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The Role of Aberrantly Spliced SHIP1 in T-Cell Acute Lymphoblastic Leukemia

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ABSTRACT OF THE THESIS

The Role of Aberrantly Spliced SHIP1 in T-Cell Acute Lymphoblastic Leukemia

by

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Of the 25% of T-cell acute lymphoblastic leukemia (T-ALL) patients who relapse or do not respond to chemotherapy, only 10-50% are ultimately curable. Genetic lesions involving the T cell receptor for antigen (TCR) have been correlated with poor prognosis, suggesting the events triggered by TCR signaling are involved in the disease. We have recently reported extensive transcript splicing of the lipid phosphatase SHIP1 in primary T-ALL which we believe may influence aberrant TCR signaling in the Ras/Erk and PI3K/Akt pathways. To determine the significance of such splicing, extensively spliced SHIP1 isoforms from primary T-ALL samples were cloned and expressed in a PMA-dependent Jurkat cell line model to study SHIP1 protein stability as well as phosphatase activity. We report that the removal of exon 8 of SHIP1 prevented the formation of stable protein while removal of exons 25 and 26 conferred protein stability.

Further, removal of exon 26 was sufficient to increasing SHIP1 phosphatase activity two fold compared to full length protein, a phenomenon we ascribe to catalytic autoinhibition. We also made PMA-independent SHIP1 expressing stable Jurkat clones to study SHIP1 involvement in the Ras/Erk pathway and report for the first time that full length SHIP1 downregulated Ras activity in T cells. Finally, in a search for novel therapeutic targets in T-ALL, we inhibited both Ras and PI3K in Jurkat cells using S-trans,trans-farnesylthiosalicylic acid and LY294002 and found synergistic effects on cell growth, confirming the efficacy of dual Ras and PI3K targeting in T-ALL.

Chapter 1: Introduction

1.1 T-cell Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is the most common pediatric blood disease and is characterized by the hyperproliferation of lymphoid progenitor cells. The result is overcrowding of the bone marrow and hematopoietic compartments, leading to fever, weight loss, impaired blood clotting, and bone pain due to reduced hematopoietic cell function [Esparza and Sakamoto, 2005]. ALL affects mostly young children, with a peak incidence between the ages of 3 and 4, and is commonly classified according to the origin of lymphoid progenitor cells as either B-cell acute lymphoblastic leukemia (B-ALL) or T-cell acute lymphoblastic leukemia (T-ALL). T-ALL accounts for 10 to 15% of all ALL and is associated with poor prognosis, poor responsiveness to chemotherapy, and relapse [Goldberg et al., 2003; Raimondi, 2007]. T-ALL is distinct from adult T-cell leukemia (ATL), which is caused not necessarily by genetic lesions but rather viral infection [Nicot et al., 2005].

1.1.1 Targeted Therapy in T-ALL

The chemotherapeutics used to treat ALL cause many adverse side effects, including fever, hepatoxicity and myelosuppression [Kim et al., 2009]. Long-term chemotherapeutic treatment can adversely affect the central nervous system, causing lasting neurocognitive deficits and thereby affecting patient quality of life [Janzen and Spiegler, 2008]. External prophylactic cranial radiation has also been used to prevent relapse caused by residual leukemia cells which may have spread to the brain, though this practice has recently been shown to be unnecessary when chemotherapy is given in a risk-specific manner [Pui et al., 2009]. In order to achieve higher event-free survival rates while limiting secondary complications from chemotherapeutics,

the goal of current leukemia research is to find molecular targets specific to leukemia cells, addressing the specific genetic lesions and aberrant signaling pathways in each patient.

As 75% of T-ALL is already curable, developing new ways to treat unresponsive and relapsing cases of T-ALL is of special interest. In Both T and B-ALL, relapse and poor prognosis have been shown to strongly correlate with the presence of cancer cells after treatment, an event termed minimal residual disease (MRD). A group of specific genetic lesions involving the T cell receptor for antigen (TCR) have been identified in T-ALL and these lesions have been shown to predict MRD at diagnosis [van der Velden and van Don, 2009]. The events following TCR stimulation are therefore of great interest when investigating new drug targets. Two major pathways induced by TCR signaling are the PI3K/Akt and the Ras/Raf-1/Erk pathways, both of which are frequently deregulated in T-ALL (see sections 1.2.1 and 1.3.1). The two pathways influence each other at many levels and show cross-pathway feedback (see section 1.3.2), suggesting that simultaneous targeting of both pathways may have synergistic effects.

1.2 The PI3K/Akt Pathway

Akt, also known as protein kinase B (PKB), is a serine-threonine kinase involved in cell growth and survival. Akt lies at a signaling hub, performing several functions. Active Akt prevents apoptosis through its ability to phosphorylate substrates such as BAD and procaspase-9 [Chang et al., 2003]. Akt also influences cell cycle progression by negatively regulating the cyclin-dependent kinase inhibitors $p27^{KIP1}$ and $p21^{WAF1}$ [Graff et al., 2000].

Akt activity is regulated by its relocation to phosphotidylinositols within the plasma membrane upon receptor stimulation. This occurs via the plextrin-homology (PH) domain of Akt, which binds the 3' phosphate of the phosphotidylinositols PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Upon binding phosphotidylinositols, Akt undergoes a conformational change, allowing it to be phosphorylated at Thr308 and Ser473 by the 3'-Phosphoinositide-Dependent Kinases 1 and 2

(PDK1/2) [Fresno et al., 2004]. The PDK kinases are thought to be constitutively active, making Akt activation dependent on the presence of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 in the plasma membrane. These lipid species are generated by Phosphatidylinositol-3 Kinase (PI3K), which phosphorylates PtdIns(4,5)P2 at the 3'-OH position in response to stimulation by receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). This action is directly opposed by Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN), which dephosphorylates PtdInds(3,4,5)P_3 at the 3'-OH position to form PtdIns(4,5)P_2 [Stambolic et al., 1998]. Once active, Akt initiates several pathways involved in cell survival, cell growth, and metabolism [Fresno et al., 2004]. A diagram of the PI3K/PIP3/Akt pathway is shown in Section 1.4.4 (Fig. 1).

1.2.1 PI3K/Akt Deregulation in T-ALL

In T-ALL, the PI3K/Akt pathway is often deregulated, mainly due to the inactivation of PTEN. Loss of PTEN can occur by gene mutation or deletion and is seen in a variety of cancers [Sansal and Sellers, 2004]. While we have shown that PTEN translational mutations are common in T-ALL cell lines, they are rare in primary T-ALL [Lo et al., 2009]. PTEN inactivation in T-ALL is rather attributed to phosphorylation of PTEN, rendering PTEN functionally inactive [Silva et al., 2008], or aberrant NOTCH1 signaling, which downregulates PTEN mRNA transcription [Palomero et al., 2007]. Our group has suggested that along with the loss of function of PTEN, inactivation of Src Homology 2-containing Inositol Phosphatase-1 (SHIP1), a lipid phosphatase which uses the same lipid substrate as PTEN, may also contribute to aberrant Akt activity [Lo et al., 2009].

1.3 The Ras/Raf-1/Erk Pathway

Ras belongs to a family of small GTPases involved in differentiation, growth and survival, and becomes active in response to stimulation by a wide variety of growth factors, cytokines, and antigenic stimulation. Ras is active when bound to GTP but becomes inactive upon hydrolysis of GTP to GDP, a process carried out with the help of RasGTPase Activating Proteins (RasGAPs). Activation of Ras is facilitated by a family of proteins called Ras Guanine Nucleotide Exchange Factors (RasGEFs) which catalyze the removal of GDP from Ras, allowing cytosolic GTP to take its place [Bourne et al., 1990].

In T cells, TCR stimulation leads to extensive tyrosine phosphorylation of membrane proteins, including Linker of Activated T cells (LAT). Once phosphorylated, LAT is bound by the adapter protein Growth factor receptor-bound protein 2 (Grb2) which binds the RasGEF Son of Sevenless (SOS), localizing it to LAT where it can activate Ras [Buday et al., 1994; Zhang et al., 1998] In addition to SOS, TCR stimulation leads to activation of another RasGEF, Ras Guanyl nucleotide-Releasing Protein (RasGRP), to activate Ras signaling. Activation of Ras by RasGRP is dependent on the production of diacyglycerol upon TCR and growth receptor stimulation [Ebinu et al., 2000]. In T cell activation, RasGRP serves as the primary activator of Ras, though once in its GTP-bound state, Ras allosterically activates SOS and creates a positive feedback loop [Roose et al., 2007]. Accordingly, full activation of Ras becomes a concerted effort between RasGRP and SOS, with SOS potentiating the effects of RasGRP. Once active, Ras can associate with downstream Raf-1 to activate the extracellular regulated kinase (Erk) cascade [Marais et al., 1995]. Erk proteins can transcriptionally activate a variety of pathways depending on the strength and duration of stimulus, from cell growth to cell cycle arrest [Marshall, 1995].

