Interleukin-2-tyrosine kinase substrate docking and its regulation by an intramolecular interaction in phospholipase Cg1

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Interleukin-2-tyrosine kinase substrate docking and its regulation by an intramolecular interaction in phospholipase C

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

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CHAPTER 1. GENERAL INTRODUCTION

General Introduction

Interleukin-2-tyrosine kinase (Itk, also known as Tsk, Emt) was initially discovered in a PCR-based molecular screen for tyrosine kinase family members that are implicated in TCR signaling present in the thymus (1-3). Along with Btk, Tec, Rlk and Bmx, Itk belongs to the Tec kinase family, the second largest non-receptor tyrosine kinase family (3-5). Tec family kinases are primarily expressed in hematopoietic lineage with the exception of Tec and Bmx, which are also found in endothelial cells. Itk and Rlk are expressed in T cells, mast cells and NK cells. Tec is also expressed in T cells, although the expression level of Tec is lower than that of Itk and Rlk. Btk is expressed in all hematopoietic cells except T cells and plasma cells.

Tec family kinases play important roles in hematopoietic cells development and function. The importance of Tec family kinases has been emphasized by the fact that genetic mutations in Btk is linked to X-linked agammaglobulinemia (XLA) in human and X-linked immunodeficiency (XID) in murine, which is characterized by reduced serum immunoglobulin and defective B cell development and function (6, 7). Itk has played an important role in TCR signaling. Itk<sup>-/-</sup> mouse showed defective TCR signaling including reduced proliferation, IL-2 production, and production of effector cytokines (8-10). Recently it is found that Itk<sup>-/-</sup> or Itk<sup>-/-</sup> Rlk<sup>-/-</sup> mice showed impaired thymic development and altered mature T cell effector functions (10-13). Growing evidence indicate that Itk also plays an important role in cytoskeletal reorganization and thus, regulates the formation of the immunological synapse (14-16).
The Tec family kinases share a similar domain structure with the Src family kinases with one Src homololy 3 (SH3) domain, one Src homology 2 (SH2) and a catalytic domain (Figure 1) \((17)\). However, the presence of the N-terminal Pleckstrin homology (PH) domain in Tec family kinases (except Rlk) and lack of the C-terminal phosphorylation tail make Tec family kinases distinct from Src family kinases. The Tec family kinases also include a unique TH (Tec homology) domain and proline rich (PR) motif. The structures of the individual Itk domains except the PH domain have been determined \((18-20)\). The PH-TH domain structure of Btk has been solved \((21, 22)\).

![Diagram of domain structures in Src and Tec kinases](image)

**Figure 1.** Src and Tec family kinases domain structure.

The non-catalytic PH, SH3 and SH2 modules mediate different protein-protein and protein-lipid interactions. In addition to mediating the intramolecular interaction with the PR motif, the Itk SH3 can also interact with the SH2 domain in a non-classical manner \((23)\). In my thesis, I extended this domain-domain interaction to the full-length Itk molecules and
presented data suggesting self-association of Itk can downregulate Itk kinase activity both in vitro and in primary T cells.

During TCR signaling, Itk phosphorylates its substrate PLCβ (5, 24). This phosphorylation activates PLCβ, which can hydrolyze PIP2 into inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) (25). In my thesis, I define the mechanism by which Itk phosphorylates PLCβ and show that a substrate docking interaction is required for efficient phosphorylation of both a PLCβ fragment and the full-length molecule. I also show that the docking interaction can be regulated by an intramolecular interaction within PLCβ. These studies provide insight into the mechanism of Itk substrate recognition and TCR signaling regulation.

Literature Review

Huge progress has been made in understanding the function of Itk in the two signaling pathways that involve Itk, the TCR signaling pathway and TCR engagement induced cytoskeletal reorganization. Due to the development of X-ray and NMR methods, the structural studies at the molecular level contributed greatly to the understanding of Itk function in the last decade.

Tec kinase structural features: PH domain

PH domains are the 11th most prevalent domains in the human genome (26) and they were first identified as structural modules that contain 100-120 amino acid residues. Proteins typically contain one or two copies of the PH domain and some proteins contain split PH
domains. Phospholipase C\(\alpha\) contains two PH domains, with one split PH domain with two halves flanking tandem SH2 domains and a SH3 domain (Figure 2).

![Figure 2. PLC\(\gamma\) domain structure. Adapted from ref. 80](image)

PH domains participate in cellular signal transduction through binding to phosphoinositides and then recruiting their host proteins to the cell membrane (26). For example, Tec family kinase PH domains specifically recognize the phosphoinositol-3-kinase (PI3K) product, phosphatidylinositol (3, 4, 5)-trisphosphate \([\text{PtdIns}(3, 4, 5)\text{P}_3]\) and then cause Tec kinase family members to associate with the cell membrane in a signaling dependent manner. However, recent studies in yeast indicate that only a small fraction of known PH domains bind phosphoinositides and most of these PH domains bind phosphoinositides with only low affinity and with little-to-no specificity (26). These studies suggest that PH domains may have binding targets other than phosphoinositides and their function is much more diverse than previously supposed.

Many protein ligands have been reported for PH domains aside from the phosphoinositides. Yao and coworkers reported that the Btk PH domain can interact with F-actin, which is important for the co-localization of Btk with actin filaments and also actin reorganization after receptor cross-linking (27). So far it has been reported that the Btk PH domain can interact with \(\zeta\) subunits of G proteins (28), protein kinase C isoforms (29), and Fas (30). Kim and coworkers presented evidence to suggest that the PH domain of another Tec kinase family member, Bmx/Etk, can interact with the small GTPase RhoA and activate
Evidence also indicates that the Etk PH domain can interact with focal adhesion kinase (FAK) (32). Therefore, the PH domain might play a diverse role by mediating many different protein-protein and protein-lipid interactions in cellular signaling transduction.

PH domain structures from many proteins have been solved, including the Btk PH domain (21, 22). All of these PH domains, including the split PH domains (33), share similar three-dimensional structures with a seven-stranded bent β-sheet with a C-terminal α-helix closing one end of the core β-barrel (34). Structures of several PH domains complexed with different phosphorylated ligands (inositol phosphates or phosphatidylinositol lipids) indicate the phospholipid binding site is not conserved in these domains (34). But all of these PH domains showed remarkable electrostatic polarization. The positively charged part of PH domains is involved in interaction with the negatively charged inositol phosphate. Removal of the positive charge in the binding pocket can significantly decrease the binding affinity of PH domain for phosphoinositides and introducing positively charged residues in the binding pocket can increase the binding affinity of PH domain for phosphoinositides. Removing a positive charge in Btk PH domain by mutating R29 to Cys destroys the interaction of PH domain with the phosphoinositides and leads to the failure of Btk membrane association (21, 35). Mutations in the Btk PH domain that lead to XLA, which is characterized by reduced serum immunoglobulin and defective B cell development and function (6, 7), are clustered at the positively charged end of the molecule around the predicted binding site for phosphatidylinositol lipids. On the other hand, increased positive potential in the gain-of-function mutant E41K leads to increased affinity for phosphatidylinositol lipids and constitutive association with the membrane and thus the activation of Btk (22, 35).
**Tec kinase structural features: SH3 domain**

SH3 domains are small binding modules that contain approximately 60 amino acids and are ubiquitous in eukaryotes. SH3 domains are found in many proteins that are involved in cellular signaling and cytoskeletal rearrangements. The function of the SH3 domain is to participate in cellular signal transduction by controlling protein-protein interactions. SH3 domains, including Itk SH3 domain, share conserved three-dimensional structures, consisting of two short β sheets that are packed against each other approximately at right angles (18, 36). Each β sheet is composed of two anti-parallel β strands and prominent connecting loops—termed the RT loop and n-Src loop.

The canonical interaction ligands for SH3 domains are proline rich motifs with the consensus sequence of PxxP (P stands for Proline and x is any amino acid), which form a left-handed polyproline-II helix (PPII) conformation in which the two invariant proline residues are found on the same face of the peptide and participate in hydrophobic contacts with conserved aromatic residues formed by the RT-loop and n-Src loop in the SH3 domain. The PxxP motif can bind SH3 domains in two different orientations. Based on the relative orientation of the basic residues to the core PxxP motif, the ligands can be classified as class I and class II. Class I proline rich ligand therefore have the consensus (R/K) xxPxxP and class II proline rich ligand have the consensus PxxPx(R/K). Residues that flank the PxxP motif can modulate the specificity and affinity of SH3/PxxP interaction not only by providing additional binding energy through electrostatic interaction with residues in the specificity pocket (a conserved Asp or Glu in RT loop of many SH3 domain), but also by orienting the ligand with respect to the binding groove on the SH3 domain (37).
In addition to interact with the PxxP motif, a large number of SH3 domains can also interact with the proline rich motif in a different manner. All Tec family kinases except the Bmx/Etk possess the proline rich motif right next to the SH3 domains (figure 1). Andreotti et al. reported Itk SH3 domain could bind proline rich motif in an intramolecular manner (18). Extensive structural studies also showed that Tec family kinase regulatory domains self-associate intermolecularly through the SH3/proline rich motif interaction in Rlk, Btk and Tec (38, 39).

SH3 domains can also participate in interactions with ligands that lack the canonical proline rich motif. For the Src family kinases, the SH3 domains interacts with the linker connecting SH2 domain and the kinase domain in an intramolecular manner (40). There is only one proline in the linker but it nevertheless forms a left-handed helix. Mutation of SH3 domain residues in the binding pocket leads to deregulated kinase activity (40).

**Tec kinase structural features: SH2 domain**

SH2 domains are structural modules that are made up of approximately 100 amino acids, conserved from yeast to eukaryotes, found in many proteins that are involved in different cellular signaling pathways including mitogenesis, motility, metabolism, immune response and gene transcription (41). Among these signaling pathways those mediated by protein kinases and protein phosphotases are the best characterized. SH2 domain mediated interactions can regulate enzyme activity in the interaction partner or by promoting protein-protein interactions (42).

All SH2 domains share a conserved three-dimensional structure with a central β sheet flanked by an α helix at each side (43). The best characterized ligand for SH2 domains are
phosphor-tyrosine (pY) containing sequences, which are critical for regulating the activity of many protein kinases and many protein-protein interactions. SH2 domains form two binding pockets, one for the phosphor-tyrosine and the other one for the residues at the C-terminal of the phosphor-tyrosine (the pY+3 residue). The universally conserved Arg in the binding pocket can bind phosphor-tyrosine by making a bidentate ionic interaction with two oxygens of the phosphate group (44, 45). The hydrophobic contact between the pY+3 side chains and the binding pocket can modulate the specificity and affinity of SH2/phosphor-tyrosine interaction.

Accumulating evidence indicates that many SH2 domains are also involved in interactions with binding partners that lack the phosphor-tyrosine motif. Our group previously reported that Asn 286-Pro 287 imide bond in the Itk SH2 domain adopts both the cis and trans conformation in solution (46). The isomerization of the cis and trans conformer regulates the ligand binding ability of the conformers (47). The cis conformer binds the Itk SH3 domain with higher affinity and the trans conformer binds phosphorylated SLP-76 with higher affinity. The noncanonical interaction surface between the Itk SH2 domain and the Itk SH3 domain has been mapped (46). The CD loop in the SH2 domain and the conserved hydrophobic binding pocket in Itk SH3 domain are implicated in the interaction. Consistent with the selective ligand interaction, NMR structure analysis indicates that the cis and trans conformers share similar three-dimensional structures with the exception of the CD loop (19). The noncanonical interaction of Itk SH2/SH3 domain can lead to dimerization/oligomerization of the larger fragment that contains both the SH3 and SH2 domains (23).
So far there are three SH2 domains have been directly implicated in human disease, signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) SH2, Btk SH2 and N-terminal SH2 domain of the nonreceptor protein tyrosine phosphoatase 2 (SHP-2) (48). Although the number of SH2 domains that are directly involved in disease is few, the importance of SH2 domains in signaling transduction makes it desirable to design some inhibitor or antagonist to selectively target specific proteins to alter specific signal transduction pathway. The availability of large numbers of high-resolution SH2 domain structures complexed with pY ligand and the importance of SH2/pY interaction laid the foundation to screen minimally sized phosphopeptide ligand of approximately 4-6 residues that can efficiently bind specific SH2 domains to selectivity inhibit cellular signal transduction pathway in deregulated cells (41). In the last decade significant accomplishments have been made in Src SH2, Grb SH2 and Zap 70 SH2 domains drug discovery (41). The discovery of noncanonical interaction ligand for SH2 domains potentially provides another template for designing new inhibitor drugs.

**Tec kinase structural features: kinase domain**

Kinase domains are the catalytic domains of protein kinases that transfer the \[\text{γ}\] phosphate group from ATP to a specific hydroxyl group in the substrate, affecting conformation and activity. The human genome encodes ~500 kinase genes, which is about 1.7% of all human genes, and ~100 of them are protein tyrosine kinases (49). Protein tyrosine kinases mediate many of the cellular signaling transduction pathways and control many cellular process. Depending on the cellular localization, there are receptor tyrosine kinases and non-receptor tyrosine kinases. Tyrosine kinase activities are strictly regulated by
complicated mechanism and loss of regulation will lead to severe disease like cancer, inflammation and autoimmune disease, which makes kinases important drug targets (50).

Biochemical analysis and structural characterization of kinase domains alone and/or full-length tyrosine kinases in both active and inactive conformation have provided significant insight into the mechanism of kinase regulation. All kinase domains share a conserved bilobal protein kinase domain fold and are composed of a smaller N lobe, and a larger C lobe (51). N lobes consist of five ⬤ strands and a critical ⬤ helix. C lobes consist of mainly ⬤ helix and an activation loop. In many tyrosine kinases, the activation loop needs to be phosphorylated to switch the enzyme to its active conformation. The N lobe and C lobe are connected by a loop, which forms a cleft for nucleotide binding and phosphotransfer.

Src family kinase regulatory mechanisms are one of the best-characterized regulation systems. Accumulating evidence indicates that Src kinase activity could be regulated by several key structural features (51, 52): the orientation of the helix C in the N-lobe, phosphorylation status of the activation loop, the relative orientation of the N lobe and C lobe, and intramolecular interactions with the Src homology domains. The Src SH2 domain interacts with the C-terminal phosphorylated Y527 tail in an intramolecular manner. As already mentioned above, the Src SH3 domain can also interact with the linker between the SH2 domain and N lobe of the kinase domain, which contains one proline yet still forms a polyproline type II helix. The intramolecular interactions mediated by the SH2 and SH3 domains lock the kinase domain in a rigid and inactive conformation (53-55). Importantly, these intramolecular interaction ligands don’t possess canonical interaction ligand property and therefore are not high- affinity ligands (51). When the SH2 and SH3 domain mediated intramolecular interactions are disengaged, helix C will be reoriented into an active
conformation for substrate binding through forming a conserved ion pair between Glu 310 (residue from helix C) and Arg 285 (Src numbering). The reorientation of helix C and phosphorylation of the activation loop are coupled (52). When the kinase domains are in an inactive conformation, the activation loop adopts a helix conformation and blocks the phosphorylation of Y416 and the access of substrate peptide. When Y416 is phosphorylated and helix C adopts an active conformation, the kinase become fully active (51).

Phosphorylation of the activation loop leads to a conformational change in the activation loop and concurrent activation of the kinase is a conserved regulation mechanism in Src family kinases. However, analysis of the crystal structures of Tec family kinase Itk indicates that the Tec family kinases might be regulated by a different mechanism. Crystal structures of the Itk kinase domain in both phosphorylated and unphosphorylated states have been solved (20). The Itk kinase domain shares the conserved bilobal structure and is similar to structures of the active states of many other kinases. Surprisingly, there is no significant conformational difference between the phosphorylated states and the unphosphorylated states in Itk. A structure has been solved for the kinase domain of another Tec family member, Btk. The Btk kinase domain structure corresponds to the unphosphorylated form (56). Overall, the Btk kinase domain structure is very similar to that of the phosphorylated Lck kinase domain except for helix C which is in an inactivated conformation (57). What is noteworthy is that the activation loop in the Btk kinase domain adopts an open conformation similar to that of Lck without limiting the access of substrate. However, biochemical data clearly indicated that transphosphorylation of Y551 (Btk) and Y511 (Itk) are critical for enzyme activation both in vitro and primary T cells (58-60). Therefore regulation
mechanism of Tec family kinases is different from that of Src family kinases and exerted by both the catalytic and the regulatory domains coordinately.

**TCR signaling pathway and the role of Itk**

Itk is involved in the downstream signaling pathway of various T cell receptors such as TCR (T cell receptor)/CD3, CD28 and CD2 (61). Among these receptors, the TCR signaling pathway is the best understood. TCR is a transmembrane protein composed of heterodimer \( \alpha \) and \( \beta \) subunits, which are covalently linked to each other by a disulfide bridge. TCR \( \alpha \) and \( \beta \) chains are noncovalently associated with CD3 \( \gamma \), \( \delta \), and TCR \( \zeta \) proteins (Figure 3) (62, 63). Each of the CD3 \( \gamma \), \( \delta \) cytoplasmic domains contains a conserved immunoreceptor tyrosine-based activation motif (ITAM) with the consensus sequence YxxL(x)_{6-8}YxxL and the \( \zeta \) chain contains three ITAM motifs. Each ITAM motif contains 2 tyrosines and there are total 10 ITAM motifs for all the CD3 chains and \( \zeta \) chain.

