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Identification Of A Novel Phosphoserine Site On Crkl Regulated By Interleukin-2 And Protein Phosphatase-1

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Abstract

CrkL, a 36 kD adaptor protein, is part of the Crk family of proteins which have been implicated in a variety of human malignancies. CrkL is of special interest in hematopoietic malignancies because of an increased level of expression in myeloid and lymphoid derived cells lines. CrkL remains understudied but it has been shown to interact with Bcr-AbI in chronic myelogenous leukemia (CML) and is currently being used as a prognostic marker for patients by evaluating phosphorylation levels. Though regulation of CrkL by tyrosine phosphorylation has been studied extensively, the role of serine and threonine phosphorylation in an IL-2 dependent has not been characterized.

CrkL has been shown to respond to IL-2 stimulation in T lymphocytes but it's role in the Jak/STAT signaling pathway has not been well defined. This research used radiolabeling and phosphoamino acid analysis to show increased levels of serine phosphorylation upon IL-2 stimulation. Mass spectrometry analysis was then performed to identify novel phosphorylated serine sites upon IL-2 stimulation and Calyculin A treatment. Sites were aligned to determine conservation among species and were chosen for site directed mutagenesis. Mutated CrkL will be characterized further in future studies.

CrkL was shown to co-immunoprecipiate with protein phosphatase 1,PP1, through mass spectrometry analysis and was further shown by completing CrkL immunoprecipitations and PP1 immunoblots. The relationship between PP1 and CrkL is important because PP1 may serve as a regulator of CrkL through serine de phosphorylation.

List of Tables

List of Figures

Chapter 1: Background and Significance

1.1.Introduction

C10 oncogene homolog-like protein, CrkL, has been shown to have increased expression in cancer cells with a resulting increase in cellular tyrosine phosphorylation levels.[1] The Crk family plays a key role in recruiting [1] multi-protein complexes within various signal transduction pathways. Multiple studies have suggested that Crk and CrkL may be involved in human cancers. Immunohistochemical studies have shown Crk overexpression in adenocarcinomas of the lung, breast, and stomach. Overexpression has also been reported in sarcomas [1]. In a study of lung carcinomas increased CrkL expression was used as a predictive factor of patient prognosis and survival.[2]

To determine the role of Crk several knockdown studies have shown Crk proteins play a role in the phenotypic behavior of human tumor cells and are involved in regulating transcription and cytoskeletal reorganization during cellular processes including growth differentiation and death [1]. Knockdown of CrkL by siRNA in lung cancer showed a decrease of tumor invasiveness, cell cycle progression, survival, and motility [2].

Crk proteins and CrkL in particular, have been understudied in hematopoietic malignancies [2]. CrkL is especially relevant in hematopoietic cancers because it is more highly expressed in myeloid- and lymphoid-derived cells [2]. CrkL known to be phosphorylated by Abl tyrosine kinase, and to interact with Bcr-AbI in granulocytes and myeloid precursor cells independent of phosphorylation [2]. CrkL phosphorylation by Abl results in auto inhibition. In Bcr-AbI positive chronic myelogenous leukemia (CML) Bcr-Abl associates with CrkL independently of phosphorylation.[3]

1.2 Cancer

Cancer is a disease that results from the transformation of normal cells to a neoplastic state.[4] It has evolved into the world's leading cause of death[5], with numbers that have doubled over the past three decades[6]. The rise in cancer related deaths has been attributed to environmental and lifestyle related choices including smoking, diet, and pollution, as well as a much longer life expectancy due to improved medical care.[6] Cancer is expected to overcome heart disease as the leading cause of death in the United States within the next few years.[7]

Cells gaining capabilities that allow them to become tumorigenic, and eventually malignant, characterize the transition of cells from normal to neoplastic.[4] These six traits have been identified as the hallmarks of cancer. The first trait is the ability to sustain constant proliferation. Cancer cells inhibit the balance between growth and death by deregulating signaling that normal cells employ. Transformed cells can employ autocrine proliferative signaling by producing growth factors themselves. They may also signal to normal cells around them to produce the growth factors they need to continue growth.[8] It is also possible for cancer cells to up-regulate receptor expression so that the cells can be hyper-sensitive to ligand, or for the cells to become independent of signaling altogether.[4]

Cancer cells have also shown to result from somatic mutations that result in constitutive signaling of downstream signaling that drives the disease. This has been shown to be the case in human melanomas where approximately 40% of cases have shown mutations in b-Raf that results in constitutive MAP signaling.[9] This has also been shown in various tumors that demonstrate PI3K mutations.[10]

Cancer cells have also shown an ability to disrupt negative feedback loops that keep the growth balance in check.[4] The most important illustration of this ability is the oncogenic mutation of Ras. Mutant Ras loses its GTPase abilities, which are necessary for maintaining the negative feedback loop that hinders uncontrolled growth. This has also been shown in the cases of PI3K signaling where PTEN phosphatase is not able to counteract PI3K activity due to mutations.[11] This type of deregulation of negative feedback loops can contribute to the development of resistance against therapeutic agents that target mitogenic signaling.[4]

Another crucial aspect to the development of cancer is the cell's ability to evade growth suppressors commonly known as tumor suppressor proteins.[4] The two prototypical tumor suppressor genes are Rb and TP53. Rb dictates whether the cell shall proceed through the cell cycle based on factors outside of the cell. When Rb experiences a mutation and results in a loss of function proliferation can continue unchecked.[12] TP53 on the other hand operates based on information from within the cell.[4] TP53 listens to signaling within the cell that signals for stress or abnormalities. This can stem from excessive genome abnormalities, low levels of glucose, and levels of available nucleotides. If conditions persist TP53 can trigger apoptosis.

Cancer cells also lose their ability for contact inhibition. In the case of normal cells, they will cease growth if there is significant cell-to-cell contact.[4] Neoplastic cells also gain an ability to resist cell death. Programmed cell death, apoptosis, is a vital part of maintaining a healthy level of cells.[13] Apoptosis is mediated by two arms of signaling within the cell, the regulatory branch and the effector branch. One branch takes in and processes signaling from outside the cell while the other processes signaling within the cell.[13] Ultimately both branches trigger proteases, caspase 8 or 9,

to initiate a cascade of caspases that handle proteolysis. The cell is taken apart by these proteases and eventually consumed by its neighboring cells or phagocytic cells.[4] On the other hand cell death by necrosis can actually be helpful to cancerous cells.[14] While abnormal cells may orchestrate evasion of apoptosis they may actually trigger necrosis of neighboring cells. The reason for this is that death by necrosis recruits inflammatory cells of the immune system.[14] Inflammatory cells have the capability to release factors that can stimulate neighboring cells to proliferate. [14] Though cell death may seem like a good opposition to unregulated cell growth, in the case of necrosis it can actually be detrimental.

Normal cells are only able to pass through a limited number of cell growth and division cycles. In contrast, neoplastic cells can progress through the cycle and unlimited number of times.[4] After a certain number of growth and division cycles cells will either enter senescence or crisis. During senescence cells remain viable and stable but no longer proliferate, while crisis leads to cell death. Cells that can avoid either of these phases and continue growing are termed immortalized.[4] Immortalized cells have shown increased telomerase activity.[15] Telomerase is an enzyme responsible for adding repeats to the end of chromosomes to prevent degradation. Eventual degradation of telomeres allow for the ends to join and to signal distress within the cell that will result in apoptosis.[15] In the case of cancerous cells telomeres degradation is greatly reduced and cells can maintain alive.

Tumors need the same requirements as normal cells, and to provide them they need a developed vasculature developed through angiogenesis. During embryonic development angiogenesis is heavily active, but soon after vasculature development these cells become quiescent.[4] With the exception of wound healing and female

reproductive cycling, angiogenesis is generally turned off. When cancerous cells begin to develop into a tumor angiogenesis is turned back on, and new vessels continually develop to supply the tumor.[16] Angiogenesis is primarily controlled by factors that act as either positive or negative regulators. Two well-known regulators of angiogenesis are vascular endothelial growth factor A, VEGF-A, and thrombospondin-1, TSP-1. VEGF-A can be upregulated by hypoxia or oncogene signaling[17] as well as held in the extracellular matrix and eventually activated by extracellular matrix-degrading enzymes. TSP-1 on the other hand is a counter balance that bind transmembrane receptors that allow for suppressive signaling.[18]

Overall cancer is the disease that occurs when normal cells acquire various abilities that allow them to continue uncontrolled proliferation, survival, and eventual invasion and metastasis. Though many mechanisms that allow for this cellular transformation have been elucidated there are a great many more that remain to be defined.