1.3.1 Ras/Erk in T-ALL

Aberrant Ras signaling is a hallmark of many cancers including adenocarcinomas, thyroid tumors, and myeloid leukemia [Bos, 1989]. While oncogenic Ras signaling is rare in B-ALL, we have reported overactive Ras in nearly 50% of primary T-ALL [Von Lintig et al., 2000], suggesting aberrant Ras activation may lend to the aggressive nature of the disease. Further, the expression of constitutively active Ras has been shown to cause aggressive T-cell leukemia in mice [Kindler et al., 2008]. Both the prevalence and the implications of oncogenic Ras signaling make it an attractive target for intervention. Ras mutations, however, are rare in primary human T-ALL, leaving the cause of overactive signaling unclear [Kawamura et al., 1999]. Without a clear cause, targeted therapy remains difficult. The matter is further complicated by the complexity of Ras/Raf-1/Erk feedback loops and cross-talk with other pathways like the PI3K/Akt pathway.

1.3.2 Ras/Raf-1/Erk and PI3K/Akt Cross-talk

Though these pathways are often separated when discussing signal deregulation in cancer, they communicate and feedback on multiple levels. Active Ras stimulates PI3K, leading to Akt activation [Orme et al., 2006]. Meanwhile, active Akt inhibits the activation of Raf-1, the effector directly downstream of Ras [Moelling et al., 2002]. Further downstream of Ras, activated Erk functions to inhibit upstream Ras activation, creating a negative feedback loop within the Ras/Erk pathway itself [Waters et al., 2005]. The multiple aspects of Ras and PI3K pathway communication reveal a great complexity in the regulation of signaling. It is therefore necessary to address both pathways when studying oncogenic signaling in T-ALL. See section 1.4.4 for a diagram of Ras/Erk and PI3K/Akt cross-talk (Fig. 1).

1.4 SHIP1

Src homology 2-containing inositol phosphatase-1 (SHIP1) was originally discovered as a 145 kDa tyrosine-phosphorylated protein with 5' phosphatase activity against the inositol lipids PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4. It holds an N-terminal SH2 domain capable of binding phosphorylated tyrosine motifs, a central phosphatase region, a C-terminal polyproline region, and two C-terminal tyrosine phosphorylation (NPXY) motifs. Upon initial characterization, the protein was appropriately named SH2 (Src homology 2)-containing inositol phosphatase, or SHIP [Damen et al., 1996].

1.4.1 SHIP1 Structure and Function

Since its initial discovery, SHIP1 has been shown to interact with several proteins. Yet because of its large size and many binding domains, the full extent of its function is still unclear. The SH2 domain in the amino terminus of SHIP1 is capable of binding phosphorylated tyrosine motifs and is responsible for recruitment of SHIP1 to surface receptors either through direct binding or through adaptor proteins. In B cells, SHIP1 is recruited though this SH2 domain to the immunoreceptor tyrosine inhibitory motif (ITIM) of FcγRIIB where it negatively regulates B cell activation [Isnardi et al., 2006]. SHIP1 is also recruited to the erythropoietin receptor in B cells in an SH2-dependent manner [Mason et al., 2000].

The central catalytic region of SHIP1 catalyzes the hydrolysis of membrane bound phosphatidylinositol (3,4,5) triphosphate [PtdIns(3,4,5)P_3, or PI(3,4,5)P_3] and cytosolic inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4 or I(1,3,4,5)P_4]. PI(3,4,5)P_3 and I(1,3,4,5)P_4 substrates are dephosphorylated by SHIP1 at the 5' position, forming PI(3,4)P_2 and I(1,3,4)P_4, respectively. A C2 domain which binds lipids in the plasma membrane is located in the C-terminal portion of the catalytic region of SHIP1. This domain can bind PI(3,4)P_2 and has been implicated in the regulation of SHIP1 phosphatase activity [Ong et al. 2007] (see section 1.4.2).

The C-terminal region of SHIP1 contains binding motifs of two categories, dynamic and static. The two NPXY sites on SHIP1 can become tyrosine phosphorylated by receptor stimulated kinases, attracting the phosphotyrosine-binding (PTB) domain of Docking proteins 1 and 2 (Dok-1/2) [Sattler et al., 2001]. In this way, the NPXY sites, along with the SH2 domain of SHIP1, represent dynamic binding capabilities in response to kinase signaling. On the other hand, the polyproline region in the carboxyl terminus of SHIP1 can statically bind the SH3 domains of the scaffolding protein Grb2 [Baran et al., 2003]. Using a combination of static and dynamic binding domains, SHIP1 participates in preassembled complexes which are recruited to sites of receptor activation. In T cells, SHIP1 is recruited to the cell surface protein LAT upon TCR stimulation and participates in a negative signaling complex involving LAT, Grb2, and Dok-2 [Dong et al., 2006].

Expression of SHIP1 is restricted to cells of hematopoietic origin, suggesting an immune specific function. A close homologue, SHIP2, is ubiquitously expressed with increased expression in heart, placenta, and skeletal muscle tissue [Pesesse et al., 1997]. SHIP2 is involved in the negative regulation of mitogenic signaling from a variety of cell surface receptors, ranging from insulin signaling in adipocytes to macrophage colony-stimulating factor (M-CSF) signaling in myeloid cells. [Wada et al., 2001; Wang et al., 2004]. SHIP1 and SHIP2 have similar structures, each with an N-terminal SH2 domain, an internal 5' inositol phosphatase region, and a C-terminal proline rich region. Unlike SHIP1, however, SHIP2 has been shown to hydrolyze as many as six different phosphoinositol species [Chi et al., 2004]. Though the two homologues appear to carry similar roles, the expression of both proteins in immune cells suggests a unique role for each.

1.4.2 Regulation of SHIP1 Phosphatase Activity

Emerging research reveals clues about the regulation of SHIP1 catalytic activity. Allosteric activation of SHIP1 phosphatase activity has been shown to depend on a C2 domain located at the C-terminal end of the phosphatase region which binds the SHIP1 catalytic product $PI(3,4)P_2$, suggesting a positive feedback mechanism for SHIP1 phosphatase activity [Ong et al. 2007]. More recently, it was shown that *in vitro* phosphorylation of SHIP1 at Ser440 of SHIP1 by Protein Kinase A (PKA) leads to increased SHIP1 activity [Zhang et al, 2010]. However, deletion of the C-terminal of SHIP1 increases SHIP1 catalytic activity and this activity becomes independent of serine phosphorylation, suggesting an autoinhibitory mechanism involving the C-terminal of SHIP1 and Ser440 [Zhang et al, 2010].

1.4.3 SHIP1 in T Lymphocyte Function

While SHIP1 function is well defined in B cells, monocytes, and macrophages, its function in T cells is still rather unclear. Bits of information exist, but they tell fragmented stories. TCR stimulation as mimicked by co-stimulation of the CD3 and CD28 cell surface receptors causes SHIP1 to localize to the plasma membrane where it becomes tyrosine phosphorylated, leading to increased catalytic activity [Edmunds et al., 1999]. SHIP1 has also been shown to inhibit T cell activation through association with Grb2 and Dok-2, two common scaffolding proteins involved in Ras signaling [Dong et al., 2006]. And while SHIP1 has been proposed to downregulate PI3K/Akt activity in B cells, T cell-specific SHIP knockout mice show no altered PI3K signaling upon costimulation of CD3 and CD28. Instead, SHIP1 knockout leads to a skewing of T helper cell subsets, upregulating the Th1-associated cytokine T-bet and subsequently increasing the cytotoxicity of CD8+ T cells [Tarasenko et al., 2007]. Meanwhile, SHIP1 knockdown T cells show reduced basal cell motility through an Akt-dependent mechanism

which cannot be rescued by treatment with a PI3K inhibitor [Harris et al., 2011], suggesting SHIP1 may not be directly opposing PI3K activity as previously thought (see section 1.4.4).

SHIP1 has also been shown to play an anti-apoptotic role in T cells. SHIP1 is drawn to the endoplasmic reticulum (ER) membrane in an SH2-dependent manner, promoting glycosylation of the Fas death receptor and thereby decreasing sensitivity to Fas ligand-induced apoptosis [Charlier et al., 2010]. SHIP1 in this case may function on the exterior of the ER as a lipid phosphatase, a scaffolding protein to recruit other enzymes to the ER, or both. This new role further demonstrates that SHIP1 is involved in normal T cell development and function but raises the question as to whether SHIP1 can be classified as a tumor suppressor.

1.4.4 SHIP1 and Akt

A review of current literature supports a role for SHIP1 as an important negative regulator of Akt activation. The bulk of studies performed, however, represent conditions which may be far from physiologically relevant. Expression of SHIP1 in Jurkat T-cell leukemia cells has been shown to decrease Akt activation as measured by phosphorylation at Ser473, but only at extremely high levels of SHIP1 expression [Horn et al., 2004]. In the same study, moderate, more physiologic levels of SHIP1 had minimal effect on Akt status. In another study connecting SHIP1 with Akt inactivation, SHIP1 is introduced into Jurkat cells as a fusion protein consisting of the catalytic core of SHIP1 and a constitutively membrane-bound domain [Freeburn et al., 2002]. Since this chimeric protein lacks the apparent inhibitory C-terminal region and was not dependent on its own domains for recruitment to the membrane, the result was a constitutively and possibly overactive SHIP1 with unrestricted access to its substrate. In any case, this chimeric SHIP1 protein only reduced Akt phosphorylation during the first two minutes of CD28 stimulation. After five minutes of stimulation, Akt was activated regardless of SHIP1 status [Freeburn et al., 2002].