TCR has no intrinsic kinase activity. When TCR binds the peptide/MHC (major histocompatibility complex) complex in APC (antigen presenting cells), co-receptor CD4 or CD8 bind different regions of the MHC complex at the same time, which also brings the associated Src family kinase, Lck into the cluster and activates Lck at the same time. Lck can phosphorylate Tyr in the ITAM motifs and then the phosphorylated ITAM motif in the \( \zeta \) chain will become a docking site for the Syk family tyrosine kinase ZAP-70 through the canonical SH2/phosphotyrosine interaction. After ZAP-70 is enrolled to the cluster, ZAP-70 can be phosphorylated and activated by Lck. Activated ZAP-70 in turn can phosphorylate downstream signaling molecules. Linker for activation of T cells (LAT) and the SH2-domain-containing leukocyte protein of 76 kD (SLP-76) are two adaptor proteins, both of
which can be phosphorylated by ZAP-70 and then they can serve as platforms for recruiting other signaling molecules like the adaptors Grb-2, GADS and Nck, the guanine-nucleotide-exchange factor (GEF) Vav1, and PLC\(\Gamma\) (17, 24, 64) (Figure 4). Lck can also phosphorylate and activate phosphoinositide 3-kinase (PI3K), which will catalyze the formation of phosphatidylinositol (3, 4, 5)-trisphosphate [PtdIns (3, 4, 5) P\(_3\)]. Itk can translocate to the plasma membrane by interacting with PtdIns (3, 4, 5) P\(_3\) through its PH domain (4, 5) as described above. When these molecules are all localized to the membrane, they will form a multimolecular signaling complex. Itk is then phosphorylated and activated by other mechanisms, which are described in detail later in this introduction.

![Figure 3. Schematic illustration of TCR. Adapted from reference 63.](image)

When Itk is activated, Itk can phosphorylate its substrate PLC\(\Gamma\) (5). When PLC\(\Gamma\) is phosphorylated and activated by Itk, it hydrolyzes PIP\(_2\) into inositol 1, 4, 5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG) (25). Then IP\(_3\) and DAG can stimulate cytosol Ca\(^{2+}\) increase and activate PKC, respectively and eventually lead to activation of transcription factor that
regulate T cell gene expression through the activation of Ras-Raf-ERK (extracellular receptor-activated kinase) pathway.

**Figure 4. T-cell receptor signaling to cytoskeleton. Adapted from reference 17.**

**TCR engagement induces cytoskeleton reorganization**

Cytoskeletal reorganization is the second pathway involving Itk that occurs following TCR engagement (14-16). After the TCR complex recognizes a particular MHC-associated peptide on an antigen presenting cell (APC), several T cell surface proteins and intracellular signaling molecules are rapidly mobilized to the site of T cell-APC contact. Concurrently, F-actin started to accumulate at the site of contact with the APC, which will lead to cell polarization. The physical contact region between the T cell and APC is called an immunological synapse (IS), or a supramolecular activation cluster (SMAC). The formation of the synapse is not required for initial TCR activation and protein phosphorylation, but the
synapse formation is important for prolonged and effective T cell signaling (65). The affinity between TCR and peptide-MHC complexes is low therefore the enrichment of adhesion molecules in the synapse will facilitate the binding of TCR to APCs promoting repeated engagement of the TCR.

Itk is implicated in actin cytoskeleton regulation, which is necessary for cell adhesion and formation of the immune synapse in T lymphocyte (66). Itk deficient T cells or Jurkat T cells with diminished Itk expression level showed disrupted TCR-induced actin polarization and deficiency in immunological synapse formation. Compared with decreased efficiency in PLCγ phosphorylation and Ca2+ mobilization, the defect in actin polymerization and formation of the immunological synapse after antigen stimulation indicates that Itk plays an essential role in cytoskeleton reorganization. Therefore Itk regulates cytoskeletal reorganization and through which regulates the formation of the immunological synapse and duration of TCR signaling.

The molecular mechanism of Itk regulating actin cytoskeleton is not clear. There is research indicating that this function of Itk is not dependent on Itk kinase activity since the TCR induced actin cytoskeleton defect in Itk-/- or Itk expression level diminished mice can be rescued by the expression of an Itk kinase inactive mutant (14). This function is related to the PH domain and canonical function of Itk SH2 domain, but not the Itk SH3 domain (16). Tec kinases might also play a critical role in TCR-mediated regulation of integrin adhesion and the recruitment of key signaling proteins involved in adhesion, including LFA-1 and talin (66).

WASP (Wiskott-Aldrich syndrome protein) is another component in the actin signaling pathway and mutation of WASP will lead to abnormal T cell cytoskeleton
organization (17). WASP can interact with the Rho family GTPase Cdc42, which will induce large conformational changes in WASP and also activate WASP. Itk deficient T cells show a profound defect in Cdc42 activation and WASP activation in the immunological synapse (15). Therefore Itk is required for recruitment and activation of both Cdc42 and WASP at the immunological synapse. Itk can also directly regulate and phosphorylate WASP (15).

Vav-1 is another protein that is rapidly phosphorylated after TCR engagement. Vav-1 can be activated by phosphorylation then Vav-1 acts as a GEF factor for the Rho family GTPases Rac, Rho and Cdc42. Vav-1 plays an important role in the signaling pathway that regulates actin cytoskeleton after TCR engagement (17). Vav-1 deficient T cells showed defective TCR mediated actin polymerization and cell adhesion. Itk-/- T cells showed defective TCR induced actin polymerization and also total abolishment of Vav-1 recruitment although for both wild type T-cells and Jurkat T cells Vav-1 is recruited to the synapse. Similar results were obtained when Vav-1 DNA and siRNA oligonucleotides for inhibiting Itk expression were cotransfected into Jurkat T cells. Therefore, the other pathway by which Itk regulates actin cytoskeleton reorganization is through regulating the localization of Vav-1. Mombroski and coworkers showed that expression of membrane targeted Vav-1 can rescue the siRNA for Itk induced defective actin cytoskeleton (14) and this data supported the regulatory role that Itk played in regulating actin cytoskeleton through Vav-1. The molecular mechanism is not clear. There is evidence showing that there is constitutive association between Vav-1 and Itk, although it is not clear whether the association is direct or indirect (14). Both Vav-1 and Itk can interact with SLP-76 through their SH2 domains.
Therefore it is possible that Itk can regulate Vav-1 localization by affecting the ability of Vav-1 to interact with SLP-76 (or LAT).

**Initial Itk activation mechanism**

When the TCR is engaged, Itk will be phosphorylated and activated within 120 seconds (67). Itk activation requires two events: membrane translocation through PH domain binding to the product of phosphoinositide 3-kinase, then transphosphorylation by the Src family kinase Lck. Phosphatidylinositol 3-kinase inhibitor can inhibit Itk membrane localization and therefore Itk phosphorylation and activation (68, 69). The PH domain is more than just a membrane translocation signal in Itk activation since Itk membrane targeting through membrane localization signal found in the N-terminus of Lck can reconstitute its inducible colocalization with TCR-CD3 but not its tyrosine phosphorylation and activation (70).

After TCR engagement, Itk participates in a multimolecular signaling complex, which includes LAT, Grb2, SLP-76, and PLC\[\text{II}\]. Itk will be recruited to the signaling complex by multivalent protein-protein interactions (71). The Itk proline rich region can bind Grb2 and LAT; both Itk SH3 and SH2 domain can interact with phosphorylated SLP-76. These multiple interactions are critical for proper localization of Itk and TCR signal transduction.

After Itk is recruited to the multimolecular signaling complex, Itk can be phosphorylated by the Src family kinase Lck at Y511 (60). Y511 is a conserved phosphorylation site in the activation loop of Tec family kinases kinase domain (Y551 in Btk) and the phosphorylation of Y511 is critical for the kinase activity of Tec family kinases (58, 59). When Btk Y511 is phosphorylated, kinase activity will be enhanced about 10 times.
Itk Y511F leads to total abolishment of Itk kinase activity in insect cells. Itk Y511F can’t rescue cytokine production and ERK activation in Itk deficient T cells while wild type Itk does (60).

When Itk is phosphorylated at Y511, Itk becomes active and phosphorylates its own SH3 domain at Y180 (72). Itk autophosphorylation occurs in cis manner although there is evidence indicating that Itk can oligomerize (73-75). In vitro kinase assays indicate that autophosphorylation on Y180 does not change Itk intrinsic kinase activity but instead changes the ability of the Itk SH3 domain to recognize substrates and participate in protein-protein interactions with ligands (74). This result provides an explanation for the early report that Itk Y180F has only partial activity in rescuing cytokine production and ERK phosphorylation in Itk deficient T cells (74). This is also consistent with the previous report that when the Btk SH3 domain is autophosphorylated, its ligand binding properties are altered compared to the nonphosphorylated protein (76, 77).

**Itk kinase activity regulation mechanism**

It is well known that intramolecular interactions mediated by SH3 and SH2 domains maintain Src family kinases in an inactive state (40, 51, 52). However the mechanisms that regulate Itk kinase activity are not clear. A detailed regulation mechanism of Itk (or Tec family kinase) is still awaiting elucidation of full-length molecule structure. Based on domain studies, Itk SH3 and SH2 domains can mediate multiple interactions. The Itk SH3 domain can interact with its own PR motif in an intramolecular manner and this interaction can also affect the ability of Itk SH3 domain to interact with other ligands (18).
In addition to interacting with the Itk proline rich region, Itk SH3 domain can also interact with its own SH2 domain in an intermolecular manner (23). The interaction between Itk SH2 and SH3 domains leads to dimerization/oligomerization of the Itk molecule, which seems to be a conserved feature in Tec family kinases although the dimerization/oligomerization is mediated by different domains for the different Tec kinases (38, 39). In the larger fragment that contains Itk proline rich region, SH3 and SH2 domains or full-length Itk, the intermolecular interaction mediated by Itk SH3 and SH2 domains seems to dominate over the intramolecular PxxP motif/SH3 interaction. We have shown that dimerization/oligomerization can decrease Itk kinase activity and mutations that diminish the dimerization/oligomerization enhances Itk kinase activity both in vitro and in vivo (Lie Min, Amy H. Andreotti, submitted to JBC, see Chapter 5), which has been talked in the Chapter 5. This is consistent with the results that deletion of TH domain in full-length Itk leads to decreased Itk kinase activity and deletion of Itk SH3 domain leads to increased Itk kinase activity (78).

Itk kinase activity can also be regulated by interaction with regulating ligands. Recently our group reported a novel substrate docking interaction that is mediated by the Itk kinase domain and substrate SH2 domains (Itk SH2 domain or PLCγ1 SH2C) (79). This substrate docking interaction is necessary for substrates to be efficiently phosphorylated. This interaction is distinct from most SH2 domains mediated interactions and it is not dependent on phosphor-tyrosine ligand. The data describes this regulatory mechanism is presented in Chapters 2 and 3.
**PLC\[g\]**

PLC\[g\] is a phospholipase that hydrolyzes PIP\(_2\) into IP\(_3\) and DAG. PLC\[g\] belongs to the phosphoinositide-specific phospholipase C (PI-PLC) family, which includes six families and 14 mammalian PLC isozymes. These are PLC\[b\] (\(\beta 1-\beta 4\)), PLC\[\gamma\] (\(\gamma 1-\gamma 2\)), PLC\[\delta\] (\(\delta 1-\delta 4\)), PLC\[\epsilon\] and PLC\[\zeta\] (\(\zeta 1-\zeta 2\)) (Figure 5) (80, 81). These PLC isozymes contain very different combinations of structural modules but all contain the conserved catalytic domain X and Y. Among these family members, PLC\[\gamma\] is the best characterized. PLC\[\gamma 2\] is expressed in B cells while PLC\[\gamma 1\] is expressed ubiquitously. PLC\[\gamma\] can be activated by many different growth factor receptors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), nerve growth factor receptor (NGF) (82) and T cell receptor (83). PLC\[\gamma\] plays an important role in cell proliferation and differentiation. PLC\[\gamma 1\] deficiency is early embryonic lethal because of defects in growth and development (84).

![Figure 5. Schematic illustration of mammalian phospholipase C (PLC) isozymes. Adapted from reference 80.](image-url)
PLC\(\gamma\) consists of an amino terminal PH domain, a number of EF hands, catalytic domain X and Y split by a PH domain \((80, 83)\). There is a large regulatory SH domain insert composed of N-terminal SH2 (SH2N), C-terminal SH2 (SH2C) and SH3 domain within the second PH domain. The SH2 domains mediated interaction is important for phosphorylation and activation of PLC\(\gamma\) \((85-87)\). SH3 domain mediated interactions play an important role in many cellular processes like cell motility, proliferation, and development that implicate PLC\(\gamma\) \((88-90)\).

Src Homology domains can also regulate PLC\(\gamma\) activity. Homma and coworkers reported that the protein fragment that includes the PLC\(\gamma\) SH2N, SH2C and the SH3 domains can inhibit PLC\(\gamma\) activity in trans and this inhibition derives primarily from a 8-mer peptide in the SH2C \((91)\). Consistent with this, Carpenter and coworkers reported that deletion of the SH domains leads to 20- to 100- fold increase in PLC\(\gamma\) activity \((92)\). Koblan and coworkers reported that a phosphotyrosine ligand could cause conformational changes in PLC\(\gamma\) and also increase lipase activity up to 85\% \((93)\).

The other important factor that regulates PLC\(\gamma\) activation is tyrosine phosphorylation. There are four phosphorylation sites in PLC\(\gamma\), Y771, Y775 \((94)\), Y783 and Y1253 \((95)\). The phosphorylation level of Y771 is about 10\% that of Y783 and is considered a minor phosphorylation site. The first three tyrosines are located in the linker between SH2C and SH3 and the phosphorylation of these three tyrosines can be detected in T cells after antigen stimulation but not the phosphorylation of Y1253, which can be detected in cells that are stimulated with PDGF or EGF \((95)\). Phosphorylation of Y783 is essential for the activation of PLC\(\gamma\), while the mutation of Y771 and Y1254 has no effect of the
phosphorylation and activation of PLC\(\Gamma\) (80, 94). After Y783 is phosphorylated, PLC\(\Gamma\) is thought to undergo conformational change to form an intramolecular interaction between PLC\(\Gamma\) SH2C and phosphor-Y783, which leads to increase in phospholipase activity of PLC\(\Gamma\) (96). The newly identified Y775 is also important for PLC\(\Gamma\) function (94).

By mutating the canonical phosphor-tyrosine binding pocket in the SH2 domains, numerous studies found that SH2N is required for membrane recruitment and tyrosine phosphorylation of PLC\(\Gamma\), while the SH2C is not critical for this event (86, 87). Rellanhan and coworkers found that SH2C could interact with phosphorylated LAT and BLNK with the same affinity \textit{in vitro} as SH2N and it can also interact with nonphosphorylated peptide from LAT (97). However, it is reported that mutation of the conserved Arg binding pocket in SH2C was not required for full length PLC\(\Gamma\) mediated interaction \textit{in vivo} (86, 87). These data suggest that an interaction surface in SH2C is possibly buried in full length PLC\(\Gamma\) before phosphorylation and activation. The fact that SH2C but not SH2N can interact with a nonphosphorylated peptide also suggests that SH2C at least mediates noncanonical ligand interactions. Thus, the commonly used criteria for judging SH2 domain function by mutating the conserved phosphor-tyrosine ligand binding pocket might be misleading and complicate the interpretation of SH2 domain function.

**Substrate docking**

Kinases and phosphatases are two key pairs of enzymes that control the signal transduction processes in the cell. Thus, it is critical to control not only the activity of these signaling proteins but also the specificity of these enzymes. One common strategy that tyrosine kinases/phosphatases utilize is to exploit protein-protein interaction modules like the
SH2 or SH3 domain, which are often fused to the core catalytic domain (98). Another strategy that Ser/Thr kinases/phosphatases use is a substrate docking mechanism in which the catalytic domain harbors a substrate-binding surface that is distinct from the catalytic site (98).

The substrate docking mechanism is well characterized in serine/threonine kinase/phosphatases such as PP1 and calcineurin, MAP kinase, CDD/cyclins, PDK1 and GSK3 (98). The structural bases for many of these docking interactions have been described. Structure data and mutagenesis analyses indicate that in most docking interactions linear peptides motifs within substrates are recognized by a groove in the catalytic domain (98).

C-terminal Src kinase (Csk) is the first tyrosine kinase for which a docking interaction is required to phosphorylate its substrate Src or Yes has been described (99, 100). Recently, our group found that the Tec family kinases also utilize a specific docking interaction to phosphorylate its substrates (79). This work is described in detail in Chapters 2 and 3.

