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PHYSICAL INTERACTIONS OF ITK WITH ITSELF AND OTHER PROTEINS IN THE TCR SIGNALING CASCADE

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Abstract

T cell receptor signaling is an intricate process. IL-2 inducible kinase (ITK) plays a pivotal role in this signaling. Itk is responsible for activating PLC gamma, which in turn triggers an infux in calcium and NFAT expression. The exact interactions that take place between ITK and other adaptor proteins within the TCR cascade have not yet been fully elucidated. Also important in this signaling pathway is the possible role that ITK dimerization plays. Using a split-YFP construct, the dimerization of ITK is examined in the context of TCR signaling. The individual domains of ITK are assessed for function, as well. The dimerization of ITK is also assessed within the context of two other molecules important to TCR signaling: PLC gamma, and SLP76. Through analysis of flow cytometry and confocal results, it was determined that the PH domain of ITK is necessary for ITK dimerization. It was also determined that PLC gamma and SLP76 by themselves cannot disrupt dimerization.

Acknowledgements

I would like to thank a few people for helping me with my thesis. Thank you very much Dr. August. Your patience and guidance helped me to finally finish my experiments and finish writing. Thank you for your constant support and help. Thank you Dr. Howell. Your advice and sound reasoning helped me manage my classes and lab time. Thank you Dr. Qian Qi, for letting me mess up your experiments, and teaching me so many techniques. Finally, a warm thank you to my friends and family.

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Introduction

In order to become activated and initiate an immune response, a T cell must signal through its T cell receptor (TCR). Depending on the signal, conditions, and type of cell, this activation can lead to a host of different cell functions including cytokine secretion, proliferation, and several others. Signaling through the T cell receptor is an elegant process. Its system of positive and negative controls allow for a precise and specific signal, while the sheer numbers and complexity of downstream effector molecules allow for a diverse array of T cell functions. The signaling cascade that results upon T cell receptor activation is continuously being studied and elucidated.

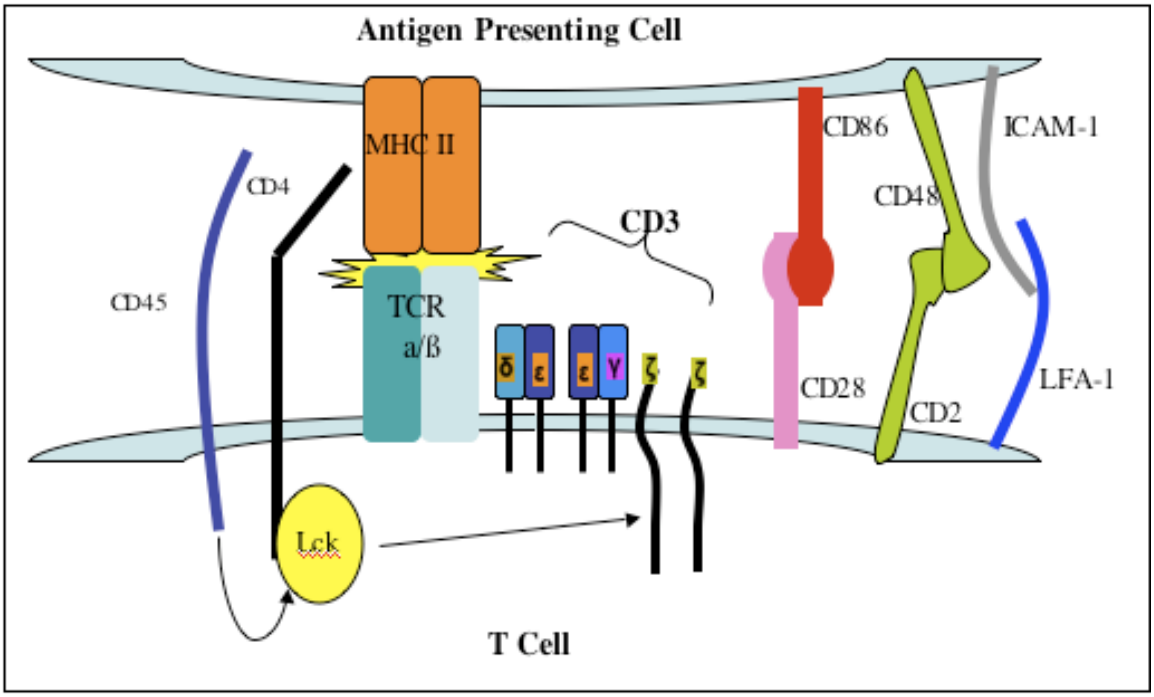

Figure 1: Immunological Synapse. TCR a/ß interact with the peptide/MHC complex. CD4 stabilizes the bond by interacting with MHC as well. CD45 activates Lck on CD4, allowing it to phosphorylate ITAMs on the CD3 molecules, especially the zeta chain. Other molecules like CD28 and CD86 provide extra adhesion and costimulation.

Table 1: Other significant molecules in the immunological synapse and their functions

Molecule on T Cell	Molecule on APC	Function	
TCR	MHC/Peptide	Recognize antigen and initiate TCR signaling	
CD4/CD8	MHC	Costimulatory signal	
CD2	CD48	Adhesion, and costimulation	
CD45	-	Dephosphorylates and activates Lck	
CD28	CD86	Costimulatory signal	
LFA-1	ICAM-1	Adhesion molecules	

This cascade begins at what is called the immunological synapse[1] (Figure 1). This term describes the interface between T cell and antigen presenting cell (in T helper cells) or normal nucleated cells (in the case of cytotoxic T cells). At the center of this synapse, and present in all T cell types is the T cell receptor. The T cell receptor is a heterodimer. In the vast majority of T cells, this heterodimer is made up of one alpha and one beta chain. However there do exist populations of T cells that express other combinations including gamma/delta, alpha/alpha etc. The T cell receptor binds to the major histocompatability complex (MHC) of the antigen presenting cell, interacting with the antigenic peptide presented by the MHC. The two major subsets of T cells, T helper and cytotoxic, have either CD4 or CD8 respectively on their cell surface. These molecules serve two major functions. First, the extracellular domain of the protein binds to the MHC, stabilizing the MHC:TCR interaction. Second, the intracellular domain of either CD4 or CD8 is associated with the Src family tyrosine kinase leukocyte-specific protein tyrosine kinase (Lck), which plays a large role in signal transduction.[2] Also present at the synapse is protein tyrosine phosphatase receptor type C, known as CD45. CD45, when in proximity with the T-cell receptor complex, is responsible for dephosphorylating and activating Lck. CD45 is an example of a negative control. At high levels, CD45 can also dephosphorylate