The specific function of SHIP1 in the Akt pathway may rely on the differential effects of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ on Akt activation. The evidence, however, points in different directions depending on the method employed and cell type used. One study involving EGF treated NIH3T3 fibroblasts showed Akt activation was more closely associated with localization of $PI(3,4)P_2$ than $PI(3,4,5)P_3$ [Yoshizaki et al., 2007], a finding in line with the observation that the PH domain of Akt has higher affinity for $PI(3,4)P_2$ then $PI(3,4,5)P_3$ [Franke et al., 1997]. This may suggest that Akt is actually activated by SHIP1 catalytic function and there may be competitive inhibition between phosphotidylinositide species during Akt activation. A closer look at effectors downstream of Akt reveals that SHIP1 can selectively inhibit the Akt activation of the growth-associated kinase Glycogen Synthase Kinase 3 (GSK-3β), lengthening the G1 stage in the cell cycle without altering apoptotic effectors downstream of Akt such as Bad and FKHR [Horn at al., 2004]. Hence, SHIP1 may be responsible for fine tuning the PI3K/Akt pathway and not necessarily abrogating it.

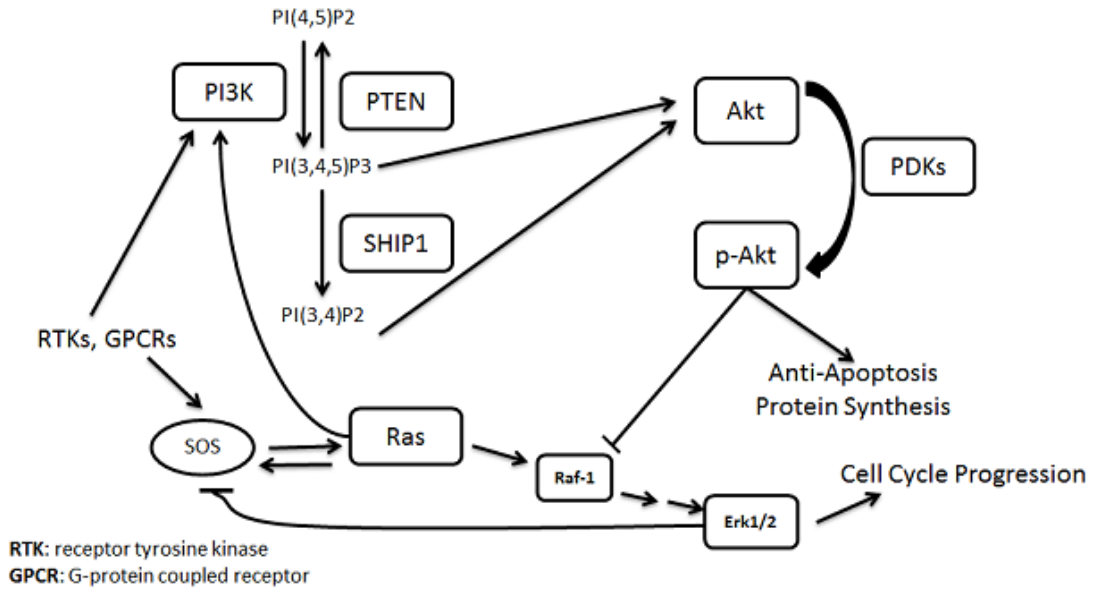

Figure 1: PI3K/Akt and Ras/Erk Pathway Crosstalk. The PI3K/Akt and Ras/Erk signaling pathways implement complex crosstalk to ensure tight regulation of cell growth and apoptosis.

1.4.5 Popular SHIP1 Isoforms

Although the SHIP1 gene encodes a 145kDa protein, shorter SHIP1 protein species are often detected. The smallest is a 110kDa species found in myeloid cells of both mouse and murine origin. When these myeloid cells are induced to differentiate, expression of this 110kDa species declines and cells begin producing full length SHIP1 as well as a 135kDa species [Geier et al. 1997]. The mechanism for generating these species can vary, with some studies reporting 3' cDNA splicing [Lucas and Rohrschneider 1999] while others report C-terminal protein cleavage [Damen et al. 1998]. Alternatively, a 110kDa SHIP1 species which is missing the SH2 domain can be created from an internal start site within the intron between exons 5 and 6 [Kavanaugh et al., 1996; Tu et al., 2001]. This SH2 truncated isoform has also been detected in virus-exposed T cells [Avota et al. 2006]. The specific function of these isoforms is still unknown, though the presence or absence of certain domains in each isoform gives clues as to the molecular interactions possible.

1.4.6 Extensive Splicing and Mutation of SHIP1 in T-ALL

Recently, we have described SHIP1 isoforms in primary T-ALL which are distinct from those previously published. In primary T-ALL, full length SHIP1 transcript is expressed, but only 25% of cases express detectable SHIP1 protein [Lo et al., 2009]. Detectable SHIP1 protein was frequently truncated at the C-terminus and we showed this to be generated through extensive splicing of SHIP1 mRNA, causing in-frame deletions as well as premature terminations. Among the isoforms most commonly found were those missing exon 8, exon 26, or exons 25 and 26 together. Other prominent isoforms included an insertion of intron 14 between exons 14 and 15, a partial insertion of intron 10 between exons 10 and 11, and a 47bp deletion in exon 12 found in a T-ALL cell line; these later three isoforms all caused translational inactivation and subsequent premature truncation [Lo et al.,2009]. The extent to which the splicing of SHIP1 contributes to T-

ALL pathology is still uncertain. Though, with an incredibly high rate of SHIP1 inactivation, the involvement of SHIP1 in T-ALL suggests more than coincidence. It was the goal of this thesis to investigate the function of these isoforms in order to understand the role of SHIP1 and SHIP1 splicing in T cell biology and cancer. Most of the focus of SHIP1 research has been placed on its lipid phosphatase function and negative regulation of Akt. Many of the SHIP1 isoforms we found in primary T-ALL, however, were not missing the phosphatase region but rather other functional binding domains. Hence, we made an effort to focus on roles of SHIP1 beyond Akt regulation.

Chapter 2: Materials and Methods

2.1 Cell Culture and Stimulation

Jurkat cells were obtained through the American Type Culture Collection. Cells were cultured in RPMI 1640 media with 10% fetal bovine serum, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin. CD3/CD28 stimulation was performed with Dynabeads Human T-Activator CD3/CD28 for T-Cell Expansion and Activation (Life Technologies) .

2.2 Western Blotting

Cells were lysed in RIPA buffer (50mM Tris-Hcl pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) with PMSF and protease inhibitors and clarified by centrifugation (14,000g x 15 min) at 4˚C. Protein lysates were seperated on 10% Bis-Tris, 4-12% Bis-Tris, or 3-8% Tris Acetate NuPAGE gels (Life Technologies) and transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA). Membranes were then blocked in 5% BSA in TBS with 0.05% Tween 20. Protein detection was performed the following antibodies: SHIP1 N-terminal (epitope: residues 6-21)(SHIP-01, #ab65807, 1:1000)(Abcam Inc., Cambridge, MA); SHIP1 C-terminal (epitope: residues 866-1020)(P1C1, #sc-8425, 1:500)(Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-Akt^{Ser473} (#9271, 1:1000), Akt(#9272, 1:500)(Cell Signaling Technologies, Danvers, MA); β-actin (#AC15, 1:50,000)(Sigma-Aldrich, St. Louis, MO). After incubation with a horseradish peroxidase conjugated anti-rabbit our anti-mouse secondary antibody (1:1000)(Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), blots were visualized using ECL substrate (Pierce) and exposed to film. Images were quantified using Image Quant (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.3 SHIP1 Expression Constructs

Spliced SHIP1 transcripts were previously cloned from primary acute lymphoblastic leukemia samples using the Invitrogen TA cloning kit [Lo et al., 2009]. Clones were seqenced using the following primers: SHIP1-5F (5′-AGGAAGTCAGTCAGTTAAGCTGGT-3′) or SHIP1-2409F (5′-CTCGAGCTGCTTGGAGAGTT-3′) and SHIP1-3863R (5′-CAGAAGCTAGGCCCTTTCCT-3′). After verification of sequence, spliced SHIP1 isoforms were digested and ligated into pcDNA3.1 mammalian expression vector (Life Technologies). SHIP1 pcDNA3.1 expression constructs were verified by sequencing with the above primers.

2.4 Transfection

Transfection was performed by electroporation using the Neon Transfection System (Life Technologies) according to the manufacturer's porotocol. Briefly, 2 x 10^6 cells were washed twice with PBS and centrifuged 10 minutes at 300xg. Cells were resuspended in 100µl Buffer R, mixed with 10µg pcDNA3.1 plasmid DNA, and electroporated with 2 pulses at 1200 volts and at a pulse length of 20 ms. After 24 hours, cells were treated with 50ng/ml phorbol 12-myristate 13-acetate (PMA) or 1µg/ml phytohaemagglutinin (PHA) for 12-24 hours to stimulate expression from the pcDNA3.1 vector. Transfection efficiency was determined by transfecting cells with pcDNA3.1-GFP and observing GFP expression under fluorescent microscopy after 24 hours.