Deregulation of kinases/phosphatases often leads to diseases like cancers; therefore the discovery of specific kinases/phosphatase inhibitors is essential for therapeutic application to combat such diseases. To date, the most common kinase inhibitors target the conserved ATP binding site. Despite some success in industry, similar structure of the kinase ATP binding site makes this type of drug inherently cross-reactive. Therefore, targeting an enzyme/substrate docking interaction provides another approach for developing small molecules that could affect enzyme activity by modulating protein-protein interactions rather than targeting an active site. Small molecule inhibitors of this type have been successfully screened for the calcineurin-NFAT interaction (101). As well, it has been reported that
peptide inhibitors containing the appropriate docking motif can selectively modulate MAPK and PP1 activity in vivo (102). These results indicate that structural and biochemical studies to define substrate docking interactions have the potential to lead to the discovery of specific and useful drugs.

**Thesis organization**

This thesis includes six chapters: a general introduction (Chapter 1), followed by four chapters (Chapter 2-5 describe scientific results) and a general conclusion (Chapter 6). The literature review in Chapter 1 contains the necessary background information and current status of the cellular singaling pathway of Itk, initial activation of Itk and its domain features. Chapter 2, which has been published in a peer-reviewed journal, describes a substrate docking mechanism in the Tec family kinases. Chapter 3, which is a manuscript in preparation for submission, extended the substrate docking mechanism discovered in Chapter 2 to full length PLC-I. Chapter 4, which is ongoing work and will be published upon the determination of the PLC-I SH2C-linker complex structure, shows that this docking interaction can be regulated by an intramolecular interaction within PLC-I. Chapter 5, which has been submitted for publication, presents data suggesting that the self-association of Itk can downregulate Itk kinase activity in vitro and in primary T cells. The general conclusion chapter (Chapter 6) includes a summary of the research work and future directions of study.

As primary author, I carried out most of the experiments in this thesis. In Chapter 2, I performed all PLC-I related experiments and Dr. Raji E. Joseph obtained data for the Itk SH3 domain. I performed all of the experiments described in Chapter 3 and 4. In Chapter 5, I
performed the all of the biochemical experiments and Dr. Wenfang Wu (University of Massachusetts Medical School) obtained the data from insect cells and primary T cells. In addition, Chapters 2-5 benefited greatly from discussion with Dr. Amy H. Andreotti, Dr. D. Bruce Fulton, and Dr. Raji E. Joseph.

References


CHAPTER 2. A REMOTE SUBSTRATE-DOCKING MECHANISM
FOR THE TEC FAMILY TYROSINE KINASES


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Abstract

During T cell signaling, Itk selectively phosphorylates a tyrosine within its own SH3 domain and a tyrosine within PLCγ. We find that the remote SH2 domain in each of these substrates is required to achieve efficient tyrosine phosphorylation by Itk and extend this observation to two other Tec family kinases, Btk and Tec. Additionally, we detect a stable interaction between the substrate SH2 domains and the kinase domain of Itk, and find that addition of specific, exogenous SH2 domains to the in vitro kinase assay competes directly with substrate phosphorylation. Based on these results, we show that the kinetic parameters of a generic peptide substrate of Itk are significantly improved by fusing the peptide substrate to the SH2 domain of PLCγ. This work is the first characterization of a substrate docking mechanism for the Tec kinases and provides evidence for a novel, phosphotyrosine-independent regulatory role for the ubiquitous SH2 domain.

KEYWORDS Itk substrate docking, SH2 domain, phosphotyrosine-independent, Tec kinases
Introduction

Transfer of the γ-phosphate group of ATP to amino acid side chains is a primary mechanism of cellular signal transduction and is carried out by a large family of enzymes termed the protein kinases. The manner in which substrate recognition is achieved by the myriad of protein kinases is not completely understood but it is clear that specificity determinants can be outside of the motif immediately surrounding a particular phosphorylation site. Docking sites have been characterized for a number of protein kinase families that include JNKs, cyclin CDKs, and MAP kinases (1-10). For the subfamily of protein tyrosine kinases, the molecular determinants of substrate recognition by the C-terminal Src kinase (Csk) have been elucidated (11, 12). For the Csk tyrosine kinase, six amino acids within the large lobe of the kinase domain comprise a remote substrate-docking motif (12) that binds to a complementary surface on the substrate (11). This docking mechanism allows Csk to recognize and phosphorylate its substrate in a specific manner.

The extent to which other families of non-receptor protein tyrosine kinases use remote docking mechanisms to achieve substrate specificity is not known. Of interest here are the Tec family kinases; immunological enzymes that comprise the second largest family of non-receptor tyrosine kinases. The Tec family kinases include Itk, Btk, Tec, Rlk and Bmx (13), and each contains a Src homology 3 (SH3) domain, Src homology 2 (SH2) domain and the catalytic domain.

The current study focuses on Itk (Interleukin-2 tyrosine kinase), the Tec kinase that participates in signaling processes following T cell receptor engagement by phosphorylating
Tyr 783 of phospholipase C\[γ\] (PLC\[γ\]) (14-19). Phosphorylation of PLC\[γ\] on specific tyrosine residues including Tyr 783 leads to activation of lipase activity (20). In addition to the PLC\[γ\] substrate, Itk also undergoes autophosphorylation on Tyr 180 within its SH3 domain (18, 21). The local amino acid sequences surrounding these two phosphorylation sites are shown in Figure 1a and reveal little sequence similarity. Moreover, the structural context of these target tyrosines differ since Tyr 180 of Itk is embedded within the SH3 domain fold (Fig. 1b) while Tyr 783 of PLC\[γ\] resides in a linker region between the carboxy-terminal SH2 domain (SH2C) and SH3 domain of PLC\[γ\] (Fig. 1c). Thus, sequence and structural differences between two known substrates of the Itk kinase raise questions related to the mechanisms by which Itk recognizes its targets in a sufficiently specific manner to maintain the fidelity of signal transduction.

In the present study, we find that the *SH2 domain* within each substrate serves a docking role for recognition and phosphorylation by Itk. In each of the substrates, the SH2 domain is remote from the site of phosphorylation and shuttles the substrate to the Itk kinase domain by enhancing the affinity of the enzyme/substrate complex. We also demonstrate that efficient substrate phosphorylation by two other Tec kinases, Tec and Btk, is equally dependent on SH2 docking indicating that this mechanism is conserved across the Tec family. Moreover, in contrast to the canonical, phosphotyrosine dependent binding behavior of SH2 domains, we show that the SH2 domains of Itk substrates perform this docking function in a phosphotyrosine-independent manner.
Results

Efficient phosphorylation by the Tec kinases requires the SH2 domain of the substrate.

In vitro kinase assays using a panel of different substrates (Fig. 1b & c) were carried out to assess the requirements for substrate phosphorylation by the Tec kinases. Tyrosine 180 within the Itk SH3 domain is the site of Itk autophosphorylation (18) yet the isolated SH3 domain does not serve as a substrate for full length Itk (Fig. 2a, lane 1). In a similar manner, the SH3 domains of Tec and Btk (containing the autophosphorylation sites Y187 and Y223, respectively) are not phosphorylated by the Tec and Btk kinases (Fig. 2b, lanes 1,2,5 & 6). Finally, a fragment of PLCγ1 that contains Tyr 783 and the adjacent SH3 domain of PLCγ1 is not phosphorylated by full length Itk (Fig. 2c, lanes 6-9). Thus, efficient phosphorylation of these Tec family substrates appears to require residues outside of the target phosphorylation site.

To further test the determinants for substrate phosphorylation we created additional Itk, Tec, Btk and PLCγ1 substrate constructs and subjected them to phosphorylation by full length Itk, Tec or Btk (Fig. 2a (lanes 2-4); 2b (lanes 3,4,7 & 8); 2c (lanes 2-5)). In every case, only substrates that contain both the site of phosphorylation (Y180 for Itk, Y187 for Tec, Y223 for Btk, and Y783 for PLCγ1) and the adjacent SH2 domain are phosphorylated by the Tec kinases. These data indicate that the SH2 domain adjacent to each phosphorylation site is required for efficient recognition and phosphorylation by the full-length Tec kinases. To test whether substrate phosphorylation can be achieved by the catalytic domain alone, the Itk SH3-SH2 substrate (Itk 32) was subjected to phosphorylation by the isolated Itk kinase domain (Itk KD). The Itk catalytic fragment leads to substrate
phosphorylation (Fig. 2d) suggesting that the kinase domain of Itk is sufficient for recognition of the SH2 domain-containing substrates.

*The SH2 domains of Itk and PLC[γ] interact with the Itk kinase domain in a phosphotyrosine-independent manner.*

Given the observation that the Itk kinase domain alone can phosphorylate substrate and that each substrate requires the presence of the SH2 domain adjacent to the target tyrosine residue, we investigated the extent to which the isolated SH2 domains of Itk and PLC[γ] interact directly with the Itk kinase domain. Myc-tagged Itk SH2 domain, GST-tagged PLC[γ] C-terminal SH2 (PLC[γ] SH2C) domain and FLAG-tagged Itk kinase domain were purified and subjected to pull-down experiments. The Itk kinase domain interacts directly with both the Itk SH2 domain and the PLC[γ] SH2C domain (Fig.3 a,b). We tested the nature of these interactions by mutating R265 in the Itk SH2 domain (22, 23) and R694 and R696 in PLC[γ] SH2C domain (24) to abolish phosphotyrosine mediated interactions of these SH2 domains. Mutation of the conserved arginines in the SH2 domains of both substrates has no discernable effect on substrate recognition and phosphorylation (Fig. 3c,d & e). To further probe phosphotyrosine requirements, we tested the effect of varying the phosphorylation state of the Itk kinase domain on its interaction with the PLC[γ] SH2C domain. Itk kinase domain that is expressed in insect cells without co-expression of Lck does not react with anti-pY antibody (Fig 3f (lane 1)) indicating an absence of phosphorylated tyrosine within the Itk molecule. In contrast, Itk kinase domain that is co-expressed with Lck is expected to be phosphorylated on Tyr 511 in the activation loop. Indeed, a phosphotyrosine blot of the Itk kinase domain that is co-expressed with Lck reveals robust
phosphorylation (Fig. 3f (lane 2)). Treatment with alkaline phosphatase effectively dephosphorylates the Itk kinase domain as evidenced by the absence of reactivity to an anti-phosphotyrosine antibody (Fig. 3f (lane 3)). Using this panel of Itk kinase domain preparations we found that PLCγ1 SH2C domain binds to the Itk kinase domain regardless of the phosphorylation status of the Itk kinase domain (Fig. 3g). Thus, several pieces of evidence point to a phosphotyrosine-independent interaction between the Itk kinase domain and the SH2 domains of Itk and PLCγ1 that mediates recognition and phosphorylation of these physiological substrates.

Free SH2 domain competes with SH2 domain-containing substrate and reduces phosphorylation of the substrate.

If the Itk SH2 domain and PLCγ1 SH2C domain are indeed docking sites required for the phosphorylation of Itk substrates, we expect that exogenous SH2 domain (either Itk SH2 or PLCγ1 SH2C) should effectively compete for the binding site on the Itk kinase domain and inhibit phosphorylation of the substrates. To address this, phosphorylation of Itk 32 and PLCγ1 SH2C-Y783- was monitored in the presence of increasing concentrations of either free Itk SH2 domain or free PLCγ1 SH2C domain (Fig. 4a,b). For both substrates, the corresponding free SH2 domain inhibits substrate phosphorylation (Fig. 4a,b) while two different control SH2 domains (derived from PI3K and Grb2) do not inhibit the phosphorylation of the substrates even at large molar excess (Fig. 4a,b). Inhibition by exogenous SH2 domain extends to phosphorylation of the full-length protein. In this case, Tyr 180 within full length Itk is the substrate and autophosphorylation at this site is greatly diminished upon addition of free Itk SH2 domain (Fig. 4c).
Finally, we tested whether addition of free SH2 domain to the *in vitro* kinase assay affects the phosphorylation of peptide B; a small model peptide substrate used previously to measure Itk activity *in vitro* (25). The levels of peptide B phosphorylation as indicated by initial velocity measurements do not change significantly with increasing concentrations of Itk SH2 domain (Fig. 4d) suggesting that SH2 binding to the kinase domain does not occur in a manner that directly interferes with peptide B binding to the active site. Furthermore, this result points to the absence of toxicity effects on Itk catalytic activity upon addition of free SH2 domain providing further support for the direct competition with substrate shown in Figure 4a-c. Thus, a specific interaction between the SH2 domain of the Itk protein substrates and the Itk kinase domain is required for efficient substrate phosphorylation and appears to be localized outside of the catalytic cleft.

*SH2 domain enhances substrate-binding affinity of a generic substrate.*

The data described above predict that the SH2 domain within the substrates of the Tec kinases serves a docking role and likely increases the affinity of substrates for the catalytic domain. To directly test this hypothesis, the PLC§SH2C domain was covalently linked to the amino terminus of Peptide B (Fig. 5a). To ensure that the desired tyrosine residue is the only site within this construct that undergoes phosphorylation, we constructed a PLC§SH2C-Peptide B mutant that replaced the putative site of tyrosine phosphorylation with serine (Fig. 5a). This mutant did not incorporate phosphate indicating that the tyrosine within the Peptide B sequence is the only site in the PLC§SH2C-Peptide B fusion that is phosphorylated by Itk (Fig. 5b).
Quantitative kinetic constants were compared for Peptide B alone and the PLC\textsuperscript{SH2}Peptide B fusion (Fig. 5c-e). Covalent attachment of the PLC\textsuperscript{SH2} SH2C domain to Peptide B increases substrate affinity as indicated by a reduced $K_m$ value; 5.64 $\mu$M for the SH2-Peptide B fusion compared to 87 $\mu$M for Peptide B alone. The $k_{cat}$ value only changed to a small extent suggesting that the SH2 domain primarily serves as a docking site to facilitate substrate recognition and binding by the Itk kinase domain. Thus, when linked to the PLC\textsuperscript{SH2} SH2C domain, Peptide B is a better substrate for Itk (exhibiting a 15-fold increase in substrate affinity) providing further support for the finding that Itk catalytic efficiency and selectively depends upon docking interactions with the substrate SH2 domain.

**Discussion**

We have demonstrated a previously unrecognized docking role for the SH2 domain within the substrates of the Tec kinases that facilitates phosphorylation at a remote tyrosine. Many kinase sequences contain SH2 domains and these binding modules are known to affect the association of the parent kinase molecule within a signaling complex (26). Additionally, processive phosphorylation mediated by SH2/phosphotyrosine interactions has been described previously (27-29). In those examples, a tyrosine kinase creates a binding site on the substrate for its own internal SH2 domain; the ensuing SH2-substrate interaction leads to efficient phosphorylation at additional sites on the substrate. In contrast to the role of the SH2 domain in the processive phosphorylation mechanism, the results presented here suggest that SH2 domains within the substrates of the Tec kinases harbor a recognition motif that is
required for efficient and selective phosphorylation of the target tyrosine. For example, the
direct substrate of Itk in T cells, PLCγ, harbors an SH2 domain (SH2C) that binds directly
to the Itk kinase domain allowing the downstream PLCγ tyrosine (Tyr 783) to be efficiently
phosphorylated by Itk. As well, autophosphorylation of Try 180 in the Itk SH3 domain
depends on Itk SH2 mediated interactions with the Itk kinase domain. This mode of
substrate recognition is evident within the full-length enzyme as addition of competing
exogenous Itk SH2 domain to full length Itk completely eliminates Itk autophosphorylation
(see Fig. 4c). To our knowledge, this direct kinase domain - SH2 interaction is a novel mode
of substrate recognition that provides significant insight into how the Tec kinases achieve
fidelity in their interactions with appropriate substrates and avoid deleterious ‘cross-talk’
with other substrates.

Our findings are consistent with a previous study of autophosphorylation within the
Tec family kinases (21) that provided a hint into the docking role of the SH2 domain. Smith
and co-workers qualitatively showed that the SH3-SH2 fragments of Btk and Itk are both
phosphorylated preferentially to the isolated Btk SH3 domain by the Btk kinase (21). The
authors speculated that the SH3-SH2 substrate may have additional interactions with the
kinase or that the site of phosphorylation in the SH3 domain becomes more accessible in the
larger SH3-SH2 substrate. They also left open the possibility that there are new
phosphorylation sites on the larger SH3-SH2 substrate but report that the SH2 domain of Btk
is not a substrate for Btk. The data we present strongly supports a model where the SH2
domain serves a direct docking role that is a significant determinant of substrate specificity
for the Tec kinases. It is especially interesting to note that Smith and co-workers report
phosphorylation levels for the SH3-SH2 fragment that are 5- to 8-fold higher than the isolated SH3 domain (21). This is in excellent agreement with kinetic data shown in Figure 5 that indicate that Peptide B is a better substrate by 7-fold \( (k_{\text{cat}}/K_m) \) when tethered to the SH2 domain of PLC\( ^{\perp} \). Finally, our use of phosphotyrosine specific antibodies and mutation of the target tyrosine supports the notion that additional phosphorylation sites in the SH2 domain containing substrates are not leading to the observed increase in substrate phosphorylation (21).