1.3 Lymphocyte Development and Activation

Lymphocyte precursors originate in the bone marrow, while development occurs either in the thymus for T cells, or the bone marrow for B cells. T-cell mediated immunity is an important component of the adaptive immune system and involves developing T-lymphocytes to specifically target viral, bacterial, parasitic infections or malignant cells.[19] Maturation in the thymus results in each T-lymphocyte expressing a unique receptor on the surface. Antigen specificity is determined early in differentiation when coding sequences doe the receptor are assembled from gene segments. [20] The receptor is then tested for recognition with ligand molecules that in its environment. If the cell interacts weakly with self antigens then it will be selected to survive in a process

known as positive selection.[20] This is important for because T cells must recognize MHC molecules when they are presented antigens in order to initiate an immune response.[20] On the other hand cells that demonstrate high affinity for self molecules will be signaled to die in a process termed negative selection.[20] This process is also necessary because these cells could mount a dangerous autoimmune response. Most cells die during development because their default course of action is death unless a signal is received to continue and continue maturation in the peripheral lymphoid organs.[20] The cells are then available for an adaptive immune response during which clonal selection is employed.[20] Lymphocytes will continually circle through the blood lymph and peripheral lymphoid organs looking for its specific antigen, and will continue to do so until it is found or the lymphocyte is ready to die.[20]

T lymphocytes continue to circulate until they encounter the antigen that corresponds to their receptor. Dendritic cells are known as antigen presenting cells, APCs, and are capable of inducing a primary immune response by activating resting nai”ve T lymphocytes. Follicular dendritic cells are also capable of maintaining immune memory by interacting with B cells.[21] Dendritic cells are present in tissues that are exposed to the environment such as skin, lungs, stomach, and intestines.[21] Once the DCs have taken up the antigen it is processed by either an endosomal or proteosomal pathway. [21] Dendritic cells process and present antigen for both CD4+ T helper cells and CD8+ T cytotoxic cells; MHC class I cells present to stimulate CD8+ T cells, while MHC class II cells present to stimulate CD4+ T cells.[21] Dendritic cells also possess co-stimulatory molecules on their cell surface that are necessary for T cell activation.

Once activated T cells undergo clonal expansion and differentiation into effector or memory T cells. The T cell receptor recognizes the antigen peptide on the MHC molecule,

and CD4 or CD8 co-receptors recognize the MHC molecule. Co-stimulation of APCs also is required for full activation of T cells [22]. Co-stimulatory receptor CD28 binds to its ligand CD80 on antigen presenting cells [22]. CD80 co-stimulatory events, along with several other co-stimulatory events, lead to the production of a variety of cytokines that can interact with cell receptors in either a paracrine or autocrine manner [23]. Interaction with antigen on MHC molecule, co-stimulatory events, and autocrine and paracrine signaling through cytokines are the three signals needed for full T cell activation. This results in cell proliferation, differentiation, and survival [24]. Upon T cell activation, expression of CTLA-4 receptors is induced as a way to temper T cell proliferation. CTLA-4 receptors outcompete CD28 receptors for CD80 on APCs to prevent further T cell expansion [25].

1.4 IL-2 Receptor and IL-2

The receptor for IL-2 is made up of three distinct subunits named alpha chain (IL-2R-a), beta chain, (IL-2R-§), and gamma chain (IL-2R-y).[26] The receptor exhibits highest affinity for its ligand, IL-2, when all three components are present but will function without alpha chain. The most critical component is IL-R-§ and it has shown to be physically and/or physically coupled with other signaling molecules.[26] IL-2R-§ has also been shown to be involved in at least two intracellular signaling pathways that mediate nuclear proto-oncogene induction.[26] The formation of the high affinity heterotrimer receptor is not preformed but is instead driven by IL-2. IL-2 is captured by IL-2R-a through a hydrophobic binding domain, which results in a relatively weak interaction.[27] This interaction results in a conformational change of IL-2 that leads to association with IL-2R-§. This complex will then recruit IL-2R-y and form the highest affinity form of the IL-2 receptor [27] and is found on activated T cells and mature dendritic cells.[28] The

intermediate affinity IL-2 receptor form includes beta chain and gamma chain and is expressed on resting T cells, macrophages, immature dendritic cells and NK cells. [28]

IL-2R-a is an affinity conferring chain and is not involved in signal transduction activity due to its short cytoplasmic chain; it is also not shared with any other interleukin receptor, though it does exhibit structural similarity to IL-15R-a. [29] IL-2R-§ on the other hand is shared with the IL-15 receptor and is involved with signal transduction. IL-2R-y is also involved in signal transduction and is shared with the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, also known as the common gamma chain cytokines or the IL-2 family of cytokines.[30] Neither y or § subunits have catalytic activity, while both rely on receptor-associated kinases to initiate cellular signal transduction. [30] The IL-2 receptor complex can then by endocytosed by a clathrin-independent, dynamin-dependent mechanism involving PI3K.[31]

IL-2R-a is made up of 272 amino acids and is 55kDa glycoprotein expressed on T cells, NK cells and B cells.[32] IL-2R§ is 551 amino acids long with a molecular weight of 75 kDa and is expressed on neutrophils, T-, B-, NK-cells, dendritic cells and some monocytes.[33] Beta chain has no catalytic activity and must be activated by receptor-associated kinases. It serves as a docking site for both Janus Kinase1 (JAK) and JAK3.[33] Gamma chain is composed of 369 amino acids, is 64kDa, and is expressed on NK, B, CD4+ and CD8+ T cells, neutrophils, granulocytes, monocytes, and macrophages.[34] Like beta chain it has no intrinsic catalytic activity and has a region used for JAK3 docking and activation.[28] Both gamma and beta chain are type I cytokine receptors, contain fibronectin-III domains, and have three disulfide bonds.[28]

Interleukin 2 is a four bundle, a-helical protein of approximately 15 kDa produced by antigen-activated T cells.[27] It can be produced by a variety of cells including NK,

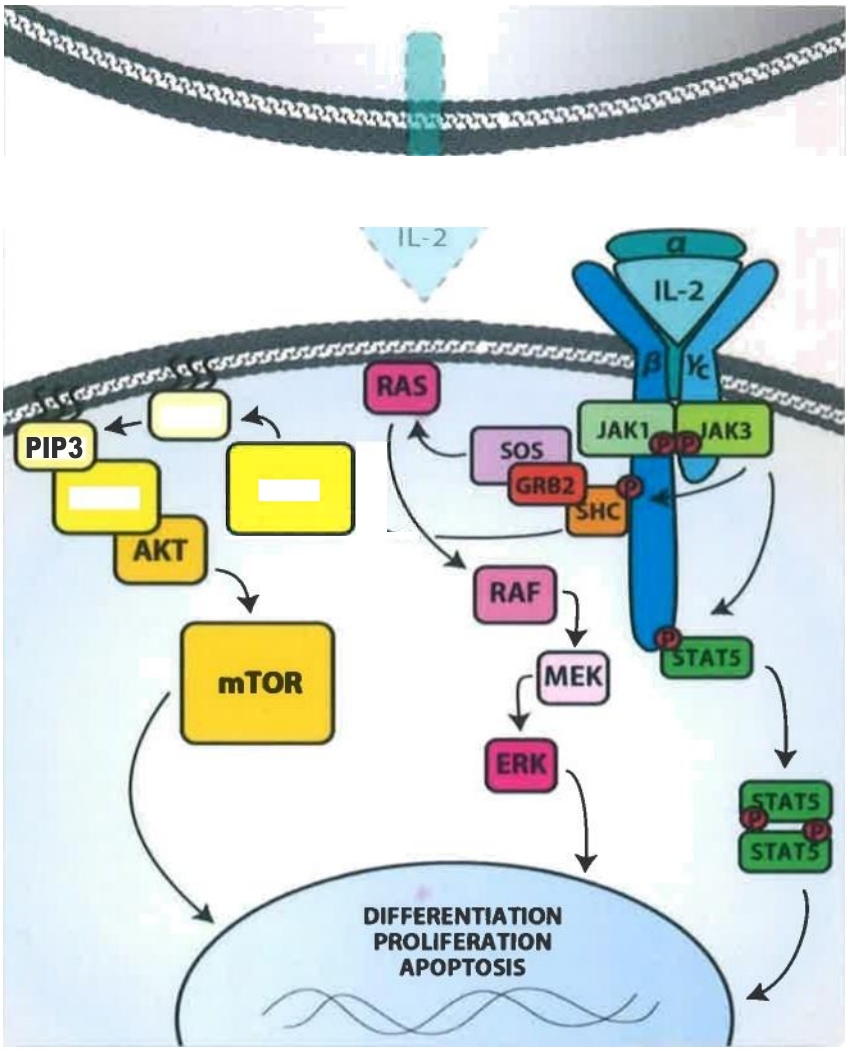
dendritic, mast, and CD8+ T cells, but is mostly produced by activated CD4+ T helper cells.[27] In CD8+ cells IL-2 can stimulate growth and differentiation into memory lymphocytes.[35] It is the main factor responsible for maintenance and differentiation of CD4+ T regulatory cells.[35]

1.5 IL-2 Signaling

Once the receptor-ligand complex is assembled there are a set of signaling pathways that may be initiated and these include the JAK/Stat, RAS/RAF/MEK/ERK, and PI3K/Akt pathways.[36] These signaling pathways are mediated by a series of phosphorylation and de-phosphorylation steps that can activate or shut off a protein.[36] Phosphorylation can take place at tyrosine, serine, and/or threonine resides. Positive regulation is mediated by the kinases that phosphorylate and negative regulation by the phosphatases that de-phosphorylate.[36] Jak1 is associated with the IL-2R§ and Jak3 is associated with y chain. Both Jaks will phosphorylate each other and when activated will phosphorylate the receptor creating docking sites for STATs. [27] The STATs will dock to phosphorylated tyrosine residues on the receptor and will then be phosphorylated themselves.[24] The two STAT molecules will then hetero- or homo-dimerize and move into the nucleus to affect gene expression. Tyrosine residues on the receptor also create a docking site for Shc, an adaptor protein, that leads to the activation of the MAPK or PI3K pathways.[27] The IL-2R complex is then internalized where IL-2R§, IL-2Ry, and IL-2 are degraded and IL-2Ra is recycled to the surface to continue to recruit IL-2.[37]

Though three pathways diverge from the IL-2R, PI3K, MAPK, and STAT pathways, they are not completely independent of each other.[37] It has been shown that a truncated version of IL-2R§, that can only recruit Shc to the receptor, will have decreased activation of downstream activators ofAkt.[38] Furthermore PI3K and STATE pathways do not show

full activation of their respective tyrosine residues are mutated. IL-2R subunits are associated with the membrane at lipid rafts.[39]



(B. Ruiz 2014)

Figure 1.1 IL-2 Signaling Pathways. Upon binding of IL-2 to IL2R-a Jak1 and Jak 3 are activated by autophosphorylation and then proceed to phosphorylate the receptor creating docking sites for a variety of pathways. Phosphorylation of IL-2R§ creates a docking site for Shc, which recruits Grb2 and Sos, which activates the Ras/Raf/MEK/ERK pathway. This pathway activates transcription of genes involved in cell survival and apoptosis. Shc also activates the PI3K/Akt pathway. Once PI3K is activated it phosphorylates PIP2, which then becomes PIP3, which will then recruit PDK to phosphorylate Akt. This pathway activates transcription of genes involved in proliferation and tumor suppression.

