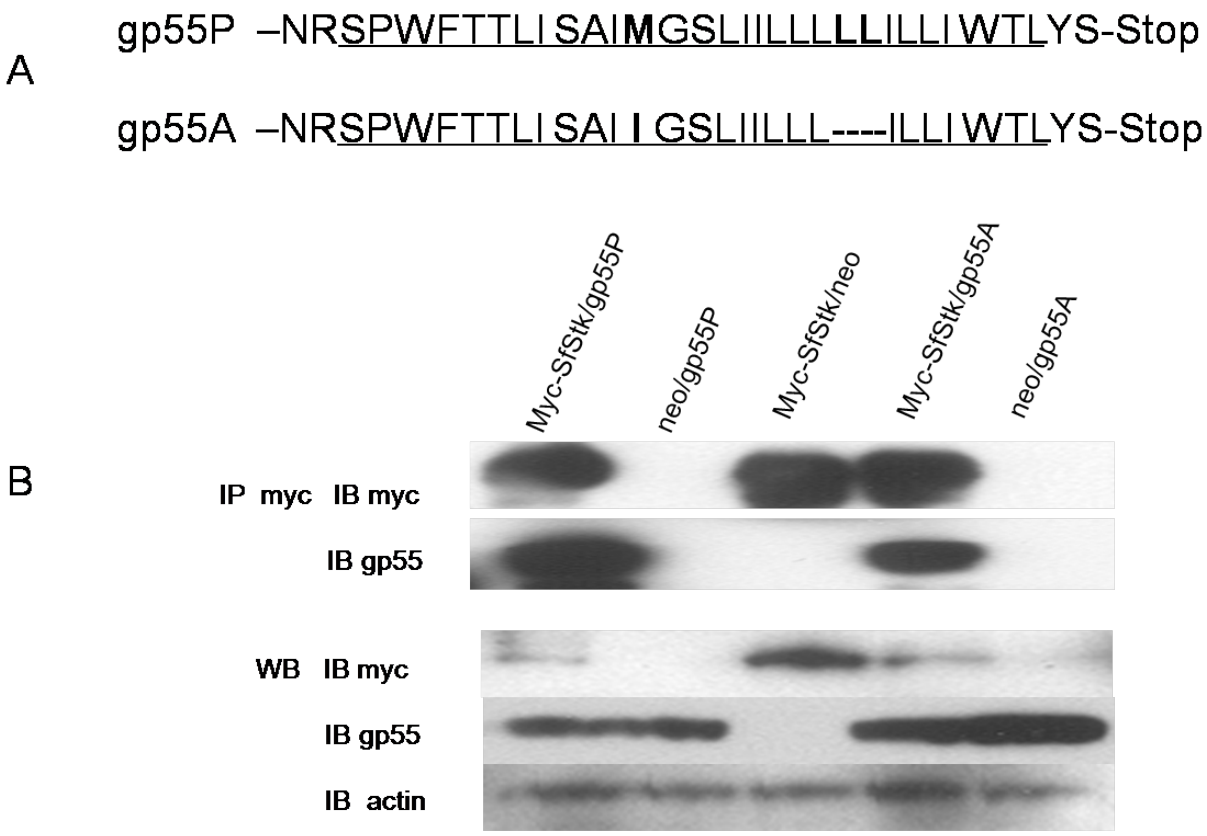

Fig 2.4 gp55 mutations in the transmembrane domain of gp55P, resembling substitutions in gp55A, do not abrogate binding with Sf-Stk.

A) The transmembrane domain of gp55P and gp55A. The transmembrane domains are underlined, with the M390I point mutation and the double deletion at Leu399,400 shown in bold. B) 293 cells were transfected with the indicated mutants of gp55 and Sf-Stk or empty vector. Cell lysates were immunoprecipitated with anti-myc antibody and blotted with anti-gp55 anti-serum.

Gp55 contains a total of 409 amino acid residues, 378 of which are in the extracellular domain, 29 residues in the transmembrane domain, and only 2 amino acid residues in cytoplasmic region[102]. In comparison, Sf-Stk has a total of 477 amino acid residues, 59 of which are in the extracellular domain[125]. Previous studies demonstrated that gp55 is capable of transforming rodent fibroblasts in the presence of Sf-Stk and that this transformation is dependent upon the extracellular domain of Sf-Stk[113]. In order to map the domain of Sf-Stk responsible for the interaction of Sf-Stk with gp55, myc tagged Sf-Stk, Sf-StkΔE and Sf-StkΔETM (Fig 2.5.A) were transfected into 293 cells with HA tagged gp55. The interaction between gp55 and the Sf-Stk mutants was investigated by co-immunoprecipitation and western blot analysis (Fig 2.5.B). The results demonstrated that gp55 co-immunoprecipitates with Sf-Stk, but not with Sf-StkΔE or Sf-StkΔETM. This indicates that the extracellular domain of Sf-Stk is essential for its interaction with gp55.

To further localize the region of the extracellular domain responsible for the gp55/Sf-Stk interaction, myc tagged Sf-StkΔ19 and Sf-StkΔ42, in which the first 19 and 42 amino acid residues, respectively, of Sf-Stk are deleted, were transfected into 293 cells with gp55 and tested for their ability to co-immunoprecipitate with gp55 (Fig 2.5.C). While Sf-StkΔ19 retains partial ability to co-immunoprecipitate with gp55, this interaction is completely abrogated with Sf-StkΔ42. There are four cysteine residues located at amino acids 8, 19, 37 and 42 of the Sf-Stk extracellular domain. In order to determine whether these cysteines mediate the interaction of Sf-Stk with gp55, we mutated the cysteines to alanine in pairs (Sf-StkC8,19A and Sf-StkC37,42A) or all four in combination (Sf-StkC4A). The Sf-Stk mutants were transfected into 293 cells with

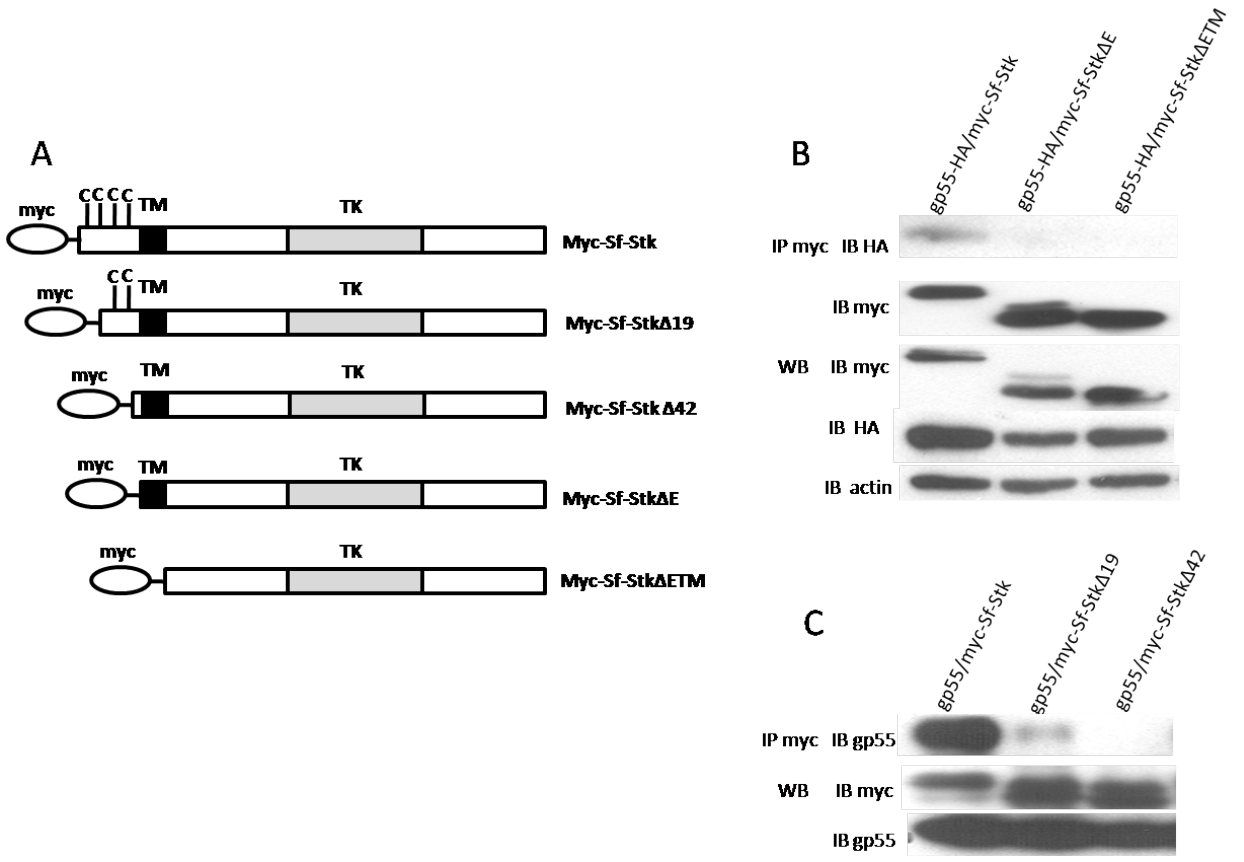

Fig 2.5 The first 42 amino acid residues of the Sf-Stk extracellular domain are required for the gp55/Sf-Stk interaction

A) Schematic representation of myc tagged Sf-Stk, Sf-StkΔ19, Sf-StkΔ42, Sf-StkΔE and Sf-StkΔETM B) 293 cells were transfected with wild type or the indicated deletion mutants of Sf-Stk and gp55-HA. Cell lysates were immunoprecipitated with anti-myc and blotted with anti-HA antibody. C) 293 cells were transfected with wild type or the indicated deletion mutants of Sf-Stk with gp55. Cell lysates were immunoprecipitated with anti-myc and blotted with anti-gp55 anti-serum.

gp55 and their interactions were assessed by co-immunoprecipitation and western blot analysis (Fig 2.6.A). Results show that, while Sf-StkC8,19A and Sf-StkC37,42A retain partial ability to co-immunoprecipitate with gp55, the ability of Sf-StkC4A to co-immunoprecipitate with Sf-Stk is completely abrogated.

Taken together, these studies suggest that sequences in the extracellular domain of gp55, rather than the transmembrane domain, likely mediate the interaction of gp55 with Sf-Stk. To determine whether the cysteines in the extracellular domain of gp55 promote the interaction of gp55 with Sf-Stk, we co-expressed Sf-Stk or Sf-StkC4A with gp55 or gp55C4A in 293 cells and assessed their ability to co-immunoprecipitate (Fig 2.6.B). Our data clearly indicate that both the cysteines in the extracellular domain of Sf-Stk and the cysteines in the ecotropic domain of gp55 are required for the interaction between gp55 and Sf-Stk. Both gp55C306,309A and gp55C337,338A retain the ability to co-immunoprecipitate with Sf-Stk (Figure 2.6.C). However, the interaction of these mutants with Sf-StkC37,42A while significantly reduced, is not abrogated. These studies indicate that the interchain disulfide bonds formed between gp55 and Sf-Stk are not limited to specific cysteines, but that the Sf-Stk/gp55 interaction can be promoted through the formation of different cysteine pairs between gp55 and Sf-Stk.

2.4.5 Cysteines in the extracellular domain of Sf-Stk are required for Sf-Stk activation and Epo^{ind} BFU-E colony formation

Our data clearly demonstrate that the cysteines in the extracellular domain of Sf-Stk mediate the interaction of Sf-Stk with gp55. In order to determine whether these cysteines are required for the enhanced phosphorylation of Sf-Stk observed in the

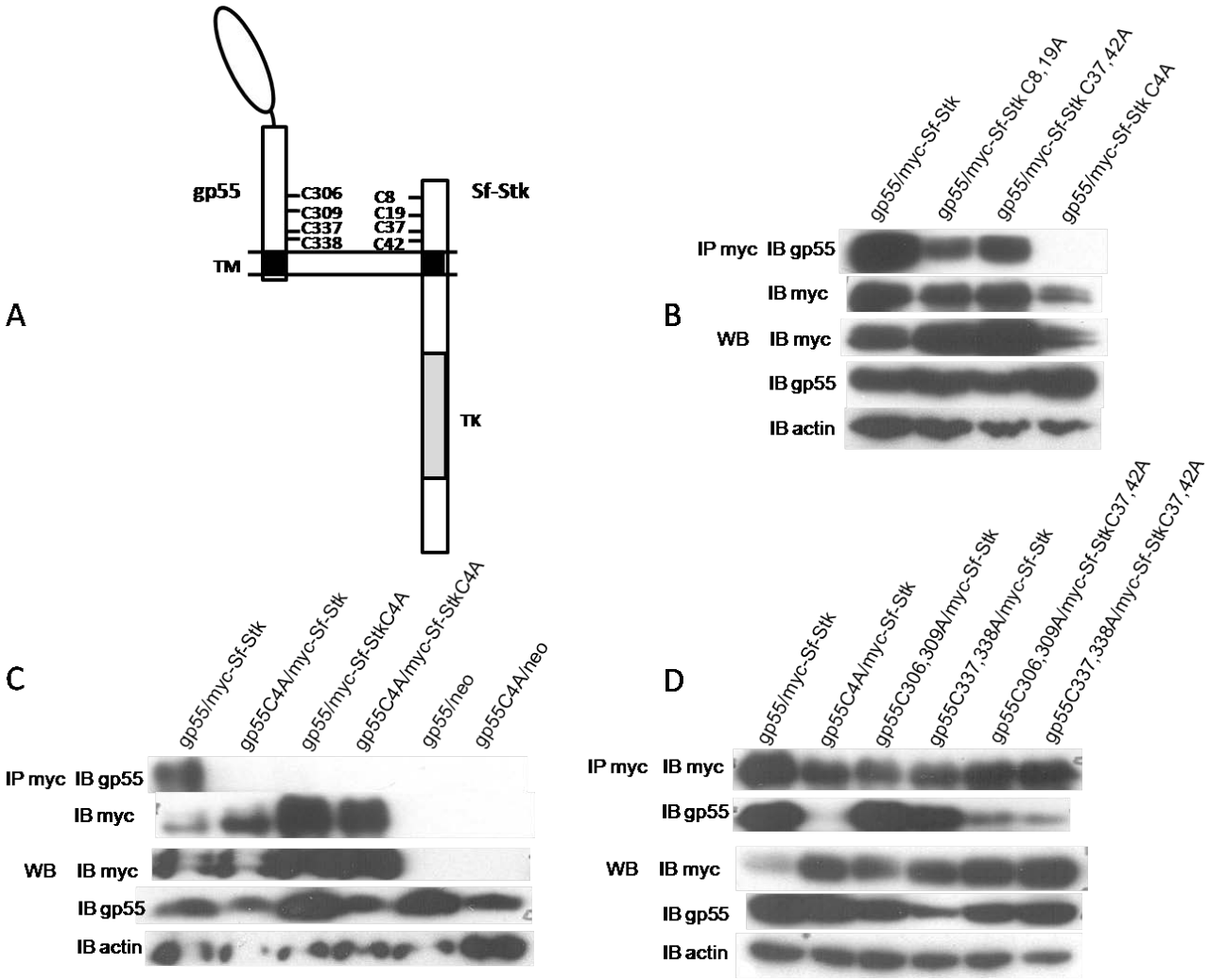

Fig 2.6 Sf-Stk interacts with gp55 through cysteine residues in the extracellular domain

A) Schematic representation of gp55 and Sf-Stk. The positions of the cysteine residues described are shown. B) 293 cells were transfected with myc tagged wild type Sf-Stk or Sf-Stk harboring cysteine to alanine mutations at the indicated positions and gp55. Cell lysates were immunoprecipitated with anti-myc antibody and blotted with anti-gp55 anti-serum. C) 293 cells were transfected with wild type myc tagged Sf-Stk or Sf-StkC4A and wild type gp55 or gp55C4A. Cell lysates were immunoprecipitated with anti-myc antibody and blotted with anti-gp55 anti-serum. D) 293 cells were transfected with myc tagged wild type Sf-Stk or the indicated Sf-Stk mutants with wild type gp55 or indicated gp55 mutants. Cell lysates were immunoprecipitated with anti-myc antibody and blotted with anti-gp55 anti-serum.

presence of gp55, 293 cells were co-transfected with wild-type or mutant forms of gp55 and wild type or mutant forms of Sf-Stk. Cell lysates were immunoprecipitated with anti-phospho-tyrosine and immunoblotted with anti-myc (Fig 2.7.A). Consistent with the co-immunoprecipitation results, these results indicate that cysteines in both the extracellular domain of Sf-Stk and in the ecotropic domain of gp55 are required for the efficient phosphorylation of Sf-Stk. While double cysteine to alanine mutants of both gp55 and Sf-Stk partially retained this activity, mutation of all four cysteines on gp55 or Sf-Stk reduced Sf-Stk tyrosine phosphorylation to baseline levels.