The phosphotyrosine-independent nature of the binding event between Itk kinase domain and SH2 domains, highlights alternative means by which SH2 domains can engage their targets. To date, targeting the conserved arginines in the phosphotyrosine binding pocket of the SH2 domain has become the traditional ‘loss-of-function’ mutation for this binding module. A full appreciation of phosphotyrosine-independent SH2 binding will lead to revised models of signaling complexes. For PLC\( ^{\perp} \) signaling in particular, traditional mutagenic approaches targeting the phosphotyrosine binding site of the SH2C domain have previously led to conclusions that this portion of PLC\( ^{\perp} \) is dispensable for PLC\( ^{\perp} \) recruitment and phosphorylation (30, 31). More recently, the extended experimental approach of Samelson and co-workers showed that all three SH domains including SH2C are required for phosphorylation of PLC\( ^{\perp} \) in T cells (32) yet these studies still relied on the mutation of arginine in the phosphotyrosine binding pocket of the SH2C domain. Certainly, the phosphotyrosine-independent docking role for the PLC\( ^{\perp} \) SH2C domain would have been missed by standard ‘loss-of-function’ mutations in the SH2 domain.
To fully characterize this novel, SH2 dependent substrate recognition mechanism, the interaction sites on both the Itk kinase domain and the substrate SH2 domains must be mapped at the molecular level. In addition to providing insight into the PLC activation mechanism, the precise arrangement of the Itk SH2 domain within the full-length Itk enzyme is of particular interest. In other tyrosine kinases, the non-catalytic SH2 domain plays a defined role in regulating kinase activity by forming direct intramolecular contacts to the small lobe of the kinase domain (33, 34). For Itk we have also found that the SH2 domain positively contributes to the regulation of catalytic activity (Joseph and Andreotti, unpublished results). Thus, the emerging bifunctional nature of the Itk SH2 domain (as substrate recognition module and regulatory domain) raises interesting questions related to how the Itk SH2 domain orchestrates its various roles during enzymatic catalysis. Does this SH2 domain interact with its neighboring kinase domain in a single mode that achieves both regulation of kinase activity and substrate recognition of the autophosphorylation site or does the SH2 domain shift between multiple interaction sites on the kinase domain to achieve these functions? With molecular level details still forthcoming, it is nevertheless clear that the data we present here point to a specific SH2 domain mediated docking mechanism by which the Tec kinases recognize and phosphorylate their substrates. One extension of this result is that screening for interactions between the Tec kinase domains and unrelated SH2 domains may provide leads to identify additional substrates for this important family of tyrosine kinases. In addition, we have shown that Itk substrates can be displaced from the active site by addition of exogenous SH2 domain. This result promises an exciting strategy to attenuate Itk mediated signaling that may have significant selectivity advantages since it
would not require the development of molecules that discriminate between very similar kinase active sites.

**Materials and Methods**

_Baculoviral and bacterial constructs_ - Full-length Itk, Btk and Tec and the Itk kinase domain fragments were PCR amplified using a reverse primer that encoded a FLAG epitope tag. The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. All of the PLC-1, Itk, Btk and Tec fragments that do not contain kinase domain were subcloned into the pGEX-2T expression vector (GE Healthcare) for production and purification from bacteria as described previously (35). The N-terminal Phosphatidylinositol 3-Kinase (p85a) SH2 domain (PI3K SH2) and Grb2 SH2 domain were cloned into pGEX-5X-1 by PCR. The pGEX-2T PLC-1 C-terminal SH2 domain-PeptideB fusion was created by PCR and included an N-terminal biotinylation sequence. For this construct and the pGEX-2T PLC-1 SH2C-PeptideB(YS) mutant, Tyr 771 was mutated to Phe to avoid phosphorylation at this site during analysis of the kinetics and Tyr 775 has been deleted by replacement with the Peptide B sequence (36). Biotinylated protein was produced in BL21 cells by co-expressing Biotin ligase. The biotinylated protein was purified by affinity purification using an Avidin resin (Pierce). For the Itk substrate constructs that include both the SH3 and SH2 domain (Itk32 and Itk32(Y180F)) we also introduced two point mutants (W208K and I282A) to eliminate complications that may arise from dimerization of the substrate as reported previously (35). The wild type Itk SH3-SH2 construct is phosphorylated by Itk to the same extent as the double mutant (data not shown). All
mutations were made using the site directed mutagenesis kit (Stratagene). All constructs were verified by sequencing at the Iowa State University DNA synthesis and sequencing facility.

_Baculovirus production_ - The pENTR vectors with various inserts were recombined _in vitro_ with BaculoDirect C-Term Linear DNA (Invitrogen) using LR Clonase II enzyme (Invitrogen). The DNA was then transfected into _Sf9_ cells using Effectene (Qiagen). Three rounds of viral selection and amplifications were carried out. For protein production, the cells were infected with a 1:1 ratio of Itk (or Btk or Tec): Lck baculovirus unless otherwise indicated. The cells were harvested 72 hrs post-infection, rinsed once with phosphate buffered saline (PBS) and stored at −80°C. Following purification each Itk enzyme construct was assessed for Tyr 511 phosphorylation using a Btk pY551 specific antibody. This step ensured that co-expression with Lck produced appropriately activated Itk (18).

_Pull-Down Assays_ - Purified 0.5 μM FLAG-tagged Itk kinase domain (Itk KD) immobilized on an anti-FLAG-resin was incubated with 1μM purified Myc-tagged Itk SH2 (Itk SH2-myc) domain in RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1mM PMSF, 1% NP40, 1mM EDTA, 1mM NaF) at 4°C overnight. The samples were washed, boiled, resolved on an SDS-PAGE gel, transferred to PVDF membrane and western blotted with an anti-Myc antibody (Invitrogen). For PLCγ pull downs 0.22 μM FLAG-tagged Itk kinase domain (Itk KD) was incubated with 3.8 μM of purified GST or 3.8 μM GST-PLCγ SH2C immobilized on glutathione beads. The samples were treated as before and western blotted using an anti-FLAG antibody. Unphosphorylated FLAG-tagged Itk Kinase domain (Itk KD) was prepared
by treating 13.6 μM Itk KD with 1 unit/μL of Alkaline phosphatase (New England Biolab) for 1 hr at 37°C.

*Protein purification* - Purification of baculovirus produced protein was carried as previously described (25). Cell pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 2 mM EDTA, 1 mM PMSF) and lysed by dounce homogenization. The homogenate was spun at 16K for 1 hr at 4°C. Glycerol was added to the supernatant to a final volume of 10%, and then incubated with anti-FLAG M2 affinity resin (Sigma) for 5 hrs at 4°C. The resin was rinsed five times in wash buffer (50 mM Tris pH 8.0, 500 mM NaCl, 1 mM PMSF, 10% glycerol), and if necessary for kinase assays, the protein was eluted in elution buffer (wash buffer with 200 μg/ml FLAG peptide) and stored at -80°C. The purified protein was quantified by measuring absorbance at 280 nm. All proteins were greater than 95% pure by Coomassie staining. We note that the Btk SH3 domain runs higher than the actual molecular weight and the closely related Tec SH3 domain (Fig. 2b). The molecular weight of both Btk SH3 domain and Tec SH3 domain were confirmed by mass spectrometry (data not shown). Additionally, identical preparations of Itk mutant enzymes that are kinase inactive (K390R) do not show any activity toward any substrate (data not shown).

*Kinase assays and Western Blotting* - Itk Full-length (FL), linker kinase domain (Itk LKD), kinase domain (Itk KD), full-length Btk or full-length Tec were incubated with the indicated substrates in an *in vitro* kinase assay buffer (50 mM Hepes pH 7.0, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 μM ATP) for one hour at RT. The samples were boiled, separated by SDS-PAGE and transferred onto a PVDF membrane. The
membranes were then blotted with either phosphotyrosine specific antibodies (anti-pY783 for PLC\(\text{g}\) or pY223 Btk antibody used to detect pY180 in Itk, pY187 in Tec and pY223 in Btk (18)), an anti-phosphotyrosine antibody (4G10 from Upstate), or an anti-FLAG antibody (Sigma) and developed by standard chemiluminescence (Pierce) methods. Quantitative kinase assays (25): \(K_m\) determinations for Peptide B [(Aminohexanoyl biotin-EQDEPEGIYGVLF-NH\(_2\)) (Anaspec Inc.)] and the biotylated PLC\(\text{g}\) SH2C-Peptide B fusion were carried out by incubating purified Itk LKD enzyme in reaction buffer (50 mM Hapes pH 7.0, 10 mM MgCl\(_2\), 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl-fluoride) and 200 \(\mu\)M ATP) and 5 mCi \[^{32}\text{P}\] ATP (GE Healthcare) at room temperature. Peptide B concentration was varied between 0-400 \(\mu\)M and PLC\(\text{g}\) SH2C-Peptide B fusion concentration was varied between 0-80 \(\mu\)M. The enzyme concentration used for kinetic analysis was 0.9 \(\mu\)M. Each assay was done in duplicate. The data obtained was fitted onto the Michealis-Menten equation using GraphFit 5 software, and the kinetic parameters were obtained.

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Figure Captions

Figure 1. Domain structures of Tec kinases and PLC\(\gamma\). (a) Sequences surrounding Y783 in PLC\(\gamma\) and Y180 in Itk SH3. Tyrosine residues that are phosphorylated by Itk are boxed. (b) Domain structure of Itk and specific Itk constructs used in this study. The autophosphorylation site of Itk, Y180 within the SH3 domain, is indicated. The domain structures of Tec and Btk are the same as Itk and the Tec and Btk domain fragments used in this study follow the same nomenclature (Btk SH3, Btk 32 (each contain Y223) Tec SH3 and Tec 32 (each contain Y187)). (c) The domain structure of PLC\(\gamma\) contains a PH domain, EF hand motif, the catalytic domain (comprising the X and Y domains), two SH2 domains (SH2N and SH2C for amino- and carboxy terminal respectively), SH3 domain, a split PH domain indicated by (P and H) and the C2 domain. The PLC\(\gamma\) fragments used in this study are shown and substrate fragments each include Y783, the target of Itk kinase activity.

Figure 2. Tec mediated phosphorylation requires the SH2 domain adjacent to the target tyrosine. (a) Only Itk fragments that contain the SH2 domain and Y180 are phosphorylated by Itk. Ten \(\mu\)M each of Itk SH3, Itk SH3-Linker (Itk 3Linker), Itk SH3-SH2 (Itk 32) or Itk SH3-SH2 with a phenylalanine in place of the Y180 phosphorylation site (Itk 32(Y180F)) were incubated with 250 nM FLAG-tagged Itk full-length (FLItk) enzyme in an in vitro kinase assay. Lanes 5-8, are no enzyme controls. Phosphorylation on Y180 in the SH3 domain is detected with an anti-pY223 Btk antibody (denoted anti-pY180 throughout). Throughout, Coomassie or Ponceau S stain of the gel, anti-FLAG blots and anti Itk blot (bottom panels) show protein levels. For panels a-c, Coomassie stain of the SH3 domain
fragments is consistently less efficient than the larger constructs but protein amounts across these experiments are uniform based on measured absorbances. Asterisks next to Comassie stained gel indicate position of Itk 32, Itk 3Linker and Itk SH3. (b) Phosphorylation of the Tec and Btk substrates was carried out in a manner similar to that shown in (a) for Itk. Btk SH3 (5 and 10 $\mu$M respectively in lane 1-2) or Btk SH3-SH2 (Btk 32) (5 and 10 $\mu$M respectively in lanes 3,4) were incubated with 100 nM FLAG-tagged Btk full-length enzyme in an in vitro kinase assay. Tec SH3 (5 and 10 $\mu$M respectively in lanes 5,6) or Tec SH3-SH2 (Tec 32) (5 and 10 $\mu$M respectively in lanes 7,8) were incubated with 100 nM FLAG-tagged Tec full-length enzyme in an in vitro kinase assay. Lanes 9-12 are the no enzyme controls at substrate concentrations of 10 $\mu$M. Phosphorylation on Y223 in the Btk SH3 and Y187 in Tec SH3 domain are detected with an anti-pY223 Btk antibody. Asterisks next to Comassie stained gel indicate position of Tec 32, Btk 32, Btk SH3 and Tec SH3. (c) Efficient phosphorylation of PLC$\gamma$ Y783 by Itk occurs only when the adjacent PLC$\gamma$ SH2 domain is present. Lane 1 is enzyme alone control. Varying concentrations (1, 3, 5 and 10 $\mu$M in lanes 2-5, respectively) of the fragment of PLC$\gamma$ that contains both the SH2C domain and the following 33 amino acids (PLC$\gamma$ SH2C-Y783-) or the PLC$\gamma$ fragment that contains the 33 residue linker followed by SH3 domain (PLC$\gamma$ -Y783-SH3, 1, 3, 5 and 10 $\mu$M in lanes 6-9, respectively) were subjected to in vitro phosphorylation by 1.2 $\mu$M FLAG-tagged Itk full-length enzyme. Phosphorylation on Y783 in the PLC$\gamma$ constructs is detected using an anti-pY783 PLC$\gamma$ antibody. (d) 2 $\mu$M purified FLAG-tagged Itk kinase domain alone (Itk KD) was incubated with 1$\mu$M (lane 2) or 10$\mu$M (lane 3) Itk 32 domain in
an in vitro kinase reaction as before. Lane 1 is 10 μM Itk 32 domain alone with no enzyme. All data shown are representative of at least three independent experiments.

Figure 3. Itk kinase domain interacts directly with PLCγ1 and Itk SH2 domains in a phosphotyrosine independent manner. (a) Empty anti-FLAG resin (lane 1) or purified FLAG-tagged Itk kinase domain (Itk KD) immobilized on an anti-FLAG-resin (lanes 2 and 3) were incubated with purified myc-tagged control protein (lane 2) or myc-tagged Itk SH2 domain (lanes 1 and 3) in a pull-down assay. Blotting with an anti-Myc antibody reveals a direct interaction between purified Itk kinase domain and the purified Itk SH2 domain (lane 3). (b) Purified FLAG-tagged Itk KD (lane 1) was incubated with GST (lane 2) or GST-PLCγ1 C-terminal SH2 domain (GST-PLCγ1 SH2C) (lane 3) each immobilized on glutathione beads in a pull-down assay. Anti-FLAG antibody reveals a direct interaction between the Itk kinase domain and the C-terminal SH2 domain of PLCγ1. (c) Interaction between the Itk kinase domain and Itk SH2 domain leading to phosphorylation of substrate does not involve the phosphotyrosine binding pocket of the SH2 domain. One μM Itk 32 (lanes 1 and 3) or Itk 32 (R265K) (lanes 2 and 4), were incubated with 250 nM FLAG-tagged full-length Itk in an in vitro kinase assay. Lanes 1 and 2 are no enzyme control. Antibody specific for Itk pY180 shows that the conserved arginine in the phosphotyrosine binding pocket of the SH2 domain is not required. (d-e) Effective phosphorylation of PLCγ1 Y783 by the Itk kinase domain does not require the phosphotyrosine binding pocket of the PLCγ1 SH2 domain. One, 3, 5 and 10 μM PLCγ1 SH2C-Y783- (d) or PLCγ1 SH2C(R964A,R696A)-Y783- (e), were subjected to phosphorylation by 1.2 μM full-length FLAG-tagged Itk. Lanes
5 and 6 (containing 1 and 10 μM substrate, respectively) are no enzyme controls. Coomassie stain of the gels and anti-FLAG blots (bottom panels) show protein levels. (f & g) Interaction between the SH2 domain and Itk kinase domain is independent of the phosphorylation status of the kinase domain. (f) FLAG-tagged Itk KD that is expressed alone (lane 1) does not react with a general phosphotyrosine (4G10) antibody while Itk co-expressed with Lck (lane 2) is phosphorylated. Itk KD that is co-expressed with Lck but also pretreated with alkaline phosphatase is not phosphorylated (lane 3). (g) Purified FLAG-tagged Itk KD that is either expressed alone (lanes 1 & 2), co-expressed with Lck (lanes 3 & 4) or treated with alkaline phosphatase (lanes 5 & 6) were incubated with immobilized GST (lanes 1, 3 and 5) or immobilized GST-PLCγ1 SH2C, (lanes 2, 4 and 6) in a pull-down assay. An anti-FLAG blot reveals binding of Itk KD to GST-PLCγ1 SH2C and not to GST alone. Ponceau S staining of the membrane (bottom panel) shows the protein levels. All data shown are representative of at least three independent experiments.