1.6 PP1 and PP2A

1.7 CrkL

CT10 regulator of kinase (Crk) is the prototypical member of the Crk family of proteins. This family is made up of Crkl, Crkll, Crklll, and CrkL[1]. They are a family of adaptor proteins with no intrinsic enzymatic activity[3]. They are responsible for recruiting proteins in a signaling cascade by forming multi-protein complexes. Crk, Crkll, and Crklll are splice variants while CrkL is its own 38kD gene product. They are made up of SH2 and SH3 domains separated by linker regions that facilitates protein interactions [1]. SH2 domains are structurally conserved protein domains that bind tyrosine phosphorylated proteins [3]. SH3 domains are also structurally conserved protein domains that bind a consensus sequence of X1-P2-p3-x4-Ps where 1 and 4 are aliphatic amino acids and 2 and 5 are always a proline [40].

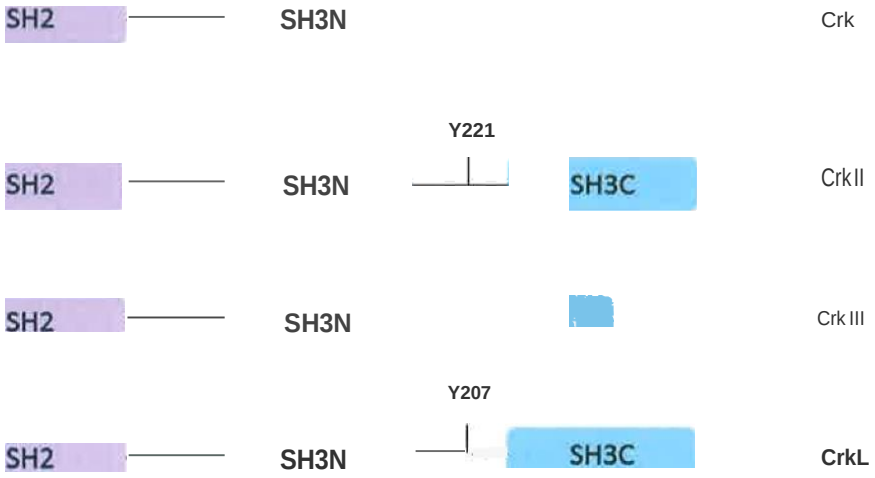


Figure 1.2: Crk Family Proteins
There are four members of the Crk family proteins and all are made up of SH2 and SH3 domains and function as adaptor proteins. Crk only contains one SH3 domain and no characterized regulatory tyrosine residues. CrkIII is the newest member to be identified and contains a truncated SH3 domain. Crk II and CrkL are the most similar of the family members as they share 56% homology and each have a regulatory tyrosine, Y221 and Y207 respectively. Crk, Crk II, and Crk III are all splice variants while CrkL is not.

CrkL and CrkII show the most similarity and are made up of SH2-SH3-SH3 domains [2]. A linker that includes a regulatory tyrosine residue separates the two SH3 domains. CrkL possesses an atypical C-terminal SH3 domain because it doesn't bind polyproline type II containing motifs [2]. The C-terminal SH3 domain is thought to be involved in allosteric regulation with Crk proteins or other proteins. It is seen in its unphoshorylated state in fetal mouse tissue except for in the lung and brain in which significant $CrkI^{P}$ is present [41]. It has also been show to associate with SOS and CG3, indicating a role in signaling pathways of small GTPases of the ras family [41]. Along with CrkL's implications in cancer *CRKL* deletion mutants have shown cardiovascular and craniofacial defects that led embryonic death [42].

Both Crk and CrkL are negatively regulated by auto inhibitory phosphorylation that blocks its ability to function as an adaptor protein. Phosphorylation occurs at Tyr221 on Crk and Tyr 207 in CrkL and results in intramolecular binding of the linker region to the SH2 domain. This binding prevents the SH2 and SH3N domains from binding other proteins [1]. This type of auto inhibition is significant because it offers an explanation for v-Crk's transforming potential through deletion of the C-terminal regulatory element [2]. This type of negative auto regulation has similarly been shown in the Src family of kinases through phosphorylation of the C-terminal domain [43]. The kinases predominantly responsible for this phosphorylation are the proto-oncogene products Abl and Arg tyrosine kinases [1]. Both the Crk and CrkL proteins are important for a broad range of biological processes which include cell proliferation, cell adhesion and migration, phagocytic and endocytic pathways for apoptotic cells, apoptosis, and regulation of gene expression [1].

The adaptor function of Crk is important for signal transduction in both physiological and pathophysiological situations [1]. CrkL shares high homology with CrkII structurally but development of knockout mice models show that they each have distinct, non-overlapping functions during embryonic development [44]. They do not have compensatory activity as it has been shown that knockout mice of each protein die perinatally [44]. Additionally, other studies have shown that in Crk -/- models there is no compensatory overexpression of CrkL, and vice versa [2]. These results indicate that there is very little molecular crosstalk between the two.

Studies have shown that during development, CrkII and CrkL do not have overlapping roles. However studies in somatic cells have the contrary. Various studies have shown that CrkII and CrkL are co-expressed and can compensate each other in cellular signaling as was showing in the Reelin signaling pathways of cortical pyramidal cells of the hippocampus. Using shRNA and a cre-lox deletion system have provided new insight into the universality of this compensation in signal transmission [1].

Crk/CrkL and Abl

Abl is a cellular homolog of v-Abl, an oncogene product of the Abelson murine leukemia virus. It has been shown to be involved in cell growth, transformation, apoptosis, cell stress, and remodeling of the actin cytoskeleton [45]. Abl phosphorylates CrkL on tyrosine 207 and tyrosine 221 on CrkII, which results in the SH2 domain associating with the phosphorylated tyrosine [3]. This auto inhibitory effect results in inhibiting the binding of effector proteins and the subsequent signaling cascades. Though the SH2 domain is blocked the $SH3^N$ domain is left available [3].

CrkL has shown to be more oncogenic than CrkII despite their structural similarities [3]. CrkL has been shown to associate with Bcr-Abl in Chronic Myelogenous Leukemia

(CML) [46]. CrkL is constitutively associated with this aberrant protein independently of phosphorylation of Y207,[3] but is shown to be constitutively phosphorylated in CML[47]. CrkL phosphorylation levels are even being used as a predictor of clinical outcome in CML patients [48]. Bcr-Abl is a fusion protein that is the product of a reciprocal translocation event resulting in the gene products of *c-abl* and *bcr* fusing. The generation of Bcr-Abl is considered to be the initial event that leads to Philadelphia (Ph)-chromosome positive leukemia [49]. The Bcr-Abl fusion protein demonstrates degregulation of the Abl tyrosine kinase that leads to constitutive activity.

Significance

Blood cancers are diseases that can affect the bone marrow, blood cells, lymph nodes, and other parts of the lymphatic system.[50] It is estimated that approximately one person every three minutes are diagnosed with a blood cancer in the United States.[50] These new cases will account for 9.3% of the cancer diagnoses that will be reported 2015. Leukemia is one form of cancer that affects white blood cells. Leukemia will account for approximately 33% of the nearly 140,000 new cases of blood cancer this year that will result in approximately 23,000 deaths.[50]

Leukemia causes more deaths than any other cancer among children and young adults under the age of 20 and Hispanic children and adolescents under this age have the highest incidence rates of this cancer.[50] This final statistic brings is of particular importance to the region because of the high number of Hispanic individuals along the border region.

CHAPTER 2: IL-2 Induces CrkL Serine Phosphorylation

2.1 Introduction

Investigating and determining the relationship between IL-2 stimulation and serine phosphorylation is important because it can elucidate important regulatory controls employed by CrkL within T cells. Because IL-2 is important for T cell differentiation and growth it is of interest in determining whether it is capable of stimulating CrkL serine and/or threonine phosphorylation. It has been previously shown that post-translational modification is important for CrkL's role in signal transduction pathways. The most studied post-translational modification has been tyrosine phosphorylation, especially at Y207, which can be stimulated by IL-2 [51]. To investigate how serine and threonine phosphorylation play a role in CrkL activity and whether IL-2 can stimulate that serine and/or threonine phosphorylation the following experiments were performed.

2.2 Materials and Methods

2.2.1 Cell Culture and Treatment

Human leukemic T cell lines Kit225 and YT were grown in RPMI 1640 (Thermo Scientific Inc.) medium with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 pg/ml, respectively) (Cellgro) (complete media) at 37 °C with 5% CO_2. YT and Kit225 cells were made quiescent by growing to exhaustion (» 5X10' cells/mL and »8X10) and then stimulated with 10,000 IU of human recombinant IL2 (NCI Preclinical Repository) for 10 minutes, and/or treated with 100 nM CA (Sigma- Aldrich) for 15 minutes. Treatments were performed at 37 °C using $20x10^6$ cells per treatment.

