THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

ROLE OF TEC FAMILY KINASES IN REGULATING MAP KINASES

HONG-HANH M. KHONG
Spring 2010

A thesis
submitted in partial fulfillment
of the requirements
for baccalaureate degrees
in Immunology and Infectious Diseases and Toxicology
with honors in Immunology and Infectious Diseases

Reviewed and approved* by the following:

Avery August
Professor of Immunology
Thesis Supervisor

James Endres Howell
Coordinator of Undergraduate Advising
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

Abstract

Itk and Txk are TEC family kinases that are important effectors found downstream of the T cell receptor which are involved in T cell signaling, function, and differentiation. While they appear to share homologous domains towards their carboxy-terminals, these two kinases are differentiated by their N-terminal domain. Txk is characterized by a palmitoylated series of cysteine residues which allows it to be constitutively associated with the plasma membrane. Itk, on the other hand, must be recruited to the plasma membrane via its PH domain upon TCR activation. It is thought that these domains played a role in facilitating the activation of the MAPK pathway by affecting kinase localization. In order to evaluate their differential roles in regulating the MAPK pathway, chimera constructs were generated by swapping the N-terminus of these two kinases. The study determined that Itk and PH-Txk phosphorylate PLC-γ at profoundly higher levels than Txk and Cys-Itk. While Itk may lead to greater levels of ERK phosphorylation than TXK, these differences in MAPK activation were not nearly as significant as those observed for phosphorylated PLC-γ. Additionally, there was no difference between the wild type kinases and their respective chimera constructs in their ability to phosphorylate ERK. Lastly, it was determined that Itk played a role in cell proliferation not only during T cell activation but during resting state as well. Thus, it was determined that the PH domain is critical in facilitating Itk's function to phosphorylate PLC-γ but not to activate MAPKs; Txk may activate the MAPK pathway via a compensatory mechanism that is independent of PLC-γ and finally, ITK may be important for cell survival and growth during resting state.

Table of Contents

Section	Page
Chapter 1: Introduction	1
1.1: Figures	6
Chapter 2: Materials and Methods	8
2.1: Cell lines	9
2.2: Media	9
2.3: Buffers	9
2.4: Transfection of HEK 293T cells	10
2.5: Generation of SH-ITK Jurkat E6.1 cells	11
2.6: Lysis of transfected cells	11
2.7: SDS-PAGE	11
2.8: Western Blotting	12
Chapter 3: Results	14
3.1: ITK leads to greater phosphorylation of ERK than TXK	15
3.2: The PH and Cys domains of ITK and TXK are not critical in ERK activation	17
3.3: The PH domain of ITK plays a critical role in positioning TEC-family kinases to phosphorylate PLC-γ	18
3.4: ITK may be important for cell survival and/or proliferation	20
Chapter 4: Discussion	21
Chapter 5: Conclusion	27
References	29

Acknowledgements

First and foremost, I have to express my sincerest gratitude to Dr. Avery August for giving me the opportunity to conduct research in his laboratory. It has been an absolute privilege and I cannot thank you enough for your patience, support, and guidance. Needless to say, I could never have finished this thesis on time without your encouragement and flexibility.

I must also thank Arun Kunnan who has literally taught me every lab technique that I've utilized to complete this thesis. Despite the fact that I came into this lab with absolutely no biomolecular lab experience, you still patiently walked me through every step of the process—multiple times if I had to troubleshoot a problem. I cannot express enough how invaluable your role has been in this entire process.

To Luis, who always offered numerous ways to troubleshoot a problem, your input has always been helpful. And equally as important, I know I can always count on you to make a trip to the Creamery whenever things don't go perfectly well—which was quite often. I'm sorry if I've increased your chances of becoming diabetic but at least we temporarily kept our sanity. Perhaps the next time around, we'll find a healthier outlet.

To Weishan, you always have an amazing attitude which keeps the rest of the lab going. Thank you for staying in lab with me until all hours of the night and for your tremendous help in quantifying my data and converting the data into something remotely logical. You'll always be my favorite lunch/dinner/linner buddy.

And last, but certainly not least, Dr. Howell: thank you for that final chat in your office. You already know how much your guidance has meant to me throughout these years. So all I can say is: domo arigato.

I would also like to thank the Schreyer Honors College for their financial support via the Career Development Summer Research Grant.

List of Abbreviations

Cys-Itk	Itk containing a cysteine residue rather than a PH domain at its N-terminus
DAG	Diacylglycerol
ERK	Extracellular signal regulated kinase
IP3	Inositol-1,4,5-triphosphate
ITK	Interleukin-2 inducible T-cell kinase
LAT	Linker for activation of T cells
MAPK	Mitogen activated protein kinase
PI3K	Phosphatidylinositol-3,4,5-triphosphate
pERK	Phosphorylated ERK
pPLC-γ	Phosphorylated PLC-γ
PH	Pleckstrin-homology domain
PH-Txk	Txk containing a PH domain rather than cysteines at its N-terminus
PI3K	Phosphotidol-inositol 3 kinase
PIP2	Phosphotidol-inositol-4,5-diphosphate
PIP3	Phosphotidol-inositol-3,4,5-triphosphate
PLC-γ	Phospholipase C- γ
PRR	Proline-rich regions
RLK/TXK	Resting lymphocyte kinase/Tyrosine-protein kinase
SH	Src-homology domain
SLP76	SH-2 domain-containing leukocyte protein of 76 kDa
TEC	Tyrosine kinase expressed in haematopoietic cells

Chapter 1: Introduction

Itk and Txk are TEC-family protein kinases that are key players in downstream signaling of the T cell receptor2. While both kinases are characterized by homologous domains including a proline-rich region (PRR), an SH3 domain, SH2 domain, and a kinase domain at their carboxy-terminal tails, they differ extensively at their amino-terminus22 (Figure 1.1). Txk is characterized by a palmitoylated series of cysteine residues which allows it to be constitutively associated with the plasma membrane4,7. Itk, on the other hand, must be recruited to the plasma membrane via its PH domain upon TCR activation1, 22 (Figure 1.2).

In fact, Itk requires two inputs for activation: recruitment to the plasma membrane and phosphorylation of its tyrosine residue[1,3,5,22] (Figure 1.3). Upon T cell activation, the Src family kinase Lck phosphorylates phosphotidol inositol 3 kinase (PI3K)[5]. Consequently, the activated PI3 kinase converts phosphotidol inositol-4,5-diphosphate (PIP2) into phosphotidol inositol-3,4,5-triphosphate (PIP3)[17]. Both Itk and PLC-γ are then recruited to the membrane and bind to PIP3 via their PH domains[1]. Once at the membrane, both Itk and Txk may become phosphorylated by Lck, ultimately leading to their activation. At this stage, Itk becomes associated with LAT and SLP76,[23] both of which are adaptor proteins that serve as docking sites for PLC-γ, among a myriad of other proteins. Recruitment of PLC-γ to this complex allows it to be phosphorylated by Itk [20]. Txk has also been shown to be involved in PLC-γ mediated pathways; however, it is unknown whether or not Txk associates with LAT and SLP76 to phosphorylate PLC-γ. Nonetheless, Itk and Txk activation are thought to lead to PLC-γ phosphorylation. This central player acts as an enzyme that converts PIP2 into IP3 (inositol-1,4,5-triphosphate) and DAG (diacylglycerol); whereas IP3 is responsible for regulating intracellular calcium

stores, DAG is ultimately responsible for activation of mitogen activated protein kinases (MAPK) including IκB, JNK, and ERK1/2[17]. Together, these two pathways lead to the transcription of essential genes that regulate T cell responses including cytokine production and T helper cell differentiation.