Figure 4. The presence of isolated SH2 domain diminishes phosphorylation of Itk Y180 or PLCγ1 Y783. Substrate used in each experiment is indicated above each set of blots. (a) (left) Itk 32 substrate was incubated with Itk full-length enzyme as before (lane 1), or with Itk 32 domain:free Itk SH2 domain in ratios of 1:10, 1:25 or 1:50 (lanes 2-4, respectively) or equivalent ratios of PI3K N-terminal SH2 domain (lanes 5-7) and subjected to an in vitro kinase assay. Anti-pY180 antibody shows that free Itk SH2 domain competes with the Itk 32 substrate while the SH2 domain from PI3K does not. Coomassie stain and an anti-FLAG blot (bottom panels) show protein levels. (right) The PLCγ1 SH2 domain competes with the
PLC\[I\] substrate in a similar manner. Lane 1 contains only Itk enzyme and PLC\[I\] SH2C-Y783- substrate without additional free SH2 domain. Ratios of free SH2 domain to substrate are 1:5, 1:10 or 1:50 in lanes 2-4, respectively and equivalent ratios of PI3K SH2 domain are used in lanes 5-7. Coomassie stain of the gel and an anti-Itk blot (bottom panels) show protein levels. (b) Same experiment as shown in (a) using the Grb2 SH2 domain as a control. (left) Itk 32 substrate was incubated with Itk full-length enzyme (lane 1), or with Itk 32 domain:free Itk SH2 domain in ratios of 1:10 and 1:25 (lanes 2 & 3) or equivalent ratios of Grb2 SH2 domain (lanes 4 & 5) and subjected to an in vitro kinase assay. (right) Identical experiment following PLC\[I\] substrate phosphorylation in the presence of the PLC\[I\] and Grb2 SH2 domains. Lane 1 is PLC\[I\] substrate alone; lanes 2 & 3 are PLC\[I\] substrate:PLC\[I\] SH2 domain in a 1:10 and 1:25 ratio, respectively and lanes 4 & 5 are identical ratios of PLC\[I\] substrate:Grb2 SH2 domain. (c) The Itk SH2 domain competes with autophosphorylation of Y180 within full length Itk. For lanes 2-4, the amount of free SH2 domain is the same as used in panel (a), lanes 2-4. No exogenous SH2 domain has been added in lane 1 and full length Itk is both the enzyme and the substrate. Coomassie stain and an anti-FLAG blot (bottom panels) show protein levels. (d) Free Itk SH2 domain does not compete with phosphorylation of a peptide substrate. Initial velocities are shown for phosphorylation of peptide B (sequence shown in Fig. 5a) by full length Itk in the absence (column 1) and presence (column 2 and 3) of Itk SH2 domain. The amount of SH2 domain used here is the same as that used in lanes 3 and 4 in (c).
Figure 5. PLCγ1 C-terminal SH2 domain can mediate docking of Peptide B into the kinase active site of Itk. (a) Construction of a PLCγ1 SH2C-Peptide B fusion protein was based on alignment of Y783 within the PLCγ1 linker and the Tyr of Peptide B. The 14 amino acids of Peptide B were used to replace the last 16 amino acids of PLCγ1 SH2C-Y783-. The Tyr that is phosphorylated in each substrate is boxed. A mutant construct is also shown that contains serine in place of the tyrosine of peptide B. This site was mutated to serine instead of phenylalanine due to solubility problems associated with the Tyr to Phe mutation. (b) FLAG-tagged Itk LKD enzyme (1 μM) was incubated with 20 μM of the PLCγ1 SH2C-Peptide B fusion protein (lane 4) or 20 μM of the PLCγ1 SH2C-Peptide B(YS) mutant protein (lane 5) in an in vitro kinase assay. Lanes 1-3 are Itk enzyme alone, PLCγ1 SH2C-Peptide B substrate alone and PLCγ1 SH2C-Peptide B(YS) substrate alone, respectively, that have each been subjected to the same in vitro kinase assay conditions. (c & d) FLAG-tagged Itk LKD enzyme was incubated with increasing amounts of biotinylated PLCγ1 SH2C-Peptide B (c) or biotinylated Peptide B alone (d) in an in vitro kinase assay. (e) Kinetic parameters describing phosphorylation of Peptide B and the PLCγ1 SH2C-Peptide B fusion protein by Itk. Data shown are the average of two independent experiments.

References


FIGURE 1

a

PLCγ1: 774 ...LYEGRNPGEYVEANPM... 789
Itk: 171 ...PEETLVIAL...YQTNDELAY... 186

b

PH-TH-SH3-SH2-KINASE

FL 1- PH TH SH3 SH2 KINASE B16 FLAG
Itk LKD 336 KINASE 619 FLAG
Itk KD 356 KINASE 619 FLAG
Itk 32 171 SH3 SH2 338
Itk 32 (Y180F) 171 SH3 SH2 338
Itk 3Linker 171 SH3 238
Itk SH3 171 SH3 232
Itk SH2-myc 230 SH2 338 myc

c

PH-EF hands-X-(P-SH2N-SH2C---SH3-H)-Y-C2-

PLCγ1 SH2C-SH3 659 SH2C Y783 SH3 863
PLCγ1 SH2C-Y783 659 SH2C Y783 789
PLCγ1 -Y783-SH3 757 Y783 SH3 863
PLCγ1 SH2C 659 SH2C 756
FIGURE 3

a

Anti-Myc
Ponceau S

b

Anti-FLAG
Ponceau S

c

Anti-pY180
Ponceau S

Anti-FLAG

---

Anti-pY783
Anti-Itk

PLCγ1 SH2C-Y783-

PLCγ1 SH2C(R694A, R696A)-Y783-

Anti-pY783
Anti-Itk

---

Itk KD
Itk KD +

Itk KD
Itk KD +

Itk KD
Itk KD +

---

Anti-FLAG
Ponceau S

---

Itk KD
Itk KD +

---

Anti-FLAG
Ponceau S
FIGURE 5

a
PLCγ1 SH2C-Y783-
Peptide B
PLCγ1 SH2C-Peptide B
PLCγ1 SH2C-Peptide B(YS)

b
![Graph showing Initial Velocity (µM/min) vs. [Peptide B] (µM)]

![Graph showing V/([Enzyme] (min⁻¹) vs. [PLCγ1 SH2C-Peptide B] (µM))]

![Graph showing V/([Enzyme] (min⁻¹) vs. [Peptide B] (µM))]

c

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<th>Substrate</th>
<th>$K_m$ (µM)</th>
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<th>$k_{cat}/K_m$ (min⁻¹·µM⁻¹)</th>
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CHAPTER 3. PHOSPHOLIPASE C\textsuperscript{g} PHOSPHORYLATION BY ITK
IS MEDIATED BY A NON-CLASSICAL SH2 SURFACE

A paper to be submitted to the Proceedings of the National Academy of Sciences

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Abstract

The manner in which protein kinases achieve substrate specificity remains elusive for most enzyme/substrate pairs. To date, substrate docking mechanisms have been described for many serine/threonine kinases but our understanding of substrate recognition within the tyrosine kinase subfamily has lagged. This has led to the notion that the tyrosine kinases accomplish specific phosphorylation of their targets through the action of modular non-catalytic domains rather than direct docking of substrate onto the kinase domain. Contrary to this model, we show that the second largest family of tyrosine kinases, the Tec kinases, recognize their substrates via a direct docking interaction between kinase domain and substrate. We define the precise docking surface on PLC\textsuperscript{g}, the physiological substrate of one of the Tec kinases, Itk. The binding surface on PLC\textsuperscript{g} that mediates recognition by Itk highlights a non-classical binding activity of the well-studied Src homology 2 (SH2) domain providing evidence that SH2 domains participate in important interactions beyond recognition of phosphotyrosine.
Introduction

Protein kinases control numerous cellular signaling networks by phosphorylating target amino acid side chains in a stringently specific manner. Based on results of combinatorial peptide library screens and structures of kinase/peptide substrate complexes, the view has emerged that the active sites of most kinases can tolerate different sequences and are therefore not stringently specific for short peptide sequences. Stringent specificity is however a requirement of cellular signaling cascades and so these enzymes must have mechanisms that control fidelity.

Two modes of achieving strict target specificity have been described for the protein kinases, one invoking a role for the modular recognition domains (such as SH3 and SH2) that are often found adjacent to the catalytic domain. For the SH2 domain in particular, processive phosphorylation mediated by SH2/phosphotyrosine interactions has been well described. In these examples, a tyrosine kinase creates a binding site on the substrate for its own internal SH2 domain; the ensuing SH2-substrate interaction leads to efficient phosphorylation at additional sites on the substrate. Alternatively, specific docking interactions between a kinase domain itself and a recognition element within the substrate have been described. Docking-based substrate interactions typically involve recognition of a substrate motif by a region of the catalytic domain that lies outside of the active site. Substrate docking has been described to date almost exclusively for the serine/threonine kinases; enzymes that often lack modular binding domains outside of the kinase domain.
Substrate docking interactions have not to date been extensively described for the tyrosine kinase family. These enzymes often contain the modular binding domains thought to mediate localization of the kinase domain to substrates and thus, in general, emerging notions of substrate specificity for the serine/threonine versus the tyrosine kinases have emphasized specific docking interactions for the former and canonical SH3 and SH2 mediated interactions controlling specificity for the latter\(^2,6\). The C-terminal \textit{Src} kinase, Csk, is the exception to these generalizations since it is a tyrosine kinase, contains an SH3 and SH2 domain, but has been shown to present a binding groove on its kinase domain that serves a direct substrate docking role\(^8,9\). Thus, the extent to which docking is a general mechanism exploited by both the tyrosine and serine/threonine kinases to achieve substrate specificity has yet to be determined.

In support of direct substrate docking as a general phenomenon among the broad class of protein kinases, we describe a specific docking interaction between the Tec family tyrosine kinases and their physiological substrates\(^10\). Like Csk, the Tec kinases (Itk, Btk, Rlk, Bmx and Tec) all contain an SH3 and SH2 domain in addition to the catalytic kinase domain. While the non-catalytic binding domains most certainly localize the Tec kinases appropriately during signaling\(^11,12\), we have shown that the kinase domain itself of each of the Tec kinases directly contacts its cognate substrate in a manner that confers significant specificity\(^10\). For example, the Itk kinase domain contacts a recognition element presented by its substrate, phospholipase C\(_\gamma\) (PLC\(_\gamma\)), that is remote from the site of phosphorylation (Y783 within PLC\(_\gamma\)).
We now identify the precise docking determinants within PLC\[\] that are required for specific phosphorylation by the Itk kinase and show that the recognition element is present in the full length PLC\[\] substrate. The docking element resides with the second SH2 domain of PLC\[\] (SH2C) and does not coincide with the classical phosphotyrosine ligand binding surface of the SH2 domain. The results therefore define a new SH2 surface that mediates protein-protein interactions and provide evidence that, in addition to the serine/threonine kinases, the general tyrosine kinase family also exploits substrate docking interactions to achieve target specificity.

**Results**

In activated T cells, Itk mediated phosphorylation of PLC\[\] leads to activation of lipase activity and subsequent hydrolysis of phosphatidylinositol (4,5)bisphosphate (PIP\(_{2}\)) to inositol 1,4,5-trisphosphate (IP\(_{3}\)) and diacylglycerol (DAG). IP\(_{3}\) and DAG in turn stimulate the release of calcium ions from the endoplasmic reticulum and activate Protein Kinase C, respectively (for a review see \(^{13}\)). Thus activation of PLC\[\] by Itk is a critical component of T cell activation and differentiation \(^{14}\) yet the molecular mechanisms by which Itk specifically targets PLC\[\] have not been described. PLC\[\] contains a PH domain, EF hand motif, the catalytic domain (comprising the X and Y domains), two SH2 domains (SH2N and SH2C for amino- and carboxy-terminal respectively), a SH3 domain, a split PH domain indicated by (P and H) and the C2 domain (Fig. 1a). The carboxy-terminal SH2 domain (SH2C) of PLC\[\] spans residues 659 to 756 within full length PLC\[\] and the site of tyrosine phosphorylation by Itk resides at position 783 \(^{15-17}\).
The PLC\(\text{g}_1\) SH2C domain is required for efficient phosphorylation of Y783 (Fig. 1b; lane 1) and fragments of PLC\(\text{g}_1\) that do not contain both SH2C and Y783 are not efficiently phosphorylated by Itk \(^1\). Moreover, PLC\(\text{g}_1\) substrate fragments that do contain both SH2C and Y783 (spanning 659 to 789; hereafter referred to as PLC\(\text{g}_1\) SH2C-linker) can be titrated away by addition of free PLC\(\text{g}_1\) SH2C domain (residues 659 to 756) (Fig. 1b, lanes 2 & 3). Competition by free SH2C domain is specific as the SH2 domains from Grb2 (Fig. 1b, lanes 4 & 5) and PI3K (not shown) have no effect on Itk mediated phosphorylation of Y783 in PLC\(\text{g}_1\). Thus, the PLC\(\text{g}_1\) SH2 domain presents a recognition motif to the Itk kinase domain that promotes phosphorylation of the remote Y783 (Fig. 1c). Our previous work also clearly demonstrated that the PLC\(\text{g}_1\) SH2C domain mediates docking to the Itk kinase domain in a non-classical manner (phosphotyrosine-independent) and that SH2C contacts the Itk kinase domain in a manner that does not occlude the kinase active site (Fig. 1c) \(^1\). Finally, it should be understood that this mechanism of substrate recognition is distinct from that of processive phosphorylation. The SH2 domain of interest resides within the substrate and the Itk kinase domain itself is responsible for substrate recognition rather than an SH2 domain within the enzyme \(^1\). Thus, despite the presence of the modular SH3 and SH2 domains within Itk, substrate recognition occurs via a direct docking interaction between the kinase domain of Itk and an SH2 domain in the substrate.

To identify the precise SH2 docking surface responsible for substrate recognition by the Itk kinase domain, we compared the primary sequences of the PLC\(\text{g}_1\) SH2C domain derived from several species with the primary sequences of two SH2 domains that do not compete
with Itk mediated substrate phosphorylation (PI3K and Grb2). Surface exposed residues that are conserved across the PLC\(^\text{G}_1\) SH2 domains from different species but differ in either PI3K or Grb2 (or both) were targeted for mutation. A total of 24 PLC\(^\text{G}_1\) SH2C candidate residues (Fig. 1d) were identified as potential substrate docking site residues. We expect that mutation of the docking motif that mediates the direct Itk/PLC\(^\text{G}_1\) substrate interaction will result in loss of substrate phosphorylation.

Using the PLC\(^\text{G}_1\) SH2C-linker substrate shown in Figure 1b & c, each of the 24 SH2C residues indicated in Figure 1d were mutated to alanine. Wild type SH2C-linker substrate and the resulting panel of mutant substrates were subjected to an *in vitro* phosphorylation assay using full-length Itk enzyme (Fig. 2a). Phosphorylation levels for each substrate were assessed by western blot using the anti-pY783 antibody. As shown in Figure 2a, mutation of E709, K711, R748, K749, K751, and R753 to alanine (lanes 19-27) either completely abolishes or significantly diminishes Itk mediated phosphorylation of Y783 in the PLC\(^\text{G}_1\) SH2C-linker substrate. These data provide evidence that these SH2 domain residues are required for phosphorylation of the remote Y783 site. Mutation of the other residues in the SH2C domain had little or no effect on the phosphorylation efficiency of the PLC\(^\text{G}_1\) SH2C-linker substrate (Figure 2a; lanes 4-18 & 28-39). Substrate phosphorylation levels that are equal to wild type substrate in this functional assay indicate that the mutated residues do not contribute to the docking interaction between PLC\(^\text{G}_1\) SH2C domain and Itk kinase domain.

Before considering the structural implications of mutational analysis, we made use of circular dichroism (CD) spectroscopy to ensure that the individual mutations do not cause large
structural changes to the SH2C-linker substrate that could account for the absence of phosphorylation on Y783 (Fig. 2b). The CD spectra acquired for wild type and each of the purified mutant substrates indicate that none of the substrate fragments were significantly altered in secondary structure as a result of surface mutation. Thus, the results of the mutational analysis shown in Figure 2a indicate a set of surface residues on the PLC\(\gamma\) SH2C domain that mediate formation of an enzyme/substrate docking complex between Itk and PLC\(\gamma\) that is required for Y783 phosphorylation.

Mapping the mutational results from Figure 2a onto the structure of the PLC\(\gamma\) SH2C domain reveals a binding surface that consists largely of basic amino acids located apart from the canonical phosphotyrosine binding site on the SH2 domain (Fig. 3a & b). K711 and E709 reside on the CD loop of the SH2C domain and R748, K749, K751 and R753 are located within the C-terminus of the domain. Residues for which mutation to alanine has no detectable effect on Y783 phosphorylation in the Itk phosphorylation assay (Fig. 2a) lie outside of this newly identified binding site and are depicted in orange in Figure 3a. Visualizing the docking site on the structure of the PLC\(\gamma\) SH2C domain bound to a phosphopeptide ligand (Fig. 3b) clearly shows why mutations in the pY pocket that disrupt classical phosphotyrosine binding of the SH2 domain have no effect on the substrate docking activity. The PLC\(\gamma\) SH2C domain contains separate and non-overlapping surfaces that control phospholigand binding and substrate docking.
We next examined the extent to which the Itk/PLC\[1\] docking interaction mediates phosphorylation of Y783 in the full-length PLC\[1\] substrate instead of the short SH2C-linker substrate fragment used thus far (Figs. 1 & 2). Given the previous observation that free PLC\[1\] SH2C domain can compete with phosphorylation of Y783 by Itk (Fig. 1b), we carried out the same experiment using full length PLC\[1\] as substrate. In a manner completely analogous to the earlier fragment study, we find that the isolated PLC\[1\] SH2C domain competes effectively with Itk mediated phosphorylation of Y783 within full length PLC\[1\] (Fig. 4a; lanes 1-3). In analogy to the earlier experiment using the PLC\[1\] substrate fragment (Fig. 1b), the same concentration of Grb2 SH2 domain is significantly less effective in competing with docking of full length PLC\[1\] (Fig. 4a; lanes 8 & 9). We also generated two separate PLC\[1\] SH2C domains carrying point mutations in the identified docking site (K749A/R753A and R748A/K751A). Like the Grb2 SH2 domain, these mutated PLC\[1\] SH2C domains do not compete with full-length PLC\[1\] phosphorylation by Itk (Fig. 4a; lanes 4-7) providing further evidence that the cluster of basic residues identified on the surface of the PLC\[1\] SH2C domain (Figs. 2a & 3a) mediate substrate docking onto Itk.