2.5 Generation of SHIP1 Stable Clones

24 hours after transfection, Jurkat cells were treated with 400µg/ml G418 for 14 days to select for construct-containing cells. Surviving cells were then diluted to single cell colonies and maintained in 200µg/ml G418. Colonies were analyzed for SHIP1 protein expression by Western blot and postive clones were maintained in 200µg/ml G418 for further experiments.

2.6 SHIP1 Immunoprecipitation and Phosphatase Assay

The assay used to determine SHIP1 phosphatase activity was performed using immunoprecipitated SHIP1 protein and is based off a modified version of the published Malachite Green Assay (Echelon Biosciences) [Zhang et al., 2010]. After SHIP1 transfection, 10^7 cells were lysed in 500µl non-denaturing lysis buffer (20mM Tris-HCl pH 7.5, 137mM NaCl, 10% Glycerol, 1% Triton X-100, and 2mM EDTA). Lysates were precleared for 1 hour at 4 ˚C using 5µg normal rabbit IgG (#sc-2027)(Santa Cruz Biotechnology, Inc.) and 25µl Protein G Sepharose 4 Fast Flow (GE Healthcare). Precleared SHIP1 lysate was then incubated onvernight at 4˚C with 1µg of polyclonal antibody for SHIP1 (N-1, #sc-6244)(Santa Cruz Biotechnology, Inc.), then precipitated with 25µl Protein G Sepharose 4 Fast Flow (GE Healthcare) for 4 hours at 4˚C. Immunocomplexes were washed in a solution of 20mM Hepes pH 7.4 and 10mM $MgCl_2$ then incubated in an enzymatic reaction buffer containing 100µM diC8-PI(3,4,5)P3, 20mM Hepes, pH 7.4, and 10mM $MgCl_2$ for 1 hour at 30˚C, rocking. Immunocomplexes were spun down and the supernatant mixed with 100µl Malachite Green solution per 20µl supernatant. After incubating at room temperature for 15 minutes to allow for color development, phosphate release was determined by measuring the absorbance at 620nm and comparing to a standard phosphate curve.

2.7 Ras Activation Assay

The Ras Activation Assay Kit was purchased from Millipore. Briefly, cells were untreated or stimulated with Human T-Activator CD3/CD28 Dynabeads (Life Technologies). 10^7 Cells were then lysed in 500µl of the provided Magnesium Lysis Buffer with protease inhibitors. To this lysate, 6µl of Ras Assay Reagent (Raf-1 RBD, agarose) was added to precipitate GTP-bound Ras. The lysate was incubated for 45 minutes at 4˚C, rocking. After washing the agarose beads in the lysis buffer above, agarose beads were resuspended in 40µl 2x LDS Sample Buffer

with 0.5M DTT and boiled for 10 minutes to release bound Ras. The sample was then analyzed by Western blot using a Ras monoclonal anitibody (RAS10, #05-516, 1:20,000) (Millipore).

2.8 Cell Growth Studies

Cell replication was measured by counting cells after 24 hours of incubation with PMA or PHA. Cells were washed with PBS, detached using trypsin, mixed with trypan blue to assess viability and counted. DNA replication was measured using a tritiated thymidine uptake assay. Cells were plated in triplicate and treated with or without PMA for 48 hours. Tritiated thymidine was added to each well and cells were incubated at 37˚C for an additional 6 hours to allow the uptake of thymidine. Cells were harvested and tritiated thymidine was measured using a scintillation counter.

2.9 XTT Cell Viability Assay

Jurkat cells were treated for 48 hours with appropriate amounts of LY294002 or transfarnesylthiosalicylic acid (FTS) and incubated in 96-well plates at 37˚C for 48 hours. Cell viability was measured using the TACS XTT Cell Proliferation/Viability Assay (R&D Systems, Minneapolis, MN). After incubating with LY294002 or FTS, 50µl XTT reagent was added to each well and incubated an additional 6 hours. Absorbance was measured at 490nm with a reference wavelength of 650nm to determine mitochondrial activity.

2.10 Drug Synergy

Drug synergy was calculated using the Loewe additivity method. This model uses the equation $I = C_{a,r}/IC_{x,a} + C_{b,r}/IC_{x,b}$, where $C_{a,r}$ and $C_{b,r}$ are the concentrations of compounds A and B when given in a combination; $IC_{x,a}$ and $IC_{x,b}$ are the concentrations of compounds A and B yielding the same level of effect when used separately; I is the resulting interactive index. An

interaction index of less than one indicates synergy while an index greater than one indicated antagonism. An index of one indicates a purely additive effect [Straetemans et al., 2005].

Chapter 3: Development of a Model to Analyze SHIP1 in T-ALL

3.1 Background: Modeling T-ALL

The T leukemic cell line Jurkat was derived from the peripheral blood of a 14 year old boy with T cell leukemia in the 1970s and since has been the most widely used model for researching T cell receptor signaling [Schneider et al., 1977; Abraham and Weiss, 2004]. The Jurkat cell line serves as a good model for studying SHIP1 *in vitro* as it does not express detectable levels of SHIP1 protein, unlike most T lymphocyte cell lines, and also lacks the lipid phosphatase PTEN which utilizes the same $PI(3,4,5)P_3$ substrate pool as SHIP1 [Lo et al., 2009]. PTEN is often downregulated in T-ALL due to activating NOTCH1 mutations and loss of PTEN has been shown to inhibit PI3K-dependent activation of Akt, promoting tumorigenesis in many cancers [Pamolero et al., 2007; Chalhoub and Baker, 2009].

Not only do these attributes allow for the specific analysis of SHIP1 phosphatase function, but they also mimic the signaling abnormalities found alongside extensively spliced SHIP1 in primary T-ALL [Lo et al., 2009].

3.2 Artificial Construct Expression in Jurkat Requires PMA Stimulation

To analyze SHIP1 function in T-ALL, SHIP1 expression constructs were created using the pcDNA3.1 vector and transfected into Jurkat cells. The pcDNA3.1 vector utilizes the cytomegalovirus (CMV) viral promoter for constitutive expression of the inserted gene and this vector was chosen because Jurkat cells have previously been show to express stronger from the CMV promoter compared to other commonly used viral promoters such as SV40 or RSV [Sutherland and Williams, 1997]. However, upon initial transfection and protein analysis, we found the transfection efficiency from pcDNA3.1 was too low in Jurkat for any functional studies (data not shown). An in-depth literature search suggested that the CMV promoter could be

stimulated with a combination of phorbol 12-myristate 13-acetate (PMA), a diacyglycerol mimic, and the superantigen phytohemagglutinin (PHA) in an NFκB-dependent manner [Sabucetti et al., 1989].

In an effort to continue using pcDNA3.1 constructs for SHIP1 analysis, we transfected Jurkat cells with full length pcDNA3.1-SHIP1 and treated cells with various combinations of PHA and PMA alone or in combination and analyzed cells for SHIP1 protein expression via Western blot. We found that 50ng/ml PMA was very effective at stimulating SHIP1 protein expression from the pcDNA3.1 vector in Jurkat cells while 1µg/ml PHA was only mildly effective (Fig. 2). No additive effect was observed when the two agents were combined. Lower concentrations of PMA and PHA were also tested to determine the sensitivity of CMV promoter stimulation. While 50ng/ml PMA was sufficient to drive CMV expression, 5ng/ml PMA had no effect on expression from the pcDNA3.1 vector (Fig. 3). Concentrations of PHA lower than 1µg/ml were also tested but were shown to be ineffective at driving expression from the CMV promoter (Fig. 3). We then tested higher concentrations of PMA to establish the upper limit of expression efficiency from the CMV promoter and found that expression from the vector did not change in the range of 50-1500ng/ml PMA (Fig. 4). Hence, 50ng/ml PMA was determined to be the ideal stimulation for pcDNA3.1 vector expression in Jurkat cells.

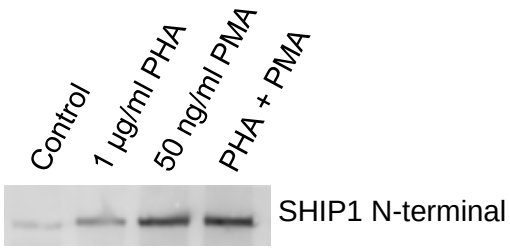

Figure 2: PMA and PHA stimulate the CMV viral promoter in Jurkat cells. Cells were transfected with pcDNA3.1-SHIP1(full length) and stimulated for 18 hours with the indicated concentrations of PMA, PHA, or the combination thereof. Cells were then analyzed for SHIP1 protein expression by Western blot using an N-terminal directed SHIP1 antibody to determine the level of CMV promoter induction. Optimal expression was obtained with 50ng/ml PMA.