Accordingly, Tec-family kinase deficient mice exhibit impaired PLC-γ phosphorylation, MAPK activation and defects in Ca^{2+} influx, resulting in a series of developmental and functional defects[22]. $Itk^{-/-}$ and $Txk^{-/-}Itk^{-/-}$ mice experience decreased positive selection of T cells, impaired activation and proliferation of T cells with corresponding decreases in IL-2 production, defects in actin polymerization and cellular adhesion and also an improper balance of $CD4^+$ and $CD8^+$ cells[9,10,11,12,15,22]. Deregulation of these two T cell subsets ultimately results in an increased susceptibility to infections from impaired immune function as well as autoimmunity[6].

Despite the central role of Itk and Txk in TCR signaling, their differential roles in regulating T cell function and differentiation remains poorly understood. Whereas Itk and Txk are expressed in all T cell subsets, they differ significantly in terms of their preferential expression levels in T_{H1} and T_{H2} cells. While Itk appears to be the primary TEC kinase expressed in naïve T cells, Txk is expressed at 3-10 times lower levels[13]. Likewise, Itk is expressed in both T helper subsets with preferential increases in T_{H2} populations. In contrast, T cell activation leads to a rapid down-regulation of Txk expression which is only reconstituted in T_{H1} differentiated cells[16]. To further substantiate the data on Itk/Txk expression patterns, studies on $Itk^{-/-}$ mice showed moderately severe defects in all T cell functions (Table 1) whereas, the only observable

defect in Txk$^{-/-}$ mice is a minor decrease in IL-2 production[8,19,20,21]. Thus, Itk appears to be the major player in T cell function.

The divergence between Itk and Txk extend beyond their structural differences and expression patterns. Early studies also suggest that Itk and Txk specifically regulate T_{H2} and T_{H1} differentiation, respectively[22]. For example, Itk$^{-/-}$ mice cannot mount a T_{H2} response to T_{H2} inducing parasites[9]. Additionally, it has been shown that the *Itk* promoter region contains multiple binding sites for GATA3, a T_{H2} transcription factor[16]. Txk, on the hand, has no such binding regions; instead, it is found to bind directly to the IFN-γ promoter thereby driving a T_{H1} response[24]. Overexpression of Txk has been shown to increase IFN-γ levels with no apparent increases in T_{H2} cytokines[14]. Given this data, Itk and Txk appear to play dichotomous roles in T cell differentiation, with each TEC-family kinase specifically inducing a T_{H2} or T_{H1} response, respectively.

However, more recent evidence suggests that Itk and Txk actually play much more redundant roles than previously thought. In a study by Sahu, et. al., Txk was shown to be able to rescue T_{H2} responses in Itk$^{-/-}$ mice if Txk was expressed at levels similar to Itk in T_{H2} cells[18]. At physiologically relevant levels, this Txk-induced T_{H2} response was observed without significant increases in IFN-γ production, a T_{H1} cytokine. Thus, the study concluded that rather than Txk specifically inducing a T_{H1} response, the selective expression levels of Itk and Txk dictate the type of T helper response observed. Furthermore, mice that are deficient in both Itk and Txk show severe perturbation of cellular functions and biochemical processes[19,20]. These defects are synergized in comparison with Itk$^{-/-}$ and Txk$^{-/-}$ mice. Thus, this data recapitulates the idea that there is a

redundancy in the roles of Itk and Txk in T cell function rather than each kinase having its distinct role.

Since these TEC-family kinases share a role in regulating T cell function, a probing question remains: “How do Itk and Txk differentially regulate the MAPK pathway given their structural differences?” Whereas Itk must be recruited to the membrane before it can become activated by Lck, it is thought that the constitutive anchoring of Txk to the plasma membrane may be more easily phosphorylated by Lck. Thus, Txk may be in a better position to phosphorylate PLC-γ and activate the MAPK pathway than Itk. However, given the data on Itk and Txk knock-out mice, the severe defects observed in $Itk^{-/-}$ mice suggest that Itk, rather than Txk, plays a larger role in regulating the MAPK pathway. To address this issue, chimera constructs of each TEC-family kinase were generated (Figure 1.4). The amino terminal regions of each kinase were exchanged such that Itk contained a cysteine residue and Txk contained a PH domain. If the PH domain of Itk and the cysteine domain of Txk are solely responsible for their functional differences, we should expect to see PH-Txk phosphorylating PLC-γ and ERK at the same levels as Itk. Likewise, phosphorylation by Cys-Itk and Txk should be at comparable levels.

1.1 Figures

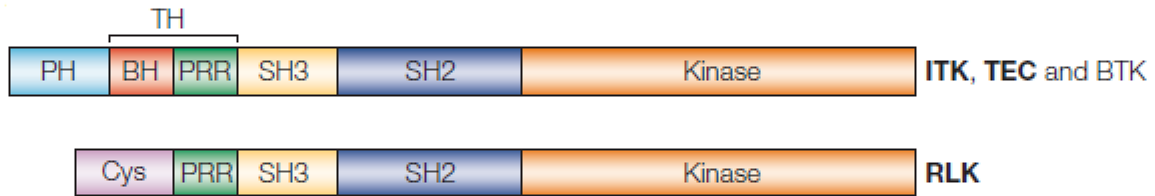

Figure 1.1: Structural Domain Organization of Itk and Rlk/Txk*
TEC-family kinases Itk, Tec, and Btk all share homologous domains comprising of a pleckstrin homology segment, a proline rich region, a src-homology 3 domain, src-homology 2 domain and finally a kinase domain. Rlk/Txk, on the other hand, possesses a palmitoylated string of cysteine residues in place of the pleckstrin homology domain.

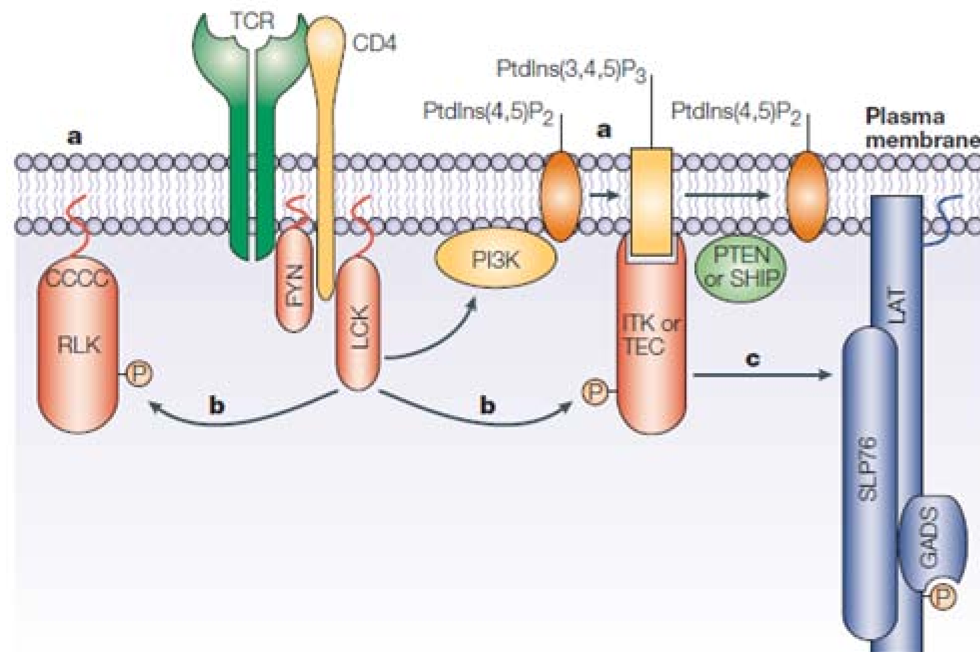

Figure 1.2: Membrane localization of Txk vs. Itk*
Because of the structural differences between Txk and Itk, their requirements for membrane localization differ as well. Whereas the PH domain of Itk requires it to be recruited to PIP3 at the plasma membrane, the cysteine residue of Txk allows it to be constitutively associated with lipid rafts on the membrane.