Lck at a different site, which inactivates Lck. Finally, the third co-receptor molecule which plays a role in signal transduction is CD3. CD3 is actually a collection of three types of homo and hetero dimmers. The first two, heterodimers of CD3-epsilon/delta and CD3-epsilon/gamma, contain large extracellular domains and small intracellular domains. The third, a homodimer of two CD3-zeta chains, contains a relatively short extracellular domain, and a long intracellular domain. Other molecules exist at the immunological synapse, and play roles in both adhesion and costimulation (see Table 1). [1]

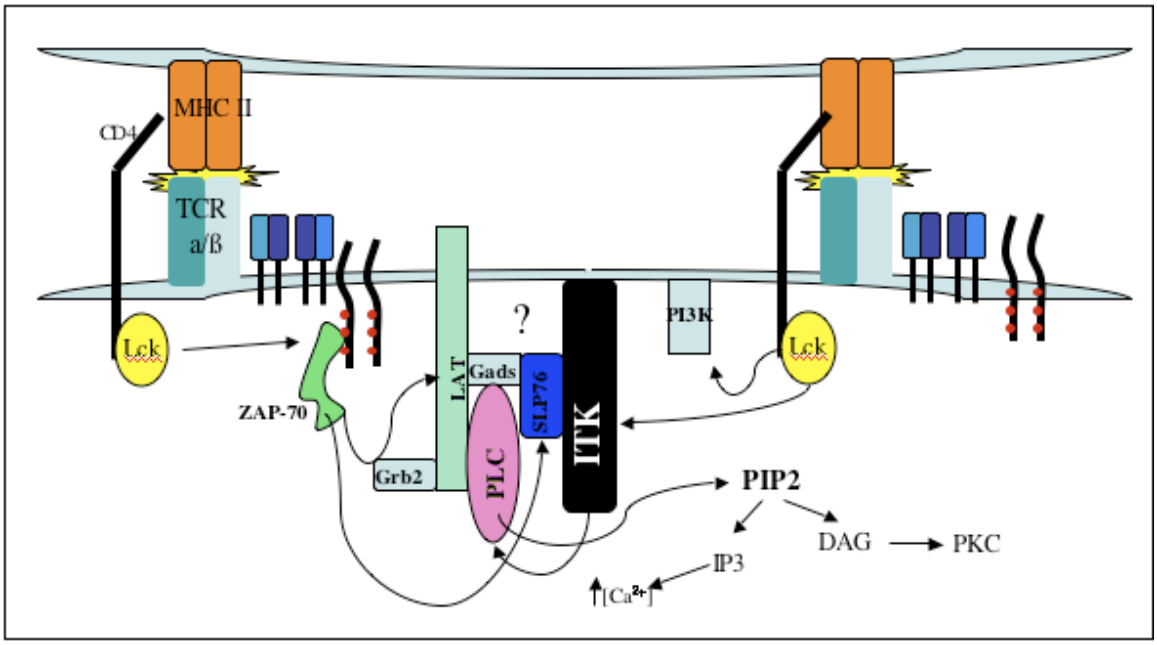

Figure 2: TCR signal transduction, and possible model for ITK signaling. The TCR signal transduction begins after Lck phosphorylates the ITAMs. ZAP-70 is recruited and phosphorylated by Lck. Now active, ZAP-70 phosphorylates LAT, which then recruits adaptor proteins like Gads+SLP76, Grb2, and the effector molecule PLC. ITK, recruited by SLP76 and PI3K and activated by Lck, phosphorylates PLC, which is then free to trigger the influx of Ca^{2+}.

Once the TCR binds its ligand, and all the appropriate costimulatory molecules bind theirs, the TCR is activated and the T-cell signaling cascade begins (Figure 2). [3] CD45, now in proximity with CD4/8, dephosphorylates and activates Lck. Lck then is free to phosphorylate

immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 molecules. This occurs primarily at the zeta chains, which contain three ITAMs each. The phosphorylated ITAMs recruit the Syk-family tyrosine kinase member Zeta-chain associated protein kinase of 70 kD (ZAP-70) via its Src-homology 2 (SH2) domains. Lck then phosphorylates the recruited ZAP-70, activating it. ZAP-70 phosphorylates and recruits the Linker of Activated T cells (LAT), an adaptor protein. [4] LAT is an essential adaptor protein in the T cell signaling cascade. LAT knockout mice have been shown to have almost complete loss of T cell signaling[3]. LAT recruits three proteins: Growth factor receptor-bound protein 2 (Grb2), Phospholipase C – gamma (PLC-gamma), and SH2 domain containing leukocyte protein of 76 kDa (SLP76) through the Gads adaptor (Grb2-related adaptor protein). Grb2 binds the guanine nucleotide exchange factor SOS, and can eventually activate the mitogen activated protein kinase (MAPK) pathway[3]. SLP76 is a versatile docking protein that is responsible for recruiting important signaling molecules. Its downstream effectors activate such pathways as the MAPK cascade, actin rearrangement, and calcium regulation[4]. Important in this study are the actions of PLC-gamma and SLP76, because they appear to be intimately involved with the Tec family kinase, IL-2-inducible Tec kinase (Itk).[4]