Previous studies from our lab demonstrated that Erk/MAPK is activated downstream of Sf-Stk. To determine whether the cysteines in the extracellular domain of Sf-Stk are required for Erk activation, 293 cells were co-transfected with wild-type or mutant forms of gp55 and wild type or mutant forms of Sf-Stk. Cell lysates were immunoblotted with anti-phosphorylated Erk1/2 antibody then stripped and blotted with anti-Erk1/2 antibody (Fig 2.7.B). Further, transcription activator protein 1 (AP1) activation, an event downstream of Erk activation, was measured by relative luciferase assay 48 hours after co-transfection of a luciferase reporter plasmid into the 293 cells together with wild type or mutant Sf-Stk and gp55 (Fig2.7.C). Consistent with the Sf-Stk tyrosine phosphorylation experiment, mutation of cysteines on either the gp55 ecotropic domain or the Sf-Stk extracellular domain resulted in the loss of Erk1/2 phosphorylation and AP1 luciferase activity.

FVP infection of primary erythroblasts promotes the Epo-independent (Epo^{ind}) formation of BFU-E colonies, due to the ability of gp55 to activate Sf-Stk and the EpoR. To determine whether the cysteines in Sf-Stk that mediate the interaction of Sf-Stk with

gp55 are required for the ability of FVP to induce Epo^{ind} colony formation, BFU-E colony assays were performed with bone marrow from C57BL/6 *Fv2*$^{r/r}$ mice, which lack Sf-Stk expression, transduced with the Sf-Stk cysteine mutations followed by infection with FVP (Fig 2.8.B). Consistent with our previous studies, FVP induced Epo^{ind} colony formation by cells expressing wild-type Sf-Stk at levels comparable to those induced by Epo. While single cysteine mutants retained partial ability to promote Epo^{ind} colony formation induced by FVP, Sf-StkC4A failed to support Epo^{ind} colony formation. Expression of gp55 alone in primary erythroblasts from *Fv2*$^{s/s}$ mice is sufficient to induce Epo^{ind} colony formation. In order to determine whether the cysteines in gp55 are required for its ability to promote Epo^{ind} colony formation, we transduced Balb/c *Fv2*$^{s/s}$ bone marrow cells with wild-type and mutant forms of gp55 and assessed BFU-E colony growth (Fig 2.8.A). While expression of wild-type gp55 was capable of promoting Epo^{ind} colony growth in *Fv2*$^{s/s}$ bone marrow, gp55C4A failed to support Epo^{ind} colony formation. As we observed with Sf-Stk, single cysteine mutants of gp55 retained partial ability to promote this response. Taken together, these data verify a crucial role for the cysteines in the ecotropic domain of gp55 and the extracellular domain of Sf-Stk in the phenotypic response of primary erythroblasts to infection with Friend virus.

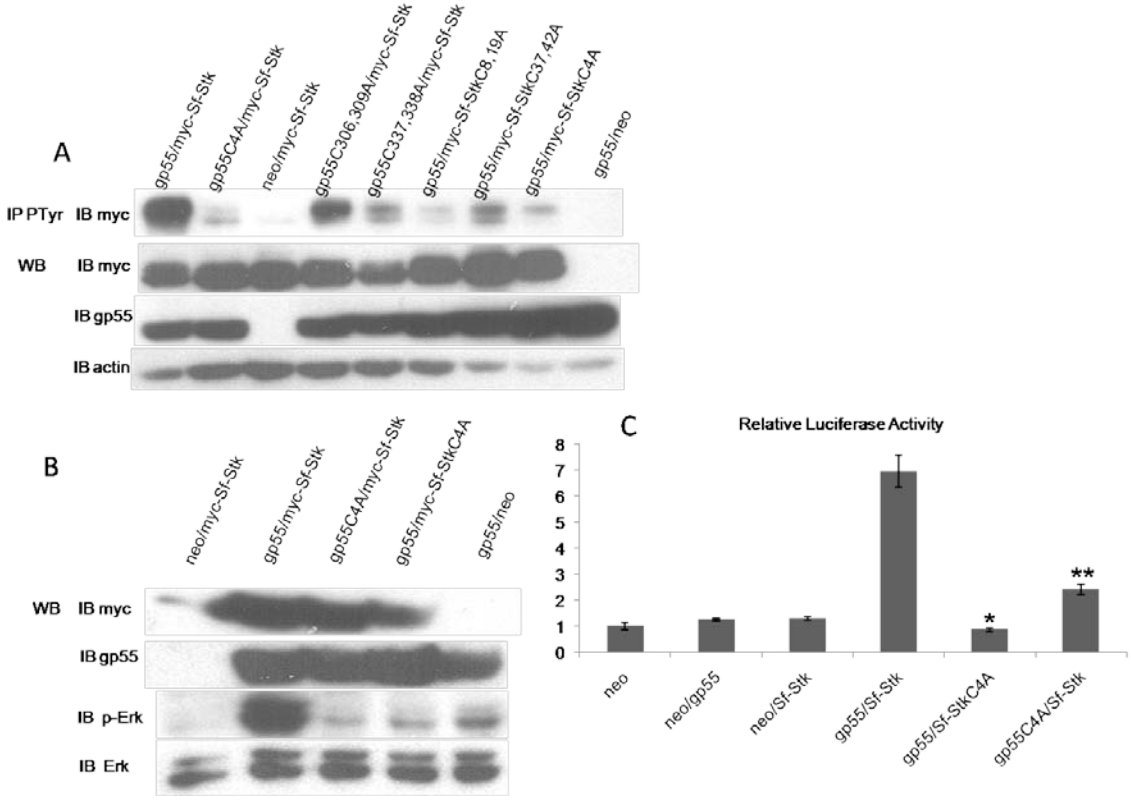

Fig 2.7 The cysteine residues in Sf-Stk and gp55 are required for Sf-Stk activation and MAPK signaling.

A) 293 cells were transfected with myc tagged wild type or mutant forms Sf-Stk with wild gp55 or mutant forms of gp55. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and blotted with anti-myc antibody. B) 293 cells were transfected with indicated wild type or mutant Sf-Stk and wild type gp55, mutant gp55, or empty vector. Cell lysates were immunoblotted with anti-PhosphoErk antibody. The membrane was stripped and reprobed with anti-Erk antibody. C) 293 cells were transfected with indicated wild type or mutant Sf-Stk and gp55, with an AP1 luciferase reporter plasmid and renilla luciferase reporter vector. 48 hours later, luciferase assay were performed according to manufacturer's instruction. * $P < 0.01$, ** $P < 0.01$

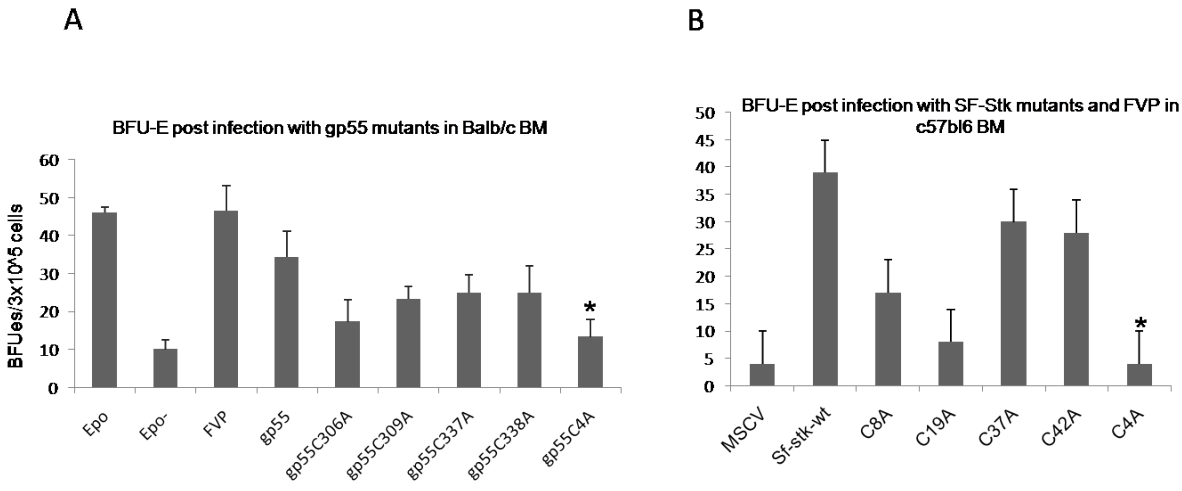

Fig 2.8 The cysteine residues in Sf-Stk and gp55 are required for Epo^{ind} BFU-E colony formation.

A) 293 cells were transfected with MSCV-gp55 and mutants with pEco. 48 hours post transfection, MSCV virus supernatant were collected and incubated over night with bone marrow cells harvested from Balb/c mice. Virus infected bone marrow cells were then plated in methylcellulose media containing IL-3(2.5ng/ml). On day 5, BFU-E colonies were stained with acid-benzidine and counted. * $P < 0.01$ B) 293 cells were transfected with MSCV-myc-Sf-Stk and mutants with pEco. 48 hours post transfection, MSCV virus supernatant were collected and incubated over night with bone marrow cells harvested from Balb/c mice. MSCV virus infected bone marrow cells were then incubated with FVP on ice for 1 hour before plated in methylcellulose media containing IL-3(2.5ng/ml). On day 5, BFU-E colonies were stained with acid-benzidine and counted. * $P < 0.01$

2.5 DISCUSSION

It has long been noted that a N-terminally truncated form of the Stk receptor tyrosine kinase is predominantly expressed in murine hematopoietic stem cells compared with expression of the full length counterpart[125]. However, since Sf-Stk lacks most of the extracellular domain and cannot bind MSP, the ligand for full length Stk, the function of murine Sf-Stk remained unexplored until its identification as the product of the *Fv2* gene, which is responsible for restriction of Friend virus susceptibility in C57BL/6 mice[110]. It has since become clear that Friend virus induced erythroleukemia is initiated by the ability of the SFFV-encoded viral glycoprotein, gp55 to interact with the Epo receptor and Sf-Stk, expressed by host hematopoietic cells. Several studies have been conducted to investigate the downstream signals generated by the gp55/ Sf-Stk interaction that promote leukemic transformation. Studies from our laboratory have demonstrated that a Sf-Stk/Grb2/Gab2/STAT3 signaling pathway is critical for this response[111, 179, 191]. Studies from our laboratory and others' have also revealed that PI3K and MAPK signaling are activated downstream of Sf-Stk and these signaling pathways are required for the Epo^{ind} growth of primary erythroid colonies by Friend virus[111, 177]. However, it has remained unclear how the interaction between gp55 and Sf-Stk results in the activation of Sf-Stk signaling. The studies described herein, shed light on the mechanism of gp55-mediated Sf-Stk activation and may provide further clues to the mechanism by which Sf-Stk activity is regulated in uninfected cells.

Here we demonstrate that the covalent interaction between gp55 and Sf-Stk is regulated through four cysteines located in the ecotropic domain of gp55 and four

cysteines located in the extracellular domain of Sf-Stk. Our data further demonstrate that the cysteines in the ecotropic domain also mediate gp55 oligomerization, confirming previous studies showing that gp55 oligomerization is mediated by intermolecular disulfide bond formation [94]. However, not all four cysteines are required for gp55 oligomerization as demonstrated by the ability of gp55C306,309A and gp55C337,338A to form dimers. This flexibility in the ability of alternate cysteines to promote gp55 oligomerization likely provides a means by which cysteines in gp55 are also free to form heteroligomers in the presence of Sf-Stk via disulfide bond formation. However, while gp55C306,309A and gp55C337,338A interact with Sf-Stk to a similar extent as wild-type gp55, we observed a much lower level of Sf-Stk tyrosine phsophorylation in these complexes compared with wild type gp55. These experiments suggest that through intermolecular disulfide bond formation, the cysteines in the ecotropic domain of gp55 can form molecular clusters of gp55 and Sf-Stk, which further enhance Sf-Stk phosphorylation. While gp55 has also been shown to interact with the EpoR through the dualtropic and transmembrane domains[77, 104, 105], our data suggest that these areas of interaction are not overlapping with Sf-Stk binding, supporting a model in which gp55 could form complexes with both EpoR and Sf-Stk molecules.

In comparison with the wild type *env* gene, there is a BB6 variant which contains a 159-bp deletion that eliminates the membrane-proximal portion of the extracellular domain (aa332-aa384)[192, 193]. Similar to its WT counterpart, BB6 immunoprecipitates as a homodimer, crosslinks with EpoR on the cell surface, and induces Epo independent growth of Ba/F3-EpoR cells[194]. However, unlike WT gp55, the BB6 variant contains a deletion that includes two of these cysteines, Cys337 and Cys338. The BB6 variant failed

to immunoprecipitate with Sf-Stk or induce its tyrosine phosphorylation[112] but was found to induce erythroleukemia in both *Fv2*$^{s/s}$ and *Fv2*$^{r/r}$ mice. However, BB6 induced erythroleukemia display milder features in *Fv2*$^{s/s}$ mice compared to the wild type Friend virus and a long extended latency in *Fv2*$^{r/r}$ mice. These observations suggest that Sf-Stk may be activated by BB6 indirectly through other proteins or that structural changes associated with the deletion in BB6 may result in the activation of other kinase.

A widely accepted model of receptor tyrosine kinase activation proposes that ligand binding promotes receptor dimerization which results in receptor phosphorylation and the initiation of downstream signaling. However, we demonstrate that Sf-Stk oligomerization is independent of gp55 and is regulated through the cytoplasmic domain. Furthermore, gp55 in which three of the four cysteines in the ecotropic domain are mutated to alanine can still be observed to immunoprecipitate with Sf-Stk and induce downstream signaling, albeit at a lower level than wild type gp55 (data not shown). Since these gp55 mutants have only one cysteine available for disulfide bond formation, this result supports the conclusion that gp55 does not promote Sf-Stk activation through the homodimerization of Sf-Stk induced by gp55 dimers.