2.2.2 Immunoorecloitation. Westam Blot Analvsla, and Masa 9oectometrv

Cells were pelleted and solubilized in lysis buffer (10 mM Tris-HCI (pH 7.6), 5 mM EDTA (pH 8.0), 50 mM NaCI, 30 mM $Na_4P_2O_7$. 50 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 pg/ml aprotinin, 2 pg/ml leupeptin, and 1 pg/ml pepstatin A, and rotating at 4 °C for 1 h. Whole cell lysates were clarified by centrifugation (15,000x g, 15 min, 4 °C). For immunoprecipitation reactions, supernatants were rotated with 3 pg of a-CrkL rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology) for 2 hours at 4 °C. Immuno-complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads where then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 4 x SDS sample buffer (50 mM Tris-HCI (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% promophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 10% SDS-PAGE and visualized by

Coomassie blue staining.

SDS-PAGE (10% Acrylamaide) analysis was used to separate protein samples once immunoprecipitated. Protein was then transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature. Western blot analysis was the performed by incubating the membrane with a-CrkL monoclonal antibody A-1 (Santa Cruz Biotechnology) or a-PP1 monoclonal antibody E-9 (Santa Cruz Biotechnology). Assays were developed with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and x-ray film. Bands corresponding to CrkL were excised from Coomassie blue stained gel and sent to the Taplin Biological Mass Spectrometry Facility (Harvard University) analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). Any protein with three or more unique peptide matches was considered confidently identified. Manual examination of the spectra as well as the probability- based score Ascore where used to determine phosphorylation site localization.

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Kit225 and YT cells were starved to quiesence and labeled with [^{32}P] orthophosphate (Perkin-Elmer) for 2 hours at 37 °C. Cells were stimulated with 100 nM IL-2 for 15 minutes and/or Calyculin A for 20 minutes. They were then pelleted and solubilized in lysis buffer and rotating at 4 °C for 1 hour. Whole cell lysates were then clarified by centrifugation (15,000x g, 15 minutes, 4°C). CrkL was immunoprecipitated with a rabbit polyclonal a-CrkLantibody C-20 as described above. All proteins were separated by 10% SDS-PAGE

and transferred to PVDF membrane and visualized by Coomassie Blue staining and autoradiography. The corresponding protein bands were excised and subjected to two dimensional phosphoamino acid analysis using the Hunter Thin layer electrophoresis apparatus[52]. Bands were excised from the membrane and hydrolyzed with 7 M HCI for 30 minutes. Samples were then dried using a SpeedVac Concentrator for 1 hour. Sample pellets were re-suspended and spotted onto TLC cellulose plates along with amino acid standards. Plates were run in one direction at 1.5 kV for 25 minutes. Plates were then rotated counterclockwise and run at 1.3 kV for 16 minutes. Plates were dried and sprayed with Ninhydrin and baked for 15 minutes at 65°C. Film was exposed to plates for 3 weeks and then developed. Labeling and phosphoamino acid analysis was repeated 6 times with the same result.

2.2.4 Mutant Development

Once a novel serine phosphosite was identified a mutant was necessary to characterize the residue. Serine 162 is in a unique location as it is right in the SH3N domain, which is important for protein docking. Serine 162 also has an interesting flanking region of EEQWWSAR, which includes two aromatic amino acids (W), two charged residues (R, E), and one polar amino acid (Q). In order to determine the role of serine 162 a serine to alanine mutant was generated by using the QuickChange site directed mutagenesis kit (Agilent) according to manufacturer's instructions. The primers used for the mutation as follows: S126A (5'-CATCCTTGTTCCGGGCAGCCCACCACTG-3'). The pcDNA human CrkL in a Pcmv6-Entry vector was purchased from Origene.

2.3 Results

2.3.1 IL-2 and Calyculin A Treatment of CrkL and Western Blot Analysis

Samples (3X10^5) of Kit225 cells were either stimulated with IL-2, IL-4, IL-7, and IL-9 for 15 minutes, Calyculin A for 20 minutes, or both. A control unstimualated sample was also included. CrkL was immunoprecipitated and samples were separated by 10% SDS-PAGE and transferred to PVDF membrane. Visualization of phosphorylated CrkL was difficult to analyze by anti-phosphoserine and anti-phospho tyrosine antibodies so molecular weight shifts were analyzed with a monoclonal a-CrkL antibody C-20. (Fig.1)

Samples showed an increased molecular weight when treated with CA, a known PP1/PP2A inhibitor, as well as an appearance of an increased molecular weight band with the treatment of the IL-2 family cytokines. The samples treated with CA or the cytokine alone showed a double band while the combination of CA and the cytokine showed a triple band indicating that the presence of both allows us to deduce that CrkL is serine or threonine phosphorylated.

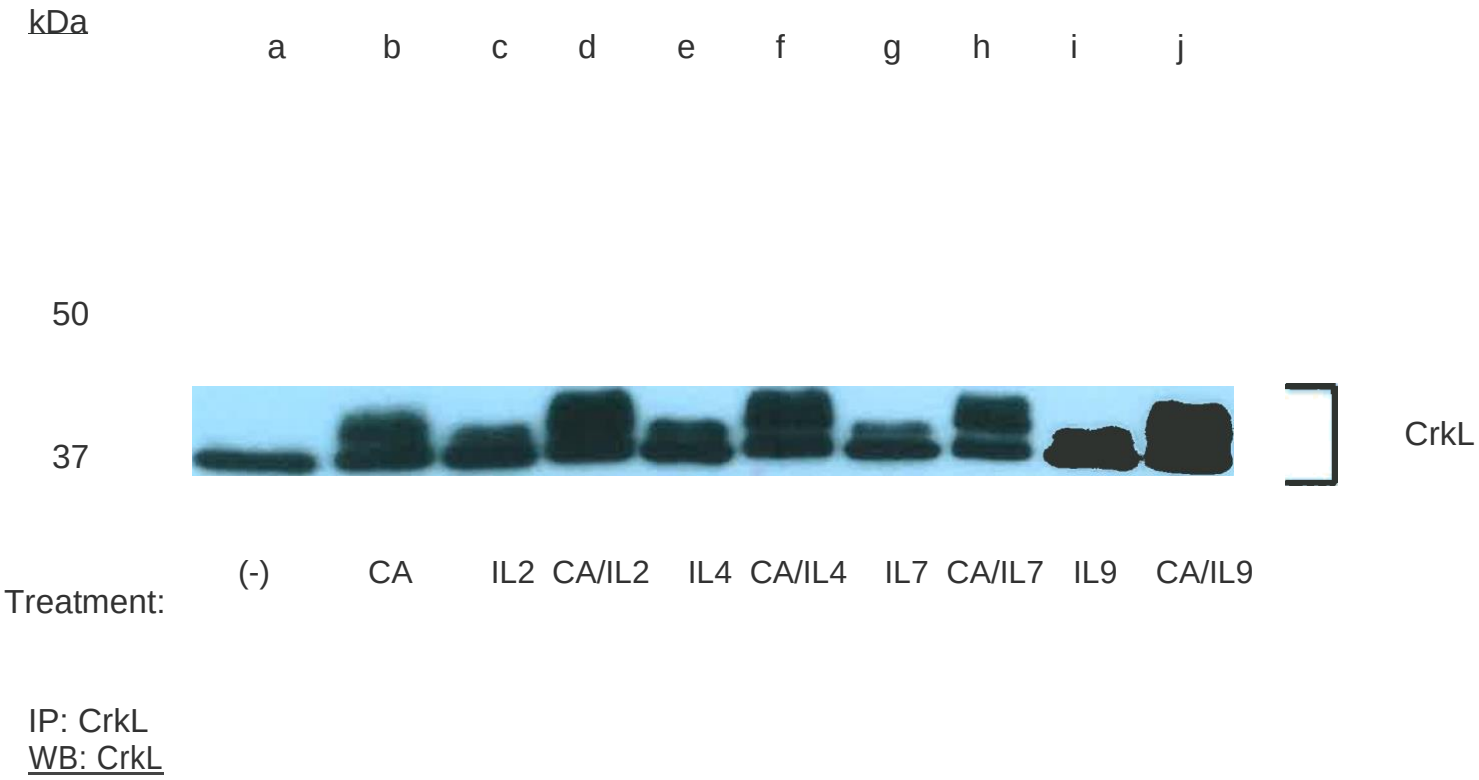


Figure 2.1: IL-2 Cytokine Family Stimulates Appearance of Higher MW CrkL Band
Kit225 cells were left untreated (-), stimulated with IL-2, IL-4, IL-7, and IL-9 for 15 min and/or treated with CA (100 nM) for 20 min. Cell lysates were immunoprecipitated (IP) with a-CrkL and separated by SDS-PAGE. Samples were transferred to PVDF membrane and immunoblotted with monoclonal a-CrkL antibody. Compared to the unstimulated (A) sample there is an increased molecular weight band that appears in the CA stimulated sample (B). Lanes with cytokine stimulation (C,G,E,I) also showed a second band of increased molecular weight, and lanes with cytokine and CA show a third band of increases molecular weight.