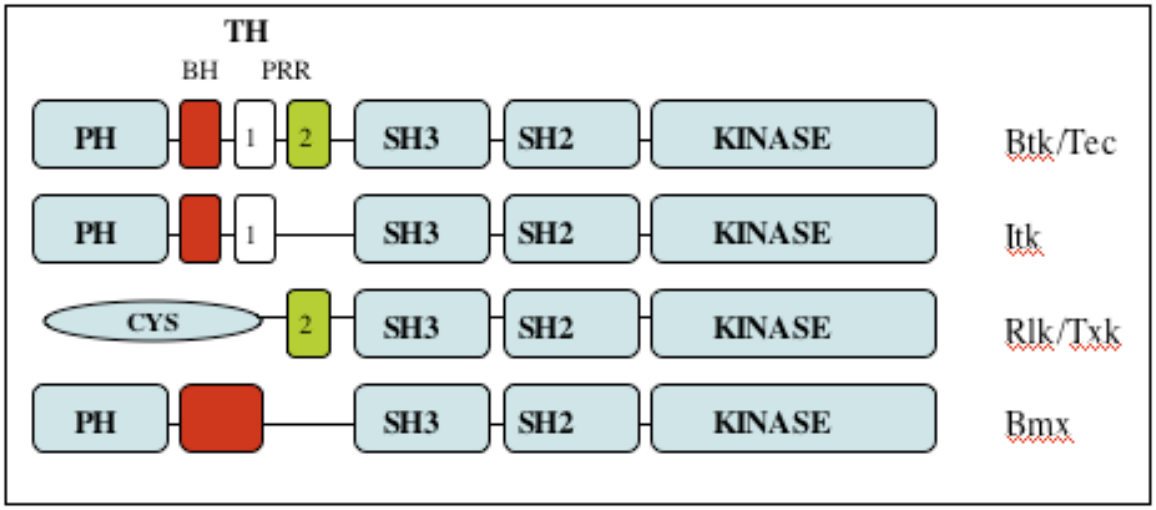

Figure 3: Tec-family kinase structures. The Tec kinases are shown here. The SH3, SH2, and Kinase domains are conserved between all Tec kinases. The TH domain consists of one or two PRR domains, and one Btk homology domain. The PH domain, used for membrane localization, is conserved in all by Rlk/Txk, which as instead a cysteine repeat region.

Itk is a member of the Tec family of non-receptor tyrosine kinases, which is the second largest family of non-receptor tyrosine kinases, with five total (Figure 3)[5]. However, with the exception of Bmx/Etk and Tec, the Tec family kinases are found almost entirely in hematopoietic cells[6]. Within these cells, Tec kinases perform a variety of functions. By acting on differential downstream molecules, Tec kinases have been shown to regulate cell processes like adhesion, actin reorganization, apoptosis, gene expression, and calcium mobilization. They are critical for signaling through many receptors, including the B and T cell receptors. Tec kinases also play a role in the production and regulation of cytokines in response to pathogens. [7] The structure of the Tec family kinases is shown in Figure 3. Each kinase has a C-terminal kinase domain, followed by an SH2 domain and an SH3 domain, much like the Src family kinases. However, Tec family kinases have differential N terminal domains. For example the Tec/Btk/Itk kinases all have a Btk homology and proline rich region (together called the Tec homology domain) followed by a pleckstrin homology (PH) domain. The proline rich region is responsible

for binding the SH3 domain of Src kinases, while the PH domain is responsible for membrane localization. The other Tec kinases, like Bmx/Etk have different N terminal regions, which can be seen in the Figure 3. Signaling through Tec kinases involves two major steps. First, the kinase must be localized to the membrane, and second, the kinase must be phosphorylated and activated by a Src-family kinase. [4,5]

Itk plays an integral role in T cell signaling. It is essential for calcium signaling and subsequent activation of nuclear factor of activated T cells (NFAT)[8]. Itk knockout mice have also been shown to have abnormal and abrogated T cell development and maturation. ITK has a C-terminal kinase domain, an SH3 domain and SH2 domain, a Tec homology domain, and an N-terminal PH domain. Like most Tec kinases, two things must happen before Itk can be activated: membrane localization, and phosphorylation[8]. Once the TCR is stimulated and Lck is activated, one of its targets is phosphoinositide 3-kinase (PI3K). Upon phosphorylation, activated PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). The PIP3 molecule recruits ITK to the membrane through its PH domain. Once at the membrane, Itk is phosphorylated at a tyrosine residue in its kinas domain by Lck, activating Itk. Itk's principal role in TCR signaling is to initiate the PLC gamma-mediated influx of Ca^{2+}. Activated Itk phosphorylates PLC-gamma, which in turn cleaves PIP2 into inositol1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG is responsible for the activation of the Ras/MAPK dependent pathways, as well as the activation of protein kinase C and subsequent expression of nuclear factor κB (NFκB). IP3 migrates to the endoplasmic reticulum where it binds to and opens Ca^{2+} channels, increasing the $[Ca^{2+}]$ in the cytoplasm. This increase in $[Ca^{2+}]$ leads to the activation of NFAT. [4]

However, even though the structure, means of activation, and major role of Itk are known, the sites of interaction between ITK and critical adaptor proteins, as well as the conformation of ITK within the cell remains largely unknown. This paper will begin to touch on two main areas of ITK research. First, signaling through the T cell receptor from ZAP-70 to PLC-gamma activation is not as clear-cut or linear as one might hope. For example, it has been shown that SLP76 binds both ITK and PLC-gamma at its N-terminal acidic region and PRR domain respectively[9]. SLP76 is recruited to LAT through Gads, bringing with it all of its attached proteins. ZAP70 phosphorylates SLP76, and is known to be required for ITK activation. [4] Finally, the guanine nucleotide factor Vav, which also binds to SLP76 and is a phosphorylation target of ITK, has been shown previously to be necessary for ITK activation and signal transduction[3]. Because all of these proteins, which some have coined the ITK signalasome, are essential to ITK function and TCR signaling as a whole, there is clearly a more complicated system at work. The following experiments brush the very tip of this iceberg, testing the various domains of ITK, and its association with PLC gamma and the adaptor SLP76. Secondly this paper will also attempt to characterize ITK dimerization within the context of these other proteins.

Materials and Methods

Plasmids and Reagents

The pCDNA3.1-YFP1-zipper and pCDNA3.1-YFP2-zipper were a kind gift from Dr. Stephen Michnick (University of Montreal, Montreal, Canada) [11]

Cell Culture/ Transfection

HEK 293T cells were cultured in Dubleco's Modified Eagles Medium supplemented with 10% FBS. Cells were split into new dishes, with new media every 3 days, and cultured at 37°C. Transfection was performed after 24 hours of culture. 3uL per 100ul of Mirus Bio TransIT-293T transfection reagent was incubated for 15 minutes. Plasmid was added after 15 minutes, and then incubated again for 25 minutes. Final plasmid/transfection reagent mix was added drop wise to its respective well.