* Source: Schwartzberg, P.L., et. al. TEC-family kinases: Regulators of T-helper-cell differentiation. *Nature Immunol*. 5: 282-295.

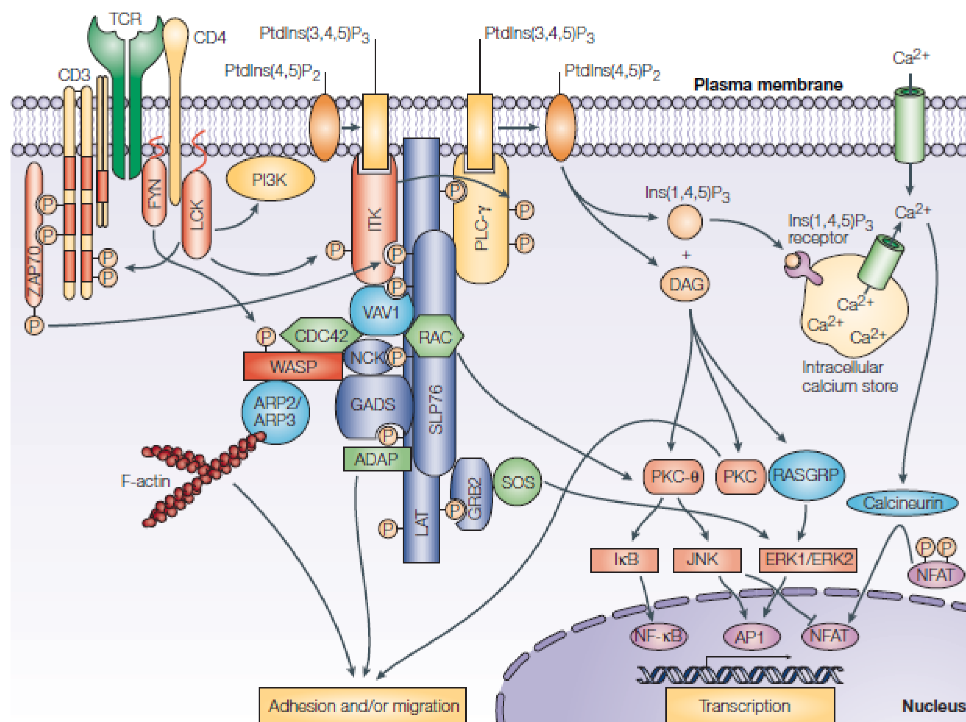

Figure 1.3: Recruitment, Activation and Downstream Signaling of Itk and Txk*
Upon T cell activation, the Src-family kinase, Lck, phosphorylates PI3K thereby activating its kinase activity. PI3K can then convert PIP2 to PIP3. This allows Itk and PLC-γ to be recruited to the membrane. Lck is also responsible for phosphorylating the ITAM motifs of the T cell receptor which allows ZAP70 to be recruited to the TCR complex. Phosphorylation of ZAP70 by Lck allows it to phosphorylate SLP76 which serves as a docking site for Itk and PLC-γ. At this point, Lck can phosphorylate either Txk or Itk. TEC kinase activation leads to PLC-γ phosphorylation which converts PIP2 into IP3 and DAG. Whereas IP3 facilitates calcium dependent cellular functions, DAG mediates the activation of a number of signaling effectors—among them is ERK, a key player of the MAPK pathway. Thus, PLC-γ is essentially a key regulator of intracellular calcium signaling and MAPK pathway activation whose activation depends on TEC-family kinase activity.

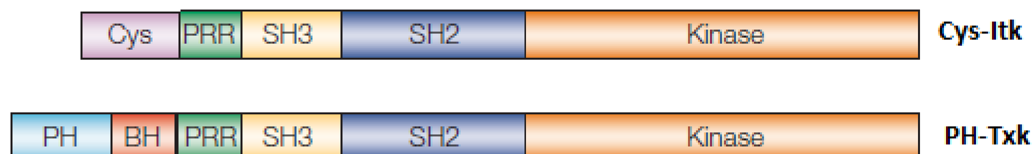

Figure 1.4: Structural Domains of TEC-family Kinase Chimera Constructs*†
Chimera constructs were generated in which the cysteine residues of Txk were swapped for the pleckstrin homology domain of Itk. Thus, Cys-Itk no longer contains its PH domain; instead, it possesses a cysteine residue which allows it to be constitutively anchored to the plasma membrane. PH-Txk, on the other hand contains the pleckstrin homology domain which requires it to be recruited to the plasma membrane and bind to PIP3.

---

†Source: modified from Schwartzberg, P.L., et. al. TEC-family kinases: Regulators of T-helper-cell differentiation. *Nature Immunol.* 5: 282-295.

Chapter 2: Materials and Methods

2.1 – Cell Lines and Plasmids:

Human Embryonic Kidney 293T cell line and Human T-cell line Jurkat E6.1 was obtained from ATCC. shITK were obtained from Sigma Aldrich. Plasmids coding for Itk, Txk, Cys-Itk, PH-Txk, and PLC-γ were established by Arun Kunnan in the Department of Veterinary and Biomedical Sciences at the Pennsylvania State University. $Itk^{-/-}$ and $Txk^{-/-}$ Jurkat cells were also establish by Arun Kunnan.

2.2 Media:

HEK 293T cells were maintained in DMEM (10% BS, v/v) including 10% (v/v) deactivated fetal bovine serum, 1% (v/v) L-glutamine, 1% (v/v) Penicillin/Streptomycin, 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, and 0.5% (v/v) HEPES buffer.

Jurkat E6.1 cells were cultured in RPMI-1640 (5% FBS, v/v) which included 5% (v/v) fetal bovine serum, 1% (v/v) Penicillin/Streptomycin, 1% (v/v) sodium pyruvate, 1% (v/v) non-essential amino acids, and 0.5% (v/v) HEPES buffer.

2.3 Buffers

2x reducing buffer: 12.5 mL 4x SDS/Tris pH 6.8, 10.0 mL glycerol, 10.0 mL 20% SDS (w/v), 0.5 mg bromophenol blue, and 1 mL β-mercaptoethanol were brought to a total volume of 50mL in ddH_2O.

Lysis buffer with phosphatase inhibitors: 50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 10mM sodium vanadate, 10 mM NaF, 10 mM PMSF, 1% NP40, 1% sodium

deoxycholate, 0.1% SDS, 10% glycerol, 80 μL 25x Roche protease inhibitor and 1μL 1000x pepstatin were brought to a total volume of 1mL in ddH_20.

Running Buffer for SDS-PAGE: 2.5mM Tris Base and 25mM glycine were brought to a total volume of 1.0L in ddH_2O and adjusted to pH 8.3.

Stripping buffer: 6.25 mL of 500 mM Tris HCl at pH 6.8, 10 mL of 10% SDS and 350 μL of β-mercaptoethanol were brought to a total volume of 50 mL in ddH_2O.

TBS/Tween: 2.5mM Tris·HCl added to 154mM of NaCl and 0.1%Tween were brought to a volume of 1L in ddH_2O.

Transfer Buffer for SDS-PAGE: 2.5mM Tris Base and 19.2mM glycine were brought to a total volume of 1.0L in ddH_2O and adjusted to pH 8.3.