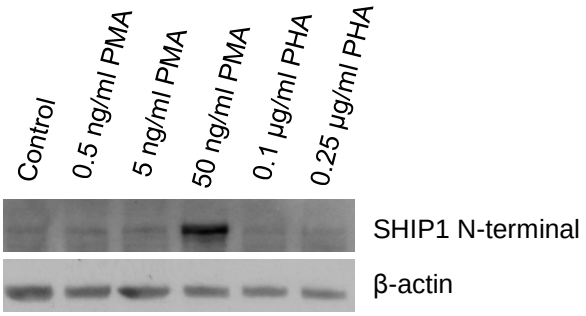

Figure 3: Lower limits of PMA and PHA required for expression from the CMV promoter in Jurkat cells. Cells were transfected with pcDNA3.1-SHIP1(full length) and stimulated for 18 hours with the indicated concentrations of PMA or PHA. Cells were then analyzed for SHIP1 protein expression by Western Blot to determine the level of CMV promoter induction. Concentrations of 5ng/ml or less of PMA and 0.25µg/ml or less of PHA were insufficient to drive construct expression.

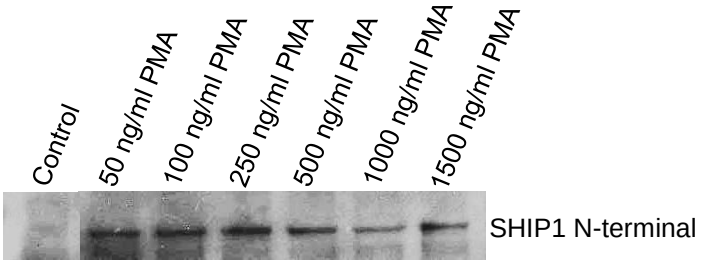

Figure 4: Stimulation of the CMV promoter using high concentrations of PMA. Jurkat cells were transfected with pcDNA3.1-SHIP1(full length) and treated with the indicated concentrations of PMA for 18 hours. Cells were analyzed for SHIP1 protein expression by Western Blot to determine the level of CMV promoter induction. Construct expression was induced but even across the range of 50-1500ng/ml PMA.

3.3 PMA and PHA Arrest the Growth of Jurkat Cells

PMA is a diacylglycerol mimic which works to activate Protein Kinase C and it has been shown to increase the proliferation of normal cells, including B and T lymphocytes [Castagna et al., 1982; van Kooten et al., 1992]. PHA, on the other hand, is a mitogenic lectin which binds the antigenic receptor on lymphocytes [Chilson and Kelly-Chilson, 1989]. It was therefore our concern that treatment of Jurkat cells with PMA or PHA in order to drive pcDNA3.1 construct expression would also cause cellular proliferation, confounding any cell growth assays involving SHIP1 constructs. To determine whether PMA would be a confounding factor in future experiments, we treated Jurkat cells with 1-100ng/ml PMA for 48 hours and assessed the ability of cells to incorporate tritiated thymidine into cellular DNA. The results of this assay were the opposite of what was expected. Treatment of cells with 5ng/ml PMA diminished DNA synthesis down to 20% of the DMSO control and this decreased DNA synthesis remained unchanged with PMA concentrations up to 100ng/ml. (Fig. 5).

To verify these results, we also measured cell proliferation over 24 hours by counting cells using Trypan blue, this time testing both PMA and PHA and using only concentrations below those which stimulated expression from the pcDNA3.1 vector (see section 3.2). Concentrations of PMA and PHA lower than those necessary to drive expression from pcDNA3.1 (under 50ng/ml PMA and 1µg/ml PHA) stimulated significant cell growth compared to the control (Fig. 6). However, treating cells with 50ng/ml PMA or 1µg/ml PHA (the concentrations necessary to stimulate expression from pcDNA3.1) reduced cell growth down to about 25% and 50% of control for PMA and PHA, respectively (Fig. 6), a finding which limited the scope of experiments we could perform when analyzing SHIP1 function in our Jurkat T-ALL model. This counterintuitive and predominantly overlooked influence of PMA on cell growth has in fact been documented and has more recently been attributed to Protein Kinase C activation, downstream

induction of the cyclin dependent kinase inhibitor $p21^{cip1}$ and subsequent G_1 cell cycle arrest [Makover et al., 1991; Detjen et al., 2000].

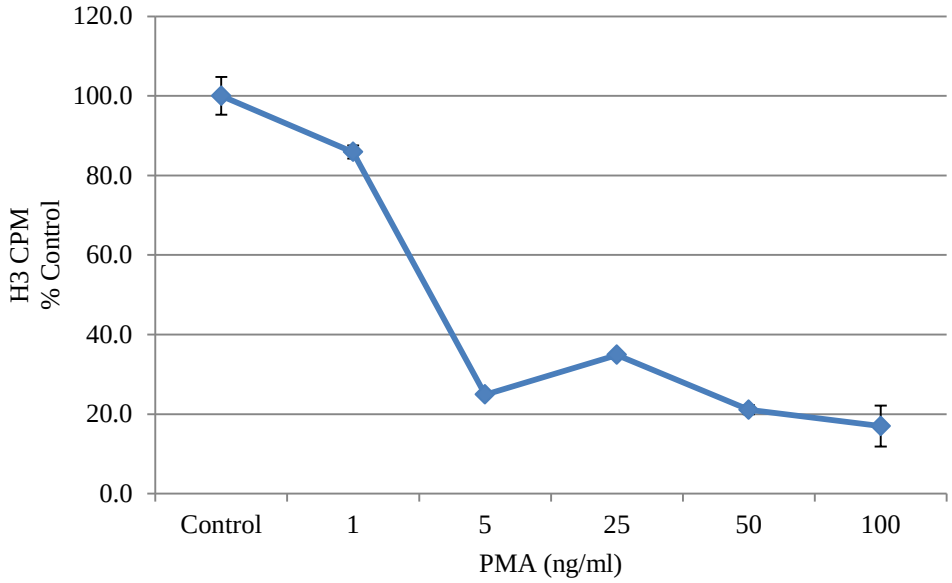

Figure 5: The effect of PMA on DNA synthesis in Jurkat cells. Cells were incubated with PMA concentrations ranging from 1-100ng/ml for 48 hours. Cells were then incubated with tritiated thymidine and analyzed using a liquid scintillation counter to measure uptake of thymidine into cellular DNA. Results are shown as a percent of control. 5ng/ml PMA was enough to reduce DNA replication down to 20% of the DMSO control.

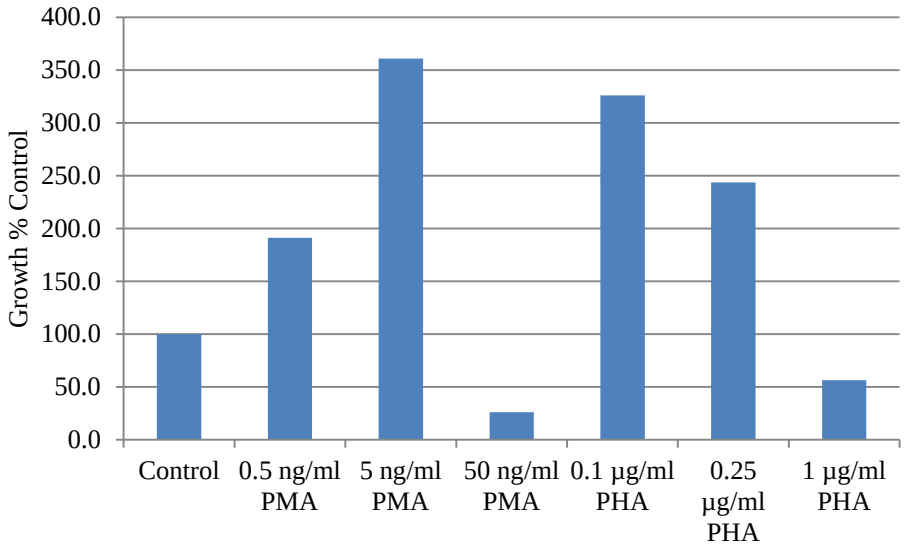

Figure 6: The effect of PMA and PHA on Jurkat cell growth. Cells were cultured with the indicated concentrations of PMA or PHA for 24 hours and counted using Trypan blue to assess viable cell number. Cell growth is represented as percent of control growth over 24 hours. Concentrations of 50ng/ml or 1µg/ml of PMA or PHA, respectively, significantly reduced cell growth while lesser concentrations of each stimulated cell growth.

Chapter 4: Characterization of Extensively Spliced SHIP1 Found in T-ALL

4.1 Background: Separating Fact from Myth

In characterizing the extensively spliced SHIP1 found in T-ALL, an effort was made to separate proposed functions from actual functions. Given the ambiguity of evidence regarding the role of SHIP1 in Akt regulation and its unfair labeling as a direct PI3K antagonist (See Section 1.4.4), alternative roles of SHIP1 were sought out. SHIP1 catalytic function is likely paramount to its function in T cells, but the consequence of its phosphatase activity is probably dependent on its ability to bind certain proteins via tyrosine phosphorylation sites, its polypropyline region, and its SH2 domain. Hence, the consequences of SHIP1 splicing are likely a combination of SHIP1 catalytic activity, its interaction with activators and effectors, as well as its protein stability. In this study, basic properties and functions were assessed for several isoforms of spliced SHIP1 in a hope to understand their role in T cells and in the development of T-ALL.