To complement the competition assays shown in Figure 4a, we next directly targeted the SH2C docking surface within full length PLC\[1\]. The same mutations in the SH2C domain (K749A/R753A and R748A/K751A) were incorporated into full length PLC\[1\] and then wild type PLC\[1\] and each mutant was then subjected to phosphorylation by the full length Itk enzyme (Fig. 4b). Both sets of mutations diminish Y783 phosphorylation by Itk; the level of phosphorylated Y783 in wild type full length PLC\[1\] is significantly higher than either
mutant. Comparing the two mutants, it is also interesting to note that the K749A/R753A mutation diminishes the pY783 level to a greater extent than the R748A/K751A mutant suggesting that these two residues might play a more significant role in binding to Itk. This is consistent with the differences between these two mutants in the fragment study described in Figure 2a. These data therefore provide evidence that a region of the PLCγ1 SH2C domain that is located outside of the canonical phosphotyrosine binding site and is remote from the site of tyrosine phosphorylation, Y783, mediates specific phosphorylation of PLCγ1 by the Itk kinase. This work ascribes a new function to the ubiquitous SH2 domain; substrate presentation of a specificity conferring recognition element to its cognate tyrosine kinase.

**Discussion**

The substrate docking mechanism that we have described for the Tec family tyrosine kinase, Itk, complements the elegant work describing a similar substrate recognition mechanism for the Csk/Src enzyme/substrate pair. These examples are beginning to suggest that, like their serine/threonine kinase counterparts, the tyrosine kinases exploit docking interactions between kinase domain and substrate and do not achieve specificity solely via the local sequence surrounding the target tyrosine and/or the canonical interactions between non-catalytic domains of the kinase and canonical motifs within the substrate. The idea of a consensus motif for particular kinase active sites has been extensively pursued and while some recognition motifs have emerged from studies with short peptides, the results largely suggest that tyrosine containing peptides are not efficient substrates for the tyrosine kinases and strict sequences preferences around the target tyrosine are not observed.\(^{1,2}\)
The discovery of long-range docking interactions for two different tyrosine kinases (Tec family and Csk) might suggest that local sequence plays little or no role in determining substrate specificity for this class of enzymes. Instead, the primary role for the amino acid sequences surrounding tyrosine phosphorylation sites might be to mediate the interactions that are induced upon tyrosine phosphorylation. For example, phosphorylation of the Src tail by Csk induces critical intramolecular interactions between the phosphorylated tail and the Src SH2 domain leading to inhibition of Src activity\textsuperscript{18-20}. Sequence changes surrounding the tyrosine in this tail have been shown to modulate the affinity of this intramolecular interaction and alter kinase regulation \textsuperscript{21}. It therefore seems likely that the sequence surrounding the Csk target tyrosine has evolved to achieve optimal regulation via interaction with the SH2 domain rather than to serve as an optimal substrate for its cognate kinase. High fidelity substrate recognition comes instead from interactions outside of the kinase active site with regions of the substrate that are removed from the target tyrosine and its neighboring sequence. Likewise, phosphorylation of Y783 in PLC\textsuperscript{g1} has been implicated in formation of an intramolecular regulatory complex with the adjacent SH2C domain \textsuperscript{22}. Thus, the amino acid sequence surrounding Y783 in PLC\textsuperscript{g1} might make little or no contribution to substrate specificity and instead has evolved to achieve the appropriate affinity with its SH2 domain binding partner following phosphorylation by Itk. The substrate docking mechanism we have described for the Itk/PLC\textsuperscript{g1} pair could therefore provide all of the specificity determining elements that are required for the Itk kinase to achieve sufficient specificity for its target during signaling.
The PLC\[I\] SH2C domain is one of the most well studied SH2 domains to date. Its role in regulating PLC\[I\] function has been extensively probed (reviewed in 23) and its structure has been solved in several contexts 24,25. However, the standard mutagenic approaches used to probe functional contributions of SH2 domains in general have not uncovered the docking role of the PLC\[I\] SH2C domain described here. The traditional loss-of-function mutagenic approach for the ubiquitous SH2 domain is to target the conserved arginines in the phosphotyrosine-binding pocket. For PLC\[I\], this approach led to conclusions that the SH2C domain of PLC\[I\] is dispensable for recruitment and phosphorylation during signaling 23,26,27. More recently, the extended experimental approach of Samelson and co-workers showed that all three SH domains including SH2C are required for phosphorylation of PLC\[I\] in T cells 28 yet these studies still relied on the mutation of arginine in the phosphotyrosine binding pocket of the SH2C domain. In addition, interactions between Itk and PLC\[I\] have been previously investigated with an emphasis on classical phosphotyrosine mediated binding by SH2 domains 29. Our results now strongly suggest that an alternative, non-canonical surface of the PLC\[I\] SH2C domain mediates phosphorylation of the downstream Y783 by docking onto the Itk kinase domain in a phosphotyrosine independent manner.

The docking site on PLC\[I\] is highly basic and therefore differs significantly from the only other tyrosine kinase, Csk, for which substrate docking has been described. In the case of the Csk substrate, Src, it has been shown that the recognition motif consists of acidic residues that are recognized by a basic patch on the Csk kinase domain 8,9. Moreover, the PLC\[I\] docking site is not restricted to a continuous stretch of primary sequence. Mutational
analysis reveals that residues in both the C-terminus (YRKMKLRYPI) and the CD loop of the SH2 domain (RAEGK) contribute to a stable docking interaction (Fig. 3a). Interspersed between the mostly positively charged residues of both the C-terminus and the CD loop are a number of hydrophobic side chains that likely contribute to stabilization of the docking interaction. Interestingly, the PLCγ docking site identified here resembles that recognized by the serine/threonine MAP kinase p38 \(^\text{30}\). In that case, alternating hydrophobic and predominately basic residues make up the recognition motif of MAP kinase targets.

The chemical nature of the PLCγ docking residues provides clues to possible docking locations on the Itk kinase domain. Specifically, it is noteworthy that the small lobe of the Itk kinase domain contains a cluster of acidic surface residues that might provide the docking site for substrate recognition. Further experiments are needed to locate the substrate docking surface on the Itk kinase domain and to evaluate the interplay between substrate docking and allosteric control of Itk catalytic activity. It is nevertheless interesting to note that for Csk and the serine/threonine kinases (reviewed in \(^\text{6}\)) distinctly different docking locations have been described on the catalytic domains (all outside of the catalytic cleft) suggesting that different kinases solve the problem of substrate fidelity using different docking platforms. As the Itk/PLCγ and additional docking mechanisms for the non-receptor tyrosine kinase family are further characterized, such differences create potential opportunities to use small molecules to modulate phosphorylation events and in turn particular signaling cascades in a highly specific manner that would not depend on discriminating between similar kinase active sites. Indeed, we have demonstrated (Fig. 4a)
that Y783 phosphorylation in full length PLC\[I\] can be completely inhibited by addition of an exogenous agent that competes with substrate docking onto Itk.

Methods

DNA constructs and virus production. Production and protein purification using baculoviral and bacterial Itk and PLC\[I\] fragments have been described in detail previously\(^{10,31,32}\). Full length PLC\[I\] (wild type or mutants) carrying a strep tag at the C-terminus were cloned into pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. All constructs were verified by sequencing at the Iowa State University DNA Synthesis and Sequencing Facility. The pENTR vectors were recombined in vitro with BaculoDirect C-Term Linear DNA (Invitrogen) using the LR Clonase II enzyme (Invitrogen). The DNA was then transfected into sf9 cells using Effectene (Qiagen). Three rounds of viral selection and amplifications were carried out.

Full-length PLC\[I\] Protein production and purification. Full-length PLC\[I\] protein production was similar as before. Briefly, the insect cells were harvested 72 h postinfection, rinsed once with phosphate-buffer saline (PBS), and stored at -80°C. Prior to purification, cell pellets were resuspended in lysis buffer (20 mM HEPES (pH 7.2), 150 mM NaCl, 2 mM EDTA, cocktail protease inhibitor) and lysed by sonication. The total lysate was cleared by spinning at 14 K at 4°C for 20 min. The supernatant was incubated with strep resin for a minimum of 2 hours at 4°C. Then the resin was washed with PBS for 4 times and HEPES
buffer [50 mM HEPES (pH 7.0), 10 mM MgCl₂] for 2 times. Then the resin was subjected to in vitro kinase assay as before¹⁰,¹³,¹².

**Pull-down assays, in vitro kinase assays and western blot.** The protocols used for pull-down assays, in vitro kinase assays and western blot have also all been described previously¹⁰.

**CD spectra.** Circular dichroism (CD) measurements of the PLCγ1 SH2C-linker variants were performed on a Jasco J-715 CD spectropolarimeter (Jasco Inc.) in the far UV region (200-260 nm) at 25°C. Samples were prepared in 10 mM KH₂PO₄, pH 7.4, at a concentration of 10 μM as determined by OD₂₈₀ using an extinction coefficient of 18910 M⁻¹cm⁻¹. CD spectra were recorded at a scanning rate of 50 nm/min with a spectral bandwidth of 2 nm and response times of 2 msec. Ten accumulations were acquired and the results were averaged. After background subtraction, all the CD data were converted from mdegree into mean residue ellipticity (deg. cm².dmole⁻¹).

**Acknowledgements:**

This work is supported by a grant from the National Institutes of Health (National Institute of Allergy and Infectious Diseases, AI43957) to A.H.A.

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**Figure Captions:**

Figure 1: (a) Domain structure of full length PLC-I. The site of PLC-I phosphorylation by
Itk (Y783) is indicated and the SH2C-linker substrate fragment (residues 659-789) is
highlighted with a grey box. The SH2C domain fragment (residues 659-756) is boxed. (b)
The free PLC-I SH2 domain competes directly with phosphorylation of PLC-I Y783. Lane
1 contains full length Itk enzyme and the PLC-I SH2C-linker substrate (SH2C-Y783-)
without additional free SH2 domain. The anti-pY783 antibody is used to detect extent of
substrate phosphorylation on Y783. Addition of isolated PLC-I SH2C domain (but not a
control SH2 domain (derived from Grb2)) to the kinase reaction reduces phosphorylation on
Y783 by competing for the docking site on the Itk kinase domain. Ratios of free PLC-I
SH2C domain to substrate are 10:1 and 25:1 in lanes 2 and 3, respectively and equivalent
ratios of the Grb2 SH2 domain are used as a control in lanes 4 and 5. Coomassie stain of the gel and an anti-Itk blot (bottom panels) show protein levels. (c) Schematic description of substrate docking mechanism. Phosphorylation of Y783 in PLC\[I\] depends on direct binding of the Itk kinase domain to the PLC\[I\] SH2C domain. The docking interaction between Itk kinase domain and PLC\[I\] SH2C domain does not occlude the active site of Itk and is independent of phosphotyrosine recognition by the SH2 domain \(^10\). (d) Sequence alignment of the PLC\[I\] SH2C domain from *Bos taurus*, *Rattus norvegicus*, *Homo sapiens* and *Xenopus* (1-4 respectively); PI3K SH2 domain (5) and Grb2 SH2 domain (6). The secondary structural elements are shown above the sequences and surface residues targeted for mutation are indicated with asterisks.

Figure 2 (a) Indicated mutations were introduced into the PLC\[I\] SH2C-linker substrate used previously \(^10\). One or five µM PLC\[I\] SH2C-linker substrate (wild type: lanes 1-3) or indicated mutants (lanes 4-39) were subjected to *in vitro* phosphorylation by 475 nM full length FLAG-tagged Itk enzyme. Lane 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 39 are no Itk enzyme controls for the wild type substrate or indicated mutants. Anti-pY783 blot indicates extent of PLC\[I\] substrate phosphorylation by Itk; Coomassie Stain shows substrate levels and anti-FLAG/anti-Itk blot shows the Itk enzyme levels. Efficient phosphorylation of Y783 within the substrate by Itk occurs only when the Itk kinase domain/PLC\[I\] SH2C domain docking interaction is intact. Mutations that disfavor or disrupt the docking interaction lead to diminished Y783 phosphorylation levels. (b) CD spectra of purified substrates (wild type PLC\[I\] SH2C domain and mutants).
Figure 3: (a) Results of the mutational analysis shown in Figure 2a are mapped onto the structure of PLC|SH2C domain (PDB: 2PLD). Blue corresponds to those amino acids for which mutation to alanine disrupts docking onto the Itk kinase domain and subsequent phosphorylation of Y783. These docking site residues are labeled and are located on the CD loop and the C-terminus of the SH2C domain. Orange indicates residues that can be mutated to alanine with no effect on Y783 phosphorylation. (b) The docking site is distinct from the phosphopeptide binding surface. The structure shown includes bound phosphopeptide in red with the phosphotyrosine (pY) and specificity pockets (pY+3) labeled and the docking site residues that were determined by mutation (Fig. 2a) in blue as in (a).

Figure 4: Full length PLC as substrate. (a) Phosphorylation of Y783 in full-length PLC is reduced by the addition of isolated PLC SH2C domain but not control SH2 domains. Full-length PLC was subjected to an in vitro kinase assay using Itk full-length enzyme alone (lane 1), or with increasing concentrations of PLC SH2C domain. Ratios of free PLC SH2C domain to substrate are 5:1 or 10:1 (lanes 2 and 3, respectively). The remainder of the gel shows the same experiment using alternative SH2 domains at concentrations that are identical to wild type PLC SH2C as controls. Lanes 4 and 5 contain, in addition to full length PLC substrate and full length Itk enzyme, the isolated PLC SH2C domain double mutant K749A/R753A, lanes 6 and 7 contain the isolated PLC SH2C domain double mutant R748A/K751A and lanes 8 and 9 contain the isolated Grb2 SH2 domain. Anti-pY783 antibody is used to detect levels of Itk induced phosphorylation of Y783, anti-strep antibody indicates the level of the full length PLC.
substrate and anti-Itk antibody is used to confirm Itk enzyme levels. Coomassie stain shows protein levels of free PLC:\(_1\) wild type or mutant SH2 domains and Grb2 SH2 domain. Strep-tactin protein on the strep resin runs at the same position as the free SH2 domains, hence a protein band is evident in lane 1 despite the absence of free SH2 domain in that experiment. (b) Itk mediated phosphorylation of Y783 in full-length PLC:\(_1\) is reduced upon mutation of specific docking residues in the PLC:\(_1\) SH2C domain. Lane 1: Strep-tagged, wild type full-length PLC:\(_1\) is incubated with full-length Itk. Top panel: Phosphorylation of Y783 is detected using a phosphospecific antibody (anti-pY783). The level of enzyme is shown in the middle panel and the level of substrate (full length strep-tagged PLC:\(_1\)) is shown in the bottom panel. Lane 2 is no Itk enzyme control. Lanes 3 & 5 are the same experiment as lane 1 using either the K749A/R753A mutant of full-length PLC:\(_1\) (lane 3) or the R748A/K751A mutant of full-length PLC:\(_1\) (lane 5) as substrate. Lanes 4 and 6 are no enzyme controls.
Figure 2

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anti-py783
Coomassie Stain
anti-FLAG/anti-itk

b

Molar Residue Ellipticity (deg cm$^2$ dmol$^{-1}$)

wave length (nm)

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-9000
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wild type
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K695A R696A S701A
C715A R716A
M681A M683A
E709A K711A
K749A R753A
D732A
N728A E730A
K743A H744A
T674A R675A
R748A K751A
N697A E698A
CHAPTER 4. A PHOSPHOLIPASE C\(^{1}\) INTRAMOLECULAR INTERACTION REGULATES THE SUBSTRATE DOCKING INTERACTION

Publication will be prepared upon completion of the PLC\(^{1}\) SH2C-linker complex structure

Lie Min\(^{1}\), D. Bruce Fulton and Amy H. Andreotti*

\(^{1}\) primary author

* corresponding author

Abstract

The SH2 domains in PLC\(^{1}\) play an important role in the membrane recruitment and phosphorylation of the PLC\(^{1}\) enzyme. It contains tandem SH2 domains, one N-terminal SH2 (SH2N) and one C-terminal SH2 (SH2C). Our data indicate that SH2C mediates an intramolecular interaction with the adjacent linker sequence regulating the substrate docking interaction with the Itk kinase domain. Like the substrate docking interaction, this intramolecular interaction is distinct from the canonical SH2/phospholigand interaction. However the intramolecular interaction does occur through the canonical binding pocket for phosphotyrosine ligand. Thus our data indicate that a non-classical interaction can occur through binding to the canonical interaction pocket of the SH2 domain of PLC\(^{1}\).
**Introduction**

Phospholipase C\(\Gamma_1\) (PLC\(\Gamma_1\)) is the enzyme that hydrolyzes phosphatidylinositol (4, 5)-bisphosphate (PIP\(_2\)) into inositol (1, 4, 5)-triphosphate (IP\(_3\)) and diacylglycerol (DAG) (1, 2). These second messengers mobilize intracellular Ca\(^{2+}\) and activate protein kinase C, respectively and subsequently lead to activation of downstream signaling that controls the cellular responses to external stimuli. PLC\(\Gamma_1\) belongs to the phosphoinositide-specific phospholipase C (PI-PLC) family, which include six families and 14 mammalian PLC isozymes. These are PLC\(\Gamma_1\) (\(\Gamma_1\)-4), PLC\(\Gamma_2\) (\(\Gamma_2\)-\(\Gamma_3\)), PLC\(\Gamma_4\) (\(\Gamma_1\)-\(\Gamma_4\)), PLC\(\Gamma_5\), PLC\(\Gamma_6\) and PLC\(\Gamma_7\) (\(\Gamma_1\)-\(\Gamma_2\)) (1, 2). These family members share conserved catalytic domain and contain very different regulatory domains.