2.4 Transfection of 239T cells

Using 6-well plates, $3.0x10^5$ 293T cells were plated per well in 2 mL DMEM and were incubated overnight at 37°C. In Eppendorf tubes, 3μL of trans-17-293 reagent from Mirus were added to 100μL of serum-free DMEM for each well. After vortexing and incubating at room temperature for 20 minutes, 1 μg of each plasmid (Itk, Txk, cys-Itk, PH-Txk or PLC-γ) were added separately to the trans-17-293 reagent, slowly mixed by pipetting and incubated at room temperature for another 30 minutes. In wells containing both PLC-γ and Itk, Txk, cys-Itk or PH-Txk, 0.5 μg of each plasmid were added for a total of 1 μg. Cells were then transfected with the plasmid(s) and incubated at 37°C for two days. The transfection was done in duplicates such that half of the wells could be starved after those 2 days in serum-free DMEM for 4 hours.

2.5 Generation of SH-ITK Jurkat E6.1 cells:

SH-ITK obtained from Sigma Aldrich was packaged into a lentiviral vector which was transfected into 293T cells. Virus particles and genome containing the SH-ITK were subsequently synthesized and packaged within the 293T cells. Supernatant containing the viruses was collected and used to tranduce Jurkat cells in order to silence the *Itk* gene.

2.6 Lysing Cells

Cells were harvested from each well, pelleted down at 12,000 rpm for 2 minutes and resuspended in 50 μL of lysis buffer. Samples were left on ice for 15 minutes and centrifuged down again at 13,000 rpm in 4^oC for 10 minutes. The supernatant was collected and reduced in 500 μL of 2x reducing buffer at 90-100^oC for 10 minutes. Samples were either stored at -80^oC or run in SDS-PAGE gel.

2.7 SDS-PAGE

Cell lysates were loaded into 10% SDS-PAGE gels and run at 100V for 2.5 hours in running buffer. Prior to transfer, membranes were equilibrated in transfer buffer for 10-20 minutes. Likewise, polyvinylidene difluoride membranes were soaked in methanol for 10-20 seconds before being equilibrated in transfer buffer for 20 minutes. Proteins were transferred electrophoretically at 20V overnight at 4^oC onto a PVDF membrane in transfer buffer with rapid stirring.

2.8 Western Blotting

Following transfer of proteins from SDS-PAGE onto the PVDF membrane, the membranes were subsequently rinsed in ddH_2O, soaked in methanol for 20 seconds, allowed to air dry, rewashed in methanol until membrane was completely wet and stored in TBS/Tween.

2.8.1 Detecting phospho-ERK1/2

Membranes were blocked in 2% Egg Albumin for one hour at room temperature. After two separate 2 minute washes in TBS/Tween, they were incubated overnight at 4°C with gentle shaking with phospho-ERK1/2 antibody (#9106) from Cell Signaling diluted 1:1000 in 5% Bovine Serum Albumin (BSA). After three 15 minute washes, they were probed with a secondary anti-mouse antibody in 5% BSA in a 1:10,000 dilution for one hour at room temperature. The membranes were again washed three times for 15 minutes each in TBS/Tween. ECL Lumigen™ PS-3 Detection Reagents from GE Healthcare was applied to the membrane for visualization.

2.8.2 Detecting phosphor-PLC-γ and PLC-γ2

Membranes were blocked in 2% Egg Albumin for one hour at room temperature. After three 5 minute washes, they were incubated overnight with gentle shaking at 4°C in phospho-PLC-γ2 or PLC-γ2 from Cell Signaling (#3871 or #3872, respectively) diluted 1:1000 in 5% BSA. Membranes were washed, probed with anti-rabbit secondary antibody diluted 1:10,000 in 5% BSA for one hour at room temperature, rewashed and detected using the ECL kit.

2.8.3 Detecting ITK

Membranes were blocked with 5% milk for one hour. Itk was detected using HA antibody from Cell Signaling (#2367) diluted 1:1000 in 5% milk overnight. After three 15 minute washes, anti-mouse secondary antibody diluted 1:10,000 in 5% milk was applied for one hour at room temperature. The membrane was again washed and detected using the ECL kit.

2.8.4 Detecting Txk, cys-ITK, PH-TXK

Membranes were blocked in 5% Milk and washed three times for 5 minutes each in TBS/Tween. Txk was detected using anti-flag antibody from Sigma Aldrich (#F7425) diluted 1:4000 in 5% BSA for two hours at room temperature. Membranes were again washed and probed using anti-rabbit secondary antibody in 5% BSA.

2.8.5 Detecting ERK1/2

Membranes were blocked in 5% milk and subsequently incubated overnight with 1:2000 dilution of Erk1/2 antibody from Cell Signaling (#4695) in 5% milk. Membranes were washed, probed with anti-rabbit secondary antibody, rewashed and detected with ECL.

2.8.5 Stripping

To reprobe after each Western Blot, membranes were incubated in50mL of stripping buffer in a water bath at 55°C for 30 minutes.

Chapter 3: Results

ITK leads to greater phosphorylation of ERK than TXK

While the signaling pathways through Itk are well known, much less is understood about Txk in regulating the MAPK pathway. It was thought that given its constitutive anchoring to the plasma membrane, Txk would be in a better position to phosphorylate PLC-γ and thus activate MAP kinases. However, when measuring for phosphorylated ERK, Itk leads to greater ERK phosphorylation than Txk (p value = .02) (Figure 3.1). While these observations are much more exaggerated in non-serum starved cells, the differences between Itk and Txk are not statistically significant under serum starved conditions (p=.16). Nonetheless, the levels of phosphorylated ERK in serum starved samples follow the same phosphorylation patterns observed in non-serum starved samples. That is, although not statistically significant, the observation that Itk leads to downstream ERK phosphorylation at greater levels than Txk is consistent and may still be biologically relevant.

a)

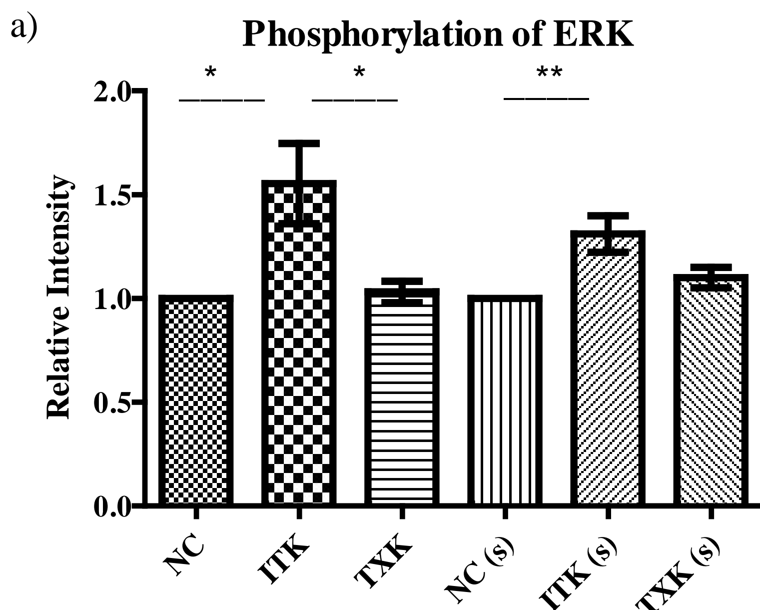

Figure 3.1.a: 293T cells were transfected with either ITK or TXK. After 48 hours, cell were lysed, run on SDS-PAGE and probed for phosphorylated ERK. Serum starved samples—labeled "(s)"—were incubated in FBS-free DMEM for 4 hours before lysing. All values are adjusted for total protein and are relative to the non-transfected 293T cells (either NC or NC starved). Values are representative of five different experiments. Error bars are calculated as SEM using Prism software. P values were obtained using a two-tailed T-test. *p=0.02; **p=0.05.