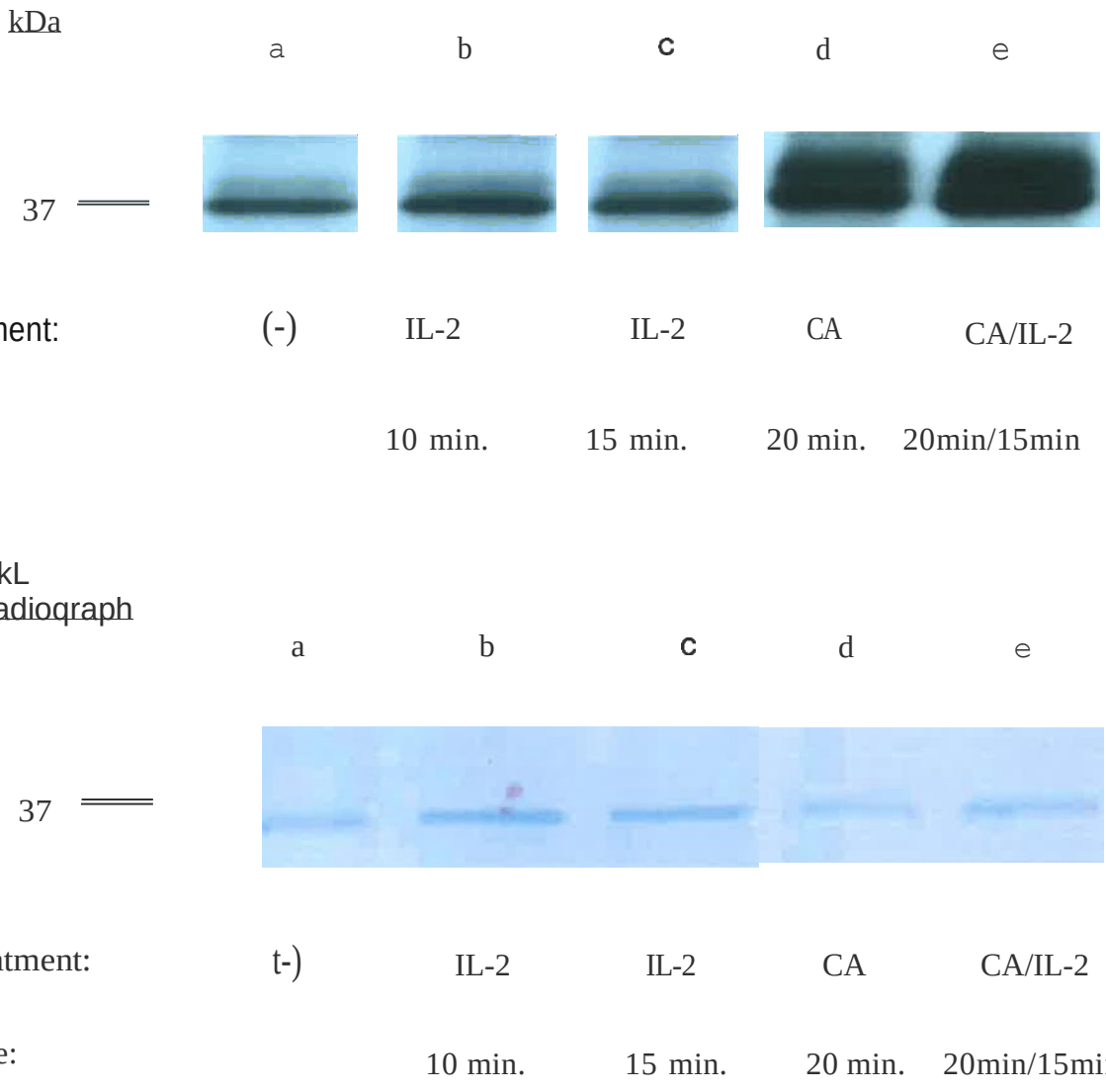

kDa
a
b
c
d
e
37
Treatment:
(-)
IL-2
IL-2
CA
CA/IL-2
Time:
10 min.
15 min.
20 min.
20min/15min
IP: CrkL
Autoradioqraph
a
b
c
d
e
37
Treatment:
t-)
IL-2
IL-2
CA
CA/IL-2
Time:
10 min.
15 min.
20 min.
20min/15min
IP: CrkL
Stain: Coomasie Blue

Figure 2.2: IL-2 and Calyculin A Stimulate Higher Phosphorylation Levels of CrkL
Kit225 cells were starved to quiescence and were treated with [^{32}P] orthophosphate for 2 hrs. at 37°C. Samples were left untreated (-), stimulated with IL2 for 15 min and/or treated with CA (100 nM) for 20 min. Cell lysates were immunoprecipitated (IP) with a-CrkL and separated by SDS-PAGE. Samples were transferred to PVDF membrane and stained with Coomasie Blue to ensure equal protein levels. Protein bands were then excised and subjected to phosphoamino acid analysis. Compared to the unstimulated sample there is an increase of phosphorylated CrkL in the sample treated with CA (d) and the sample treated with IL-2 for 15 min. (c) Sample treated with both showed the greatest amount of phosphorylation.

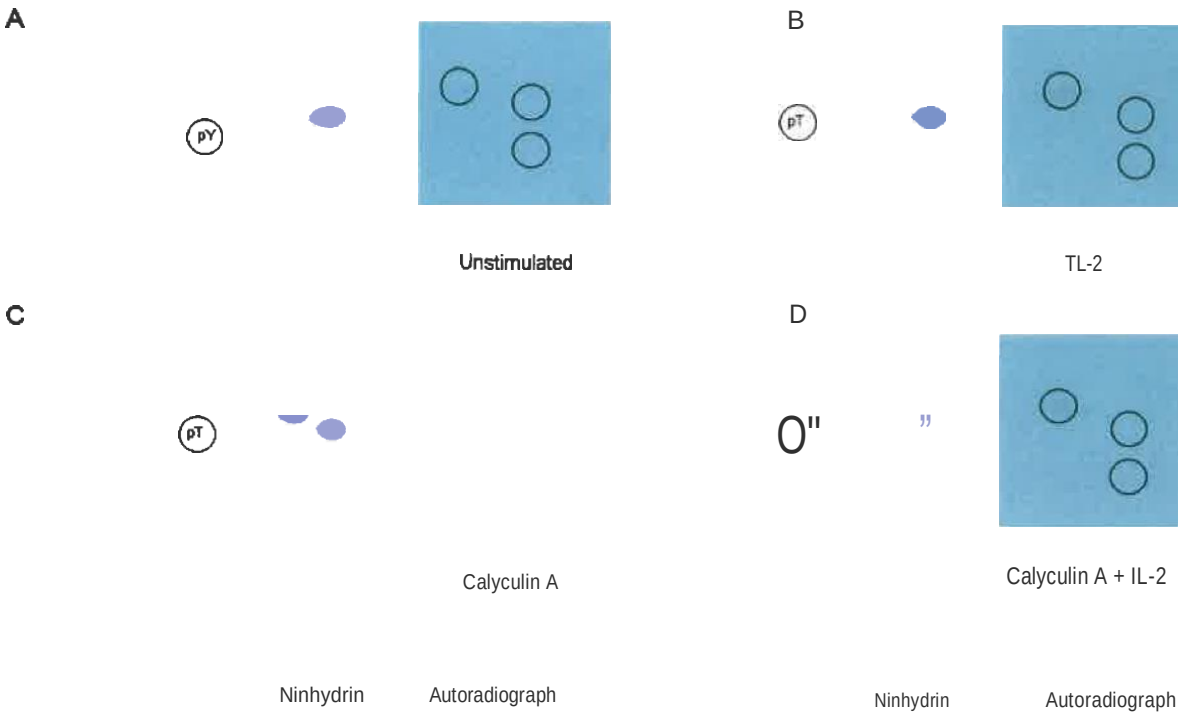


Figure 2.3: IL-2 and Calyculin A Stimulate Increased Serine Phosphorylation of CrkL
Kit225 cells were starved to quiescence and were treated with [^{32}Pj orthophosphate for 2 hrs. at 37°. Samples were left untreated (A), stimulated with lL2 for 15 min (B) and/or treated with CA (100 nM) for 20 min (C)(D). Bands were excised from PVDF membranes hydrolyzed with HCI for 30 min and transferred onto TLC cellulose plate. Phosphoamino acid analysis was assessed for CrkL phosphorylation status.

2.3.2 Identification of Novel Phosghoserine Sites in CrkL

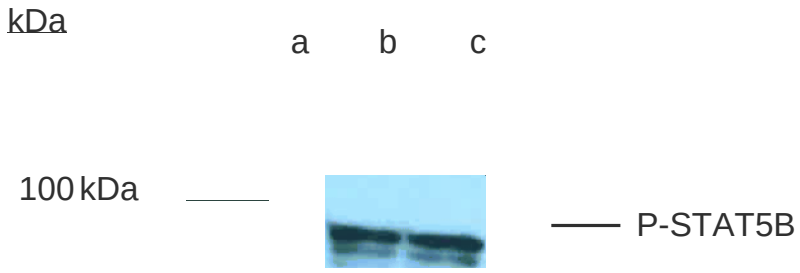
In preparation for LC-MS/MS analysis YT cells (2X10^7) cells either left unstimulated or treated with IL-2 for 10 minutes, IL-2 for 15 minutes, CA for 20 minutes, or a combination of CA for 20 minutes followed by IL-2 for 15 minutes. Cells were lysed and immunuprecipitation was performed with a-CrkL rabbit polyclonal and protein was separated by 10% SDS-PAGE and visualized by Coomassie Blue staining. (Figure 2.4a) To ensure cells were quiescent a small aliquot of the sample was analyzed for STAT5b activation with a phospho-specific a-STAT5b (Figure 2.4). Bands corresponding to CrkL (37kD) were excised, subjected to trypsin and Asp-N digestion, and analyzed by liquid chromatography-tandem mass spectrometry. YT Spectra analysis using the Sequest search algorithm revealed a combined protein coverage of 73% for the non-treated sample, 76% for IL2 stimulated, 76% for CA treated cells, and 68% for IL-2 + CA, as well as the identification of several novel CrkL phosphorylated peptides. (Figure 2.5) Phosphopeptides were identified which included phosphorylated S184, which appears to be constitutively phosphorylated and has been previously identified. Y127, Y207, the regulatory tyrosine located in the linker region between the SH3 domains, appear phosphorylated in the IL-2 sample. S185 appears phosphorylated in the Fsk sample which has not been previously identified. S162 appears in the IL-2 and CA stimulated samples but not in the unstimulated or Fsk sample indicating it is IL-2 and CA inducible. S162 is a novel phosphoserine site.