There is increasing evidence that some receptor tyrosine kinases can form homo or heterodimers in the absence of ligand, and that ligand binding stabilizes the dimer and drives a conformational change in the receptor which promotes receptor activation. Using bimolecular fluorescence complementation (BiFC) assays, Tao demonstrated that all EGFR family members form inactive ligand-independent dimers in the ER which are translocated to plasma membrane[195], and a two-hybrid assay suggests that intracellular domains are responsible for this spontaneous dimerization. Further, EpoR is found to

form an inactive dimer through the transmembrane domain in the absence of Epo [196]. Though scanning cysteine mutations in the EGFR and EpoR juxtamembrane and transmembrane domains demonstrated the potential for the generation of many disulfide linked dimers, only certain cysteines can promote signaling downstream of the EGFR and EpoR resulting in cell transformation. These data suggest that a tightly controlled conformational change in the EGFR and EpoR upon ligand binding leads to dimer activation[197, 198].

In studies of the human homologue of Stk, Ron, Lu et al demonstrated that Ron dimerization was dependent upon stimulation with a Ron agonist mAb, but failed to detect the dimerization of a C-terminally deleted mutant of Ron following stimulation with the Ron agonistic[149], suggesting that ligand binding and the C terminal tail of Ron are critical for Ron dimerization. These data are in contrast to our results suggesting that Sf-Stk spontaneously dimerizes in the absence of gp55. Thus, sequences in the extracellular domain of Stk, deleted in Sf-Stk, could inhibit the spontaneous dimerization of the receptor, and this inhibition could be overcome by ligand binding. Alternatively, we have shown that Stk is missing an exon, present in Ron, that encodes part of the juxtamembrane domain, and that this deletion results in ligand-independent activation of the receptor. It is therefore also possible that the inclusion of these sequences in the juxtamembrane domain inhibits spontaneous dimerization, whereas in the absence of these juxtamembrane sequences, the full length receptor spontaneously dimerizes in the absence of ligand. The internal promoter that drives the expression of Sf-Stk is conserved in the human gene, and a comparable Sf-Ron transcript exists in human cells. It will be interesting to determine whether Sf-Ron also spontaneously dimerizes or

whether dimerization of Sf-Ron is dependent on its ability to interact with gp55 or other cellular proteins.

In summary, our studies support a model in which Sf-Stk forms an inactive oligomer in the cytoplasm. Through cysteine mediated disulfide bond formation, gp55 recruits Sf-Stk and promotes an active conformation of the Sf-Stk oligomer, resulting in enhanced receptor activation and downstream signaling. Our previous studies indicate that the Stk receptor, in response to stimulation by its ligand, MSP, regulates macrophage activation and that Stk-/- mice are more susceptible to septic shock. Sf-Stk is also expressed in macrophages (data not shown) and, intriguingly, a recent study in which Sf-Stk, but not full-length Stk, was deleted in mice indicates that Sf-Stk plays a crucial role in the regulation of macrophage activation and susceptibility to septic shock *in vivo* Furthermore, our unpublished observations indicate that Sf-Stk plays a critical role in promoting short-term radioprotection of mice following bone marrow transplantation. Taken together, these studies highlight the importance of understanding how Sf-Stk activity is regulated, not only by gp55, but also by other cellular factors. The studies described here will lay the foundation for addressing this important biological question.

Chapter 3

Gp55 promotes the cell surface localization of Sf-Stk

3.1 Abstract

Friend virus induces an erythroleukemia in susceptible mice initiated by the interaction of the Friend virus encoded glycoprotein, gp55, with the erythropoietin receptor and the product of the host *Fv2* gene, a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). We have previously demonstrated that gp55 induces the activation of Sf-Stk through disulfide bond formation mediated by cysteines in the extracellular domain of Sf-Stk and in the ecotropic domain of gp55. We further demonstrated that this interaction does not affect dimerization of Sf-Stk, but results in enhanced Sf-Stk tyrosine phosphorylation. Here we demonstrate that the interaction between gp55 and Sf-Stk induces the cell surface translocation of Sf-Stk. We also show that mutation of the cysteines in the extracellular domain of Sf-Stk results in enhanced cell surface localization in the absence of gp55, but not enhanced phosphorylation of Sf-Stk. Alternatively, while a constitutively activating mutation in the kinase domain of Sf-Stk is not sufficient to induce receptor phosphorylation and downstream signaling, mutation of the cysteines in the extracellular domain of Sf-Stk in this context restores full activity of Sf-Stk in the absence of gp55. Taken together, these results suggest that cell surface localization of Sf-Stk by gp55 is critical for receptor phosphorylation and activation of downstream signaling by Sf-Stk.

3.2 Introduction

Two forms of the Stk cDNA were originally cloned from murine erythroleukemia (MEL) cells. The longer cDNA encoded the 1378aa full length Stk receptor tyrosine kinase, while the shorter cDNA encoded a 477aa N-terminally truncated form of the Stk receptor (Sf-Stk)[125]. Full length Stk is the murine receptor for macrophage stimulating protein, MSP, and is the murine homologue of the human Ron receptor tyrosine kinase. Stk is expressed as a single chain precursor and then cleaved at a specific site into a 30kDa α chain and a 140kD β chain linked by a disulfide bond, forming the mature heterodimeric Stk receptor. The full-length Stk gene encodes an N-terminal signal peptide and processed Stk is located on the plasma membrane. Upon MSP binding through the extracellular αβ chain, Stk becomes tyrosine phosphorylated and activates downstream signaling. The gene encoding Stk contains 19 exons flanked by 18 introns. Transcription of Sf-Stk starts from an internal promoter in intron 10 and translation is initiated from an ATG codon in exon 11. Sf-Stk lacks the sequences encoding the α chain and most of the extracellular part of the β chain, but retains an intact transmembrane domain and cytoplasmic domain. Sf-Stk is the predominant transcript in hematopoietic cells, however the function of Sf-Stk in these cells remained unstudied until its identification as the product of Friend virus susceptibility-2, *Fv2*, gene[110].

Friend virus is a complex of two viruses, the spleen focus forming virus (SFFV) and the ecotropic helper Friend murine leukemia virus (F-MuLV). SFFV is responsible for the rapid splenomegaly and acute erythroleukemia induced by Friend virus infection[63-66], while F-MuLV provides helper function and can be substituted for by

other murine leukemia viruses[67]. Specifically, the glycoprotein gp55, encoded by the SFFV *env* gene, acts as the transforming viral oncoprotein. *Fv2* encoded Sf-Stk, is required for Friend virus susceptibility. $Fv2^{r/r}$ mice, including C57BL/6 mice, contain mutations in the internal promoter, lack expression of Sf-Stk and are resistant to Friend virus infection. However, expression of full length Stk is not affected and these mice exhibit normal steady state erythropoiesis. *In vitro* and *in vivo* expression of Sf-Stk in C57BL/6 bone marrow cells has been shown to confer Friend virus susceptibility to $Fv2^{r/r}$ mice[111]. We have demonstrated that Sf-Stk covalently interacts with gp55, mediated by disulfide bond formation through cysteines in the extracellular domain of Sf-Stk and cysteines in the ecotropic domain of gp55, resulting in constitutive activation of Sf-Stk. However, the subcellular localization of the gp55/Sf-Stk interaction has not been examined.

The gp55 glycoprotein is not assembled into the virion structure. The majority of gp55 is retained intracellularly in association with ER membranes. Only 3% to 5% of the total cellular gp55 is further processed in the Golgi apparatus, and the mature protein is then transported to the cell surface [93, 97, 199-201]. The Sf-Stk transcript lacks a consensus signal sequence[110, 125], thus Sf-Stk is presumably located in the ER and cannot be processed to the plasma membrane. Interestingly, Rulli et al reported that an activating M330T point mutation in the kinase domain of Sf-Stk is sufficient to induce Epo independent growth of erythroid colonies without gp55[202], indicating that interaction with gp55 may only be required to promote kinase activation of the truncated receptor, but not for regulating subcellular localization, since Sf-StkM330T is not expressed on cell surface. Though the bulk of gp55 and EpoR were found to co-

immunoprecipitate in the ER[95], a productive interaction between EpoR and gp55 requires localization on the cell membrane[96, 97]. Current models regarding the gp55/Sf-Stk interaction, based on the gp55/EpoR interaction, suggest that Sf-Stk may be brought to the cell membrane by gp55. Nishigaki et al reported that Sf-Stk immunoprecipitated with EpoR in the presence of gp55 in transfected Ba/F3 cells, suggested that Sf-Stk may interact indirectly with the EpoR through gp55[112, 113]. Therefore, an interaction between Sf-Stk and the EpoR, either direct or indirect through gp55, could be required for Epo independent proliferation and differentiation of FV infected erythroblasts.

Here we demonstrate that, through disulfide bond formation between gp55 and Sf-Stk, gp55 induces the cell surface translocation of Sf-Stk. Furthermore, we show that subcellular localization of Sf-Stk is regulated by the cysteines in the extracellular domain of Sf-Stk, and mutation of these cysteines promotes cell surface localization of Sf-Stk, but not receptor phosphorylation, in the absence of gp55. Finally, we demonstrate that full Sf-Stk activity requires both kinase activation and cell surface localization. Taken together, our results suggest that cell surface localization of Sf-Stk plays a critical role in mediating the activation of Sf-Stk by gp55.

3.3 Materials and Methods

3.3.1 Antibodies and cell culture reagents

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The Mirus-293 transfection reagents were purchased from Mirus Bio, LLC (Madison, WI). The Dual-Luciferase Reporter Assay System was purchased from Promega Corporation (Madison, WI). Antibodies against the Myc tag, HA tag, phospho-tyrosine, phosphor-Erk1/2, Erk1/2 and HRP linked anti-rat IgG were purchased from Cell Signaling (Danvers, MA). Antibody against actin, HRP linked anti-mouse IgG was purchased from Sigma-Aldrich, Inc (St.Louis, Mo). Mouse True Blot ULTRA HRP anti-mouse IgG was purchase from eBiosciences (San Diego, CA). HRP linked anti-rabbit IgG was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rat anti-serum against gp55 was kindly provided by Dr. Sandra Ruscetti (National Cancer Institute). All PCR Primers were ordered from Operon Biotechnologies, Inc (Huntsville, AL)**.** PfuTurbo DNA polymerase was purchased from Stratagene (La Jolla, CA). ECL plus Western blotting detection reagents were purchased from GE Healthcare (Piscataway, NJ). Pierce Cell Surface Protein Isolation Kit was purchased from Thermo Fisher Scientific Inc (Rockfold, IL).

3.3.2 Gene construction and mutagenesis

MSCV-myc-Sf-Stk cysteine mutagenesis and MSCV-gp55 cysteine mutagenesis was described previously in chapter 2. MSCV-myc-Sf-Stk M330T and MSCV-myc-Sf-

Stk C4AM330T were generated from MSCV-myc-Sf-Stk and MSCV-myc-Sf-Stk C4A, respectively by using the primers 5'-CCTGCCAGTCAAATGGACGGCACTGGAGAGCCTGC-3' and 5'-GCAGGCTCTCCAGTGCCGTCCATTTGACTGGCAGG-3'.

3.3.3 Cell Transfection and Luciferase Assay

$5x10^4$ HEK 293 cells were plated into 24-well plates. 20 hrs later, a designated mixture of 20 ng Sf-Stk or gp55, 20 ng of AP1 luciferase reporter plasmid and 0.5 ng renilla reporter plasmid were used for transient transfection in each well with the Mirus-293 transfection reagent according to the manufacturer's protocol. For control vector, 20 ng MSCV-neo plasmid was used in the transfection of each well. 48hrs after transfection, luciferase assays were performed according to the manufacturer's instructions.

3.3.4 Immunoprecipitation and Western Blot Analysis

$3x10^5$ HEK 293 cells/well were plated into 6-well plates. 20 hours later, the cells were transiently transfected with a 300 ng mixture of the designated plasmids per well and 2 wells were transfected for every set of samples. 40-48hrs post transfection, cells were suspended in 500ul ice cold cell lysis buffer containing 150mM NaCl, 20mM Tris·HCl(pH7.5), 5mM EDTA, 1% NP-40, 1mM phehylmethylsulfonyl fluoride, 1mM Na_3VO_4, 10mM NaF. Cell lysates were centrifuged at 1500 rpm for 20 mins. Following centrifugation, the supernatant lysates were transferred to prechilled tubes. 40 ul cell

lysates were mixed with 4X denaturing SDS loading buffer and heated to 100 degrees for 5 mins. 800 ul of lysates were used for immunoprecipitation as follows: Cell lysates were incubated with the appropriate amount of primary antibody at 4 degrees for 30 mins, incubated with protein G magnetic beads for 2 hrs and washed by ice cold PBS buffer three times. Beads were resuspended in 40 ul 1X SDS denaturing loading buffer and heated to 100 degrees for 5 mins. Western blot and immunoprecipitation samples were separated by SDS-PAGE gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% non fat milk TBST buffer for 1 hr and probed with primary antibody at 4 degrees overnight. Membranes were washed 3 times in TBST and incubated with horseradish peroxidase-conjugated IgG for another hour. Membranes were washed 3 times in TBST before applied to ECL plus western blotting detection reagents for visualization. For reprobing, membranes were stripped with 62.5mM Tris·HCl (pH6.8), 2% SDS and 0.7% β-mercaptoethanol and 0.7% β-mercaptoethanol at 55 degrees for 30 mins.

3.3.5 Cell Transfection and Cell Surface Protein Isolation

1-2x10^6/ml CHO cells were plated into 10cm plates. 20hrs later, a designated mixture of 1-2ug plasmid were used for transient transfection in each plate with the Mirus-CHO transfection reagent according to the manufacturer's protocol. For control vector, 1-2ug MSCV-neo plasmid was used in transfection of each plate. 48hrs after transfection, cell surface biotinylation and protein isolation were performed according to

the manufacturer's instructions. Prepared cell surface and cytosolic proteins were directly applied to a standard Western Blot assay.

3.3.6 Cell Transfection and Confocal Microscopy

$3x10^5$/ml CHO cells were plated into 6-well plates. 20hrs later, a designated mixture of 0.5ug plasmid were used for transient transfection in each well with the Mirus-CHO transfection reagent according to the manufacturer's protocol. 48hrs after transfection, Confocal Microscopy was performed at excitation 488 nm.

3.3.7 Flow Cytometry

$3x10^5$ cells/well 293 cells were plated into 6-well plates. 20hrs later, the cells were transiently transfected with a 300 ng mixture of the designated plasmids per well. 40-48hrs post transfection, cells were treated with EDTA/Trypsin for 20 seconds and resuspended in 2ml ice cold washing buffer containing PBS and 2%FBS. 1 million cells were transferred to new tubes and centrifuged for 5 mins. at 1500rpm. For 293 cells transfected with YFP, cells were resuspended in 1ml ice cold washing buffer for analysis by flow cytometry. Cells requiring staining were resuspended in 100ul washing buffer and incubated with 10ul Alexa Fluor 647 conjugated Myc-tag mouse antibody (Cell Signaling) for 20mins on ice. Cells were then washed and centrifuged twice before being resuspended in 1ml washing buffer for analysis by flow cytometry.

3.4 Results

3.4.1 Co-expression of gp55 promotes the localization of Sf-Stk to the plasma membrane

To investigate the subcellular location of the gp55/Sf-Stk interaction, plasmids expressing EGFP or a Sf-Stk-EGFP fusion protein were transfected into CHO cells in the presence or absence of gp55. 48 hours later, the distribution of EGFP fluorescence was examined by confocal microscopy (Fig 3.1.A). In cells expressing the Sf-Stk-EGFP fusion protein alone, fluorescence was observed widely distributed in the cytoplasm. However, in the presence of gp55, fluorescence distribution on the plasma membrane was significantly enhanced. These data suggest that expression of gp55 changes the subcellular distribution of Sf-Stk. Western blotting was also applied to study these changes, myc tagged Sf-Stk was expressed in CHO cells along with gp55 or empty vector. 48 hours later, cell surface proteins were biotinylated and isolated, followed by SDS-PAGE electrophoresis and western blot analysis. Immunoblotting with a myc tag antibody confirmed that cell surface expression of Sf-Stk was increased in the presence of gp55 (Fig 3.1.B).