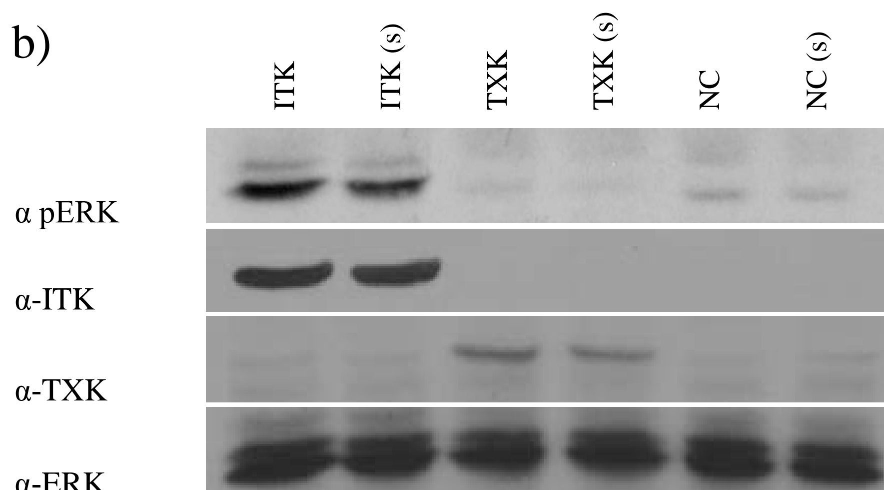

Figure 3.1.b: Western Blot representative of one of five experiments which evaluated phosphorylated levels of ERK by ITK vs. TXK. 293T cells were transfected with either ITK or TXK, incubated at 37°C for 48 hours and subsequently serum starved for 4 hours. Cell lysates were run on SDS-PAGE and Western Blotted for phosphorylated ERK. The membrane was stripped and probed for ITK, TXK and ERK½ to ensure proper transfection and equal protein loading.

The PH and Cys domains of Itk and Txk are not critical in ERK activation

To investigate whether the PH and Cys domains of Itk and Txk were responsible for their differences in ERK activation, chimera constructs were generated in which the PH domain of Itk was switched for the Cys domain of Txk (Figure 1.4). Given the data in Figure 3.1, it was thought the PH domain could enhance Txk's ability to activate ERK whereas the Cys domain impaired Itk's function in causing ERK phosphorylation. Indeed, there was an increase in PH-Txk function compared to Txk; likewise, a parallel decrease in ERK activation by Cys-Itk compared to Itk was observed (Figure 3.2). However, these values were not statistically significant—given p values of .24 and .34, respectively. Furthermore, if the PH domain was solely responsible for the differences in Itk and Txk function, we should expect to see Itk and PH Txk to phosphorylate ERK at equal levels. However, pERK levels in PH Txk transfected cells were still lower than levels observed in Itk. Additionally, when comparing phosphorylated levels of ERK in serum starved cells, the differences observed between the wild type kinases and their chimera constructs were completely diminished. While Itk and cys-Itk transfected cells maintained slightly elevated levels of pERK compared to Txk and PH Txk, there is no statistical significance between Itk vs. cys-Itk and Txk vs. PH Txk. The only significant values were of those observed between the negative controls and Itk and between Itk and Txk. Thus, the PH and Cys domains do not play a significant role in affecting downstream ERK phosphorylation.

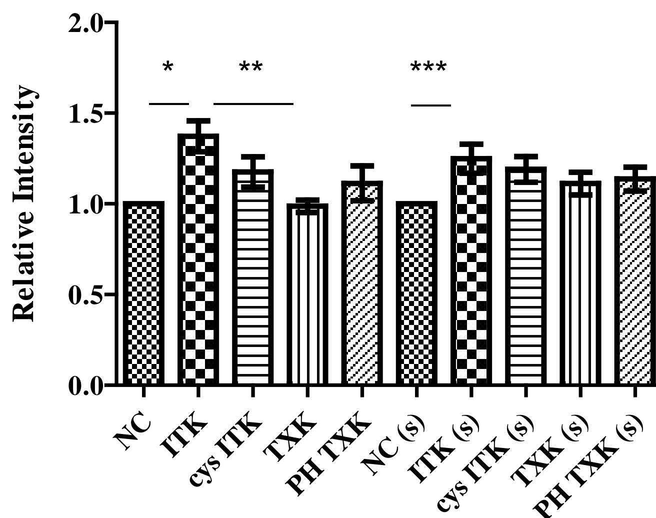

Figure 3.2: Cells were again transfected with either Itk or Txk in addition to chimera constructs cys-Itk or PH-Txk. After 48 hrs, cells were lysed, run on SDS-PAGE and probed for pERK. Results are the average of four separate experiments. Error bars were calculated as SEM via Prism. P values were generated using a two-tail T test. *$p<0.03$; **$p<0.01$; ***$p=0.05$.

The PH domain of ITK plays a critical role in positioning TEC-family kinases to phosphorylate PLC-γ

While the PH and Cys domains did not play a significant role in downstream ERK activation, we wanted to determine whether or not they had an effect upstream of ERK. Upon Tec family kinase phosphorylation by Lck, Itk and Txk become activated and are thought to phosphorylate PLC-γ. Recall that PLC-γ is a central effector that is involved in mediating calcium dependent cellular functions as well as facilitating MAPK activation. While it is has been shown that both Itk and Txk are involved in PLC-γ mediated processes, Txk's capacity to phosphorylate PLC-γ relative to Itk's remains elusive. Thus, 293T cells were co-transfected with PLC-γ and one of the four kinases. Strikingly, replacement of the PH domain for the Cys domain completely knocked out Itk's ability to phosphorylate PLC-γ (Figure 3.3). Likewise, addition of the PH domain to Txk allowed for significantly greater PLC-γ phosphorylation compared to Txk. Thus, Itk and PH-Txk had significantly higher capacity to phosphorylate PLC-γ whereas Txk and Cys-Itk had significantly lower levels of phosphorylated PLC-γ. Thus in this regard, the PH domain clearly plays a critical role in allowing ITK to phosphorylate PLC-γ.