2.3.3 PPI Interacts With CrkL

LC-MS/MS results showed that CrkL co-immunoprecipitated with protein phosphatase 1 (Table 2.2). Considering the increased level of CrkL serine phosphorylation when treated with Calyculin A and the physical interaction demonstrated through mass spectrometry there could be a relationship between PP1 and CrkL. Kit225 cells (3X10^6) were left either unstimulated or treated with IL-2 for 15 minutes, CA for 20 minutes, or both IL-2 for 15 minutes and CA for 20 minutes. Samples were lysed and CrkL was immunoprecipitated with a-CrkL antibody and protein was separated by 10% SDS-PAGE. Western blot analysis with a mouse monoclonal a-PP1 antibody showed an increased amount of PP1 with IL-2 stimulation, and no detectable PP1 with Calyculin A present indicating a role for PP1 in CrkL negative regulation. (Figure 2.7)

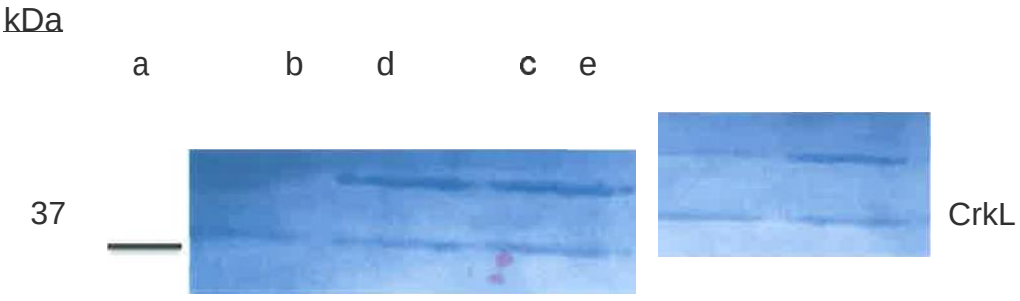
Calyculin A is a PP1/PP2A inhibitor and due to that we also wanted to look at any interaction between PP2A and CrkL. Mass spectrometry samples did not show any co-immunoprecipitation with PP2A in any of the treatments. Kit225 cells (3X10^6) were left either unstimulated or treated with IL-2 for 15 minutes, CA for 20 minutes, or both IL-2 for 15 minutes and CA for 20 minutes. Samples were lysed and CrkL was immunoprecipitated with a-CrkL antibody and protein was separated by 10% SDS-PAGE. Western blot analysis with a mouse monoclonal a-PP2a antibody showed no detectable presence of PP2A with any of the treatments. (Figure 2.8) Furthermore samples were probed with PP2A antibodies (Cell Signaling) against either subunit A, B, C with no detectable presence of PP2A.

A.



Total Cell LYsate
WB: pSTAT5

B.



IP: CrkL
Stain: Coomassie

Figure 2.4: Immunoprecipitation of CrkL for Mass Spectrometry
YT cells were starved to quiescence and were left untreated (-), stimulated with IL2 for 15 min and/or treated with CA (100 nM) for 20 min. Sample was separated by 10% SDS-PAGE and sent for LC-MS/MS analysis.

Table 2.1: LC-MS.MS Identification of Phosphorylation Sites in CrkL

Treatment	Coverage	Phoepho Residues
No Treatment	72.94%	S184
IL-2 1O min.	7006	S184,S&6],Y127
	.58	162, Y207
Calyculin A	74.59•#	S162
in	69%	S18

Table 2.1: LC-MS.MS Identification of Phosphorylation Sites in CrkL
YT cells were starved to quiescence and were left untreated, stimulated with IL2 for 15 min and/or treated with CA (100 nM) for 20 min, or treated with Forskolin for 20 min. Phosphopeptides were identified which included phosphorylated S184, which appears to be constitutively phosphorylated and has been previously identified. Y127, Y207, the regulatory tyrosine located in the linker region between the SH3 domains, appear phosphorylated in the IL-2 sample. S185 appears phosphorylated in the Fsk sample which has not been previously identified. S162 appears in the IL-2 and CA stimulated samples but not in the unstimulated or Fsk sample indicating it is IL-2 and CA inducible. S162 is a novel phosphoserine site.

Phosphopeptide: KGEILVIIEKPEEQWWSAR

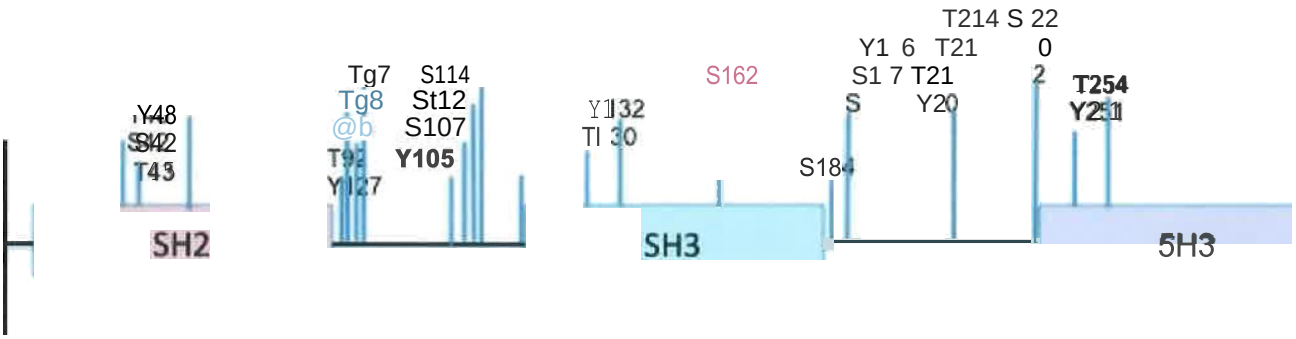
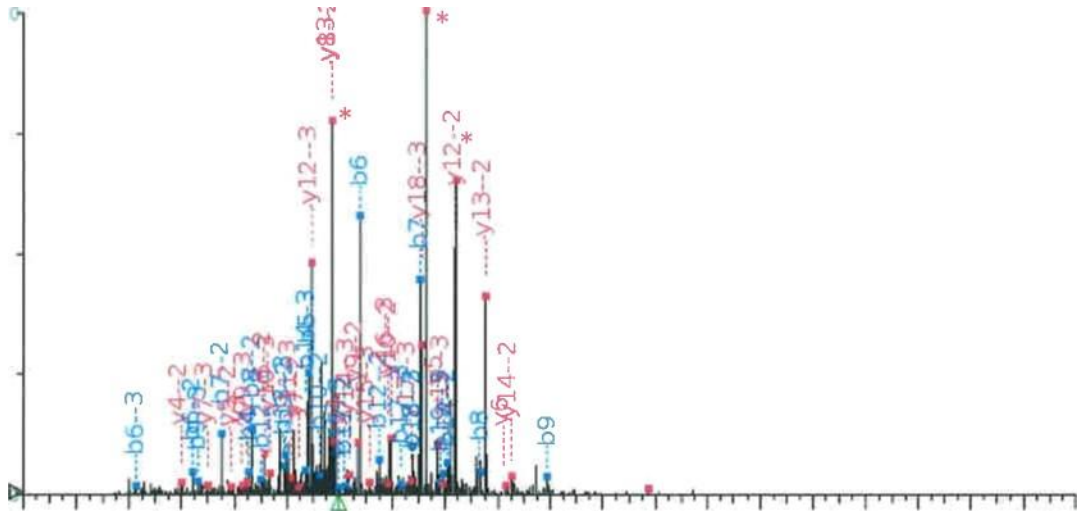


Figure 2.5: Identification of Novel Phosphorylation Sites on Human CrkL
LC-MS/MS analysis showed an IL-2 and Calyculin A inducible site on CrkL at serine 162 indicated in red. Previously identified phosphorylated amino acid residues are shown in black. Serine 162 is located within the SH3N domain.

A

Phosphopeptide: KGEILVIIEKPEEQWWSAR



B

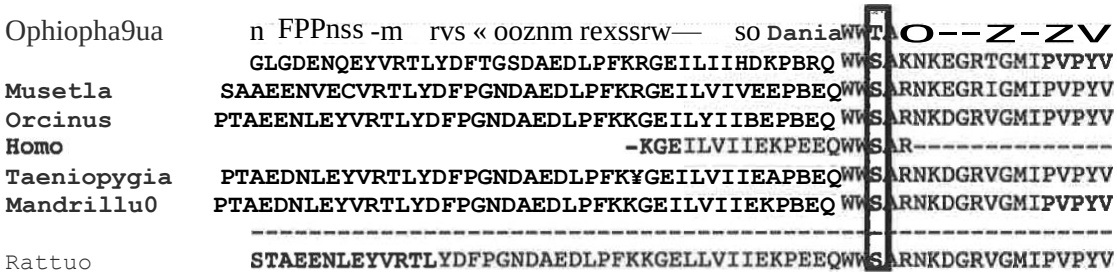


Figure 2.6: Tandem Mass Spectra and Sequence Conservation of CrkL S162
(A)Tandem mass spectra of a monophosphorylated peptide showing site localization of S162 on IL2 and CA stimulated YT cells, as indicated by asterisks. (B) Amino acid sequence alignment of the region surrounding S162 from different organisms using CLUSTAL OMEGA program. Conservation of S162 across species as well as the surrounding sequence containing aromatic tryptophan (W) make it an interesting site for involvement in regulation.