Unlike the rest of the PLC family members, PLC\(\Gamma_1\) contains Src homology domains, which include an N-terminal SH2 domain (SH2N), a C-terminal SH2 domain (SH2C) and a SH3 domain. The SH2N domain is critical for the membrane recruitment and phosphorylation of PLC\(\Gamma_1\) (3, 5), which are important for PLC\(\Gamma_1\) activation. Furthermore, the Src Homology domains can also directly regulate PLC\(\Gamma_1\) activity. Homma and coworkers reported that a protein fragment that includes the PLC\(\Gamma_1\) SH2N, SH2C and the SH3 domain can inhibit PLC\(\Gamma_1\) activity and this inhibition derives primarily from an 8-mer peptide in the SH2C domain (6). Consistent with this, Carpenter and coworkers reported that deletion of the SH domains results in a 20- to 100- fold increase in PLC\(\Gamma_1\) activity (7). Koblan and coworkers reported that a phosphotyrosine ligand for SH2 domains causes a conformational change in PLC\(\Gamma_1\) and also increases lipase activity up to 85% (8, 9).
By mutating the canonical phosphor-tyrosine binding pocket in the SH2 domains, numerous studies have found that SH2N is required for the membrane recruitment and tyrosine phosphorylation of PLC\(\beta\), while SH2C is not critical for this event (4, 5). Our group previously reported a substrate docking interaction that is mediated by the PLC\(\beta\) SH2C domain. This docking interaction is required for efficient phosphorylation of the full-length PLC\(\beta\) Y783 and it is mediated by an alternative interaction surface that is different from the canonical phospholigand interaction surface of the SH2C. Using NMR spectroscopy, we find an intramolecular interaction within PLC\(\beta\) that alters accessibility of this docking site. Analysis of the truncation mutants by NMR spectroscopy indicates that the intramolecular interaction is mediated by the A\(^{786}\)NPM\(^{789}\) sequence in the linker region and residues in the SH2C domain form a hydrophobic binding pocket that overlaps with the canonical binding pocket for phosphotyrosine ligand although there is no phosphor-tyrosine in this linker region. Thus our data indicate that a non-classical interaction can occur through the canonical interaction pocket of the SH2 domain of PLC\(\beta\).

**Results**

*The PLC\(\beta\)/Itk interaction is regulated by an intramolecular interaction within PLC\(\beta\).* As previously reported (10) and demonstrated here in Figure 1a and 1b, the PLC\(\beta\) SH2C domain interacts directly with the Itk kinase domain in a pull down assay using purified proteins (Fig. 1b, lane 3, 5 and 7). Further analysis of the PLC\(\beta\) SH2C/Itk kinase domain interaction reveals that the PLC\(\beta\) SH2C-linker construct (contains both the SH2C domain and the 33 amino acid linker region, Fig. 1a) reproducibly interacts to a lesser extent
with the Itk kinase domain than does the isolated PLC$\gamma$1 SH2C domain in pull down assays conducted under a variety of conditions (Fig. 1b, lane 4, 6 and 8). The diminished binding of SH2C-linker to the Itk kinase domain could indicate a regulatory mechanism by which the Itk kinase/PLC$\gamma$1 SH2C domain interaction is modulated by conformational changes within PLC$\gamma$1 or steric blocking of the docking site.

Nuclear Magnetic Resonance (NMR) is a useful tool to monitor individual amino acid residues and possible conformational changes and/or changes in chemical environment induced upon ligand binding. A comparison of separately acquired Heteronuclear Single Quantum Coherence (HSQC) spectra for the PLC$\gamma$1 SH2C domain alone and SH2C-linker reveals significant chemical shift differences that suggest either interaction between the SH2C domain and the adjacent linker sequence and/or a conformational change in the SH2C domain that is induced by the linker sequence (Fig. 1c, 1d and 1e). The absence of linewidth changes as a function of concentration of SH2C-linker as well as the measured linewidths in each of the HSQC experiments (16-19 Hz) provide evidence that the interaction between the SH2C domain and the adjacent linker occurs in an intramolecular sense. In addition, we synthesized a peptide that corresponds to the linker region (Fig. 1a) to assess the extent to which this peptide sequence interacts in an intermolecular sense with the isolated SH2C domain. Minimal chemical shift changes are observed for $^{15}$N labeled SH2C upon titration with the linker peptide (data shown in Fig. 1f and 1g). Following previously reported methods (11, 12) we used the small but observable chemical shift changes to extract dissociation constant and find that the affinity of this peptide for the SH2C domain is quite low (2.5 mM Kd). Thus, measured linewidths, the absence of spectral changes with
concentration and the low affinity of the intermolecular interaction between linker peptide and SH2C domain are all consistent with an intramolecular interaction between linker and SH2C domain in the PLC\[I\] SH2C-linker construct.

Figure 1d and Figure 1e show the SH2C residues that have significant chemical shift differences upon the association of the adjacent linker. The linker extends from the C-terminal of SH2C and both the C-terminus and N-terminus are close in space, therefore it is reasonable to see the residues from the \[A\]A, A helix, AB loop and \[B\]B (we call this region cluster I) exhibit significant chemical shift differences. These residues are highlighted in orange in Figure 1e. In addition to the residues in cluster I, residues from the CD loop, \[D\]D, EF loop and BG loop also exhibit significant chemical shift differences between SH2C and SH2C-linker (we call this region cluster II). These residues are highlighted in blue in Figure 1e. Interestingly, residues in cluster II also exhibit significant chemical shift differences upon titration of the linker peptide (Fig. 1f). These residues form a continuous surface (Fig. 1g) that partially overlaps with the surface formed by residues in cluster II in Figure 1e. Therefore the linker sequence itself can associate with the SH2C domain to some extent independent of the covalent bond linking the SH2C domain and the following linker sequence.

To further define the contribution of the linker sequence and the minimal fragment responsible for regulating the Itk kinase/PLC\[I\] SH2C domain interaction, four truncated PLC\[I\] SH2C-linker mutants were created (Fig. 2a). For each of the truncation mutants and the SH2C-linker protein, the interactions with the Itk kinase domain were analyzed using a pull-down assay. Comparing the amount of Itk kinase domain that is bound to the various
immobilized GST tagged PLC[I] SH2C fragments (Fig. 2b, lane 3 to 8), it can be seen that all of these truncated mutants (Fig. 2b frome lane 5 to lane 8) pull down more Itk kinase domain than does the PLC[I] SH2C-linker (lane 4). The smallest difference between the PLC[I] SH2C-linker and the longest truncation mutant PLC[I] SH2C-785 (Fig. 2a) indicated that the last 4 amino acids A^786NPM^789 in the linker region are critical for regulating the PLC[I] SH2C/Itk kinase domain interaction.

To reveal any possible interaction and/or conformational change in the series of truncation mutants, HSQC spectra for two of the truncated mutants, PLC[I] SH2C-770 and PLC[I] SH2C-785 were chosen to compare with the isolated PLC[I] SH2C domain, SH2C-linker and also with each other (Fig. 3a, 3b, 3d, 3e and 3g). The comparison indicate that the chemical shift differences between the PLC[I] SH2C and PLC[I] SH2C-785 and the differences between the PLC[I] SH2C domain and PLC[I] SH2C-770 are less than that of SH2C and SH2C-linker and no chemical shift differences between the PLC[I] SH2C-770 and PLC[I] SH2C-785 are evident. Therefore, data here indicate that the last 4 residues of the linker region A^786NPM^789 and the first 14 residues of the linker region interact with and/or induce a conformational change in the PLC[I] SH2C while there is no interaction between the PLC[I] SH2C and the linker residues spanning 771 to 785.

Figure 3b and 3e show the observed chemical shift differences between SH2C and SH2C-770 and chemical shift differences between SH2C and SH2C-785 and the surface plot of these chemical shift differences are shown in Figure 3c and 3f. Some residues in cluster I showed significant chemical shift difference compared with SH2C in both of these truncation
constructs, therefore the existence of the 14 residues at the N-terminal of the linker sequence induces the chemical environment change for the residues in cluster I (the $\square$A, $\square$A, AB loop and $\square$B of SH2C). Equally interestingly, residues in cluster II exhibit no significant chemical shift differences from that of SH2C for both of these truncation mutants. Therefore the significant chemical shift differences that we observed for residues in cluster II comes from association with the last 4 residues $A^{786}NPM^{789}$ in the linker and the significant chemical shift differences for residues in cluster I come from contacting the 14 residues (or less) at the N-terminal of the linker. Consistent with this, the titration of the linker peptide into the SH2C shows that only cluster II residues (the $\square$C, $\square$D, EF loop and BG loop) exhibit significant chemical shift in the absence of the covalent link between the PLC$\square$ SH2C and N-terminus of the linker (Fig. 1f, highlighted in blue in 1g).

To confirm that the residues $A^{786}NPM^{789}$ are associating with cluster II residues (highlighted in blue in the SH2C, in Fig. 1e and 1g), we created a mutant in which N$^{787}MP^{789}$ were mutated to Ala. If indeed $A^{786}NPM^{789}$ are responsible for the association, the mutation should break up the interaction and therefore the residues from cluster II region (the $\square$C, $\square$D, EF loop and BG loop) should have similar chemical shift resonance as those in the isolated SH2C domain. An HSQC spectrum of this mutant was acquired and compared with those of the SH2C and SH2C-linker revealing that the residues in cluster II in the mutant have almost same chemical shift resonances as in the isolated PLC$\square$ SH2C, while residues in cluster I (the $\square$A, $\square$A, AB loop and $\square$B strand) still have different chemical shift resonances from both SH2C and SH2C-linker (Fig. 4a, b, c and d). Thus the last 4 residues in the linker are indeed critical for interacting with the PLC$\square$ SH2C domain.
Because of the putative regulatory role of the last 4 amino acids $A^{786}NPM^{789}$ played in the substrate docking interaction, we mapped the residues in the SH2C that are associating with $A^{786}NPM^{789}$ (the residues that showed significant chemical shift in Fig. 1d and 1f and highlighted in blue in Fig. 1e and 1g) onto the structure of the SH2C domain (Fig. 5a) (13). F706 is located at the [C strand of the SH2C domain; E709 resides on the CD loop; K713, H714, C715 and V717 are from the [D strand; G727 is from the EF loop, and H744, L746 and Y747 are from the BG loop. Comparison of the surface that is mapped here with the substrate docking surface (Fig. 5b, also see Chapter 3 for detailed information) indicates they are partially overlapped, which might explain the diminished interaction between the SH2C-linker and the Itk kinase domain. Meanwhile, it is also plausible that the association of the residues at the C-terminal of Y783 with SH2C restricts the access of Y783 and therefore we can speculate that the mutation that disrupts the intramolecular interaction will make the SH2C-linker or full-length PLC$\gamma_1$ a better substrate for Itk.

Viewing the residues that are mediating the intramolecular interaction on the SH2C structure indicates that these residues form a hydrophobic groove in the SH2C domain (Fig. 5a). Comparing the intramolecular interaction surface with a phosphotyrosine peptide bound SH2C domain (Fig. 5c) reveals that the intramolecular interaction surface overlaps with the canonical binding pocket for phosphotyrosine ligand. This suggests that association of the linker residues with the SH2C domain could regulate the canonical SH2C/phosphotyrosine interaction and vice versa.
Discussion

The NMR structure of PLC\[\text{II} \] SH2C bound to a phosphotyrosine peptide from platelet-derived growth factor (PDGF) was one of the first SH2 domain structures solved \((13)\). More recently PLC\[\text{II} \] SH2C bound to a peptide that contains two phosphotyrosines has been solved by NMR \((14)\). Both of these structures represent phosphotyrosine mediated SH2 interactions. Unlike the canonical SH2/phosphotyrosine interaction, the intramolecular interaction between PLC\[\text{II} \] SH2C and the adjacent linker does not involve phosphorylated Tyr. Therefore the intramolecular interaction is mediated by an interaction mechanism that is distinct from the canonical SH2/phospholigand interaction. Solving the structure of SH2C-linker to understand the detailed molecular mechanism of intramolecular interaction and its regulation of the substrate docking interaction will be important for us. However, the solving the structure of this SH2C-linker complex presents challenges due to the exchange broadening that diminishes the quality of the NMR spectra. Different pulse sequences to suppress relaxation during transfer are currently being explored. We will also design mutations to attempt to modify the dynamic exchange regime based on our data for the intramolecular interaction. As well, we have attempted crystallization of SH2C-linker complex but thus far been unsuccessful. The inability to crystallize SH2C-linker complex is consistent with the dynamic exchange behavior observed by NMR.

Our data indicate that conformational rearrangements and/or steric blocking by the linker sequence within the PLC\[\text{II} \] substrate can mask the docking site for the Itk kinase domain contributing to a complex set of regulatory interactions that maintain fidelity in TCR signaling. Intramolecular association of the PLC\[\text{II} \] SH2C domain and the neighboring linker
The function of SH2 domains is most often probed by mutating the universally conserved Arg in the phosphotyrosine binding pocket. For PLCγ1 SH2C, this traditional loss-of-function mutagenesis approach has led to the conclusion that SH2C is not critical for many cellular events involving PLCγ1 (4, 5). It is interesting that the interactions that we described for PLCγ1 SH2C, the docking interaction that is described in Chapter 2 and 3 and the intramolecular interaction between PLCγ1 SH2C domain and the adjacent linker...
described in this chapter, are both independent of phosphotyrosine. Our results support the idea that the function of PLC\[\text{SH2C} \] domain is versatile and SH2C can mediate both canonical SH2/phosphotyrosine interactions and noncanonical interactions with ligands that do not contain phosphotyrosine. Thus the commonly used criteria for judging SH2 domain function by mutating the conserved phosphotyrosine ligand-binding pocket might be misleading and misguide the interpretation of SH2 domain function in general.

Materials and Methods

Protein production and purification. Production and protein purification using baculoviral and bacterial Itk and PLC\[\text{SH2C} \] constructs have been described in detail previously (\textit{10}).

Pull-down assays and western blot. The protocols used for pull down assays and western blotting have also all been described previously (\textit{10}). All peptides were obtained from Genscript. Peptide concentrations were confirmed by amino acid analysis at the Iowa State University protein facility.

NMR spectra acquisition. NMR Spectra were acquired using a Bruker AVII 700 spectrometer equipped with a 5mm HCN cryoprobe operating at 700.133 MHz \[^1\text{H} \] frequency. All spectra were obtained at 303K. Backbone NH resonances were confirmed for PLC\[\text{SH2C} \] and assigned for SH2C-linker using \[^{15}\text{N}-\text{edited NOESY, TOCSY, HNCA, HNCOCA,}
HNCACB and CACBCONH. 2D \[^1\text{H}-^{15}\text{N} \] HSQC spectra (\textit{17}) acquired for PLC\[\text{SH2C,}

SH2C-linker and truncation mutant were compared and differences in $^1$H and $^{15}$N chemical shifts were quantified using the formula (18): $D_{ave} = \left\{ \left[ \frac{1}{2} \left( D_{HH} \right)^2 + (0.2 D_{HN})^2 \right] \right\}^{1/2}$. Residues with chemical shift changes above the mean plus one standard deviation were considered significant. $^1$H and $^{15}$N chemical shifts were externally referenced to DSS in identical buffer. Additionally, 3D $^{15}$N-edited NOESY spectra were obtained for both SH2C and SH2C-linker. Full analysis of these data is underway in the context of structure determination but clear differences for certain residues are noted herein. NMR samples were prepared in identical buffer: 50 mM KH$_2$PO$_4$, pH 6.4, 150 mM NaCl, 2 mM DTT, 0.02% NaN$_3$.