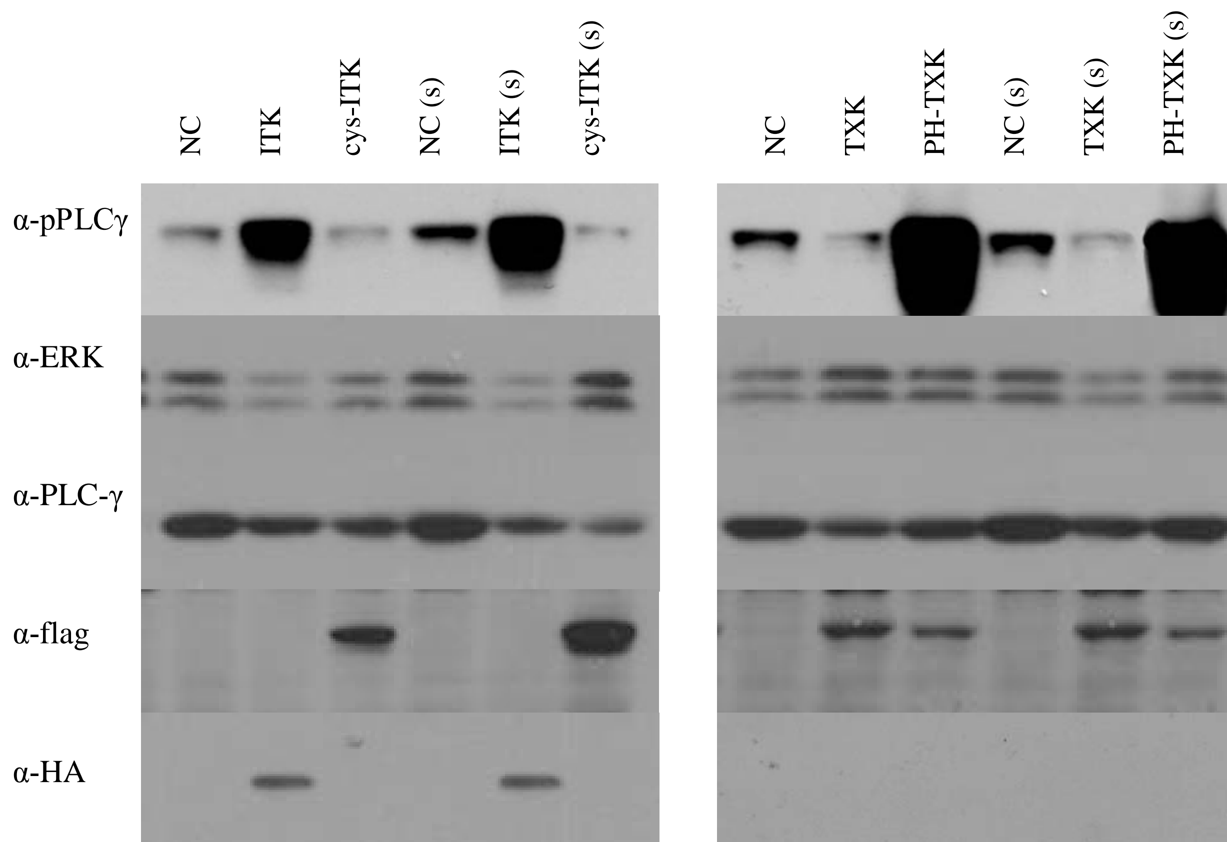

Figure 3.3: Negative controls were transfected with 1.0 μg of PLC-γ alone whereas all other samples were co-transfected with 0.5 μg of PLC-γ and another 0.5 μg of their respective kinases. Lysates were probed for phosphorylated PLC-γ to determine level of PLC-γ phosphorylation by each kinase. Western Blotting for ERK and PLC-γ was done to ensure even loading and proper transfection of PLC-γ. Kinases cys-Itk, Txk, and PH-Txk were all tagged with flag. ITK was tagged with HA. These were all Western Blotted to ensure that the phosphorylated PLC-γ levels were due to their associated kinases.

ITK may be important for cell survival and/or proliferation

The effect of inhibiting Itk expression has a greater effect than just impairment of cellular function and differentiation. $Itk^{-/-}$ cell populations were observed to grow at significantly slower rates than wild type populations (data not shown). Thus, cell populations that were transfected with shRNA specific for Itk become overgrown by wild type cells (Figure 3.4A). Figure 3.4B represents the restoration of Itk expression levels in $Itk^{-/-}$ cell populations after some time due to the proliferation of wild type cells over $Itk^{-/-}$ cell. Together, these observations suggest that signaling through Itk may be important for cell function which could affect survival and proliferation. These observations were paralleled in $Txk^{-/-}$ cells although cell growth was not as dramatically impeded.

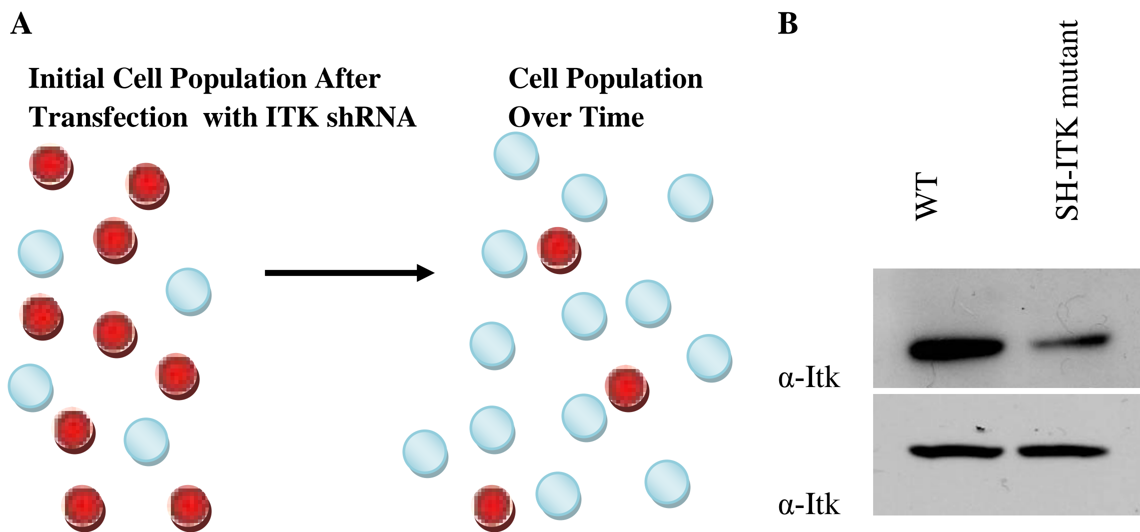

Figure 3.4: A) $Itk^{-/-}$ are represented by red cells whereas wild type cells are depicted as blue cells. Because less than 100% of cells within a culture will be transfected with shRNA, wild type cells expressing Itk will overgrow $Itk^{-/-}$ cells after a period of time. B) Jurkat cells were transfected with shRNA specific for Itk, thereby generating $Itk^{-/-}$ Jurkat cells. The upper bands are representative of Itk expression levels in wild type versus $Itk^{-/-}$ cell population one week after transfection with shRNA. The lower bands represent Itk expression levels after one month of transfection.

Chapter 4: Discussion

TEC-family kinases Itk and Txk are important mediators of T cell function and differentiation[2,22]. Whereas Itk contains a PH domain at its N-terminus thereby requiring it to be recruited to the membrane[1,22], Txk consists of palmitoylated string of cysteine residues which allows it to be constitutively anchored to the plasma membrane[4,7]. While both kinases are involved in PLC-γ phosphorylation and subsequent MAP Kinase activation[20], it is unclear how these kinases differentially regulate these pathways given their structural differences. Early studies show that Itk is the major kinase that regulates these pathways[13,22], with moderate to severe biochemical and functional defects seen in $Itk^{-/-}$ mice including decreased calcium signaling, deficiencies in positive selection, proliferation, and IL-2 secretion, as well as dysregulation of $CD4^+$ vs. $CD8^+$ T cell subsets[9,10,11,12,15,22]. While these defects are potentiated in $Itk^{-/-}Txk^{-/-}$ mice, deficiencies in Txk alone do not lead to any observable defects other than slightly lowered levels of IL-2 production[8,19,20,21]. It was also thought that Itk and Txk differentially induced a $CD4^+$ and $CD8^+$ response, respectively[22]. However, more recent data show that, when expressed at equivalent levels as Itk in wild type mice, Txk can rescue a T_{H2} response in $Itk^{-/-}$ deficient mice[18]. This data suggests that Txk and Itk not only have overlapping functions, but also that T cell differentiation depends on the level of expression rather than differential functions of Itk and Txk[18]. Thus, to provide further insight into their role of T cell function and differentiation, this study sought to evaluate the roles of Itk and Txk in regulating the MAPK pathway and the upstream effectors.