Table **2.2 LC-MS/MS Analysis CrkL Co-Immunuprecipitation**

t Unique	Total	reference	AVG	Gene Symbol	gWT{kDa)
30	362	**P461O9_CRKL HUMAN**	2.8424	CRKL	33.76
16	17	**P35579_MYH9_HUMAN**	3.5251	MYH9	226.39
16	16	**PO7355_,ANXA2 HUMAN**	3.2350	ANXA2	38.58
14	38	**P539O7_CAZA** 1 HUMAN	3.1374	CAPZA1	32.90
12	22	**P22626_ROA 2 HUMAN**	2.6179	HNRNPA2B1	37.41
12	12	PO4O4O CATA HUMAN	3.2753	CAT	59.72
11	12	**PO278B_TRFL HUMAN**	3.0706	LTF	78.13
11	11	**QO2B78_RL6_HUMAN**	2.5480	RPL6	32.71
10	11	**QO9666 AHNK_HUMAN**	2.5268	AHNAK	628.70
9	9	P42357_HUTH_HUPgAN	2.8925	HAL	72.65
8	26	PO44OS_G3P HUMAN	2.8968	GAPDH	36.03
8	14	**P461O8_CRK_HUMAN**	2.9049	CRK	33.81
8	10	**Q5M7ZO_RNFT** 1_HUMAN	2.7967	RNFT1	49.68
7	17	IGHG RABIT	2.3776		35.38
7	8	**PO4OB3 ANAA1_.HUMAN**	3.6017	ANXA1	38.69

Table 2.2: **LC-MS/MS Analysis CrkL Co-Immunuprecipitation**
Kit225 cells were left untreated (-), stimulated with IL-2 for 15 min and/or treated with CA (100 nM) for 20 min. Cell lysates were immunoprecipitated (IP) with a-CrkL and separated by SDS-PAGE. Samples were transferred to PVDF membrane and immunoblotted with monoclonal a-PP1 antibody. IL-2 stimulated lane (B) shows an much larger amount of PP1 associated with it when compared to the unstimulated lane (A). Samples with CA treatment show an absence of PP1 signal indicated that the inhibitor disrupted the interaction between CrkL and PP1.

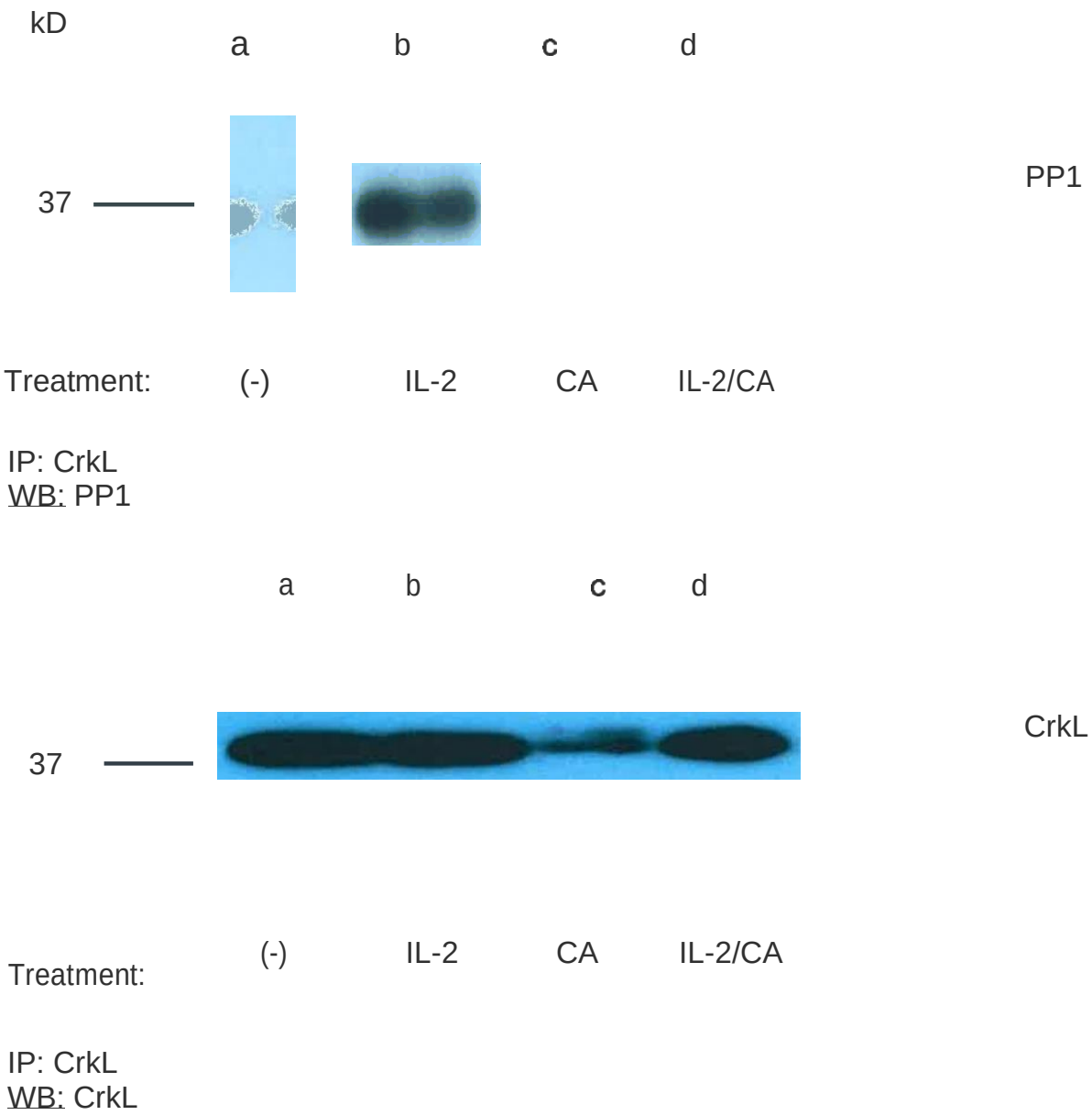


Figure 2.7: IL-2 Stimulated Kit225 Cells Co-Immunoprecipitatewith PP1
Kit225 cells were left untreated (-), stimulated with IL-2 for 15 min and/or treated with CA (100 nM) for 20 min. Cell lysates were immunoprecipitated (IP) with a-CrkL and separated by SDS-PAGE. Samples were transferred to PVDF membrane and immunoblotted with monoclonal a-PP1 antibody. IL-2 stimulated lane (B) shows an much larger amount of PP1 associated with it when compared to the unstimulated lane (A). Samples with CA treatment show an absence of PP1 signal indicated that the inhibitor disrupted the interaction between CrkL and PP1.

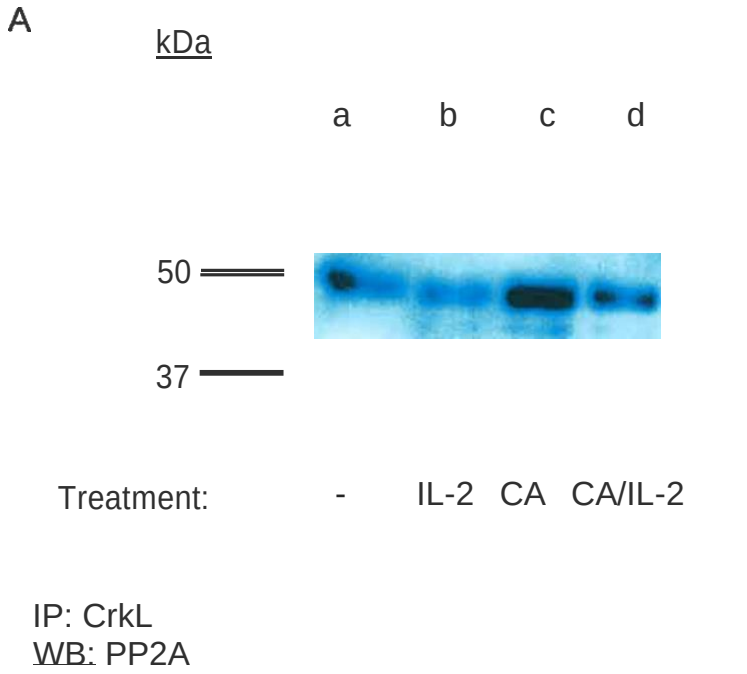


Figure 2.7: IL-2 Stimulated Kit225 Cells do not Co-Immunoprecipitate with PP2A Kit225 cells were left untreated (-), stimulated with IL-2 for 15 min and/or treated with CA (100 nM) for 20 min. Cell lysates were immunoprecipitated (IP) with a-CrkL and separated by 10% SDS-PAGE. Samples were transferred to PVDF membrane and immunoblotted with monoclonal a-PP2A antibody. All lanes show no signal in the range of PP2A molecular weight.