3.4.2 Gp55 promotes the plasma membrane localization of Sf-Stk through cysteine mediated disulfide bond formation

Previously we demonstrated that with the interaction of gp55 with Sf-Stk is mediated by cysteines in the ecotropic domain of gp55. In order to test whether this

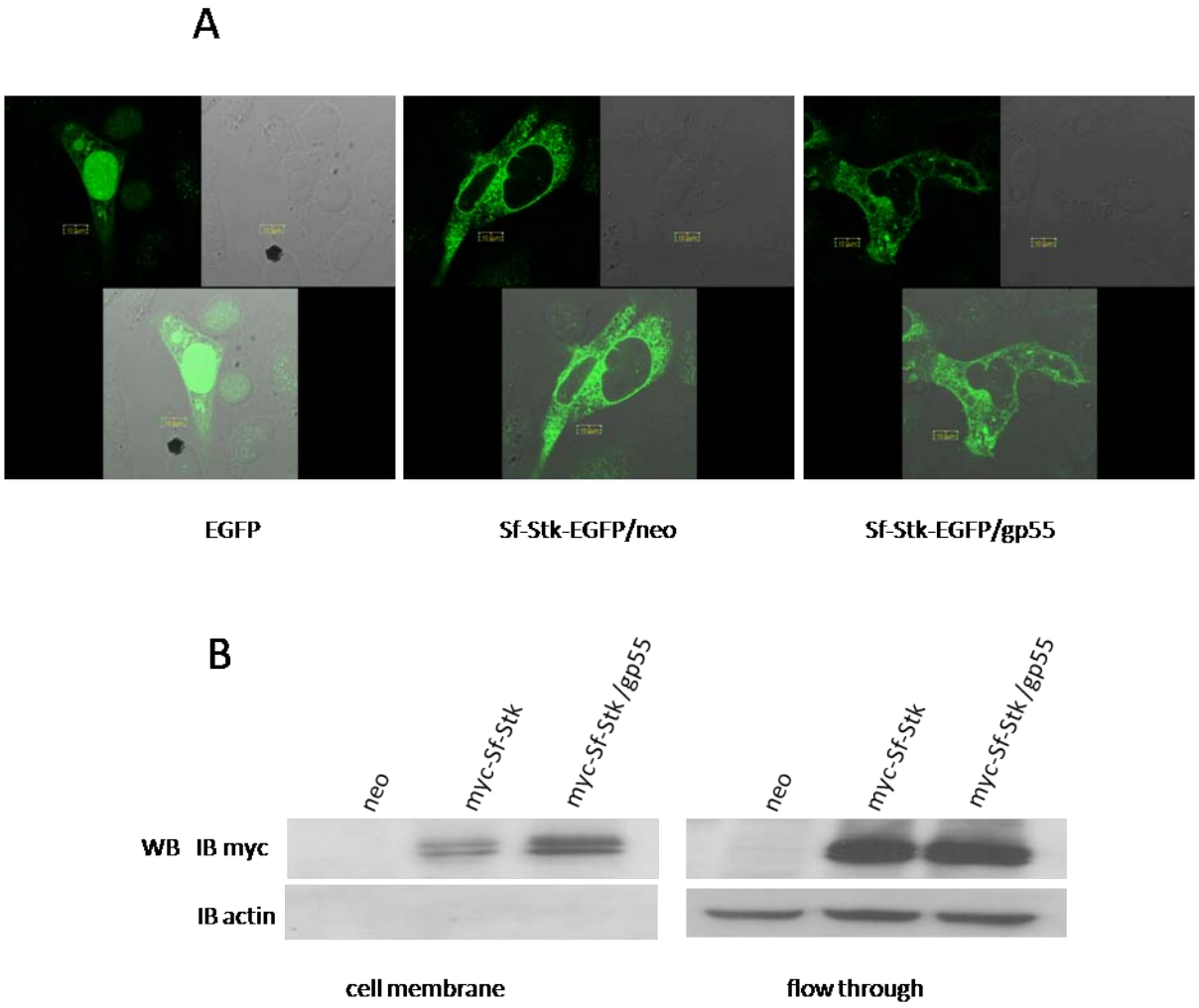

Fig 3.1 gp55 promotes the cell surface localization of Sf-Stk

A) CHO cells were transfected with a Sf-Stk-EGFP fusion protein in the presence or absence of gp55. Cells were examined for EGFP fluorescence using confocal microscopy. B) CHO cells were transfected with myc-Sf-Stk with gp55 or empty vector. 48 hours later, cell surface proteins were biotinylated and isolated. Collected proteins were analyzed to SDS-PAGE electrophoresis. Membranes were immunoblotted with anti-myc antibody.

interaction is required for cell surface localization of Sf-Stk, we employed flow cytometry using Sf-Stk tagged at the N-terminus with myc, to evaluate the surface expression of Sf-Stk in the presence of wild type gp55 or gp55 harboring mutations in the four cysteins in the ecotropic domain (gp55C4A). MSCV-myc-Sf-Stk plasmids were co-transfected into 293 cells with gp55, gp55C4A or empty vector. 48 hours later, cells were collected and stained with Alexa fluor 647 conjugated Myc tag antibody without permeabilizing the cells in order to visualize only the myc-Sf-Stk located on the plasma membrane (Fig 3.2.A). Results from this experiment confirm the cell surface localization of wild type Sf-Stk in the presence of gp55. Gp55C4A, which cannot interact with Sf-Stk, loses its ability to recruit Sf-Stk to the plasma membrane. Using the same approach, we mutated pairs (Sf-StkC8,19A, Sf-StkC37,42A) or all four (Sf-StkC4A) of the extracellular cysteines in Sf-Stk and examined plasma membrane localization of these mutants in the presence of wild-type gp55 (Fig 3.2.B). We have demonstrated that Sf-StkC8,19A and Sf-StkC37,42A retain partial ability to interact with gp55 while this interaction is abrogated with Sf-StkC4A. Flow cytometry analysis demonstrated that, compared with wild type Sf-Stk, Sf-StkC8,19A and Sf-StkC37,42A exhibit lower levels of cell surface localization. However, surprisingly, Sf-StkC4A, which can not interact with gp55, was localized on the plasma membrane.

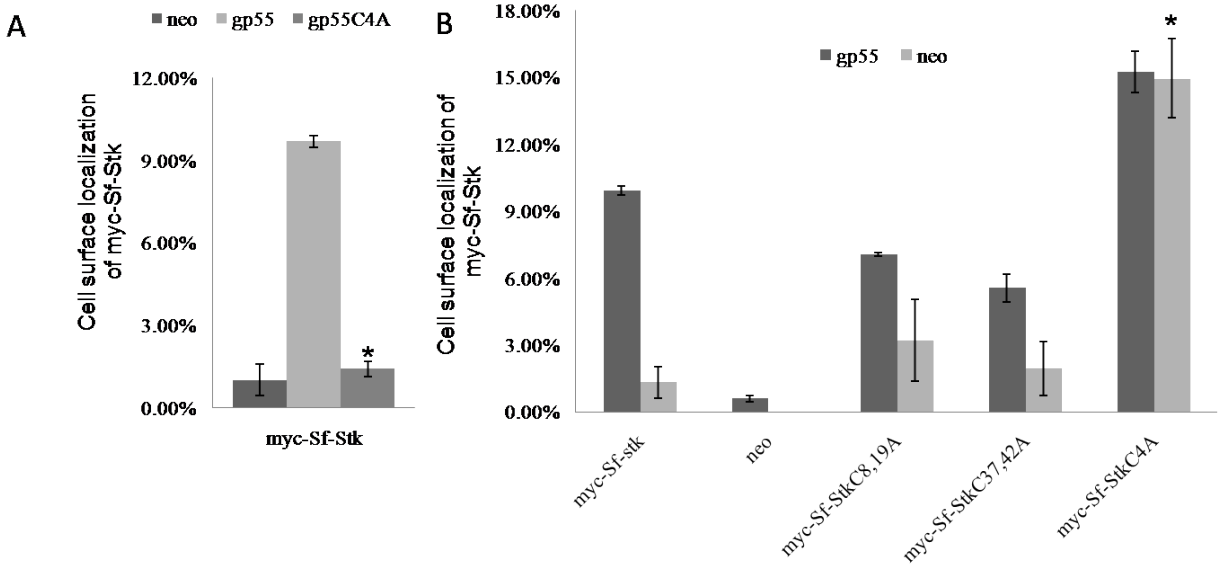

Fig 3.2 Cysteine residues in gp55 and the extracellular domain of Sf-Stk regulate Sf-Stk plasma membrane localization

A) 293 cells were transfected with myc-Sf-Stk in the presence of gp55, gp55C4A or empty vector. 48 hours later, cells were resuspended and stained with Alexa fluor 647 conjugated myc tag antibody. Cells were washed and applied to flow cytometry. * $P < 0.01$ B) 293 cells were transfected with the indicated myc tagged wild type or mutant Sf-Stk in the presence of gp55 or empty vector. 48 hours later, cells were resuspended and stained with Alexa fluor 647 conjugated myc tag antibody. Cells were washed and sorted by flow cytometry. * $P < 0.01$

3.4.3 Plasma membrane localization is required for Sf-Stk activation.

Though Sf-StkC4A is expressed on the plasma membrane, our previous studies clearly demonstrate that Sf-StkC4A is not tyrosine phosphorylated or capable of supporting Epo^{ind} BFU-E formation in response to FVP. Taken together, these data indicate that, while gp55 promotes the cell surface localization of Sf-Stk, this cell surface localization alone is not sufficient to activate Sf-Stk signaling. In order to address the role of plasma membrane localization of Sf-Stk in mediating the activation of Sf-Stk, we utilized a mutant of Sf-Stk containing a M330T mutation, which has previously been reported to have constitutive activity[177]. We introduced the M330T mutation into both Sf-Stk and Sf-StkC4A (Sf-StkM330T, Sf-StkC4AM330T) and transfected these constructs into 293 cells in the presence or absence of gp55. Flow cytometry data indicated that Sf-StkM330T is translocated to plasma membrane when co-expressed with gp55 while Sf-StkC4AM330T is constitutively localized to the plasma membrane in the absence of gp55 (Fig 3.3.A). We further tested the ability of gp55 to promote tyrosine phosphorylation of the Sf-Stk mutants. Consistent with previous studies, Sf-StkM330T exhibits a higher level tyrosine phosphorylation than wild type Sf-Stk. However, this phosphorylation is significantly increased when gp55 is co-expressed with Sf-StkM330T. Alternatively, introduction of the M330T mutation in the context of Sf-StkC4A, which localizes to the plasma membrane but does not promote downstream signaling, resulted in full activation of the receptor in the absence of gp55 (Fig 3.3.B). We also tested the induction of Erk phosphorylation and AP1 luciferase activity downstream of Sf-Stk, Sf-StkM330T or Sf-StkC4AM330T in the presence or absence of gp55(Fig3.3.C).

Consistent with the phosphorylation data, Sf-StkM330T requires coexpression of gp55 in order to stimulate Erk phosphorylation or AP1 luciferase activity while Sf-StkC4AM330T alone is capable of activating Erk/MAPK signaling. These findings suggest that gp55 promotes both the activation of Sf-Stk and its translocation to the plasma membrane, and that both of these activities of gp55 are required for full activation of Sf-Stk. Re-creation of either of these functions alone by mutations in Sf-Stk (Sf-StkC4A which localizes to the plasma membrane or Sf-StkM330T which results in constitutive activation of the kinase) fails to result in full receptor activation, while re-creation of both activities (Sf-StkC4AM330T) fully restores the level of Sf-Stk activity observed in the presence of gp55.

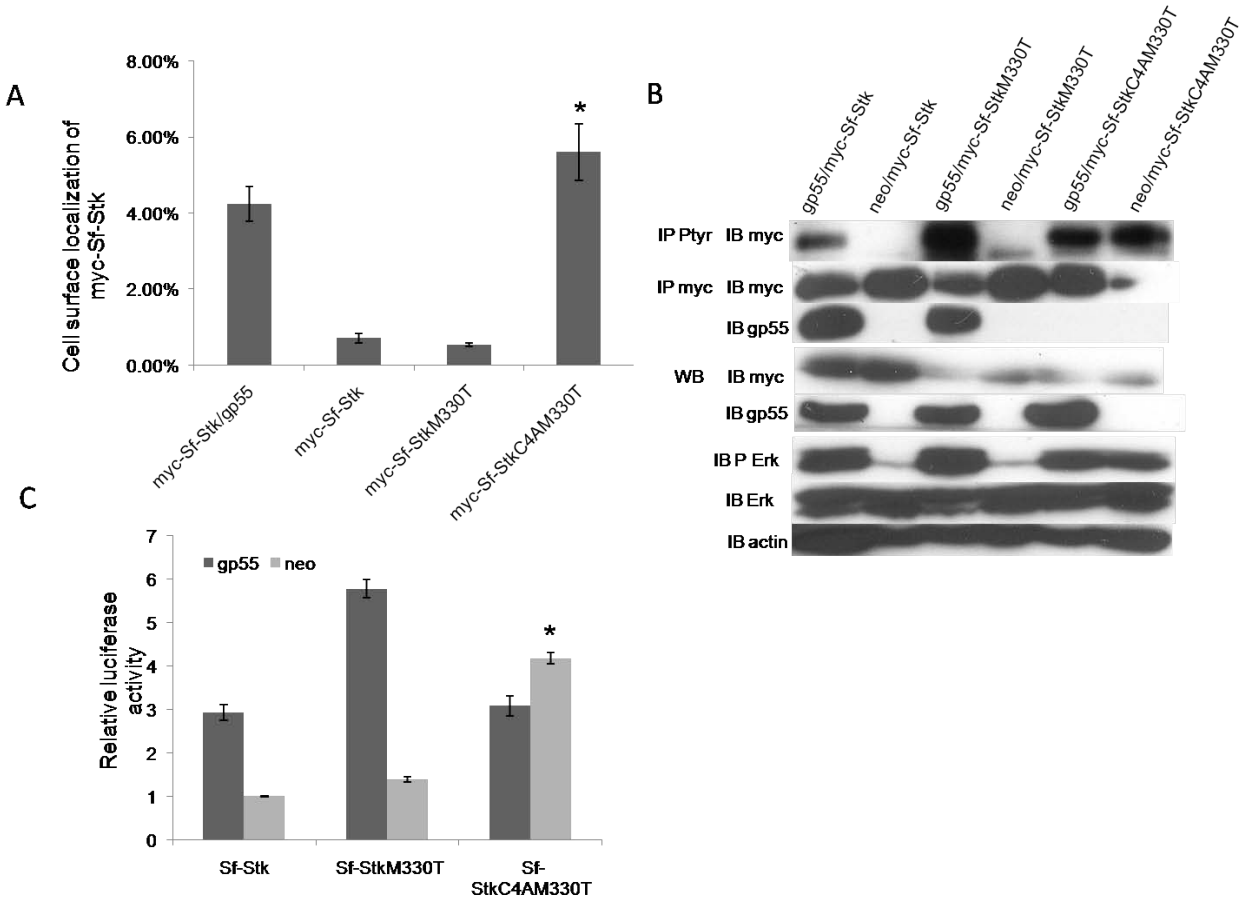

Fig 3.3 Cell surface expression is required for full activation of Sf-Stk

A) 293 cells were transfected with the indicated myc tagged wild type or mutant forms of Sf-Stk in the presence of gp55 or empty vector. 48 hours later, cells were resuspended and stained with Alexa fluor 647 conjugated myc tag antibody. Cells were washed and sorted by flow cytometry. * $P < 0.01$ B) 293 cells were transfected with the indicated myc tagged wild type or mutant forms of Sf-Stk in the presence of gp55 or empty vector. Cell lysates were either immunoprecipitated with anti-phosphotyrosine antibody and blotted with anti-myc antibody or immunoprecipitated with anti-myc antibody and blotted with anti-gp55 antiserum. C) 293 cells were transfected with the indicated wild type or mutant forms of Sf-Stk in the presence of gp55 or empty vector and AP1 luciferase reporter and renilla reporter plasmids. 48 hours later, AP1 activities was assessed using a luciferase assay according to manufacturer's instruction. * $P < 0.01$

3.5 DISCUSSION

Friend virus induced erythroleukemia is initiated by the ability of the SFFV-encoded viral glycoprotein, gp55, to interact with the Epo receptor and Sf-Stk, expressed by host hematopoietic cells. Several studies have been conducted to investigate the downstream signals generated by the gp55/ Sf-Stk interaction that promote leukemic transformation. Studies from our laboratory have demonstrated that a Sf-Stk/Grb2/Gab2/STAT3 signaling pathway is critical for this response[111, 179, 191]. Work from our lab and other researchers have also revealed that PI3K and MAPK signaling are activated downstream of gp55/Sf-Stk and required for disease development [111, 177]. However, it has remained unclear how the interaction between gp55 and Sf-Stk results in the activation of Sf-Stk signaling.