4.2 SHIP1 Isoform Protein Stability

To characterize spliced SHIP1, expression constructs were created for several of the SHIP1 isoforms found in T-ALL [Lo et al., 2009]. Theses spliced isoforms included full length (FL) SHIP1; SHIP1 with in-frame deletions of exon 8 (dx8), exon 26 (dx26), exons 25 and 26 (dx25x26), or exons 8, 25 and 26 (dx8x25x26); and SHIP1 with a 47 bp deletion in exon 12 (d47bp) which results in a premature stop codon in the same exon. Figure 7 shows the six constructs created and their respective binding domains. Each construct has the SH2 domain of SHIP1 while the number of tyrosine phosphorylation sites and the presence of a polyproline region varies depending on the presence of exons 25 or 26; exon 25 contains one tyrosine phosphorylation site while exon 26 contains the other tyrosine phosphorylation site as well as the polyprolene region of SHIP1. All constructs except for d47bp SHIP1 maintain the phosphatase

domain. To determine whether these spliced SHIP1 transcripts created stable protein, SHIP1 constructs were transfected into Jurkat cells and construct expression was stimulated with PMA. Cells were harvested and analyzed for SHIP1 protein expression by Western blot using an antibody directed toward the amino terminus of SHIP1 (Fig. 8). Full length SHIP1, dx26, dx25x26, dx8x25x26 and d47bp SHIP1 ran with apparent molecular weights of 145kDa, 125kDa, 115kDa, 115kDa and 55kDa, respectively. Interestingly, dx8 SHIP1 produced no stable protein. This result suggests that exon 8 plays a role in SHIP1 protein stability and in-frame removal of exon 8 may be a mechanism for regulating SHIP1 during either normal development or tumorigenesis. It follows that the amino acids encoded by exon 8 do not seem to be part of any promenent functional domains, yet exon was found specifically removed in certain cases of primary T-ALL. However, the issue was complicated by the fact that while dx8 SHIP1 did not form stable protein, the dx8x25x26 SHIP1 construct did. It may be that exon 8 confers protein stability while exons 25 and 26 confer a certain level of instability. This would be in agreement with the observed stability of d47bp SHIP1 which contains exon 8 but not exons 25 or 26. This may also explain why the dx25x26 SHIP1 construct expressed protein more robustly than any of the other SHIP1 constructs while dx8x25x26 SHIP1 expressed weakly compared to full length SHIP1 (Fig. 8). Together, these results suggest that much of the role of SHIP1 splicing in T-ALL may be to regulate SHIP1 activity by affecting protein stability. Though, given the absence of certain binding domains in the exon 25 and 26 deleted SHIP1 isoforms, the overall effect of splicing on SHIP1 activity is likely a combination of this functional domain loss and the affected stability of each protein.

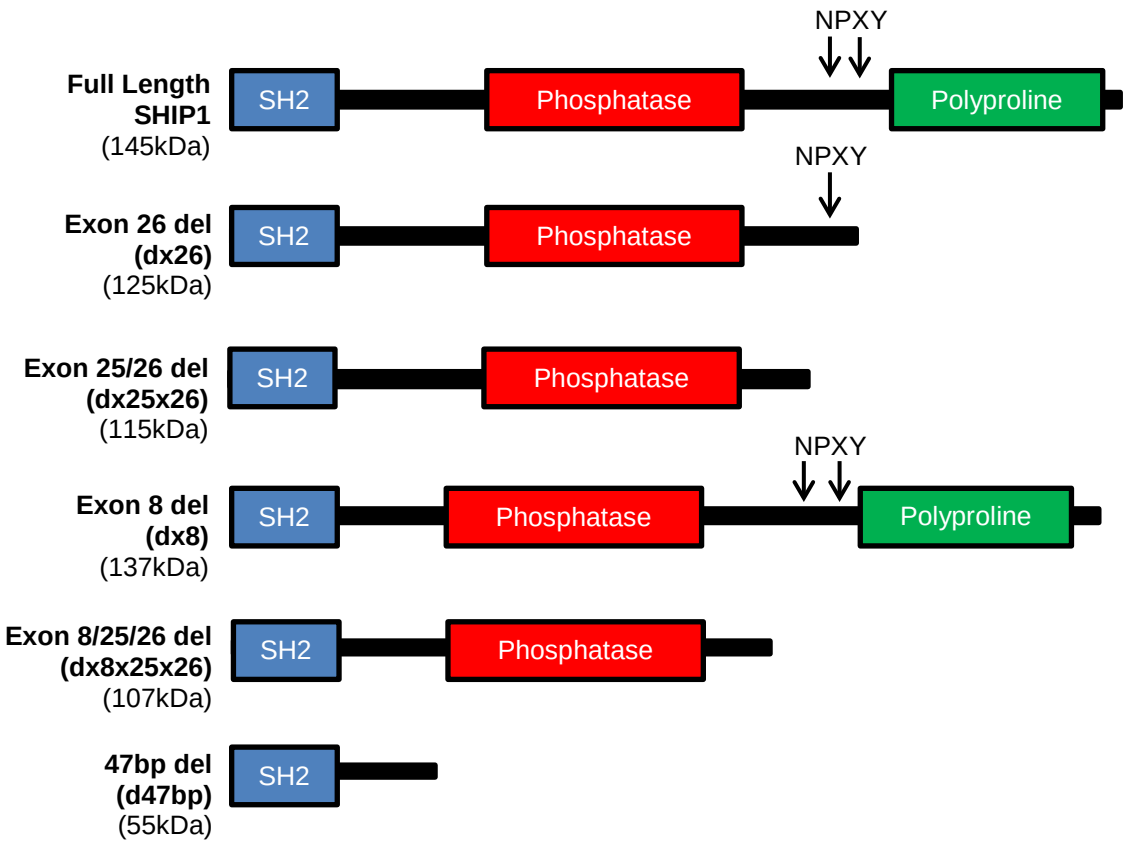

Figure 7: Protein diagram of spliced SHIP1 isoforms cloned from primary T-ALL. Clones were made for full length SHIP1 and five different spliced isoforms. Boxed domains are shown for the SH2 region, the lipid phosphatase enzymatic domain, two possible tyrosine phosphorylation sites (NPXY), and a polyproline region. Protein domains are not necessarily drawn to scale.

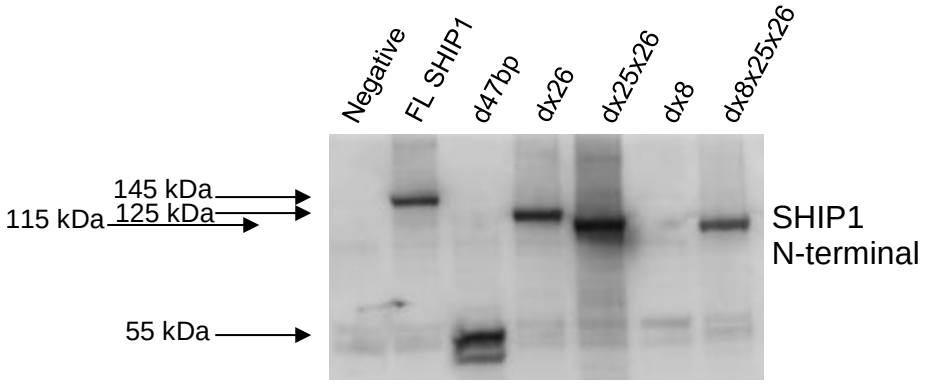

Figure 8: SHIP1 isoform construct expression. Jurkat cells were transfected with SHIP1 constructs for full length (FL SHIP1), exon 12 47bp deleted (d47bp), exon 26 deleted (dx26), exon 25/26 deleted (dx25x26), exon 8 deleted (dx8) or exon 8/25/26 deleted (dx8x25x26) SHIP1 and analyzed for SHIP1 protein expression by Western Blot. SHIP1 protein was detected with an antibody to the amino terminus of SHIP1. Only the exon 8 deleted isoform did not translate into stable protein. The observed molecular weights of SHIP1 isoforms are designated by arrows (left).

4.3 Deletion of Exon 26 Increases SHIP1 Phosphatase Activity

Though this report aimed to focus on roles of SHIP1 outside of regulating Akt phosphorylation, these roles may still rely on the enzymatic activity of SHIP1. Moreover, SHIP1 splicing in T-ALL may serve to regulate this activity. Deletion of the carboxyl terminal of SHIP1 has been shown to increases SHIP1 phosphatase activity [Zhang et al., 2010] and similar findings have been found for the SHIP1 analogue SHIP2 [Prasad et al., 2009]. The findings on SHIP2 describe a mechanism of catalytic autoinhibition involving the amino terminal SH2 domain and the carboxyl terminus, with deletion of the C-terminus being sufficient to increase catalytic activity. As both the SHIP2 study and the SHIP1 study from Zhang et al. focused on SHIP deletions of the entire carboxyl terminus, it remains unclear what carboxyl regions of SHIP1 are directly responsible for catalytic inhibition.