Chapter 3: Overview and Future Directions

3.1 Overview

IL-2 is essential for activation of T, B, and NK cells as well as being the main driver for T cell proliferation. IL-2 signaling has been shown to be mediated by the IL2R which is tyrosine and threonine phosphorylated to create a docking site for STATs and adaptor molecules such as Shc, which will continue down the PI3K and/or the MAPK pathways. Adaptor molecules, such as CrkL, involved in these pathways are necessary for assembling protein networks and mediating protein interactions. These major pathways leading to T cell proliferation serve as targets for therapeutic agents against malignancies.

The Crk family proteins are adaptor proteins implicated in various types of malignancies including glioblastoma and gastric, prostate, ovarian, breast, and lung cancers. CrkL is of unique importance in hematopoietic malignancies because of an increased level of expression in myeloid and lymphoid derived cells lines. CrkL remains understudied in hematopoietic cancers and could function as a possible therapeutic target.

It has been previously reported that CrkL is tyrosine phosphorylated with IL-2 stimulation and work in our lab has shown increased levels of serine phosphorylation with IL-2 stimulation (Fig 2.3). Considering the role of adaptor proteins in signaling networks we sought to investigate whether IL-2 can stimulate serine phosphorylation of CrkL and the regulation of CrkL by serine/threonine phosphatases PP1 and PP2A.

The first objective of this thesis was to determine whether IL-2 can induce serine or threonine phosphorylation, and then to determine which residues if so. The first step was stimulating quiescent Kit225 and YT cells with IL-2 and the IL-2 family of cytokines (Figure 2.1) which share the common gamma chain, as well as including samples pre-

treated with Calyculin A, a known serine/threonine phosphatase inhibitor. Western blot analysis showed the **appearance of a double band correlating to a higher MW CrkL** when stimulated with IL-2 family cytokines, and a triple band when treated with Calyculin A (Figure 2.1). This result indicated that IL-2 family cytokines stimulate the appearance of a modified CrkL and that CA enhances the result, pointing to a relationship between CA and the modification.

To investigate the effect of IL-2 and CA treatment on CrkL [^{32}P] orthophosphate labeling and phosphoamino acid analysis was performed on CrkL bands. Results showed an increased amount of CrkL phosphorylation in IL-2 and IL-2 with CA samples. Furthermore PAA showed **an increased amount of serine phosphorylation** in IL-2 and IL-2 with CA samples. (Figure 2.3)

To map which serine residues were being phosphorylated we employed immunoprecipitation of CrkL and identification of phosphopeptides through LC-MS/MS. We identified S162 to become phosphorylated in response to IL-2 stimulation as well with CA treatment. The novel IL-2 inducible S162 site was then examined via sequence alignment software to determine whether the residue and the region surrounding was conserved across different species. **The phosphopeptide was shown to be highly conserved across** species. In order to characterize this site a mutant S162A was generated using site directed mutagenesis.

LC-MS/MS analysis showed a variety of proteins that co-immunoprecipitated with CrkL and one of them was protein phosphatase 1. Due to response to CA the appearance of PP1 was further investigated by co-immunoprecipitations of CrkL with PP1. Results showed that when Kit225 cells were stimulated by IL-2 there is an increased amount of PP1 associated with CrkL while the introduction of CA

reduces the amount of PP1 associated with CrkL. Calyculin A is a PP1/PP2A inhibitor so PP2A was also analyzed by co-immunoprecipitation. Co-immunoprecipitation results show no PP2A associated with CrkL for any of the **treatments.** Furthermore PP2A was not shown to co-immunoprecipitate with CrkL by LC-MS/MS.

Kinase consensus predictive software (KinasePhos 2.0) determined the phosphopeptide contains a sequence that could be phosphorylated by Akt 1 or **MAPK with MAPK having the higher SVM score.** The software predicted serine kinases that are involved IL-2 signaling. Using this as a starting point we could use selective inhibitors of MAPK and Akt1, such to see if there is a decreased level of CrkL phosphorylation.

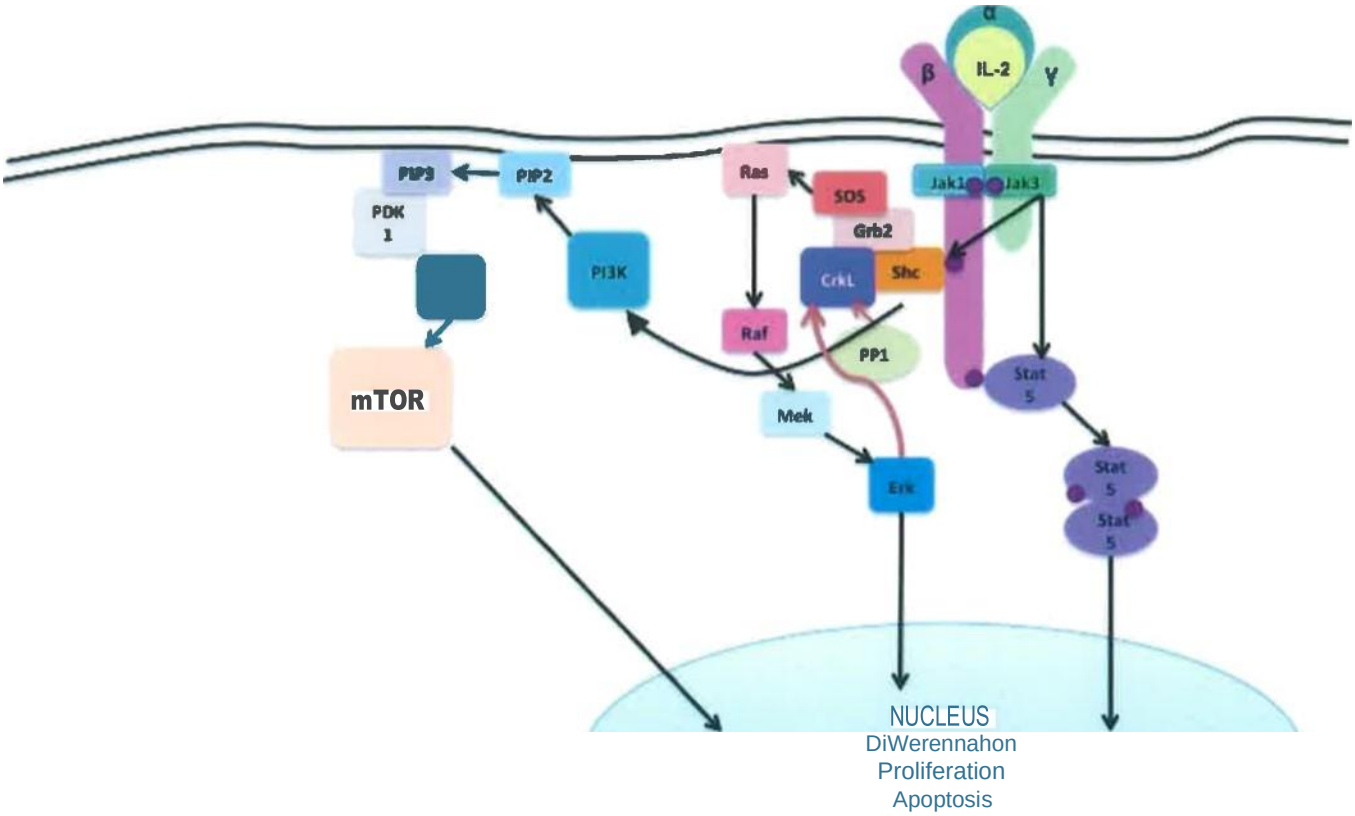


Figure 3.1: Model of CrkL in IL-2 Signaling Pathway
CrkL has been shown to form a complex with Grb2, SOS, and Shc. Our work has shown that IL-2 stimulation results in increased serine phosphorylation. Kinase prediction software determined that a MAPK consensus sequence exists within CrkL indicating a possible role for either ERK 1 or ERK2 as a kinase for CrkL. PP1 has also been shown to have a regulatory relationship involving CrkL phosphorylation.

3.2 Future Directions

The previous data demonstrated that CrkL serine phosphorylation is inducible by IL-2 but the role of the phosphorylation needs to be studied. Characterization of the mutant by radiolabeling, PAA, and co-immunoprecipitations should be performed in the future. Furthermore the kinase responsible, our target being ERK1/2, for phosphorylating the IL-2 inducible serine will need to be determined to further understand where along the cascade CrkL plays a role. To do this selective kinase inhibitors should be used to target certain known serine/threonine kinases and determine the effect on CrkL serine phosphorylation. Data from these experiments can be taken further by knockdown experiments of the kinase by siRNA and looking at levels of CrkL phosphorylation

To further investigate the relationship between CrkL with and PP1 selective phosphatase inhibitors can be used to determine the effect on CrkL. Immunoprecipitation, Western blot analysis, and radiolabeling could be used to visualize the effect. These experiments would need to be followed with knockdown of PP1 to corroborate the relationship with CrkL by looking at the serine phosphorylation levels of CrkL. If there are increased levels of phosphorylation when PP1 is knocked down we could confidently state that PP1 plays a negative regulatory role of CrkL serine phosphorylation.

Lastly, and most importantly, would be to generate a phospho-specific antibody against CrkL serine 162. An antibody would allow one to readily characterize the role that serine phosphorylation of CrkL can play in cellular signaling networks. A specific antibody can be used for testing in primary malignant cell samples to determine if serine 162 phosphorylation levels differ from non-cancerous cell samples. Ultimately this work

could be used to determine the role of CrkL S162 phosphorylation in disease and how it can be targeted for therapeutic purposes in hematopoietic malignancies by exploring options that could inhibit or enhance CrkL serine phosphorylation depending on the role it is playing in signaling networks.

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CURRICULUM VITA

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