Here we demonstrate that the covalent interaction between gp55 and Sf-Stk promotes the relocalization of Sf-Stk to the plasma membrane. Subcellular localization plays a critical role in regulating the effector function of many signaling proteins. Sf-Stk transcription starts at exon 11 and lacks most of the extracellular domain, including the projected signal sequence. Hence it has remained unclear whether Sf-Stk is a membrane protein. Our data demonstrates that Sf-Stk primarily localizes inside the cell and relocates to the plasma membrane through its association with gp55. It is reported that an N-terminally truncated EGFR cannot be expressed on the cell surface, but its cell surface localization can be rescued by co-expression of wild type EGFR[203]. We tested the hypodissertation that full-length Stk could similarly promote the cell surface localization of Sf-Stk by co-expressing the full-length receptor and Sf-Stk in 293 cells. However, we

failed to detect cell surface expression of Sf-Stk in the presence of full-length Stk (data not shown).

Our studies indicate that the cysteines responsible for the interaction of Sf-Stk with gp55 also regulate the cellular localization of Sf-Stk. Interestingly, mutation of all four cysteines in the extracellular domain of Sf-Stk resulted in cell surface localization of Sf-Stk in the absence of gp55, suggesting that these cysteines may play a role in maintaining Sf-Stk in the cytoplasm. A similar phenomenon was reported in the assembly of secretory immunoglobulin M (IgM), which is retained in the ER through Cys575 by thiol retention until this cysteine is occupied by forming a disulfide bond with other IgM monomers or the Joining Chain[204, 205]. Also, palmitoylation on cysteine residues has been shown to regulate the subcellular trafficking of many membrane targeted proteins. A proposed model suggests that palmitoylation on cysteine residues could mask sites that mediate potential protein/protein interactions. Palmitoylation close to the membrane may also force flanking residues into closer membrane proximity, thus changing the conformation of the protein. Palmitoylation on GluR subunits of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor promotes retention of the receptor in the Golgi, preventing cell surface delivery. When at the cell surface, palmitoylation of the AMPA receptor inhibits 4.1N interaction and facilitates internalization of the receptor[206]. In the case of Sf-Stk, binding of gp55 to the extracellular cysteines in Sf-Stk could displace these inhibitory interactions, thus promoting cell surface localization of Sf-Stk. However, the reduced phosphorylation and inability of the Sf-StkC4A mutant to transform primary erythroblasts indicates that cell surface localization alone is not sufficient to promote receptor activation.

We also set out to test whether plasma membrane localization is necessary for Sf-Stk activation and its ability to promote Epo independent colony formation in response to Friend virus. Toward that end, an ER retention peptide[96] was added to the C terminus of Sf-Stk (Sf-StkER). Western blotting confirmed the successful expression of Sf-StkER. However, flow cytometric analysis revealed that Sf-StkER could still be translocated to cell surface when coexpressed with gp55 (data not shown). Therefore, to address this question, we turned to another approach. A tyrosine kinase domain point mutation in the GDNF receptor family member Ret (RetM918T) was first found in multiple endocrine neoplasia type 2B (MEN 2B) patients[207, 208] and results in constitutive activation of Ret. This constitutively activating mutation was later observed in Met and Ron (MetM1268T, RonM1254T)[136, 137]. Generation and expression of the M1231T mutation in Stk (StkM1231T) resulted in stronger phosphorylation and metastasis than wild type Stk, but did not affect the transforming ability of Stk[142]. A Sf-StkM330T mutant was reported to promote a constitutive increase in receptor tyrosine phosphorylation compared with wild type Sf-Stk[202]. We have demonstrated that, like wild-type Sf-Stk, Sf-StkM330T is not located on the plasma membrane in the absence of gp55. However, while this mutant harbors some constitutive phosphorylation, it failed to promote Erk1/2 tyrosine phosphorylation or AP1 luciferase activity. When Sf-StkM330T was co-expressed with gp55, we found that Sf-StkM330T relocated to plasma membrane and its tyrosine phosphorylation was further enhanced. Interestingly, mutation of M330T in Sf-Stk in the context of Sf-StkC4A, which locates to the plasma membrane but fails to promote downstream signaling, restored full activity to the receptor suggesting that

activation of the kinase and cell surface localization both play a critical role in the activation of Sf-Stk by gp55.

Several critical signaling molecules are localized to the plasma membrane. Our previous work demonstrated that Gab, PI3K and Src proteins are all involved in mediating Stk signaling. The Src protein contains an N-terminal 14-carbon myrsitoyl group which facilitates its membrane targeting. Mutational studies showed that a correlation exists between N-myristoylation, subsequent membrane association, and the ability of v-Src to transform cells into a neoplastic state[157]. Membrane localization is also important in mediating the function of Gab proteins. Gab2 does not support Met induced Erk activation and morphogenesis in epithelial cells unless a myristoylation group and a Met binding site is added to it, suggesting that both membrane localization and a strong interaction with Met is required to promote morphogenic signaling[209]. The membrane localization of Gab proteins is mediated through the PH domain, which can bind with PIP3, the product of activated PI3K. In addition, Erk mediated phosphorylation of Ser551 on Gab1 also contributes to the membrane localization of Gab1[210]. Thus, membrane localization of Sf-Stk by gp55 could be required for the ability of Sf-Stk to form a complex with these or other signaling molecules.

While gp55 can interact with the EpoR in the ER, the activation of the EpoR by gp55 requires cell surface localization of the gp55/EpoR complex. It has been suggested that EpoR and Sf-Stk can form a trimolecular complex in the presence of gp55[112], but the function of this EpoR/Sf-Stk interaction in the progression of Friend disease remains unclear. Full-length Ron has also been shown to interact with the EpoR, and stimulation of Ron with MSP results in the phosphorylation of the EpoR in trans[211]. Thus the cell

surface localization of Sf-Stk by gp55 could be important in promoting its association with the EpoR, and this association could be required to promote the Epo independent growth of primary erythroblasts in response to Friend virus infection.

Our previous studies support a model in which Sf-Stk forms an inactive oligomer in the cytoplasm. The data presented here suggest that, through cysteine mediated disulfide bond formation, gp55 recruits Sf-Stk to the plasma membrane and promotes an active conformation of the Sf-Stk oligomer, resulting in enhanced receptor activation and downstream signaling. Recent studies indicate that Sf-Stk plays an important role in the regulation of septic shock, a phenotype previously attributed to the full-length Stk receptor (Unpublished observation by Dr Robert F. Paulson). In addition, several studies have identified a similar Sf-Ron transcript in both normal and transformed human cells [130, 212]. However, it is unclear how Sf-Stk or Sf-Ron activity and subcellular localization might be regulated under normal physiological conditions or in the development of cancer in the absence of viral infection. It will, therefore, be critical to identify cellular factors that are involved in regulating the activation and/or subcellular localization of Sf-Stk. The studies described herein lay the foundation for addressing this important biological question.

Chapter 4

Signaling downstream of gp55/Sf-Stk requires Src family kinases

4.1 Abstract

Friend virus induces an erythroleukemia in susceptible mice initiated by the interaction of the Friend virus encoded glycoprotein, gp55, with the erythropoietin receptor and the product of the host *Fv2* gene, a naturally occurring truncated form of the Stk receptor tyrosine kinase (Sf-Stk). We have previously demonstrated that gp55 promotes phosphorylation and cell surface localization of a pre-formed Sf-Stk dimer. Here we demonstrate that the phosphorylation of Gab1 by Sf-Stk is enhanced by gp55 and is mediated through the C terminal docking tyrosines, Tyr429 and Tyr436, which are also required for Erk/MAPK activation downstream of Sf-Stk and gp55. Further, we demonstrate that Src kinase activity is required for the phosphorylation of Gab1, Gab2 and Erk and the activation of an AP1 luciferase reporter by Sf-Stk and gp55. However, Src kinase activity is not required for the enhanced phosphorylation of Sf-Stk in the presence of gp55, suggesting that Src functions downstream of Sf-Stk in this signaling pathway. Finally, we demonstrate that the recruitment of Src to Sf-Stk is critically dependent on the presence of gp55, and that Src kinase activity is essential for Epo independent colony formation by FVP infected primary erythroblasts. Taken together, our results suggest the possibility that recruitment of Sf-Stk to the plasma membrane by gp55 results in the activation of Sf-Stk as well as its association with Src family kinases,

which mediate the phosphorylation of Gab1 and Gab2, and the downstream activation of Erk and AP1.

4.2 Introduction

Since Friend disease was first reported in 1957[62], the acute erythroleukemia induced by the various strains of Friend virus have provided an excellent model to study multistage carcinogenesis[184]. In the first stage, the virus infects erythroid progenitor cells and a viral glycoprotein, gp55, interacts with both the erythropoietin receptor (EpoR) and a naturally occurring truncated form of the stem cell derived tyrosine kinase (Stk), Sf-Stk, resulting in the Epo-independent (Epo^{ind}) expansion of erythroid progenitor cells. Sf-Stk is encoded by the Friend virus susceptibility gene, *Fv2*[110]. $Fv2^{r/r}$ mice, including C57BL/6 mice, lack expression of Sf-Stk and are resistant to FV infection, while full-length Stk expression is unaffected. An internal promoter within the Stk locus drives Sf-Stk expression, and $Fv2^{r/r}$ mice harbor mutations in the internal promoter. Sf-Stk lacks the N-terminal ligand binding domain of full-length Stk, but retains the transmembrane and tyrosine kinase domains. *In vitro* and *in vivo* expression of Sf-Stk in C57BL/6 bone marrow cells has been shown to confer Friend virus susceptibility to $Fv2^{r/r}$ mice[111].

Sf-Stk has been shown to covalently interact with gp55, resulting in constitutive activation of Sf-Stk[112]. We previously demonstrated that the interaction of gp55 and Sf-Stk is mediated through the interaction of cysteines located in the ecotropic domain of gp55 with cysteines in the extracellular domain of Sf-Stk. In the absence of gp55, Sf-Stk

is retained in cytoplasm as an inactive dimer. However, in the presence of gp55, Sf-Stk is relocated to plasma membrane and phosphorylation of Sf-Stk is enhanced. We further demonstrated that both cell surface expression and activation, induced by either gp55 expression or a point mutation in the kinase domain, are required for full Sf-Stk activation and the induction of downstream signaling events.

Previously, by generating point mutations in the Sf-Stk C terminal docking sites, we demonstrated that Tyr436 of Sf-Stk is required for Grb2 binding and Epo^{ind} expansion of Friend virus infected erythroid progenitor cells[111]. Mutational analysis of Grb2 demonstrated that the Grb2 C-terminal SH3 domain is essential for the formation of FVP derived Epo independent BFU-E[175]. This SH3 domain binds to a number of large adaptor proteins including the Gab family of adaptors, and we have shown that $Gab2^{-/-}$ mice exhibit significantly smaller spleen sizes than wild type mice following infection with Friend virus. Exogenous expression of Gab2 rescues Friend virus derived Epo independent BFU-E formation in Gab2-/- bone marrow, demonstrating that Gab2 expression in Friend virus target cells is required for the progression of Friend erythroleukemia. A Sf-Stk/Gab2 fusion protein containing no Grb2 binding site transfected into erythroid progenitor cells from $Fv2^{r/r}$ mice successfully supported the growth of Friend virus derived Epo independent BFU-E[175], suggesting Gab2 is the primary signal downstream of Grb2 activation in this system. Further, we showed that Gab2 recruits STAT3 through a YXXQ binding motif, which is required for Epo independent colony formation following Friend virus infection. This work suggested that gp55 mediates the Epo independent growth of erythroid progenitor cells through a Sf-Stk/Grb2/Gab2/STAT3 signaling pathway.

We also demonstrated that Erk/MAPK and PI3K/Akt signaling are activated downstream of Sf-Stk. Both the MEK inhibitor, PD98059, and the PI3K inhibitor, wortmannin, block Epo^{ind} colony formation in response to Friend virus, and co-expression of Sf-Stk with a dominant negative p85 in transduced bone marrow cells from $Fv2^{r/r}$ mice failed to rescue the response of these cells to Friend virus infection, suggesting that Erk/MAPK and PI3K/Akt signaling play essential roles in Friend virus induced erythroblast growth[111, 175, 179, 180].

Stk is the murine homologue of the human Ron receptor. Previous studies have demonstrated that the non-receptor tyrosine kinase, Src, plays a critical role in the regulation of this family of receptors. Extracellular matrix induced integrin aggregation mediates Fak activation and triggers the activation of Src, which targets phosphorylated Ron and further enhances Ron phosphorylation[162]. Further, PP2, a Src inhibitor, was reported to reduce both MSP dependent and independent Ron phosphorylation in MCF-10A breast epithelial cells, and co-expression of a dominant negative Src blocked MSP dependent and independent Ron phosphorylation[163]. We also demonstrated that Src is required for the ligand-independent activation of Erk by full-length Stk [164].

Here, we demonstrate that Src family kinases play an essential role in mediating the activation of signals downstream of Sf-Stk and gp55 as well as the Epo independent growth of primary erythroblasts in response to Friend virus infection. Based on these and other findings, we propose a model in which gp55 promotes the cell surface localization and phosphorylation of Sf-Stk, resulting in its association with Src at the plasma membrane. Src then mediates the phosphorylation of downstream signaling molecules, including Gab1 and Gab2, resulting in the activation of the Erk/AP1 signaling pathway

and promotes the Epo independent colony formation of Friend virus infected bone marrow progenitors.