Flow Cytometry

Four days prior to flow cytometry analysis, cultured HEK 293T cells were split into 6 well plates. After 24 hours of incubation in 37 degrees C, the cells were transfected with the desired plasmid (see transfection protocol). Transfected cells were once again incubated in 37 degrees C for 24 hours. After 24 hours, the cells were transferred from 37 to 30 degrees C incubator for enhanced fluorescence. After 12 hours of incubation in 30 degrees C, the cells were removed. Excess media was aspirated. To each cell-containing well, 1 ml of phosphate buffered saline (PBS) with 2% FBS was added, and the cells were suspended and transferred to 1.5ml eppendorf tubes. At the flow cytometry facility, 300 mL of the cell suspension was added to cytometry tubes, which

are used in the flow cytometer. FITC was used as a filter for the YFP fluorochrome Any unused cell suspension was transferred back to the eppendorf tubes.

Confocal Microscopy

Glass coverslips were placed in each well of a 6-well plate. Cells were split and transfected in the same manner as the above flow cytometry protocol. After the final 12 hour incubation, the coverslips were removed from the wells and the cells were analyzed using the Olympus FluoView FV1000.

Results

Use of split-YFP construct to detect dimerization

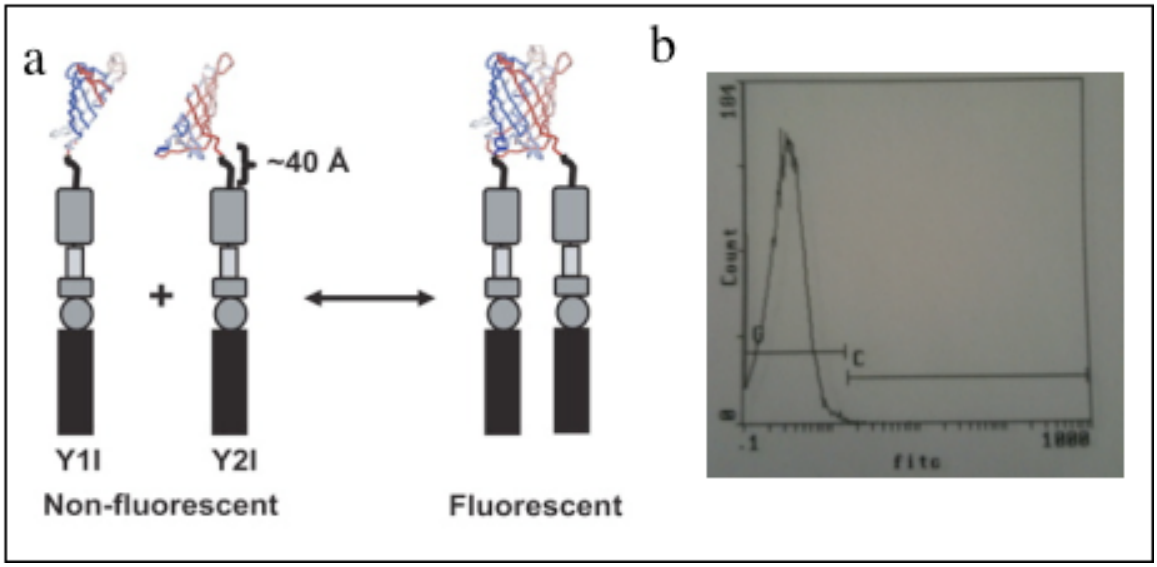

Figure 4: A Split-YFP construct. a) diagram representing split-YFP construct[8]. One half of a YFP is placed on the N terminus of each ITK. If they come within 80 angstroms of one another, they fluoresce b) cells transfected with just YFP1-ITK, no significant fluorescence

In order to detect the presence of ITK dimers in the cell, a split-YFP ITK construct was utilized. One YFP molecule was split in half to YFP1 and YFP2. Each of these was attached to an ITK molecule via a 10 amino acid linker at the N terminus. In this model, if the two ITK molecules, now dubbed YFP1-ITK and YFP2-ITK come close to one another the YFP will fluoresce. Because the linkers are 10 amino acids long, and the length of each amino acid is roughly 4 Å, the YFP fluorescence would be detected when the two ITK molecules are within 80 Å of each other [8]. Significant fluorescence indicates that the ITK molecules are either dimerizing or oligimerizing. To demonstrate that there is no fluorescence unless the two halves are in close

proximity, cells were transfected with YFP1ITK only. No significant fluorescence was found (Figure 4b**)**, which established that any fluorescence detected in subsequent experiments would be due to dimerization.

ITK forms dimers on the cell surface

The next step was to transfect cells with both YFP1-ITK (Y1I) and YFP2-ITK (Y2I). 293T cells were co-transfected with YFP1-ITK and YFP2-ITK. In order to see if the polarity of the ITK was a factor in dimerization, another set of 293T cells was co-transfected with YFP1-ITK and ITK-YFP2. The ITK-YFP2 protein has the YFP2 molecule fused to the C terminus rather than the N terminus. The cells were first analyzed by flow cytometry. YFP fluorescence was detected in both combinations, at significantly higher levels than non transfected 293T and YFP1 transfected 293T cells (see Figure 5 a**).** In order for the YFP to fluoresce, these ITK molecules must be within 80Å of each other. This indicates that they must be forming dimers, or possibly larger groups. In order to look at the localization of these dimers in the cell, the same transfection was performed and analyzed by confocal microscopy. It is clear from the confocal results that these dimers are occurring only on the cell surface, and nowhere else within the cell.