The first set of experiments sought to compare the ability of each TEC-family kinase to lead to ERK phosphorylation. On the one hand, it was thought that constitutive association of Txk with lipid raft fractions at the plasma membrane[4,7] would increase the

ability of Txk to phosphorylate PLC-γ and subsequently lead to greater ERK activation compared to Itk. On the other hand, the severe immunologic defects observed in $Itk^{-/-}$ but not in $Txk^{-/-}$ suggest that Itk plays a much greater role in ERK activation[9,19,20]. Results from Figure 3.1 demonstrate that Itk induces slightly higher phosphorylation of ERK than Txk. While the pattern of ERK phosphorylation by Itk and Txk were consistent, it was not enough to be statistically significant. Under serum starved conditions, pERK levels were barely lower in Txk transfected cells than Itk transfected cells. Because Txk activates the same pathway as Itk at comparable levels, it is possible to see why Txk could rescue Itk mediated functions if Txk were expressed at similar levels to Itk. Thus, it is consistent with the idea that T cell function and differentiation depends on the level of expression and not necessarily specific function of these kinases[18]. In fact, the severe biochemical defects observed in $Itk^{-/-}$ mice but not in $Txk^{-/-}$ can be partially explained by their different expression patterns. Because Itk is expressed three- to ten-fold higher than Txk in naïve T cells[13], the effects of knocking out Itk expression would be significantly more severe than inhibiting Txk function.

Given the data in Figure 3.1 in which Txk did not lead to greater ERK phosphorylation, it would suggest that the position of each kinase before T cell activation would not play a significant role in causing downstream ERK phosphorylation. Because the PH and Cys domains determine the positioning of Itk and Txk in the T cells, these domains were exchanged on each kinase such that Itk contained a Cys residue and Txk contained a PH domain. The plasmids of these chimera constructs were subsequently transfected into 293T cells in order to test this secondary hypothesis. The results from Figure 3.2 indicate that, contrary to our initial hypothesis, addition of the Cys domain to

Itk actually caused a slight decrease in ERK phosphorylation. Likewise, there was a parallel increase in phosphorylation events by PH-Txk compared to wild type Txk. However, while this trend of phosphorylation events is consistent with the associated kinases, the values are not statistically significant. Thus, no definitive conclusion can be made thus far.

However, if there really is a difference between the wild type kinases and their respective chimera constructs, it is possible to speculate that the PH domain of Itk not only allows Itk to bind to PIP3[1] but also facilitates Itk's subsequent association with SLP76 and LAT. Because these two adaptor proteins recruit PLC-γ to the complex, Itk can very easily phosphorylate PLC-γ and ultimately lead to phosphorylation of ERK[20]. At this point, it is unclear whether or not Txk becomes associated with these critical adaptor proteins[23]. It may be that Txk comes into the vicinity of PLC-γ simply by being constitutively associated with the plasma membrane. However, due to the association of Itk with the SLP76 and LAT adaptor complex[23], the length of time Itk is associated with PLC-γ may be much longer than the association seen with PLC-γ. Additionally, because PLC-γ also possesses a PH domain, both Itk and PLC-γ may be simultaneously recruited to the membrane and subsequently, to the multimeric complex. Thus, under this scenario, the timing and duration of the interaction may be the key factor that leads to greater phosphorylation of PLC-γ by Itk than Txk—a factor potentially mediated by the PH domain. Ultimately, this difference in PLC-γ phosphorylation would translate—to some degree—into lower levels of MAPK activation.

To partially test this hypothesis, 293T cells were co-transfected with plasmids for PLC-γ and one of the four kinases. Proteins from the cell lysates were examined for phosphorylated PLC-γ. Interestingly, phosphorylation of PLC-γ was not only significantly higher in Itk transfected cells but also in cells expressing PH-Txk (Figure 3.3). Likewise, these levels of pPLC-γ were significantly lower in both Txk and Cys-Itk. This is strong evidence that the PH and Cys domains of these TEC-family kinases play crucial roles in facilitating PLC-γ phosphorylation events.

However, considering that PLC-γ phosphorylation is a key pathway that leads to ERK activation, one should expect to see a parallel increase in MAPK by Itk and PH-Txk compared to Txk and Cys-Itk. However, the profound differences in PLC-γ phosphorylation (Figure 3.3) do not mirror those in ERK activation (Figure 3.2) and thus presents an interesting caveat. That being said, it is possible that Txk may actually have a compensatory mechanism of activating the MAPK, independent of PLC-γ. The exact mechanisms through which these processes occur, however, is mere speculation.

As a final experiment to recapitulate the findings in previous studies, Itk$^{-/-}$ Jurkat cells were generated using shRNA specific for Itk (Figure 3.4). It was found that Itk$^{-/-}$ cells grew at a much slower rate than wild type cells. As evidence of this, Itk expression levels were restored in Itk$^{-/-}$ deficient cell populations. This suggested that wild type Jurkats outgrew the Itk$^{-/-}$ cells and thus caused the subsequent restoration of Itk levels. Ultimately, this data indicates that deficiencies in Itk not only impair cell proliferation upon T cell activation—as evidenced by previous studies—but may also play a role in cell survival and proliferation in actively growing cells. Likewise, Txk$^{-/-}$ strains also

grew at a slower rate than wild type but twice as fast as $Itk^{-/-}$ strains (data not shown). These results are consistent with the idea that, because of the higher levels of Itk expression, defects in Itk would lead to more severe cellular dysfunction compared to that of Txk.

Chapter 5: Conclusion

The study found that Itk led to slightly greater MAPK activation than Txk. These differences between Itk and Txk are even more exaggerated when examining PLC-γ phosphorylation. Whereas Itk and PH-Txk phosphorylated PLC-γ at significantly higher levels, Txk and Cys-Itk had much lower capacities to phosphorylate PLC-γ. This suggests that the PH domain plays a prominent role in positioning TEC kinases to phosphorylate PLC-γ. However, these differences between the TEC-family kinases and their respective chimera constructs were not mirrored in their capacities to facilitate ERK phosphorylation. Lastly, the study determined that Itk also plays a role in cell survival and proliferation in actively growing T cells.

A potential future direction would be to determine whether or not Txk bound to SLP76 and LAT. This feature may explain the significant difference between Itk and Txk's ability to phosphorylate PLC-γ. Another potential study could explore other potential pathways that Txk is involved in. Additional studies on the role of Itk in naïve T cells in their resting state could also be conducted.

References:

1. August, A., A. Sadra, B. Dupont and H. Hanafusa. 1997. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc. Natl. Acad. Sci. U S A.* 94: 11227-11232.

2. Berg, L.J., L.D. Finkelstein, J.A. Lucas and P.L. Schwartzberg. 2005. Tec family kinases in T lymphocyte development and function. *Annu. Rev. Immunol*. 23: 549-600.

3. Bunnell, S.C., M. Diehn, M.B. Yaffe, P.R. Findell, L.C. Cantley and L.J. Berg. 2000. Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. *J. Biol. Chem*. 275: 2219-2230.

4. Chamorro, M., M.J. Czar, J. Debnath, G. Cheng, M.J. Lenardo, H.E. Varmus, P.L. Schwartzberg. 2001. Requirements for activation and RAFT localization of the T-lymphocyte kinase Rlk/Txk. *BMC Immunol.* 2: 3.

5. Ching, K.A., J.A. Grasis, P. Tailor, Y. Kawakami, T. Kawakami and C.D. Tsoukas. 2000. TCR/CD3-induced activation and binding of Emt/Itk to linker of activated T cell complexes: requirement for the Src homology 2 domain. *J. Immunol.* 165: 256-262.

6. Cohn, L., Elias, J.A., and G.L. Chupp. 2004. Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* 22: 789-815.

7. Debnath, J., M. Chamorro, M.J. Czar, E.M. Schaeffer, M.J. Lenardo, H.E. Varmus and P.L. Schwartzberg. 1999. Rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. *Mol. Cell Biol.* 19: 1498-1507.