4.3 MATERIALS AND METHODS

4.3.1Antibodies and cell culture reagents

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The Mirus-293 transfection reagents were purchased from Mirus Bio, LLC (Madison, WI). The Dual-Luciferase Reporter Assay System was purchased from Promega Corporation (Madison, WI). Antibodies against the Myc tag, HA tag, phospho-tyrosine, phosphor-Erk1/2, Erk1/2 and HRP linked anti-rat IgG were purchased from Cell Signaling (Danvers, MA). Antibody against actin, HRP linked anti-mouse IgG was purchased from Sigma-Aldrich, Inc (St.Louis, Mo). Mouse True Blot ULTRA HRP anti-mouse IgG was purchase from eBiosciences (San Diego, CA). HRP linked anti-rabbit IgG was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Rat anti-serum against gp55 was kindly provided by Dr. Sandra Ruscetti (National Cancer Institute). All PCR Primers were ordered from Operon Biotechnologies, Inc (Huntsville, ALlocation)**.** Restriction enzymes and Protein G magnetic beads were purchased from New England Biolab (Ipswich, MA). PfuTurbo DNA polymerase was purchased from Stratagene (La Jolla, CA). ECL plus Western blotting detection reagents were purchased from GE Healthcare (Piscataway, NJ). Pierce

Cell Surface Protein Isolation Kit was purchased from Thermo Fisher Scientific Inc (Rockfold, IL).

4.3.2 Gene construction and mutagenesis

To construct MSCV-myc-Sf-StkY3F, the Sf-StkY3F fragment was PCR amplified form MSCV-StkY3F by using the primers 5'-GGGGAATTCCATGACCGTGGGTGGTGAG-3' and 5'-CTGAATTCAAGTGGGCAGGGGTGGCTCTG-3'. Sf-StkY3F PCR fragment was then digested with *EcoR* I and cloned into *EcoR* I digested MSCV-myc-Sf-Stk vector.

4.3.3 Cell Transfection and Luciferase Assay

$5x10^4$ HEK 293 cells were plated into 24-well plates. 20hrs later, a designated mixture of 20ng Sf-Stk or gp55, 20 ng of AP1 luciferase reporter plasmid and 0.5ng renilla reporter plasmid were used for transient transfection in each well with the Mirus-293 transfection reagent according to the manufacturer's protocol. For control vector, 20ng MSCV-neo plasmid was used in transfection of each well. 48hrs after transfection, luciferase assays were performed according to the manufacturer's instructions.

4.3.4 Immunoprecipitation and Western Blot Analysis

$3x10^5$ HEK 293 cells/well were plated into 6-well plates. 20hrs later, the cells were transiently transfected with a 300 ng mixture of the designated plasmids per well

and 2 wells were transfected for every set of samples. 40-48hrs post transfection, cells were suspended in 500ul ice cold cell lysis buffer containing 150mM NaCl, 20mM Tris·HCl(pH7.5), 5mM EDTA, 1% NP-40, 1mM phehylmethylsulfonyl fluoride, 1mM Na_3VO_4, 10mM NaF. Cell lysates were centrifuged at 1500 rpm for 20 mins. Following centrifugation, the supernatant lysates were transferred to prechilled tubes. 40 ul cell lysates were mixed with 4X denaturing SDS loading buffer and heated to 100 degrees for 5 mins. 800 ul of lysates were used for immunoprecipitation as follows: Cell lysates were incubated with the appropriate amount of primary antibody at 4 degrees for 30 mins, incubated with protein G magnetic beads for 2 hrs and washed by ice cold PBS buffer three times. Beads were resuspended in 40 ul 1X SDS denaturing loading buffer and heated to 100 degrees for 5 mins. Western blot and immunoprecipitation samples were separated by SDS-PAGE gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% non fat milk TBST buffer for 1 hr and probed with primary antibody at 4 degrees overnight. Membranes were washed 3 times in TBST and incubated with horseradish peroxidase-conjugated IgG for another hour. Membranes were washed 3 times in TBST before applied to ECL plus western blotting detection reagents for visualization. For reprobing, membranes were stripped with 62.5mM Tris·HCl (pH=6.8), 2% SDS and 0.7% β-mercaptoethanol and 0.7% β-mercaptoethanol at 55 degrees for 30 mins.

4.3.5 Retrovirus generation and in vitro infection of Primary Murine Bone Marrow Cells

To generate MSCV retroviral supernatants, $3x10^5$ cells/well 293T cells were plated into 6-well plates. 20hrs later, the cells were transiently transfected with 300ng pEco and 700ng MSCV plasmids for each well. 40-48hrs post transfection; viral supernatants were collected and filtered through 0.45um sterile syringe filters (Pall Life Sciences, Ann Arbor, MI) before use. For FVP supernatant generation, FP63 cells (a kind gift from Alan Bernstein, Mount Sinai Hospital, Toronto, Ontario, Canada.) were cultured in DMEM medium with 10% FBS. The FVP supernatants were collected and filtered through 0.45um sterile syringe filters, aliquoted and stored at -80 degrees. For *in vitro* infection of primary bone marrow, cells were harvested from femurs of 8 weeks old mice and resuspended in DMEM medium with 40% FBS. The bone marrow cells were infected by incubation with retroviral supernatants for 20hrs along with 10ng/ml interleukin-3. For Epo-independent CFU-E and BFU-E colony assays, the bone marrow cells were resuspended with FVP supernatants and incubated on ice for 1hr. The cells were then added to Methocult Medium M3234, along with 2.5ng/ml IL-3, in triplicate with or without 1U/ml Epo. Cultures were incubated for 3 days or 5 days respectively for CFU-E and BFU-E in 5% CO_2 at 37 degrees. Erythroid colonies were visualized by acid-benzidine staining. For PP1 treatment, different concentration of PP1 (20nM, 10nM, 5nM, 1nM) were mixed in before bone marrows were plated into Methocult Medium M3234.

4.4 Results

4.4.1 The phosphorylation of Gab1, Gab2 and Erk1/2 downstream of Sf-Stk is enhanced by co-expression of gp55.

It's suggested that activation of the MAPK pathway by overexpression or constitutive activation of Ron is necessary and sufficient to promote cell differentiation, proliferation and transformation in multiple cell lines [138, 146-148]. While studies from our laboratory demonstrated that Erk signaling is required for the ability of Friend virus to induce Epo^{ind} colony formation, studies from other laboratories demonstrated that inhibition of p38 or JNK of MAPK had little or no effect on the transformation of NIH3T3 fibroblasts by Sf-Stk in response to Friend virus[177]. Here, we examined the ability of Sf-Stk to promote phosphorylation of Erk and p38 in the presence and absence of gp55 (Fig 4.1). Erk phosphorylation was slightly induced by Sf-Stk alone and this activation was strongly enhanced in the presence of gp55, however phosphorylation of p38 was not promoted by Sf-Stk in the presence or absence of gp55. We also failed to detect enhanced phosphorylation of Jnk under these conditions (data not shown). These studies confirm that Erk is the primary MAPK activated by Sf-Stk and highlights a critical role for gp55 in this response.

The classic signaling pathway by which Erk/MAPK is activated downstream of growth factor receptor tyrosine kinase signaling is through the recruitment of a Grb2/SOS complex. Alternatively, Gab1 has been reported to play a critical role in the sustained activation of Erk downstream of cMet [168, 169]. While the details of this signaling pathway are not entirely clear, the association of Shp2 with Gab1 plays an essential role

in this process[213]. Previously we have demonstrated that recruitment of a Grb2/Gab2 complex to the second docking site tyrosine of Sf-Stk is critical for promoting Friend virus induced Epo^{ind} colony formation, however the role of Gab1 in this process has not been investigated. To determine whether gp55 promotes the phosphorylation of Gab1 or Gab2 downsteam of Sf-Stk, Sf-Stk was transfected with or without gp55 into 293 cells and endogenous Gab1 and Gab2 phosphorylation were assessed. We found that, while Sf-Stk expression alone induced some Gab1 and Gab2 phosphorylation, coexpression of gp55 resulted in enhanced phosphorylation of both Gab1 and Gab2 (Fig 4.1). The size of the phosphorylated Gab1 band was also increased in the presence of gp55, indicating that the phosphorylated band observed in the absence of gp55 likely does not represent the fully phosphorylated form of Gab1.

We previously demonstrated co-immunoprecipitation of Gab2, but not Gab1, with Sf-Stk in 293 cells. However, these experiments were performed in the absence of gp55. Therefore, we set out to determine whether Gab1 can be recruited to the Sf-Stk receptor in the presence of gp55. Toward that end, we co-transfected 293 cells with Sf-Stk in the presence or absence of gp55 and examined the ability of Sf-Stk to co-immunoprecipitate with Gab1 (Fig 4.2B). We found that the presence of gp55 promoted the interaction of Sf-Stk with Gab1.

4.4.2 Both Tyr429 and Try436 in Sf-Stk mediate Gab1 binding and Erk/MAPK signaling

Both Gab1 and Gab2 contain proline rich motifs that promote their interaction with the C terminal SH3 domain of Grb2, thus Gab1 and Gab2 can indirectly associate

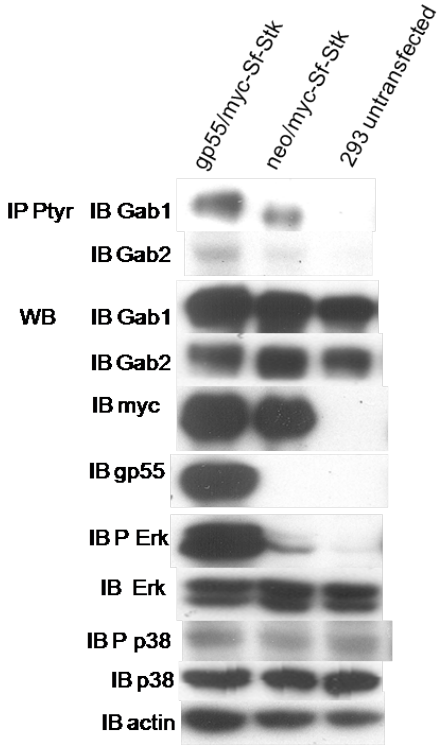

Fig 4.1 Coexpression of gp55 and Sf-Stk promotes the phosphorylation of Gab1, Gab2 and Erk.

293 cells were transfected with Sf-Stk in the presence or absence of gp55. 48 hours later, cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with Gab1, Gab2 antibodies. Cell lysates were also immunoblotted with anti-phsophoErk, anti-phosphop38 antibodies. Membranes were stripped and probed again with anti-Erk and anti-p38 antibodies.

with activated Sf-Stk through Grb2. Previous studies from our laboratory have demonstrated that Grb2 binding is mediated by the C terminal YVNV docking site of Sf-Stk starting with Tyr436. However, Gab1 can also directly bind with Met via the Met binding domain (MBD). This binding involves Tyr1249 on the c-Met C terminus and requires intact upstream residues. In Sf-Stk, the counterpart of Met Tyr1249 is Tyr 429. We hypothesized that Gab1 could be involved in the sustained activation of Erk downstream of Sf-Stk either through the indirect association with Sf-Stk via Grb2 or through direct MBD mediated binding. To test this hypodissertation, Sf-Stk in which the individual docking site tyrosines are mutated to phenylalnine (Sf-StkY429F, Sf-StkY436F) or a double mutant (Sf-StkY2F) were transfected into 293 cells and the phosphorylation of endogenous Erk was examined (fig 4.2.A). The results demonstrate that Erk phosphorylation decreased in the presence of both Sf-StkY429F and Sf-StkY436F mutants, and is completely abolished downstream of Sf-StkY2F. Consistent with these results, AP1 luciferase activity is decreased in both Sf-StkY429F and Sf-StkY436F mutants, and is further reduced to baseline levels in cells expressing Sf-StkY2F (Fig 4.2C). These data confirm that both Tyr429 and Tyr436 docking sites can mediate the activation of the Erk/MAPK signaling pathway downstream of Sf-Stk.

In order to determine whether one or both docking sites leads to the recruitment and activation of Gab1, we co-expressed gp55 and wild-type Sf-Stk, Sf-StkY429F, Sf-StkY436F or Sf-StkY2F and examined the ability of Sf-Stk to co-immunoprecipitate with Gab1 and promote Gab1 phosphorylation. Consistent with the Erk phosphorylation data, we found that mutation of either docking site tyrosine resulted in decreased association of Sf-Stk with Gab1 (Fig 4.2B). Further, we found that phosphorylation of Gab1 was more

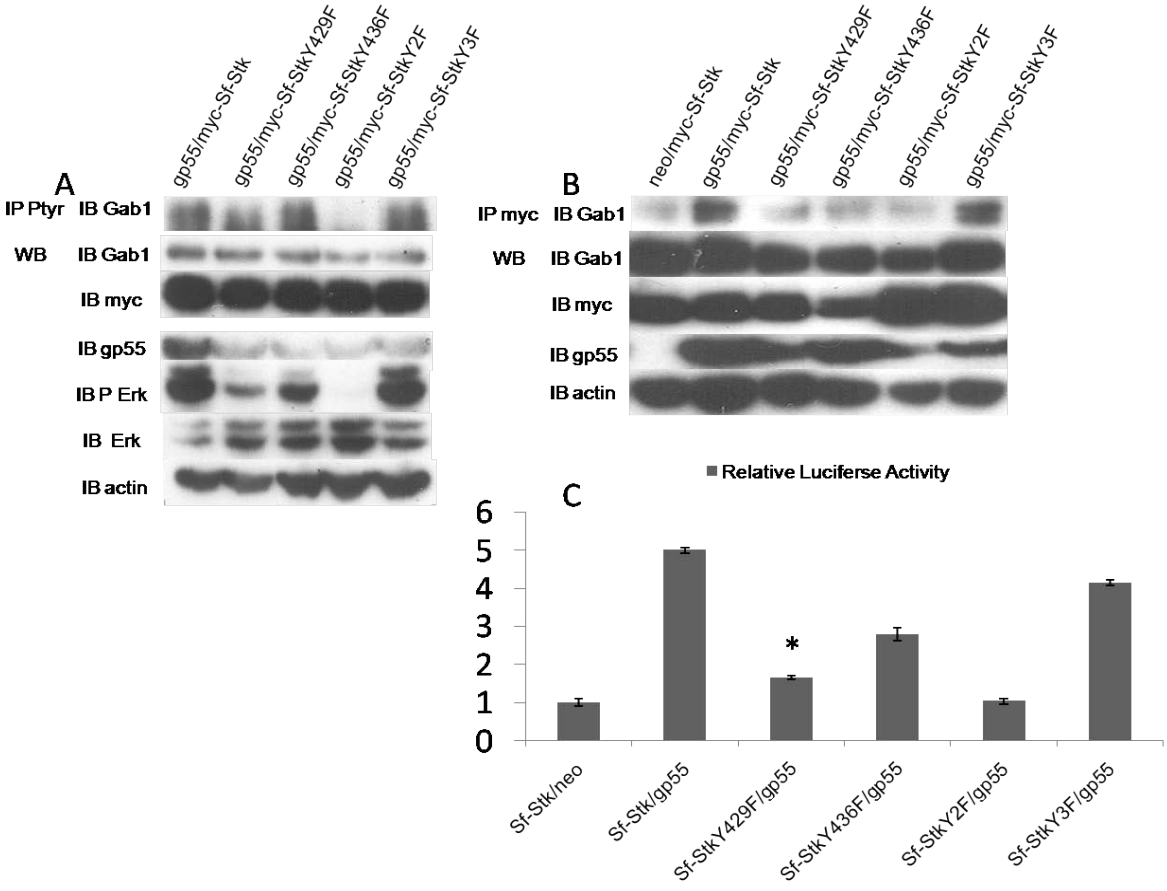

Fig 4.2 gp55 promotes the recruitment and activation of Gab1 by Sf-Stk through the docking site tyrosines

A) 293 cells were transfected with the indicated wild type or mutant forms of Sf-Stk and gp55. 48 hours later, cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with Gab1 antibody. Cell lysates were also immunoblotted with anti-phospoErk antibody. The membrane was then stripped and blotted with anti-Erk antibody. B) 293 cells were transfected with the indicated wild type or mutant forms of Sf-Stk and gp55. 48 hours later, cell lysates were immunoprecipitated with anti-myc antibody and immunoblotted with Gab1 antibody. C) 293 cells were transfected with the indicated wild type or mutant forms of Sf-Stk and gp55 with AP1 luciferase reporter and renilla luciferase reporter vectors. 48 hours later, AP1 activity was assessed using a luciferase assay according to manufacturer's instruction. * $P < 0.01$

dependent on Y429 than Y436 (Fig 4.2A), however mutation of both docking site tyrosines fully abrogated both the binding of Gab1 to Sf-Stk and the tyrosine phosphorylation of Gab1 in the presence of Sf-Stk and gp55 (Fig 4.2A and 4.2B).