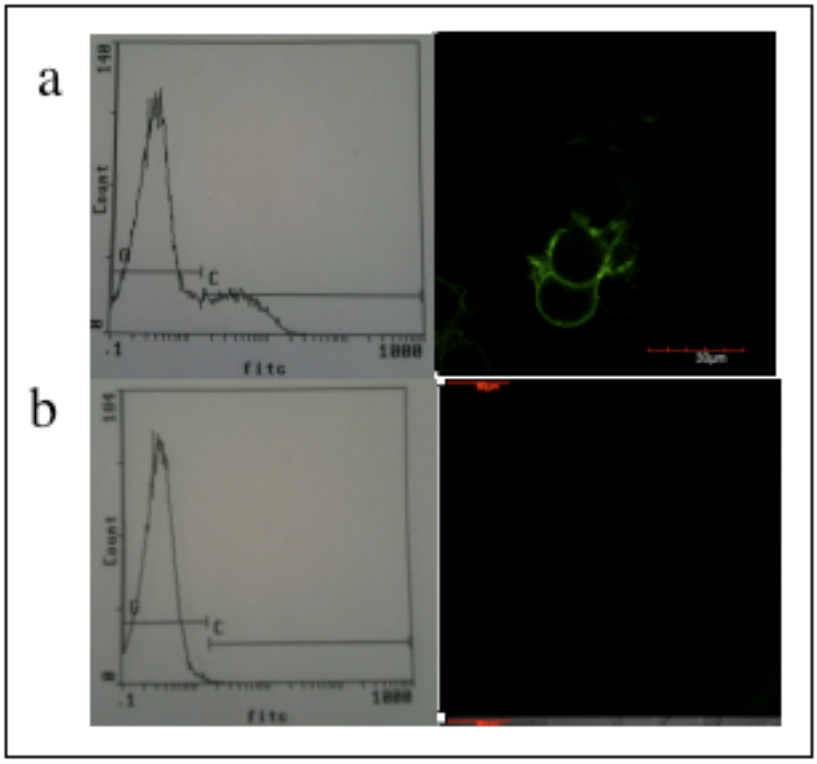

Figure 5: Cells transfected with both YFP halves. a) flow cytometry and confocal results for YFP1-ITK+YFP2-ITK b) flow cytometry and confocal results for YFP1-R29C-ITK+YFP2-R29C-ITK.

ITK with mutant PH domain does not form dimers on cell surface

The next step was to see whether or not these dimers were dependent on their localization to the cell membrane. In order to test this, an ITK mutant with a point mutation in the PH domain was developed. This mutant R29C ITK is unable to interact with PIP3, and thus should be unable to attach to the cell membrane. 293T cells were transfected with YFP1-R29C-ITK + YFP2-R29C-ITK as well as YFP1-R29C-ITK + R29C-ITK-YFP2. Flow cytometry analysis showed that there was no measurable fluorescence when compared to regular 293T cells (see Figure 5b). Confocal microscopy confirmed these results.

ITK with missingt ΔSH2 or ΔSH3 domain dimerizes with WT ITK but not with PH mutant

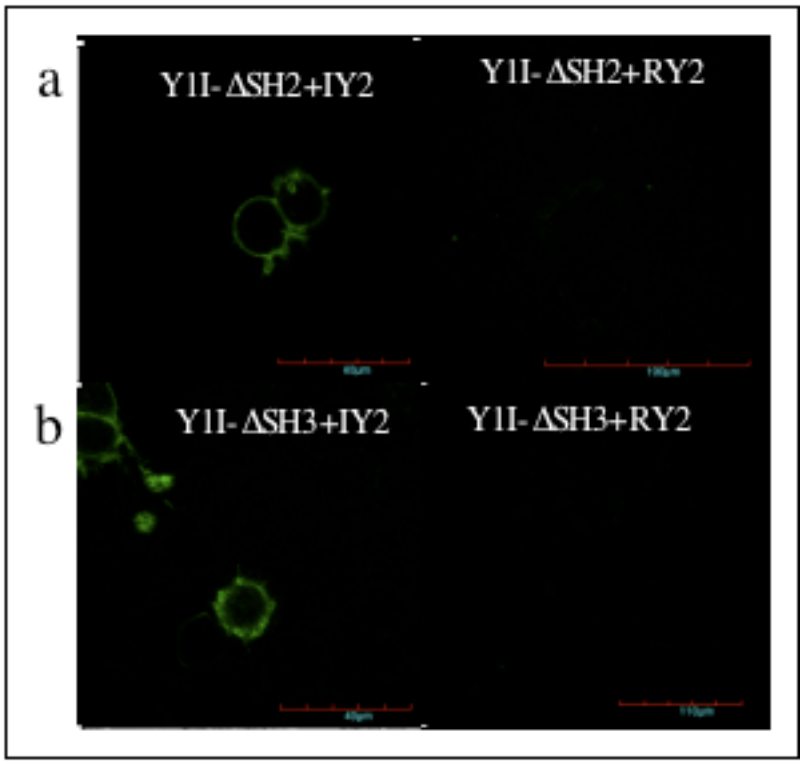

Figure 6: Confocal Microscopy SH2/SH3 mutants a) SH2 mutant ITK can dimerize with WT ITK, but not PH mutant. b) SH3 mutant ITK can dimerize with WT ITK but not PH mutant. The green fluorescence for both mutants indicates ITK dimers on the cell membrane.

In order to investigate whether ITK's protein-protein interaction domains play a role in its dimerization, two mutant ITKs lacking either SH2 and SH3 were developed. The SH2 domain has been shown to bind SH3 domains of neighboring ITK, causing head to tail dimers, and SH3 can bind to the PRR domain on the same ITK, resulting in a folded conformation. Four groups of 293T cells were transfected as follows: YFP1-ΔSH2-ITK + YFP2-ITK; or YFP1-ΔSH2-ITK + ITK-YFP2; or YFP1-ΔSH3-ITK + YFP2-ITK; or YFP1-ΔSH3-ITK + ITK-YFP2. Four more groups of 293T cells were transfected the same way except with R29C-ITK replacing wild type

ITK. The cells were analyzed by flow cytometry (data not shown), which revealed high levels of YFP fluorescence with wild type ITK partners, and no significant fluorescence with PH mutant ITK partners. Confocal microscopy confirmed these results (Figure 6), and revealed that the ΔSH2 and ΔSH3 mutants still dimerized only on the cell surface. These results confirmed earlier studies by Dr. Qian Qi, who also showed that mutations in the remaining ITK domains (TH and PRR) still resulted in YFP fluorescence, aka ITK dimerization, on the cell surface[8].