Given that exon 26 of SHIP1 contains the polyproline region along with one tyrosine phosphorylation sites and an exon 26 deleted SHIP1 isoform was found in T-ALL, we wondered whether this exon held a specific catalytic inhibiting function. A modified version of the published Malachite Green assay for phosphatase activity [Zhang et al, 2010] was utilized using $PI(3,4,5)P_3$ as the lipid substrate to test SHIP1 catalytic activity. Dx26 SHIP1 was tested alongside full length SHIP1 and d47bp SHIP1 which is missing the catalytic domain and therefore served as a negative control in the assay. Deletion of SHIP1 exon 26 was sufficient to increase SHIP1 phosphatase activity two fold compared to full length SHIP1 (Fig. 9). D47bp SHIP1 had negligible activity and served to indicate the background signal of the assay. The increased activity of dx26 SHIP1 revealed not only that exon 26 is specifically involved in regulating phosphatase activity but also that certain SHIP1 splicing in T-ALL is functioning to increase this activity. As the cellular function of SHIP1 phosphatase activity is still largely unclear, the implications of an increase in SHIP1 catalytic activity are unknown, though the

overall effect is likely dependent on the different interactive domains of SHIP1 as well as the cellular context in which the protein is placed.

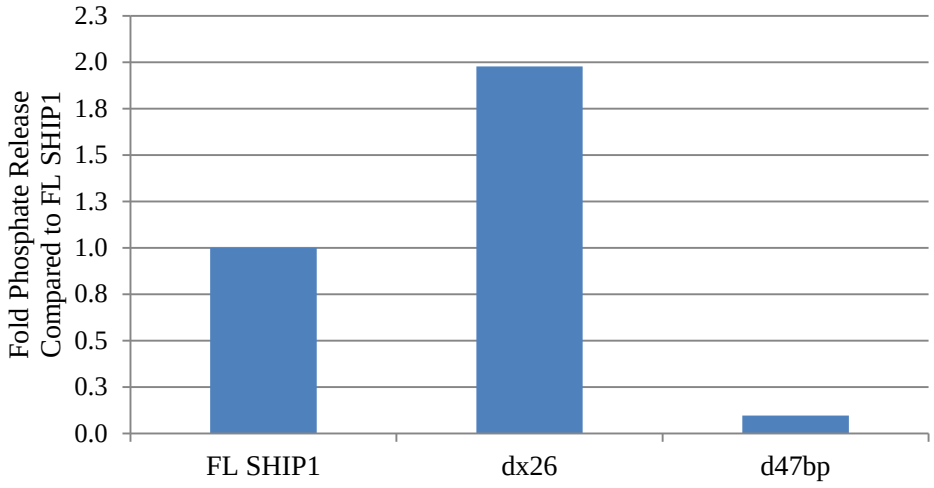

Figure 9: Phosphatase activity of the exon 26 deleted SHIP1 splice variant. Constructs for full length (FL SHIP1), exon 26 deleted (dx26), or 47bp deleted (d47bp) SHIP1 were expressed in Jurkat cells and immunoprecipitated with a polyclonal antibody recognizing the N-terminal of SHIP1. SHIP1 immunoprecipitates were then incubated with PI(3,4,5)P3 substrate and released phosphate was measured using the Malachite Green assay. Phosphate release rates were normalized to the SHIP1 protein amount in the immunoprecipitates as measured by Western blot band quantification. Results are shown as activity compared to full length SHIP1. Dx26 SHIP1 showed twice the catalytic activity of FL SHIP1 while d47bp SHIP1, which is missing the catalytic region, had negligible activity.

4.4 Connecting SHIP1 and Ras in T-ALL

We have reported that Ras is overactive in about 50% of primary T-ALL [Von Lintig et al., 2000], though Ras mutations are rare in the disease [Kawamura et al., 1999]. We wondered if this deregulation was an effect the extensively spliced SHIP1 found in T-ALL [Lo et al., 2009]. Though a link between Ras and SHIP1 has not yet been established in T cells, there is evidence for the involvement of SHIP1 in attenuation of Ras signaling in other hematopoietic cell types. In B cells, SHIP1 has been documented to interact with the adaptor protein DOK-1 to mediate Ras activation in FcγRIIB activation [Tamir et al., 2000]. SHIP1 has also been shown to negatively regulate Ras/ERK signaling in macrophages upon Toll-Like Receptor 4 (TLR4) stimulation [An et al., 2005] as well as Fcγ receptor stimulation [Ganesan et al., 2006]. As the Toll-like receptors (TLRs) and Fcγ receptors activate cell signaling patterns similar to those activated upon TCR signaling, we hypothesized that SHIP1 may be functioning in T cells to negatively regulate TCR-induced Ras signaling.

4.4.1 Creating Stable Clones to Study Ras and SHIP1 in T-ALL

The necessity to use PMA to stimulate SHIP1 construct expression in Jurkat cells confounded looking at Ras activation because PMA mimics the signaling molecule diacyglycerol, potently activating Ras [Ebinu et al., 2000]. To overcome this limitation, we created Jurkat clones which stably expressed spliced SHIP1 isoforms. SHIP1 isoform constructs were transfected into Jurkat cells and subjected to antibiotic selection for 3 weeks wherafter antibiotic-resistant cells were clonally selected and analyzed with and without PMA stimulation for SHIP1 protein expression. Stable clones were generated which expressed full length (SHIP1 FL); exon 26 deleted (dx26) SHIP1; or exon 8, 25 and 26 deleted (dx8x25x26) SHIP1, though only the full length SHIP1 clone expressed the protein without PMA stimulation (Fig. 10). Future efforts will continue to focus on a way around the PMA limitations, such as using viral methods for preparing

stable clones or the use of timed PMA pulses to stimulate SHIP1 production without affecting assay results. As we generated at least one clone to express full length SHIP1 without PMA stimulation, we were able to test whether SHIP1 attenuates Ras in T cells.

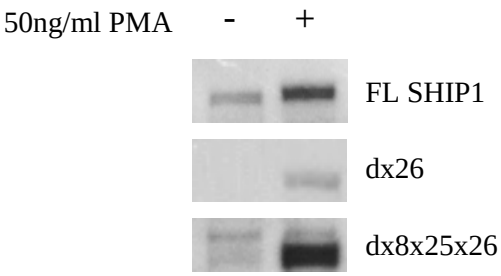

Figure 10: Stable SHIP1 expressing Jurkat clones. After SHIP1 isoform transfection and clonal selection under 400µg/ml G418 for 3 weeks, clones were treated with or without 50ng/ml PMA for 24 hours and protein expression was determined by Western blot, probing for SHIP1 using an antibody directed toward the amino terminus of SHIP1. Clones were generated which expressed full length SHIP1 (FL SHIP1), exon 26 deleted (dx26) SHIP1, or exon 8/25/26 (dx8x25x16) deleted SHIP1, though only the clone expressing full length SHIP1 expressed significant protein without PMA stimulation.

4.4.2 SHIP1 Negatively Regulates Ras Activity in Jurkat Cells

To test our hypothesis that SHIP1 may be regulating Ras in T cells, Jurkat cells which stably expressed SHIP1 (FL SHIP1) or a vehicle control (control) were assayed for Ras activity under untreated or TCR-stimulated conditions. TCR signaling was induced by treating cells for 30 minutes with beads coated with antibodies to the cell surface molecules CD3 and CD28, costimulation of which mimics TCR stimulation by an antigen. Ras activity was measured using the Ras Activation Assay Kit from Millipore, a method which isolates active, GTP-bound Ras and compares it via immunoblot to total Ras levels. Ras activity was quantified by measuring band intensities of GTP-bound Ras and total Ras and normalizing the levels of GTP-bound Ras to total Ras for each experimental condition. Fold Ras activity as compared to untreated control cells is depicted in a bar graph (Fig. 11). Cells expressing SHIP1 had reduced overall Ras activity compared to control cells, though we did not observe specific TCR-induced Ras inhibition (Fig. 11). To our knowledge, this is the first report of SHIP1 inhibiting Ras in T cells. Our observations suggest that in T cells, SHIP1 does not function to regulate TCR-specific Ras signaling but rather overall levels of Ras activity. It remains unclear, however, how SHIP1 is precisely involved in the attenuation of such Ras signaling. There are a number of undefined growth factors in the serum-supplemented cell culture media, any one of which could be responsible for recruiting SHIP1 to attenuate Ras. Future experiments with our spliced SHIP1 constructs will focus on whether SHIP1 requires its central phosphatase domain or carboxy-terminal tyrosine phosphorylation sites and polyproline region to regulate Ras. This information may lead to the discovery of proteins which SHIP1 is interacting with in order to attenuate Ras, and subsequently the cellular context and mechanism by which SHIP1 is recruited.