4.4.3 Src family kinase activity is required for the phosphorylation of Gab1, Gab2 and Erk downstream of Sf-Stk and gp55.

PP1 has been reported to block EGF induced Gab2 phosphorylation without inhibiting phosphorylation of the EGF receptor[214]. In addition, overexpression of Src and Gab2 in the MCF-10A epithelial cell line promoted EGF independent acinar growth[215]. To determine whether Src mediates the phosphorylation of Sf-Stk or the phosphorylation of Gab1 and Gab2 downstream of Sf-Stk, wild-type Src or a dominant negative Src (SrcDN) in which the ATP binding site K295M is mutated, were co-transfected into 293 cells with wild type Sf-Stk in the presence or absence of gp55 and the phosphorylation of Sf-Stk, Gab1, Gab2 and Erk phosphorylation were examined (Fig 4.3.A). Results from these studies clearly demonstrate that coexpression of Src with gp55 and Sf-Stk promotes not only Gab2 but also Gab1 tyrosine phosphorylation. Also, overexpression of Src alone is sufficient to induce Gab1, Gab2 and Erk phosphorylation and coexpression of Src with gp55 and Sf-Stk further enhances these effects. Alternatively, expression of the SrcDN completely abrogates gp55/Sf-Stk induced Gab1, Gab2 and Erk phosphorylation. However, SrcDN does not inhibit gp55 induced Sf-Stk phosphorylation, suggesting that Src functions upstream of Gab1 and Gab2, but downstream of Sf-Stk. Consistent with these results, we found that while wild-type Src enhanced AP1 luciferase activity in the presence of Sf-Stk and gp55, expression of

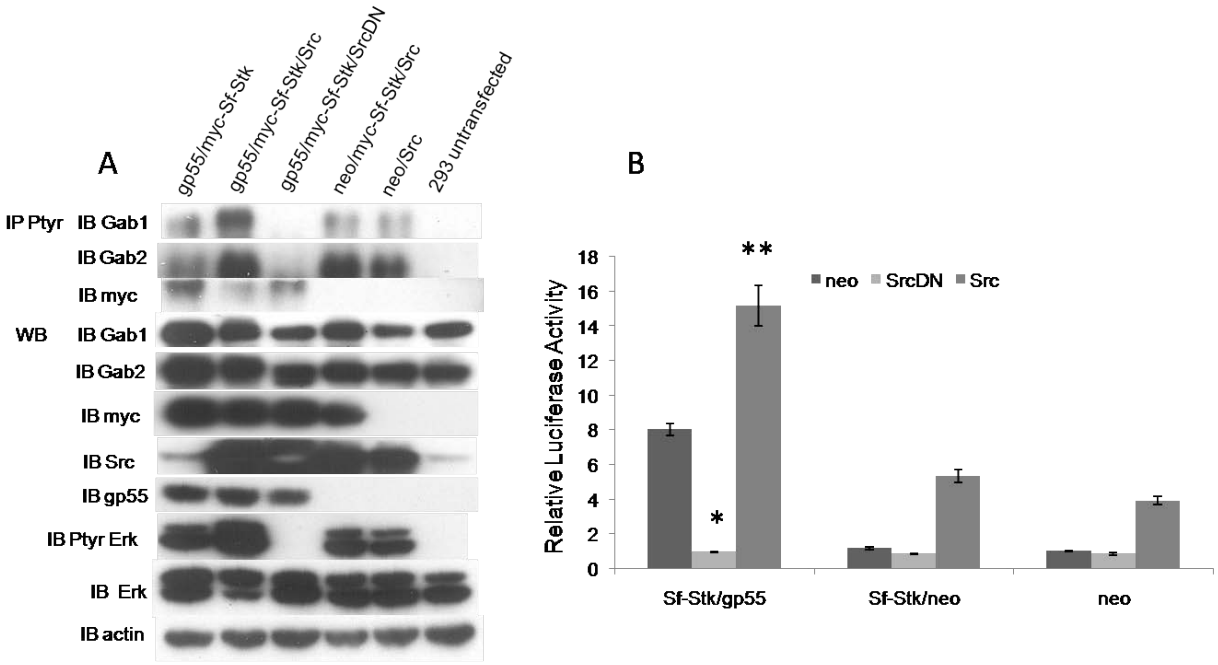

Fig 4.3 Src kinase activity is required for gp55/Sf-Stk mediated Gab1 and Gab2 phosphorylation and Erk/MAPK activation

A) 293 cells were transfected with wild type or dominant negative Src with Sf-Stk and gp55. 48 hours later, cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with Gab1, Gab2 and myc antibodies. Cell lysates were also immunoblotted with anti-phospoErk antibody. The membrane was then stripped and blotted with anti-Erk antibody. B) 293 cells were transfected with wild type or dominant negative Src with Sf-Stk and gp55 with AP1 luciferase reporter and renilla luciferase reporter vector. 48hrs later, AP1 activity was assessed by using a luciferase assay according to manufacturer's instruction.* $P < 0.01$ **$P < 0.01$

SrcDN completely inhibited this activity (Fig 4.3.B). Overexpression of Src alone was also sufficient to induce AP1 activity in the presence or absence of Sf-Stk, however the induction was significantly greater in the presence of Sf-Stk and gp55. Taken together, these data indicate that gp55/Sf-Stk induced Gab1 and Gab2 phosphorylation, as well as the downstream activation of Erk and AP1, are mediated by Src.

4.4.4 Src interacts with Sf-Stk in a gp55-dependent manner and is required for the Epo-independent growth of primary erythroblasts infected with Friend virus

Our previous studies demonstrated that Tyr1175, Tyr1265 and Tyr1294 in the kinase domain of full-length Stk are required for MSP independent Stk activation and can mediate the interaction of Stk with Src. Alternatively, the SH2 domain of Src could be recruited to the C terminal docking sites of Sf-Stk[159]. To determine whether Src interacts with Sf-Stk in a gp55-dependent manner, Src was co-transfected into 293 cells with myc tagged wild type Sf-Stk, Sf-StkY2F or Sf-Stk in which tyrosines 274,364 and 393 are mutated (Sf-StkY3F) in the presence or absence of gp55. As demonstrated in Figure 4.4, the interaction of Src with Sf-Stk was only detected in the presence of gp55. In the presence of Sf-StkY2F or Sf-StkY3F, Src binding was reduced by not abrogated, suggesting that Src can be recruited to Sf-Stk in multiple ways. Consistent with these results, Sf-StkY3F retained the ability to induce Erk phosphorylation (Fig. 4.2A) and AP1 activation (Fig. 4.2C), albeit to a slightly lower level than wild-type Sf-Stk. These data demonstrate that, while the three tyrosines in the kinase domain of Sf-Stk provide a primary docking site for Src, this mechanism of association is dispensable for Src mediated Erk/MAPK signaling downstream of Sf-Stk.

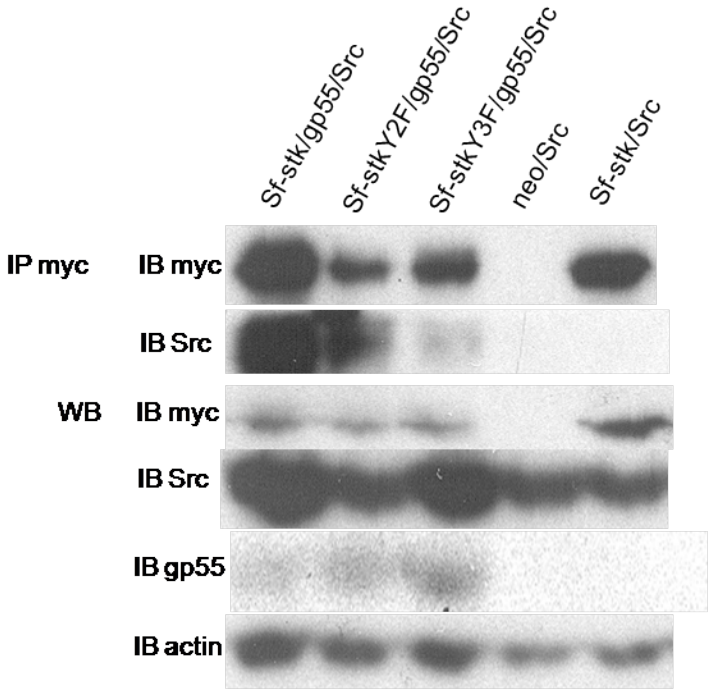

Fig 4.4 gp55 promotes the interaction of Sf-Stk with Src

293 cells were transfected with Src, wild type or mutant forms of Sf-Stk and gp55. 48 hours later, cell lysates were immunoprecipitated with anti-myc antibody and immunoblotted with Src antibody.

To test whether Src plays a role in promoting Friend erythroleukemia, SrcDN was transduced into bone marrow cells from $Fv2^{s/s}$ BALB/cJ mice followed by FVP infection, and the cells were scored for Epo^{ind} CFU-E colony formation (Fig 4.5.A). Our results show that Friend virus induced Epo^{ind} CFU-E colony formation was completely blocked by the expression of SrcDN. In addition, PP1, an inhibitor of Src family kinases, was utilized to inhibit Src activity in this assay(Fig 4.5.B). Consistent with the SrcDN results, PP1 displayed a dose depenent ability to block Friend virus induced Epo^{ind} BFU-E colony formation. Taken together, these data suggest that Src may play an important role in the progress of the early stage of Friend disease.

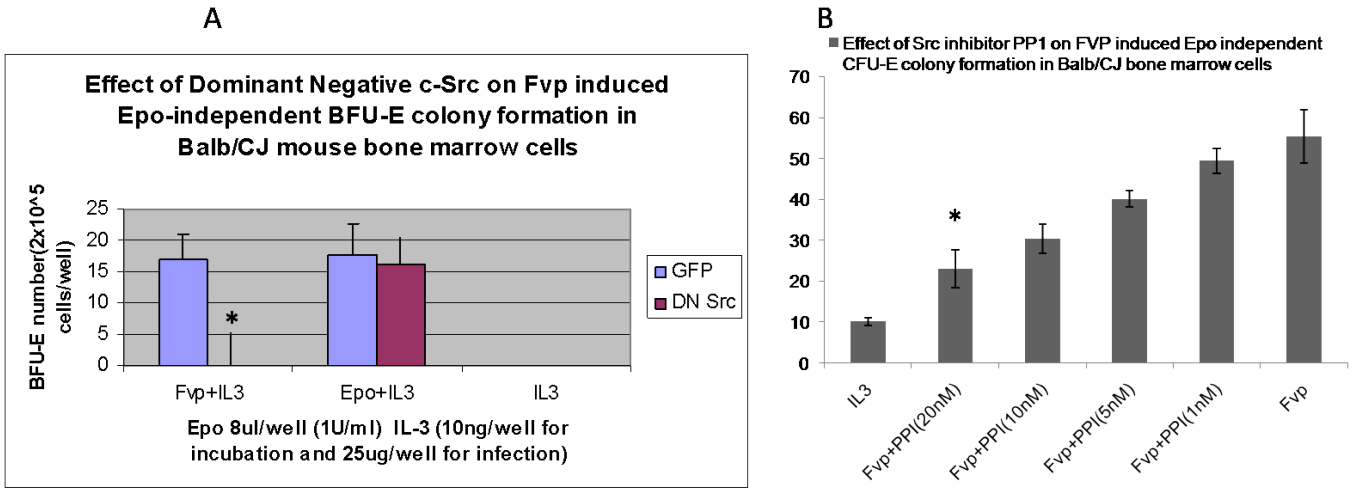

Fig 4.5 Src is required for the Epo^{ind} growth of Friend virus infected erythroblasts

A) 293 cells were transfected with MSCV-SrcDN and pEco. 48 hours post transfection, MSCV viral supernatant were collected and incubated over night with bone marrow cells harvested from Balb/c mice. Virus infected bone marrow cells were then plated in methylcellulose media containing IL-3(2.5ng/ml). On day 5, BFU-E colonies were stained with acid-benzidine and counted. * $P < 0.01$ B) Bone marrow cells harvested from Balb/c mice were incubated with FVP on ice for 1 hour then plated in methylcellulose media containing IL-3(2.5ng/ml) and the indicated concentration of PP1. On day 3, CFU-E colonies were stained with acid-benzidine and counted. * $P < 0.01$

4.5 Discussion

It is well established that Friend disease is initiated by the interaction of the SFFV-encoded viral glycoprotein, gp55, with the Epo receptor and Sf-Stk, expressed by host hematopoietic cells. We have demonstrated that gp55 interacts with Sf-Stk through cysteine residues located in the extracellular domains of gp55 and Sf-Stk, promoting Sf-Stk phosphorylation and the relocalization of Sf-Stk from the cytosol to the plasma membrane. Here, we set out to determine the effect of gp55 binding to Sf-Stk on the downstream signals initiated by Sf-Stk.

We have demonstrated previously that Erk/MAPK signaling is activated in Sf-Stk expressing 3T3 cells and that the MEK inhibitor, PD98059, inhibits Epo^{ind} colony formation in response to Friend virus infection, suggesting that the ability of Sf-Stk to activate Erk/MAPK signaling is necessary in the progression of Friend disease[111]. In addition to Erk activation, others have demonstrated that JNK and p38 are activated in epithelial cells overexpressing wild-type or constitutive active mutants of Stk or Ron[138, 149, 154]. Our data demonstrate that only Erk phosphorylation downstream of Sf-Stk is strongly induced in the presence of gp55. This observation is consistent with the report that JNK and p38 inhibitors have little or no effect on gp55/Sf-Stk induced transformation of NIH 3T3 cells[177].

Our previous studies indicated that Tyr436 in Sf-Stk is essential for the Epo independent growth of Friend virus infected cells and further suggested that recruitment of a Grb2/Gab2/STAT3 signaling complex to this site is critical in promoting this response[111, 175]. However, the potential role of Gab1 in Friend disease has not been

investigated. Gab1 was first identified through its homology to the insulin receptor substrate (IRS), and overexpression of Gab1 enhanced cell growth and resulted in transformation of 3T3 cells[216].

Both Gab1 and Gab2 contain binding motifs for Shp2, which has been shown to be critical for sustained Ras-Erk activation downstream of cMet [168, 169]. Gab1, in which the Shp2 binding motif is mutated, failed to promote the activation of Erk or transcriptional reporter downstream of Met [170]. However, overexpression of Gab1, but not Gab2, promotes epithelial morphogenesis and lemallipodia formation and is localized to the membrane of lamellipodia upon Met activation. Sustained Erk activation was shown to be a requirement for lamellipodia formation, and activated Erk localized to lamellipodia in Gab1, but not Gab2, expressing cells. Moreover, addition of a MBD domain and a myristoylation signal to Gab2 conferred the ability to promote lamellipodia formation and morphogenic signaling upon Gab2 and activated Erk was localized to the leading edge of the lamellipodia in these cells[209]. These studies indicate that subcellular localization of Gab1 and Gab2 is a critical determinant of the function of these adaptors.