Expression of PLCgamma does not inhibit dimerization of ITK, nor does it rescue dimerization in PH mutants

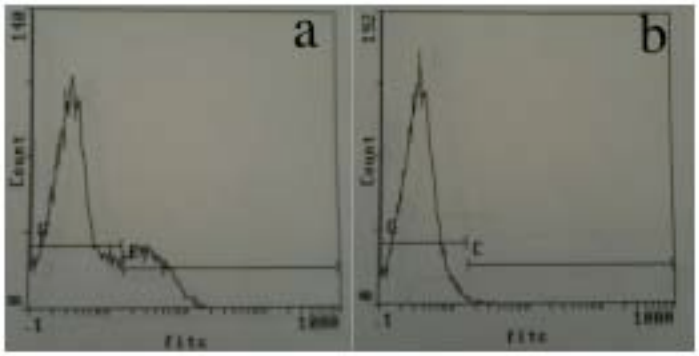

Figure 7: PLC gamma does not inhibit dimerization of ITK a) YFP1-ITK + YFP2-ITK + PLC gamma, expression of PLC gamma does not inhibit dimerization of ITK b) YFP1-R29C-ITK+YFP2-R29C-ITK + PLC gamma, expression of PLC gamma does not rescue dimerization in PH mutants

Now that we know that ITK dimerizes at the cell membrane, it's important to look at how the different adaptor proteins and effector molecules in the TCR signaling cascade affect this dimer. Other than its obvious Ca^{2+} inducing functions, PLC gamma also plays a structural role in the ITK signalasome. Along with the appropriate controls, 293T cells were transfected with four groups of plasmids: YFP1-ITK + YFP2-ITK + PLC; YFP1-ITK + ITK-YFP2 +PLC; YFP1-

R29C-ITK + YFP2-R29C-ITK + PLC; and as YFP1-R29C-ITK + R29C-ITK-YFP2 + PLC. The transfected cells were analyzed using flow cytometry (Figure 7), and the presence of PLC was verified via SDS PAGE (data not shown). The addition of PLC to both sets of plasmids did not seem to have any real effect. Dimers still occurred for WT ITK in the presence of PLC as evidenced by the presence of fluorescence. Dimers were still located on the cell surface, as evidenced by confocal microscopy (data not shown). In the PH mutant, there was still no dimerization, as evidenced by the lack of fluorescence.

Expression of SLP76 does not inhibit dimerization of ITK, nor does it rescue dimerization in PH mutants

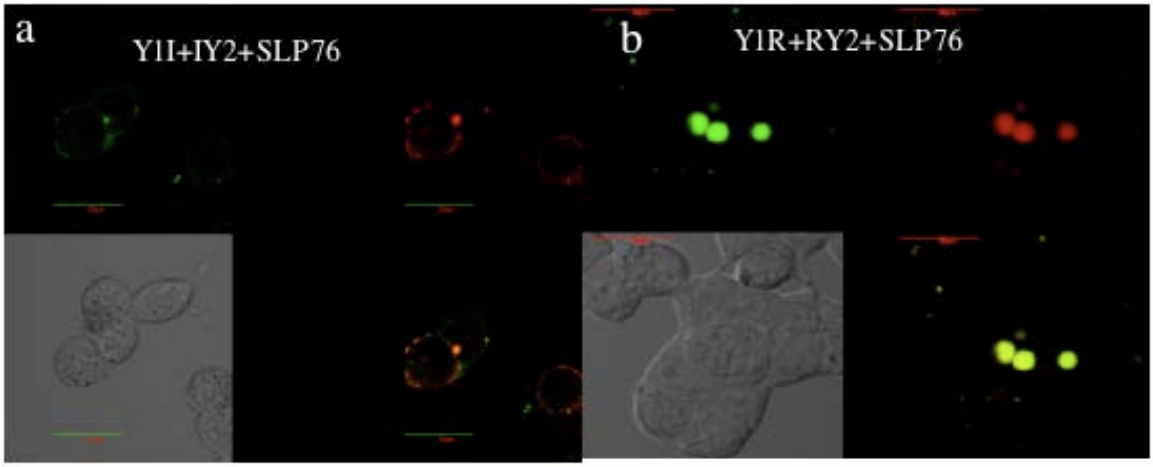

Figure 8: SLP76 expression does not inhibit ITK dimerization a) YFP1 -ITK+YFP2-ITK+SLP76, SLP76 does not inhibit dimerization. The red spots prove that SLP76 has indeed been transfected into the cells, while the green fluorescence indicates that ITK is dimerizing on the cell surface b) YFP1-R29C-ITK+YFP2-R29C-ITK+SLP76, SLP76 does not rescue dimerization in PH mutants. Red spots indicate successful SLP76 transfection, and there is no indication of ITK dimerization.

SLP76 clearly plays an integral role in the TCR and ITK signaling cascades. ITK binds SLP76, which in turn binds PLC gamma, bringing the two in close proximity. Knockouts of

SLP76 result in almost complete abrogation of T cell signaling and function. In this experiment, 293T cells were transfected with the same group of plasmids as the PLC experiment, only PLC was replaced with SLP76. The main difference between the two experiments was that while the PLC plasmid encodes PLC and PLC only, the SLP 76 plasmid encodes the SLP76 protein with an attached dsRED molecule for fluorescence assays. As it turns out, this creates a variety of problems. Most of these will be analyzed in depth in the discussion section, but the most pressing problem is that when dsRED is analyzed by flow cytometry, it fluoresces in both PE *and* FITC, the two channels used in these experiments to recognize and separate YFP fluorescence. Therefore, flow cytometry cannot be used to distinguish the presence of dimerization, because it cannot distinguish YFP from dsRED. For this reason, the transfection results were analyzed using confocal microscopy only.