8. Ellmeier, W., S. Jung, M.J. Sunshine, F. Hatam, Y. Xu, D. Baltimore, H. Mano and D.R. Littman. 2000. Severe B cell deficiency in mice lacking the Tec kinase family members Tec and Btk. *J. Exp. Med*. 192: 1611-1624.

9. Fowell, D.J., K. Shinkai, X.C. Liao, A.M. Beebe, R.L. Coffman, D.R. Littman and R.M. Locksley. 1999. Impaired NFATc translocation and failure of TH2 development in Itk-deficient $CD4^+$ T cells. *Immunity*. 11: 399-409.

10. Labno, C.M., 2003. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr. Biol*. 13: 1619-1624.

11. Liu, K.Q., S.C. Bunnell, C.B. Gurniak, and L.J. Berg. 1998. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *J. Exp. Med*. 187: 1721-1727.

12. Lucas, J.A., L.O. Atherly and L.J. Berg. 2002. The absence of Itk inhibits positive selection without changing lineage commitment. *J. Immunol*. 168: 6142-6151.

13. Lucas, J.A., A.T. Miller, L.O. Atherly and L.J. Berg. 2003. The role of Tec family kinases in T cell development and function. *Immunol. Rev.* 191: 119-138.

14. Kashiwakura, J., N. Suzuki, H. Nagafuchi, M. Takeno, Y. Takeba, Y. Shimoyama and T. Sakane. 1999. Txk, a nonreceptor tyrosine kinase of the Tec family, is expressed in T helper type 1 cells and regulates interferon γ production in human T lymphocytes. *J. Exp. Med.* 190: 1147-1154.

15. Miller, A. and L. Berg. 2002. Defective Fas ligand expression and activation-induced cell death in the absence of IL-2-inducible T cell kinase. *J. Immunol*. 168: 2163-2172.

16. Miller, A.T., H.M. Wilcox, Z. Lai and L.J. Berg. 2004. Signaling through Itk promotes T helper 2 differentiation via negative regulation of T-bet. *Immunity*. 21: 67-80.

17. Noh, D.Y., S.H. Shin and S.G. Rhee. 1995. Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochem. Biophys. Acta*. 1242: 99-113.

18. Sahu, N., A.M. Venegas, D. Jankovic, W. Mitzner, J. Gomez-Rodriguez, J.L. Cannons, C. Sommers, P. Love, A. Sher, P.L. Schwartzberg, and A. August. 2008. Selective expression rather than specific function of Txk and Itk regulate Th1 and Th2 responses. *J. Immunol.* 181: 6125-6131.

19. Schaeffer, E.M., J. Debnath, G. Yap, D. McVicar, X.C. Liao, D.R. Littman, A. Sher, H.E. Varmus, M.J. Lenardo and P.L. Schwartzberg. 1999. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. *Science*. 284: 638-641.

20. Schaeffer, E.M. and P.L. Schwartzberg. 2000. Tec family kinases in lymphocyte signaling and function. *Curr. Opin. Immunol.* 12: 282-288.

21. Schaeffer, E.M., G.S. Yap, C.M. Lewis, M.J. Czar, D.W. McVicar, A.W. Cheever, A. Sher and P.L. Schwartzberg. 2001. Mutation of Tec family kinases alters T helper cell differentiation. *Nature Immunol*. 2: 1183-1188.

22. Schwartzberg, P.L., L.D. Finkelstein, and J.A. Readinger. 2005. TEC-family kinases: Regulators of T-helper-cell differentiation. *Nature Immunol*. 5: 282-295.

23. Su, Y.W., Y. Zhang, J. Schweikert, G.A. Koretzky, M. Reith and J. Wienands. 1999. Interaction of SLP adaptors with the SH2 domain of Tec family kinases. *Eur. J. Immunol*. 29: 3702-3711.

24. Takeba, Y., H. Nagafuchi, M. Takeno, J. Kashiwakura, and N. Suzuki. 2002. Txk, a member of nonreceptor tyrosine kinase of Tec family, acts as a Th1 cell-specific transcription factor and regulates IFN-γ gene transcription. *J. Immunol.* 168: 2365-2370.

Academic Vita of

Hong–Hanh Khong
316 Fourth Ave
Newtown Square, PA 19073
Phone: 610 969 5006
hmk5043@psu.edu

EDUCATION:

The Pennsylvania State University, University Park, PA 16802
Schreyer Honors College
Bachelor of Sciences in Immunology and Infectious Diseases
Bachelor of Sciences in Toxicology
with honors in Immunology and Infectious Diseases
Minor in International Studies

THESIS TITLE: Role of Tec Family Kinases in Regulating MAP Kinases
Thesis Supervisor: Avery August, Ph.D.

WORK EXPERIENCE:

Research Assistant - Spring 2009 to Present
Dr. Avery August, The Pennsylvania State University
Department of Veterinary and Biomedical Sciences

- Determined the differential functions of Itk vs. Txk in phosphorylating PLC-γ and regulating MAPK pathways.
- Analyzed data using Western Blotting, transfections, transformations, and PCRs

Research Assistant – Fall 2007-Spring 2008
Dr. John Tierney, The Pennsylvania State University
Department of Chemistry

- Synthesized and modified compound showing anti-carcinogenic effects against ovarian cancer in CHO cells
- Analyzed integrity of compound using NMR, IR, MS, and TLC techniques

SERVICE:

Mexico

- Participated in Project Mexico, laying the foundation for and restoring six schools in Tijuana
- Raised money for Project Mexico, personally exceeding expected funds by $2,000
- Volunteered at a catholic orphanage
- Coordinated with Los Niños, a grass roots organization, to educate the community on nutrition and conservation

Spain

- Explored Spain's political and religious history and contrasted it to the ongoing crisis on the Gaza Strip

Guatemala

- Analyzed the effects of U.S. imperialism on Guatemalan culture and politics
- Volunteered at ADISA, a non-governmental organization that works to improve the lives of disabled youths and victims of the Guatemalan Civil War
- Visited a community funded elementary school that provides free education to indigenous children

Philadelphia

- Volunteered over 100 hours at the Indochinese American Council as a member of the PA Literacy Corps
- Tutored young adults who wished to attain their GEDs and find a stable job
- Donated $100 to March of Dimes, a foundation that works to prevent premature births, birth defects, and infant mortality

University Park

- Donated 17'' of hair and over $300 to THON
- Worked as a Lion Ambassador at Penn State Brandywine, raising money for THON, delivering over 15 tours throughout the semester, producing banners for social events, managing the Fall Fair
- Donated $100 to Project Haiti along with crutches and a leg brace

HONORS:

- Featured in the Delaware County Times for service works including efforts for victims of Katrina and earthquake in Pakistan 2006
- Dean's List 2007-2010

GRANTS:

- Academic Competitiveness Grant 2007-2008
- Supplemental Educational Opportunity Grant 2008-2010
- Smart Grant 2008-2009
- Penn State Tuition Assistance Grant 2008-2010
- Schreyer Honors College Career Development Summer Research Grant 2009

SCHOLARSHIPS:

- Schreyer Honors Special Project 2009
- Mary M. Tharp Scholarship 2006-2010
- Academic Excellence Scholarship 2007-2009
- Schreyer Honors College Study Scholarship 2008
- Kimmel Scholarship 2008-2009
- Hartman, A. Trustee Scholarship 2008-2009
- Lighthouse Trustee Scholarship 2009-2010
- Dreibelbis Endowment Scholarship 2009-2010

LANGUAGES: Vietnamese, Spanish (proficient)