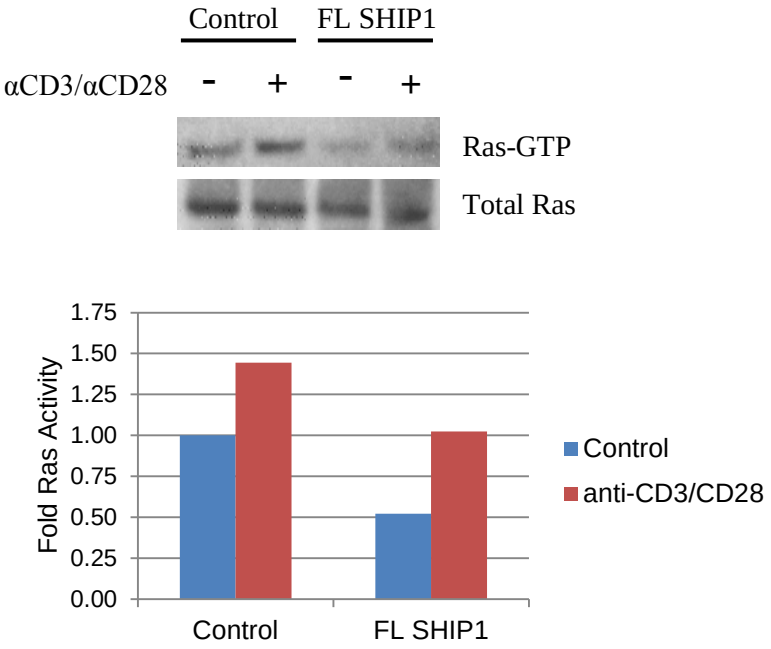

Figure 11: SHIP1 inhibits Ras activity regardless of TCR stimulation. Jurkat cells stably expressing full length SHIP1 (FL SHIP1) or a vehicle control (Control) were stimulated for 30 minutes with beads coated with anti-CD3 and anti-CD28 antibodies to activate TCR signaling. Active, GTP-bound Ras was pulled down with Raf-1 RBD agarose and immunoblotted alongside total protein, detecting with an anti-Ras antibody. Active Ras was quantified by measuring band intensities of Ras-GTP and normalizing these values to Ras levels in total protein. The graph below depicts fold Ras activity compared to untreated control cells. Ras activity was reduced by the presence of full length SHIP1 both in untreated and CD3/CD28 stimulated conditions.

Chapter 5: Pathway Inhibition in the Treatment of T-ALL

5.1 Background: Targeting Ras and PI3K/Akt in T-ALL

Though current treatment methods in ALL have achieved nearly an 80% event-free survival rate for patients, cases of T-ALL continue to have higher relapse rates than those of B-ALL. The occurrence of minimal residual disease is higher in T-ALL than B-ALL and correlates with poor prognosis [Willemse et al., 2002]. The persistence of residual leukemic stem cells may be attributed to Ras deregulation, as mutational activation of Ras has been shown to deregulate signaling and induce hyperproliferation of hematopoietic progenitor cells [Van Meter et al., 2007]. Ras is therefore a prime target for therapeutics treating severe or relapsed cases of T-ALL. Likewise, the frequent PTEN downregulation and subsequent PI3K-dependent Akt activation seen in T-ALL make PI3K and Akt attractive targets as well [Gutierrez et al., 2009].

5.2 Synergistic Effects of FTS and LY294002 in Jurkat

Along with their frequent deregulation in T-ALL, the inherent cross-talk between the Ras/Erk and PI3K/Akt pathways further support their dual targeting in the treatment of ALL. We therefor considered targeting the two pathways in combination as an efficacious new approach to T-ALL therapeutics. Jurkat cells were treated with various combinations of S-trans,trans-farnesylthiosalicylic acid (FTS), an agent which works to inactivate Ras by dislodging it from the cell membrane, and LY294002, a potent but reversible inhibitor of PI3K which prevents Akt activation by depleting the $PI(3,4,5)P_3$ substrate pool. After 48 hours of drug treatment, cell metabolism and viability were assessed by XTT assay. The XTT assay is a derivative of the MTT assay and it measures the reduction of tetrazolium salts to determine overall mitochondrial function and therefore cell viability. Results are shown as percent XTT activity compared to untreated cells (Fig. 12). Synergy between FTS and LY294002 was then determined using the

Loewe additivity model [Straetemans et al., 2005]. A Loewe interaction index of less than one indicates synergy while an index of 1 indicates only an additive effect. For a detailed description of the Loewe additivity model, see Materials and Methods. Treating Jurkat cells with either 50µM FTS or 10µM LY294002 alone resulted in a 30% reduction of viability (Fig. 12). Maximal effect on viability was achieved with 100µM FTS or 50µM LY294002, while a combination of 50µM FTS and 10µM LY294002 resulted in reduced viability which when applied to the Loewe model yielded an interaction index of approximately 0.7, showing a synergistic effect between FTS and LY294002 at these concentrations.

Since the 5 year event-free survival rate for T-ALL patients on modern treatment protocols is around 75% [Goldberg et al., 2003], there is little demand to add new therapeutics to standard treatment. However, only 10-50% of relapsed cases of ALL are curable [Bailey et al., 2008] and targeting Ras and Akt together may be useful in treating cases which relapse or do not respond to traditional chemotherapy.

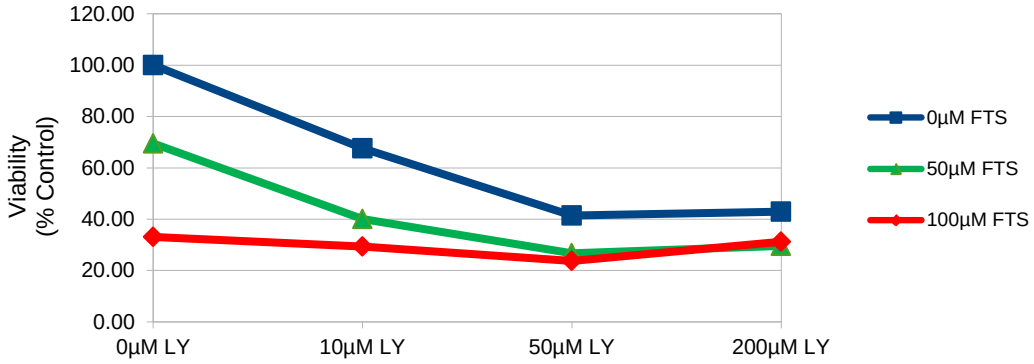

Figure 12: Synergistic effect of FTS and LY294002 in Jurkat. Jurkat cells were incubated with the indicated concentrations of the Ras inhibitor S-trans,trans-farnesylthiosalicylic acid (FTS) and the PI3K inhibitor LY294002 (LY) alone or in combination for 48 hours. Cell viability was measured by XTT assay. Viability is shown as a percent of untreated cells. A combination of 50µM FTS and 10µM LY294002 yielded a Loewe interaction index of about 0.7, indicating synergy between the two compounds.

Chapter 6: Setting Sail: The Future Voyages of SHIP1

6.1 Fine Tuning of a T-ALL model for Spliced SHIP1

The majority of the work in this study contributed to the development of a model to appropriately analyze SHIP1 in T-ALL. The conditions necessary to express our spliced SHIP1 constructs in the Jurkat cell model were determined and optimized, as were assays for characterizing multiple levels of SHIP1 function. Along the way, we also discovered the inherent limitations of the PMA-dependent model. Mainly, PMA induced both the Ras/Erk and the PI3K/Akt pathways and thereby masked any effects SHIP1 may have had on either pathway. For this purpose, stable SHIP1 positive Jurkat clones were created to facilitate SHIP1 characterization. Future studies will focus on generating and using Jurkat clones which stably express other SHIP1 isoforms to clarify the role of SHIP1 in T lymphocyte function and T-ALL.

6.2 Relevance of SHIP1 Splicing in T-ALL

Given the prevalence of extensive SHIP1 splicing found in primary T-ALL, it is logical that SHIP1 may contribute to a disease phenotype. We have shown that SHIP1 isoforms with intact phosphatase regions can have different catalytic activities depending on truncations of the C-terminus. Furthermore, evidence in this study suggests that full length SHIP1 is a regulator of Ras in T cells. Depending on the spliced isoform, alternatively spliced SHIP1 may or may not have the binding domains necessary to attenuate Ras signaling, which may still be dependent on SHIP1 phosphatase activity. Hence, the binding properties and catalytic activities of the different spliced isoforms of SHIP1 likely contribute to very specific functionalities, each with a distinct role in cell signaling. Because of crosstalk in mitogenic signaling, deregulation due to extensively spliced SHIP1 is complicated and likely involves the combination of all present SHIP1 isoforms.

Finally, inhibition of the Ras/Erk and PI3K/Akt pathways revealed synergistic targets in the treatment of T-ALL. Along with current treatment, dual Ras and PI3K inhibition may prove useful in preventing relapse in cases of T-ALL with poor prognosis. Future studies involving more spliced SHIP1 stable Jurkat clones will determine if this sensitivity is due to the loss or extensive splicing of SHIP1.

6.3 Looking Forward

The end goal of characterizing extensively spliced SHIP1 in T-ALL is to be able to use SHIP1 status to predict the specific pathways affected in T-ALL and target them accordingly. With a T-ALL model for SHIP1 in place, future work will focus on the effects of different spliced SHIP1 isoforms on Ras activity. Specifically, it is hypothesized that deletion of exon 26 of SHIP1 is sufficient to relieve SHIP1 of its function as a regulator of Ras. There is also interest to look at proteins downstream of Ras in the Ras/Erk cascade, including Raf-1 and Erk. The complex feedback mechanisms in this cascade suggest SHIP1 may function to regulate this pathway at more than one level. It remains to be seen if poor prognosis within T-ALL can be linked to excessive splicing or loss of SHIP1, but future studies connecting SHIP1 status and T lymphoid progenitor markers may reveal that improper splicing of SHIP1 at a specific stage of development contributes to leukemogenesis.

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