In these confocal experiments, the dsRED SLP76 fluoresced red, while the YFP-ITK construct fluoresced green. Ignoring the flaws in this experimental set up for the time being, the confocal microscopy yielded good initial results. ITK dimerized on the cell membrane, fluorescing green, while the SLP76 showed up as individual sharp points of fluorescent red (Figure 8). The initial images made it look like there was some significant colocalization of SLP76 and ITK dimers. However, this hypothesis was quickly dispelled, because green spots of fluorescence were showing up even in the SLP76 control cells. That being said, there was clearly membrane-dimerization of ITK in cells that were simultaneously expressing small dots of red fluorescence, which indicates that ITK dimerization occurs even in the presence of SLP 76. The PH mutant ITKs still had zero fluorescence in cells that had been transfected with SLP76.

SLP76 and PLC gamma together seem to have the same effect together as apart

The next logical step to take was to see if co-expressing both SLP76 and PLC in 293T cells would effect the dimerization of ITK. This turned out to be fairly challenging. As the number of plasmids increases, the ratio of each individual plasmid to the total volume of transfection reagent decreases. This makes the odds of getting all four plasmids transfected into one cell much worse. That being said, 293T cells were transfected as in the previous experiment except both SLP76 and PLC were transfected instead of one or the other. Once again, green fluorescence showed up in the SLP76 (red fluorescent) control, so all colocalization must be disregarded. The results from this set of experiments were varied. Some cells had dimerization and YFP fluorescence but no SLP76 fluorescence, while others had just the opposite. The majority of the cells had either green ITK dimers *or* red SLP76 spots. The vast majority fluoresced much weaker than the two and even three-plasmid transfections of earlier experiments. The discrepancy from result to result within this experiment makes any sort of conclusion nearly impossible. Methods to improve this experiment in order to get useable results will be discussed later in the paper.

Dimerization of ΔSH2 and ΔSH3 ITK mutants was also unaffected by PLC gamma and SLP76 expression

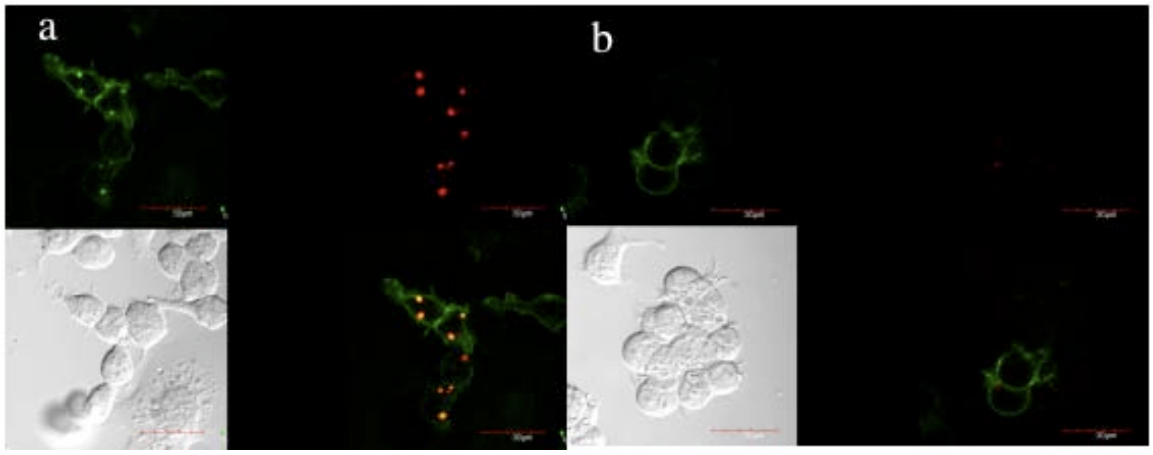

Figure 9: ΔSH2 and ΔSH3 ITK mutants dimerize in presence of PLC or SLP76 a) Y1IΔSH2+IY2+SLP76, dimerization of SH2 mutant ITK is unaffected by SLP76. b) Y1IΔSH3+IY2+SLP76, dimerization of SH3 mutant ITK is unaffected by SLP76. In both transfections, the green fluorescence at the cell membrane indicates ITK dimerization, while the red points within the cytosol indicate the presence of SLP76.

For the sake of completion, the ΔSH3 and ΔSH2 mutants were also transfected with PLC gamma or SLP76. The same transfection set-up from the previous ΔSH2 and ΔSH3 ITK experiment was used in this experiment. Two groups were set up, identical except that one group added PLC gamma to each transfection, and the other group added SLP76 to each transfection. The PLC-only results were analyzed by flow cytometry. Both ΔSH2 and ΔSH3 ITK mutants formed dimers and fluoresced with WT ITK (data not shown). Furthermore, both ΔSH2 and ΔSH3 ITK failed to rescue dimerization and fluorescence with PH mutant ITKs. The SLP76-only results were measured using confocal microscopy (see Figure 9). Again, the ΔSH2 and ΔSH3 ITK mutants behaved almost exactly like WT ITK. The cells which received ΔSH2/ΔSH3 ITK

and SLP76 exhibited green fluorescence and dimerization on the cell membrane, and sharp red fluorescing SLP76 in the cytosol. The PLCgamma + SLP76, four plasmid transfection was not repeated for the ΔSH2 and ΔSH3 ITK because it produced such disparate results in the previous experiment.

Discussion:

T cell receptor signaling is incredibly sophisticated. If the sheer number of molecules involved in this cascade weren't enough, the fact that they can be differentially expressed and altered structurally makes this pathway a veritable treasure chest of undiscovered signaling mechanisms. ITK clearly plays an essential role in this pathway. Although much is known about ITK already, there are still major pieces of the puzzle missing.

Dr. Qian Qi has previously demonstrated that ITK forms dimers on the cell membrane[8]. In this paper, those results were replicated and verified, and once again it was proven that ITK forms dimers at the cell membrane. It was also shown that these dimers are PH-domain dependent, and *only* PH-domain dependent. Mutations in all ITK domains, except for the PH domain, had no effect on the ability of the ITK to dimerize and fluoresce. The question remains: what role does this dimerization play in TCR signaling? By testing the ITK dimers with different TCR signaling molecules, the goal is to find one that may change the dimer; either by inhibiting its formation, or by rescuing its formation in mutants like the R29C ITKs. In this paper, it was shown that PLC gamma does not abrogate dimer formation, or rescue it. SLP76 had identical results. Although these results are a far cry from a complete picture, they point to an adaptor-independent dimerization of ITK.