Here, we demonstrate that gp55 plays a critical role in promoting the recruitment of Gab1 and the activation of Erk downstream of Sf-Stk. Further, we demonstrate that Y429, a potential binding site for the MBD of Gab1, contributes more substantially to the phosphorylation of Gab1 and Erk than Y436, which can promote the recruitment of both Gab1 and Gab2 in a Grb2-dependent manner. These studies suggest that Gab1 may play a critical role in mediating Erk activation downstream of the gp55/Sf-Stk signaling complex. Interestingly, while the recruitment of Gab1 to PIP3 in the plasma membrane

has been regarded as a major regulatory step for the activation of Gab1, recent studies suggest that PI3K activity is not sufficient to promote Gab1 binding to the plasma membrane, but that MAPK-dependent phosphorylation of Ser551 in Gab1 is crucial for the recruitment of Gab1 to the plasma membrane[210]. Thus membrane localization of Sf-Stk by gp55 could play a pivotal role in the recruitment of Gab1 to the plasma membrane, and the activation of Erk by Sf-Stk could provide a feed forward mechanism to promote Gab1 recruitment and sustained Erk signaling. Our previous observation that fusion proteins between Sf-Stk-Gab1 and Sf-Stk-Gab2 were equally capable of inducing Erk phosphorylation were likely due to the artificial recruitment of both Gab1 and Gab2 to the receptor complex[175].

Src is a proto-oncogene that plays key roles in cell proliferation, differentiation and survival. Src is overexpressed in breast cancer cells and has been reported to enhance Ron activation in epithelial cells[162, 163]. The colony assays described herein indicate that Src may also play a role in the progression of Friend disease. Our studies indicate that Src is required for the phosphorylation of Gab1, Gab2 and Erk downstream of Sf-Stk, but is not required for the phosphorylation of Sf-Stk itself in the presence of gp55. Src contains an SH3 domain and is suggested to directly associate with Gab2 through a proline rich motif on Gab2[214]. Thus, while we have shown that Src is primarily recruited to Sf-Stk in the presence of gp55 via tyrosines in the kinase domain of Sf-Stk, Src could also be recruited indirectly to the signaling complex indirectly through Gab2. This is consistent with our observation that the tyrosines in the kinase domain of Sf-Stk are not required for the activation of downstream signals, while Src activity is critical in promoting these responses. Previous research found that another Src family

kinase, Lyn, is also involved in Friend disease. Lyn is not required for FVP susceptibility but for the terminal differentiation. Lyn-/- mice do not develop Polycythemia following FVP infection[50]. Lyn directly interacts with EpoR and promote the differentiation signal, while Sf-Stk activated STAT3 in early phase inhibit erythroid differentiation [217]. It's interesting to study how these two signaling events are spatiotemporally regulated.

Taken together, the observations described here and elsewhere support a model in which the plasma membrane localization of Sf-Stk promoted by gp55 results in the recruitment and activation of Gab1, which promotes the activation of Erk. The localization of Sf-Stk to the plasma membrane also promotes the association of the signaling complex with Src, which is required for the phosphorylation of both Gab1 and Gab2. The activation of Erk could also lead phosphorylation of Gab1 resulting in its enhanced recruitment to the plasma membrane. Our previous studies identified a critical role for Gab2 in the development of erythroleukemia induced by Friend virus, through its ability to promote the recruitment and activation of Stat3. While the Stat3 binding site in Gab2 is not present in Gab1, the activation of Gab1 by the gp55/Sf-Stk signaling complex may be critical in promoting Erk activation in these cells. Studies are currently underway to test the role of Gab1 and Src in the progression of Friend disease in vivo.

Chapter 5

Conclusion and Discussion

Cell signaling is a complex communication system that regulates the processing and integration of environmental information and intracellular signals to promote cellular activities. Hence, abnormal cell signaling events play an integral part in the development of many diseases including cancer. Studies regarding the mechanisms which drive these abnormal signaling activities not only provide insight into the complex regulation of cell fates but also aid in the identification of potential treatment targets and strategies. In this dissertation, we utilize the model of Friend virus induced acute erythroleukemia to study mechanisms governing receptor tyrosine kinase regulation mediated by the Friend virus envelope protein, gp55. The regulated activation of Sf-Stk by gp55 and the downstream signaling events mediated by this interaction will have broader implications in the study of both normal and leukemic hematopoiesis.

The signaling pathways activated by Stk/Ron have been the subject of intensive studies, as the abnormal activation of this receptor, as a result of overexpression or constitutive activation, has been linked to the development of cancer in both mice and humans. Sf-Stk and Sf-Ron are both generated from an internal promoter in intron 10 and thus lack most of the extracellular domain, but retain an intact transmembrane domain and cytoplasmic domains. This dramatic structural alternation could results in significant changes in their cellular functions. Though Sf-Stk expression is the predominant Stk transcript in murine hematopoietic cells, Sf-Stk cannot be activated by MSP or other

known ligands and mouse strains that lack Sf-Stk expression exhibit normal steady erythropoiesis. Thus, studies regarding the function of Sf-Stk have long been neglected, until the identification of Sf-Stk as the product of *Fv2* gene.

Our studies demonstrate that the cysteine residues in the ecotropic domain of gp55 and the extracellular domain of Sf-Stk are responsible for the gp55/Sf-Stk interaction. This interaction not only promotes the translocation of Sf-Stk from the cytoplasm to plasma membrane, but also promotes the activation of Sf-Stk. We show that plasma membrane localization is critical for the function of Sf-Stk, as a constitutive activating mutation in Sf-Stk (Sf-StkM330T) requires plasma membrane translocation in order to achieve full receptor activity. We further studied the mechanism of Sf-Stk subcellular localization and found that mutation of the cysteines in the extracellular domain of Sf-Stk results in its localization to the cell surface without the aid of gp55. This suggests that cysteine residues in the Sf-Stk extracellular domain are responsible for its intracellular retention.

The detailed mechanisms governing this retention are not clear, however studies regarding the subcellular localization of other proteins have demonstrated a similar role for cysteines in regulating intracellular retention. One example is the assembly of secretory polymerized IgM. IgM is retained in the ER through Cys575 by thiol retention until this cysteine is occupied by forming a disulfide bond with other IgM monomers or Joining Chain. Another example is palmitoylation on cysteine residues of GluR subunits of the AMPA receptor. Palmitoylation promotes retention of the receptor in the Golgi and prevents cell surface delivery. When at the cell surface, palmitoylation of AMPA receptor inhibits 4.1N interaction and facilitates internalization of the receptor. It not clear what is

occurring in the case of Sf-Stk, but binding of gp55 to the extracellular cysteines of Sf-Stk could displace these inhibitory interactions, thus promoting cell surface localization of Sf-Stk.

We also show that cell surface expression alone is not sufficient to promote Sf-Stk activation as Sf-StkC4A, which does not interact with gp55, is not phosphorylated and cannot recruit downstream signals. Also, we show that the Sf-Stk forms a dimer independent of gp55 expression, and this oligomerization is regulated through the Sf-Stk cytoplasmic domain. It has been show that many receptors such as the EGFR and EpoR form inactive dimers in the absence of ligand, and that ligand binding can transform the preformed dimers into an active form. Our findings suggest that Sf-Stk activation occurs via a similar mechanism, that gp55 binding does not promote Sf-Stk dimerization but perhaps promotes a conformation change resulting in enhanced receptor phosphorylation. Since dimerization of the full-length Ron receptor has been demonstrated to require ligand binding, it will be important to determine how multimerization of these different forms of the receptor is differentially regulated.

It is likely that plasma membrane localization permits Sf-Stk and full length Stk/Ron to recruit many important signals which are also membrane localized. Upon kinase activation and phosphorylation on the C-terminal docking sites, multiple downstream signals are recruited to Stk/Ron. The MAPK and PI3K/Akt pathways are the two major signaling pathways activated downstream of this and other receptor tyrosine kinses. Our previous studies demonstrated that the Erk/MAPK and PI3K/Akt signaling pathways are also activated downstream of Friend virus induced Sf-Stk activation. Here we further demonstrate that Erk phosphorylation downstream of Sf-Stk is strongly induced by gp55.

Our data suggest that the Sf-Stk C-terminal docking site tyrosines, and the direct association of Sf-Stk with Gab1, rather than its indirect asscoation through Grb2, plays a major role in the activation this signaling pathway. Gab1 contains multiple phosphorylation sites and can recruit many important downstream signals including Shp2 and PI3K. However, Gab1, unlike Gab2, does not contain a consensus STAT3 binding motif, thus a Sf-Stk-Gab1 fusion protein cannot rescue Epo^{ind} colony growth induced by Friend virus in $Fv2^{r/r}$ erythroid progenitors. On the other hand, we suspect that the Sf-Stk-Gab2 fusion protein can rescue Epo^{ind} colony growth in $Fv2^{r/r}$ erythroid progenitors because it not only recruits STAT3, but the enhanced direct association of Sf-Stk and Gab2 in the context of the fusion protein could substitute for Gab1 in the induction of Erk/MAPK signaling. Future studies using Gab1 fl/fl mice will determine whether endogenous Gab1 plays an important role in the progression of Friend erythroleukemia.

Our previous studies indicated that, in the context of the Sf-Stk/Gab2 fusion protein, the kinase activity of Sf-Stk was dispensable for the ability of this fusion to support the Epo independent growth of Friend virus infected cells. It was therefore likely that kinases, other than Sf-Stk, could result in the phosphorylation of Gab1 or Gab2 downstream of Sf-Stk under normal physiological conditions. Therefore, we tested the role of the non-receptor tyrosine kinase, Src, in Sf-Stk signaling. We demonstrate that expression of Src alone can stimulate Gab1 and Gab2 phosphorylation and Erk/MAPK signaling, while expression of a dominant negative Src inhibits gp55/Sf-Stk induced Gab1 and Gab2 phosphorylation as well as the downstream activation of Erk and AP1. However, expression of a dominant negative Src does not inhibit Sf-Stk phosphorylation in response to gp55. These data support a model in which the enhanced activation of Sf-

Stk by gp55 results in the autophosphorylation of the docking site tyrosines and recruitment of Gab1 or Gab2, while the recruitment of Src to the receptor complex, either directly or indirectly through its association with Gab, results in the Src-mediated phosphorylation of Gab, resulting in the activation of sustained Erk/MAPK signaling (Fig 5.1). The co-overexpression of Src and Ron has been reported in breast cancer. Our study indicates that these two kinases may play a cooperative role in cancer development, and that the treatment of such cancers could be enhanced by targeting both kinases.

Recently, Sf-Ron expression has also been reported in multiple cancers. However, it is unclear how Sf-Ron activity might be regulated in these cells and whether other cellular proteins may substitute for gp55 in the activation of Sf-Ron under these conditions. Though Friend virus cannot infect human cells, we adopted the research methods described here to study signaling events downstream of Sf-Ron, and found that gp55 can effectively bind to and activate Sf-Ron. Furthermore, exogenous expression of Sf-Ron can rescue the Epo^{ind} colony growth of $Fv2^{r/r}$ murine erythroid progenitor cells in response to Friend virus infection. This suggests that Sf-Ron may share similar mechanisms of inhibition/activation as Sf-Stk. Thus, our future studies will build a bridge from understanding the function of Sf-Stk in mice to understanding the function of Sf-Ron in humans, not only by gp55, but also by other cellular factors. The studies described herein lay the foundation for addressing these important biological questions

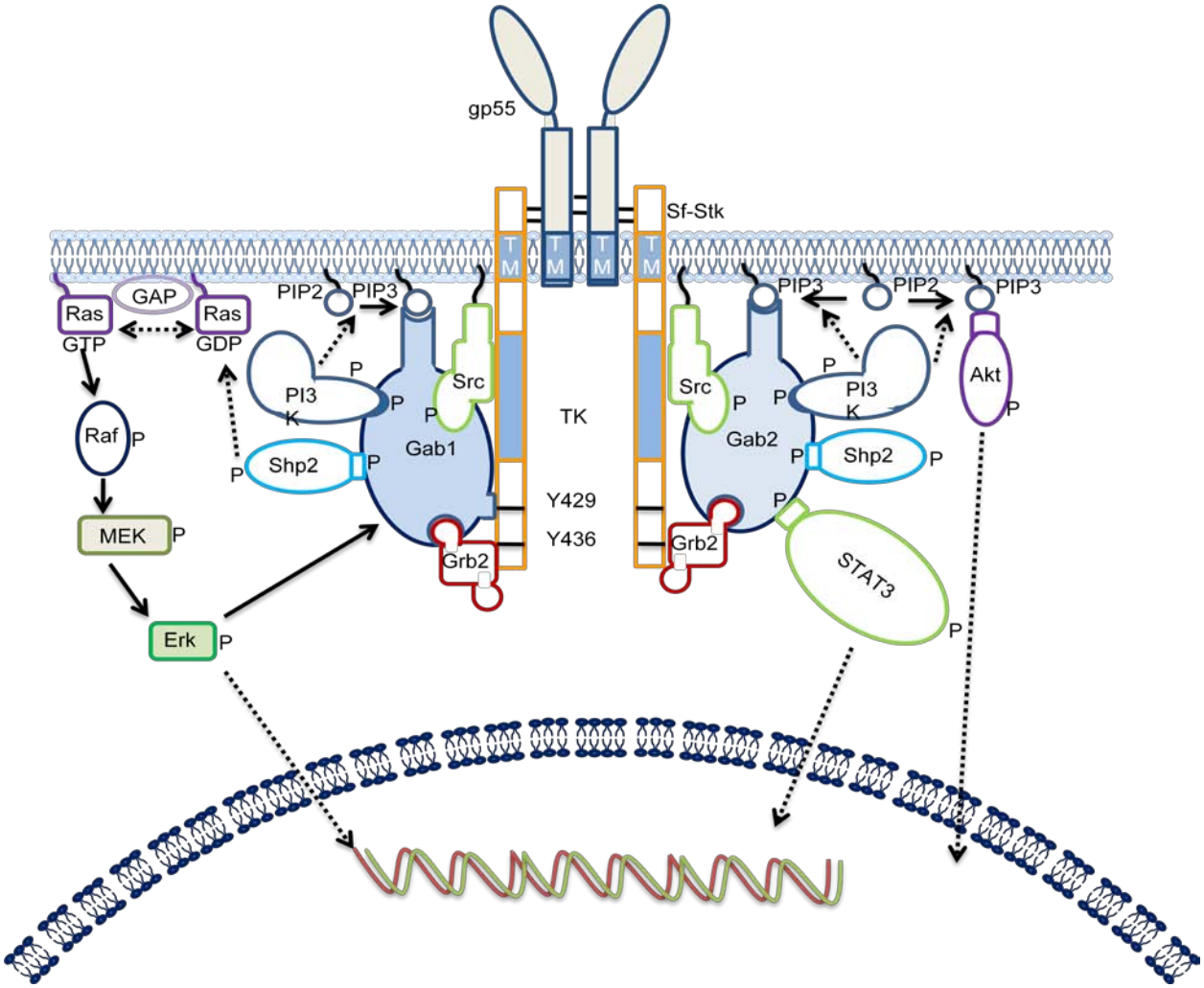

Fig 5.1 gp55 induced Sf-Stk activation and downstream signaling

The cysteine mediated interaction of gp55 and Sf-Stk relocates Sf-Stk to plasma membrane and induces constitutive activation of Sf-Stk. Activated Sf-Stk recruits Gab1 and Gab2 directly or indirectly through the C-terminal docking sites, Src is also recruited to the Sf-Stk kinase domain tyrosines. Erk/MAPK and PI3K signaling pathways are activated by Gab1 and Gab2; STAT3 is also recruited and activated by Gab2.

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