However, some of the experiments that were performed could have yielded more meaningful information had they been performed differently. The first and foremost is that a different dye matchup should have been used for ITK and SLP. DsRed and YFP's spectra overlap far too much for any sort of colocalization study. In the confocal experiments involving SLP 76, the dsRED attached to the SLP76 was able to fluoresce in the YFP filter. Obviously this

caused problems, because it then it was unclear whether a spot of fluorescent green was actually an ITK dimer in the cytosol, or if it was just bleed through from SLP76. Because the ITK-YFP constructs are complicated and also very effective at showcasing ITK dimers, the easiest thing to do to fix this problem would be to find a naked SLP76 plasmid, and either attach a different color fluorochrome to it, or just use antibodies. The use of antibodies would also supply some method to alter adjust the intensity of a certain signal, which is not present in plasmid-borne fluorochromes. Colocalization studies with SLP76 and ITK could produce some very interesting results, but so could any other pair of proteins in the TCR signaling cascade.

The four-plasmid transfections should also have been performed differently. Firstly, four-plasmid transfections should have been avoided if possible. It soon became clear that it was unlikely for all four plasmids to make it into one cell. But should the need arise, each plasmid has to be marked somehow. In the YFP1-ITK + ITK-YFP2 +PLC gamma +SLP76 transfections the PLC gamma should have been tagged. Because it was not tagged, not only was it unclear whether it had been transfected or not without doing an SDS-PAGE, but possible patterns of cell localization could have been observed. It's possible that coexpression of PLC gamma and SLP76 leads to the disruption of ITK dimers. But because the PLC gamma was not tagged, it would not have been possible to see.

Previous mistakes aside, there are plenty of exciting future projects that can build off of this research. Of particular interest is the elucidation of the intricate signaling that occurs between Zap-70 and PLCgamma activation. It is becoming clear that this part of the TCR signaling pathway is far from linear. The fact that both SLP76 and PLC gamma bind to ITK, but neither one by itself has an impact on ITK dimerization points to a complex array of intermolecular interactions. To analyze these interactions, the first step would be to do

colocalization studies for all proteins. The absorption and emission spectra would have to be distinct for each fluorochrome in order to avoid the same mistakes that plagued the experiments of this paper. Presumably, all molecules believed to interact with ITK should be examined, in order to get a more complete picture of which molecules are involved with ITK dimerization. An interesting next step, or possibly even a goal, would be to study these molecules in actual T cells. Then perhaps instead of adding molecules via transfection, the strategy would be to knockdown certain molecules, and assess their function in that manner.

Another interesting next step would be to investigate the role that dimerization plays in TCR signal transduction. It has recently been shown that ITK exists in vivo as an inactive monomer[10]. It has not been shown whether the ITK dimers that form on the cell membrane are necessary for calcium influx, or even for the ITK signalasome formation. To test this, ITK knockout T cells could be transfected with PH mutant ITK. The cells could then be analyzed to detect both ITK dimerization (which would presumably be abrogated) and calcium influx.

As with most signaling pathways, there is usually more to it than meets the eye. In this paper, it has been shown that ITK dimerizes on the cell surface. It has also been shown that this dimerization is independent of all domains but the PH domain on ITK. Finally, it was shown that at least on their own, PLC gamma and SLP76 have no affect on the dimerization of ITK. These data suggest a complex and non-linear series of interactions that allow TCR signaling to occur. The protein-protein interactions of ITK along with its conformation in the cell play a huge role in TCR signaling. This study, along with other, show that there is much room for further experimentation regarding this complex and intriguing process.

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Academic Vita

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Education

The Pennsylvania State University, University Park, PA Schreyer's Honors College
Major: Immunology and Infectious Disease
Minor: Biochemistry and Molecular Biology
Anticipated Graduation in May 2010

Research Experience

August Laboratory

- Worked as a research assistant from May 2007 to May 2010
- Premiere immunology lab in the Biomedical Sciences department at PSU
- Involved in graduate student projects
- Managed my own thesis project, "Physical interactions of ITK with itself and other proteins involved in the TCR signaling cascade"
- Experience in lab techniques including DNA ligation, plasmid construction, plasmid transfection, fluorescence micrcoscopy, and flow cytometry

Baums Laboratory

- Worked as a research assistant from September 2007 to December 2007
- Molecular ecology lab
- Gained experience with population genetics, gel electrophoresis, and DNA extraction

Scholarships and Awards

- Raymond F. Russel Scholarship 2008/2009
- Deans List Fall 2006, Spring 2007, Spring 2008, Fall 2008, Spring 2008, Fall 2009
- Schreyer Honor's College Discovery Grant Summer 2009, and Summer 2008

Service and activities

- Mount Nittany Medical Center September 2006 to May 2010
    - Hospital volunteer at both the emergency department and surgical center
    - Aided doctors and nurses with day-day tasks
    - Personal interaction with medical staff and patients
    - Trained other volunteers

- Homes of the Indian Nation (HOINA) Summer 2009
    - Summer service learning trip to India
    - Worked in an orphanage in rural village outside of Vishakhaputnam
    - Helped with day to day operations as well as classroom teaching for the children
    - English lessons for the staff
    - Community outreach programs
- Atlas Thon September 2006 to May 2010
    - Independent organization participates in Penn State's Dance Marathon
    - Raises money for kids with cancer
- Biochemistry Society September 2006 to May 2010
    - Professional society promoting discussion of pertinent materials
    - Provide education and tutoring for younger students
    - One of four seniors who ran the club
    - Held position of Secretary from September 2009 to May 2010

Work Experience

- Penn State Environmental Health and Safety – Infectious Waste/Chemical Waste management, January 2006 to August 2008
- Facilitated transfer and removal of infectious and chemical waste from various labs on campus

Computer/Lab Skills

- Gel electrophoresis
- SDS/Page electrophoresis
- Polymerase Chain Reaction
- Microsoft Excel
- Confocal Microscopy
- DNA Extraction
- Plasmid Transfection
- Flow Cytometry
- Antibody Staining

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- References available upon request