Acknowledgements

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References


Figure Caption

Figure 1: PLC\textsuperscript{g} SH2C and the adjacent linker interact intramolecularly. (a) Schematic of PLC\textsuperscript{g} SH2C, PLC\textsuperscript{g} SH2C-linker and the linker peptide. The 33 amino acid linker located at the carboxy-terminus of the SH2C domain contains Y783 (boxed) that is phosphorylated by Itk. (b) The Itk kinase domain (KD) interacts with PLC\textsuperscript{g} SH2C-linker to a lesser extent than with SH2C at various conditions. Purified FLAG-tagged Itk KD (lane 1) was incubated with GST (lane 2), GST-SH2C (lanes 3, 5 and 7), GST-SH2C-linker (lanes 4, 6 & 8). The conditions of each pull-down experiment were varied. Specifically, the SH2C and SH2C-linker were purified either using PBS buffer (lanes 3 & 4) or HEPES buffer (lanes 5-8) and the salt concentration varied from 150 mM NaCl (lanes 2, 3 and 4) to 0.5 M NaCl (lanes 5 & 6) to 1 M NaCl (lanes 7 & 8). The amount of flag tagged Itk KD bound to immobilized GST, GST-SH2C or GST-SH2C-linker is detected using an anti-FLAG antibody and the levels of GST, GST-SH2C and GST-SH2C-linker are detected by Ponceau S stain. (c) \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra of PLC\textsuperscript{g} SH2C (black) and PLC\textsuperscript{g} SH2C-linker (red). Backbone resonance frequencies differ significantly for these two proteins indicating an association between the SH2C domain and the adjacent linker sequence. (d) Chemical shift differences between SH2C and SH2C-linker (derived from HSQC spectra shown in c). The differences in chemical shift between SH2C and SH2C-linker were quantified using the formula (18): 

\[
\delta_{\text{ave}} = \left\{ \frac{1}{2} \left[ (\delta_1)^2 + (0.2\delta_N)^2 \right] \right\}^{1/2}
\]

The horizontal lines indicate mean plus one standard deviation. Chemical shift differences greater than the mean plus one standard deviation are considered significant. Proline residues at positions 686, 699, 746 and 755 do not appear in the HSQC data. N700 and K749 are not assigned and are therefore not included in this analysis. Secondary structures are indicated above the protein sequence. Residues from the
A, □A, □B and AB loop are in cluster I; residues from the CD loop, □D, EF loop and BG loop are in cluster II. All of the chemical shift difference data are processed in the same way throughout this chapter. (e) A surface plot of the chemical shift differences between SH2C and SH2C-linker. The SH2C (PDB ID 2PLD) is shown in grey and the residues that exhibit significant chemical shift in cluster I are highlighted in orange; residues that exhibit significant chemical shift in cluster II are highlighted in blue. The color scheme is same for all of the surface plots throughout this chapter. (f) Chemical shift changes upon titrating linker peptide into SH2C. The presence of linker peptide induced chemical shift changes for residues in cluster II. (g) A surface plot of the chemical shift changes upon linker peptide binding. It can be seen that all the residues that exhibit significant chemical shift changes are in cluster II.

Figure 2: The last 4 residues in the linker sequence are critical for regulating the docking interaction. (a) Schematic of PLC\[\] SH2C-linker, the truncation mutants and SH2C. The number above the linker residues indicates the amino acid number in the full-length PLC\[\] protein. (b) All of the truncation mutants interact with the Itk kinase domain (KD) to a greater extent than does the SH2C-linker. Purified FLAG-tagged Itk KD (lane 1) was incubated with GST (lane 2), GST-SH2C (lanes 3), GST-SH2C-linker (lanes 4) and different truncation mutants (lane 5, 6, 7 and 8 respectively). The conditions of each pull-down experiment were same as that of lane 2-4 in figure 1b. Specifically, the SH2C and SH2C-linker were purified either using PBS buffer (lanes 3 & 4) and the salt concentration 150 mM NaCl. The amount of flag tagged Itk KD bound to immobilized GST, GST-SH2C, GST-
SH2C-linker and different truncation mutant is detected using an anti-FLAG antibody and the levels of GST, GST-SH2C and GST-SH2C-linker are detected by Ponceau S stain.

Figure 3. The last 4 residues in the linker sequence associate with the SH2C domain. (a) $^1$H-$^{15}$N HSQC spectra of PLC$^\beta$ SH2C (black) and truncation mutant PLC$^\beta$ SH2C-770 (blue). The backbone resonance frequency difference between SH2C and SH2C-770 is less than that of SH2C and SH2C-linker. (b) Chemical shift difference between SH2C and SH2C-770 (derived from HSQC spectra shown in a). All the residues that exhibit significant chemical shift are in cluster I and they are highlighted in orange in Fig. 3c. (c) A surface plot of the chemical shift differences between SH2C and SH2C-770. (d) $^1$H-$^{15}$N HSQC spectra of PLC$^\beta$ SH2C (black) and truncation mutant PLC$^\beta$ SH2C-785 (purple). The backbone resonance frequency difference between SH2C and SH2C-785 is less than that of SH2C and SH2C-linker. (e) Chemical shift difference between SH2C and SH2C-785 (derived from HSQC spectra shown in d). Like Fig. 3b, only residues in cluster I show significant chemical shift difference and these residues are highlighted in orange in Fig. 3f. (f) A surface plot of the chemical shift differences between SH2C-785 and SH2C. (g) $^1$H-$^{15}$N HSQC spectra of the two truncation mutants PLC$^\beta$ SH2C-771 (blue) and SH2C-785 (purple). Backbone resonance frequencies are same for these two proteins except there are more peaks in SH2C-785 (the purple spectrum) indicating no association between the SH2C domain and the middle 15 amino acids in the linker sequence.

Figure 4: Mutation of NPM partially disrupts the association of linker with SH2C domain. (a) $^1$H-$^{15}$N HSQC spectra of PLC$^\beta$ SH2C (black), SH2C-linker (red) and SH2C-linker
NPM_AAA mutant (magenta). In SH2C-linker NPM_AAA mutant, some residues have similar chemical shift resonance with that in the SH2C and some residues have different chemical shift resonances from both the SH2C and SH2C-linker. (b) Residue H714 in the δD strand in SH2C-linker NPM_AAA (magenta) has almost same chemical shift resonance with that in the SH2C. (c) Residue G727 from the EF loop in SH2C-linker NPM_AAA has almost same chemical shift resonance with that in the SH2C. (d) Residue V683 in the AB loop that has different chemical shift resonance from that of both SH2C and SH2C-linker.

Figure 5: The interaction surface for the last 4-linker residues partially overlaps with the substrate docking interaction surface. The association of the last 4 residues in the linker region with SH2C is mediated through a surface that partially overlaps with the canonical binding pocket for phosphotyrosine peptide. (a) A surface plot of the binding pocket for the last 4 residues. The highlighted surface is composed of all of the residues that are highlighted in blue in Fig 1e and 1g. (b) A surface plot of the docking site residues. The SH2C is shown in grey and the docking residues are shown in green. (c) A surface plot of structure SH2C bound to a phosphotyrosine ligand (2PLD). The SH2C is shown in grey and the phosphotyrosine ligand is shown in red.
Figure 1

a

**PH-EF hands-X-(P-SH2N-SH2C-...SH3-H)-Y-C2**

PLCγ1 SH2C-linker

PLCγ1 SH2C

PLCγ1 linker peptide

---

b

Anti-FLAG

Ponceau S

1 2 3 4 5 6 7 8

Itk KD alone  GST  GST-SH2C-linker  GST-SH2C  GST-SH2C-linker  GST-SH2C  GST-SH2C-linker

Itk Kinase Domain

GST-SH2C-linker

GST-SH2C

GST

---

c

PLCγ1 SH2C

PLCγ1 SH2C-linker
Figure 1 (continued)

[Diagrams and graphs showing molecular structures and residue numbers with annotations for clusters and termini.]
Figure 2

a

**PH-EF hands-X-(P-SH2N-SH2C---SH3-H)-Y-C2-**

<table>
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<th>Protein</th>
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<th>Sequence</th>
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<td>SH2C</td>
<td>NEEALEKIGTAEPDYGALYEGRNPGFYVEANPM</td>
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<td>PLCγ1 SH2C-785</td>
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</tr>
<tr>
<td>PLCγ1 SH2C</td>
<td>SH2C</td>
<td></td>
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</table>

b

**Anti-FLAG**

- Itk KD alone
- GST
- GST-PLCγ1 SH2C-linker
- GST-PLCγ1 SH2C-763
- GST-PLCγ1 SH2C-770
- GST-PLCγ1 SH2C-779
- GST-PLCγ1 SH2C-785

**Ponceau S**

- GST-SH2C-linker
- GST-SH2C
- GST

1 2 3 4 5 6 7 8
Figure 3

(a) 

(b) 

(c) 

(d) 

(e) 

(f)
Figure 3 continued
Figure 4
CHAPTER 5. INTERMOLECULAR SELF-ASSOCIATION INHIBITS THE ACTIVITY OF INTERLEUKIN-2 TYROSINE KINASE

A paper submitted to the Journal of Biological Chemistry

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*These authors contributed equally

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Abstract

Protein tyrosine kinases are responsible for producing phospho-tyrosine sites in many signal transduction processes. The regulation of the immunological non-receptor tyrosine kinase, Interleukin-2 tyrosine kinase (Itk), is not yet well understood. Here we show that full length Itk self-associates in an intermolecular fashion and that sequence changes in the SH3 domain reduce the extent of self-association. Results of in vitro kinase assays indicate that the activity of wild-type, full-length Itk is reduced at higher enzyme concentrations while the full-length Itk-SH3 mutant exhibits enzymatic activity that increases linearly with increasing enzyme concentration. When expressed in insect cells, the full-length Itk-SH3 mutant phosphorylates the Itk substrate PLCγ1 better than wild-type Itk. Furthermore, expression of the full-length Itk-SH3 mutant in primary T cells induced higher ERK activation following TCR stimulation than wild-type Itk. Our results suggest that the protein tyrosine kinase Itk can be negatively regulated by intermolecular clustering.
Introduction

Interleukin-2 tyrosine kinase (Itk) is a non-receptor protein tyrosine kinase of the Tec family that is expressed in T cells, mast cells and NK cells (1-5). This kinase participates in signaling processes following T cell receptor engagement by phosphorylating phospholipase C-\(\gamma\) (PLC-\(\gamma\)) (6-9). In addition to Itk, the Tec family kinases include Btk, Tec, Rlk and Bmx, each of which shares a similar domain structure with the Src kinase family (10). Both families contain a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain and the catalytic domain. With the exception of Rlk, the Tec kinases also contain a pleckstrin homology (PH) domain and a tec homology (TH) domain at the amino terminus. A significant difference between the Src and Tec kinases is that the Tec kinases lack the carboxy-terminal autoinhibitory sequence that serves to negatively regulate the Src kinases. The absence of a Src-like regulatory tail in Itk and related family members raises questions as to how the Tec kinases are turned off during the course of T cell signaling.

We and others have previously reported detailed structural studies for regulatory domain fragments of Itk, Btk, Tec and Rlk (11-19). An emerging theme for each of these kinases is that the non-catalytic domains form dimeric and higher order oligomeric structures in solution. For Itk, self-association of the regulatory domains occurs via intermolecular interactions between the SH3 domain and the SH2 domain (12). An intermolecular interaction has also been described for the Itk PH domain (20). The isolated PH domain interacts both with itself and with the PH domain within full length Itk in co-immunoprecipitation experiments. Finally, a split YFP system has previously allowed
visualization of intermolecular interactions between full-length Itk molecules in cells (21). Thus, abundant data point to intermolecular clustering of Itk, most likely mediated by multiple Itk regulatory domains; yet to date, the functional significance of this self-association has not been investigated.

Here, we first extend the earlier studies of Itk domain fragments and demonstrate that full-length Itk self-associates in an intermolecular fashion in vitro. To evaluate the functional significance of the observed self-association, we then designed a mutant Itk molecule that retains all of the structural features of the wild-type enzyme, yet exhibits diminished self-association. Analysis of the catalytic activity of the non-clustering mutant compared to wild-type indicates that the self-association of wild-type Itk results in decreased kinase activity. Given the necessary concentration dependence of intermolecular Itk clustering, we examined the signaling properties of wild type and mutant Itk following expression in Itk−/− primary CD4+ T cells, thus avoiding complications that would arise from over-expression conditions. In addition, this system allowed us to test Itk function in T cells expressing homogeneous populations of mutant or wild-type Itk, instead of a mixed population of Itk molecules, as would arise following expression of the mutant Itk construct in Itk-sufficient T cells. The results of these experiments indicated that the Itk mutant exhibiting diminished self-association and increased in vitro kinase activity also had increased signaling function upon T cell receptor engagement in primary T cells. We discuss mechanistic explanations for this observation and a model for the control of Itk activity during T cell receptor signaling.
**Experimental Procedures**

*Constructs and baculovirus production.* V5 or myc tagged proteins were cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). Itk \(_{(BkSH3)}\) chimera was generated by replacing Itk SH3 domain sequence from Pro171 to Asn232 with the human Btk sequence spanning Ser214 to Ser275 by PCR. Flag tagged full-length wild-type (mouse Itk) or Itk \(_{(BkSH3)}\) chimera were cloned into the pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. The point mutations were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The pENTR vectors with various inserts were recombined *in vitro* with BaculoDirect C-Term Linear DNA (Invitrogen) according to the manufacturers instructions (Invitrogen) for virus production. The PLC\(\alpha\) baculovirus has been described previously (9).

*Immunoprecipitation and western blot.* NIH 3T3 cells were transfected with myc or V5 tagged DNA using Effectene\textsuperscript{TM} transfection reagent from Qiagen. Twenty-four hours post transfection, cells were lysed in buffer containing 0.5% NP 40, 50mM Tris (pH 7.4), 150mM NaCl, 10mM MgCl\(_2\), and protease inhibitors (Roche Applied Science). Immunoprecipitaiton, western transfer and western blotting were performed using standard techniques.

*Protein expression and purification.* The purification method for bacterially expressed protein has been described previously (12). For S/9 protein expression, baculoviruses for full-length wild-type Itk or the Itk\(_{(BkSH3)}\) chimera along with Lck virus were used to infect S/9 cells in a 1:1 ratio. Proteins were purified using methods reported previously (22-24). The concentration of purified protein was measured by absorbance at 280 nm using an extinction coefficient for Itk of 118570 M\(^{-1}\)cm\(^{-1}\).

*NMR spectroscopy.* NMR spectra were recorded at 298 K on a Bruker DRX500 spectrometer operating at a \(^1\)H frequency of 499.867 MHz. Protein concentrations were adjusted to 1 mM.
Global least squares parameter fitting of the titration data was performed using the Matlab (version 5.3.1, The Mathworks Inc.) suite of programs.

Phosphorylation status. Purified wild-type full-length Itk, Itk(BtkSH3) and Y180F full-length Itk were subjected to autophosphorylation assays by incubation at room temperature in a buffer containing 50mM Hepes (pH 7.0), 10mM MgCl₂, 1mM DTT, 1mg/ml BSA, 0.2mM ATP at RT for 30min at 0.8 μM final enzyme concentration. The reaction was stopped by the addition of SDS-loading dye, run on an SDS-PAGE gel and western blotted.

Kinase assay. The method (described in (22)) is adapted from previous in vitro kinase assays developed for Itk (23,24). Biotin labeled peptide-B (AnaSpec Inc.), was used as a substrate in all of the in vitro kinase assays. For the determination of Kₘ for peptide B, substrate concentration was varied from 10 μM to 400 μM. The wild-type and chimeric Itk exhibit similar Kₘ and kₑₐₜ at low concentrations of enzyme (100 nM). For initial velocity measurements at varying enzyme concentrations, the peptide B concentration was maintained at 400 μM. Kₘ and Vₘₐₓ were determined by fitting the data to the Michaelis-Menten equation using the enzyme kinetics module of the Graph Fit 5 program.

Purification of primary CD4⁺ T cells. Spleen and lymph node cells were isolated from Itk⁻⁻ (6) mice, and CD4⁺ T cells were purified by positive selection using anti-CD4 antibody-coated magnetic microbeads (Miltenyi Biotec).

Retrovirus production. Itk and Itk(BtkSH3) were cloned into the retroviral mouse stem cell virus vector MSCV2.2-IRES-GFP (25). Phoenix-E retroviral packaging cells were transfected with each retrovirus construct plus the pCL-Eco retrovirus Packaging vector (Imgenex) as described previously (9), and virus particles were harvested and stored at
80°C. For each infection, 2 x 10^6 primary CD4⁺ T cells were stimulated with 5ng/ul of PMA (Sigma) and 375ng/ul of Ionomycin (Calbiochem) for 24 hrs and then incubated with 2ml of viral supernatant plus IL-2 (30ng/ml) and lipofectamin (Invitrogen). After 3-4 days, CD4⁺GFP⁺ infected cells were sorted by flow cytometry and cultured a further two weeks in IL-2. For analysis, 3 x 10⁶ cells were restimulated by incubation with biotinylated-anti-CD3 Ab (25 μg/ml) for 10 min, followed by strepavidin (50 μg/ml) crosslinking for 5 min. Cell lysates were analyzed for phospho-ERK by immunoblot; alternatively cells were permeabilized and stained with anti-phosphoERK antibody (Cell Signaling) followed by flow cytometry.

Results

*Full-length Itk self-associates via intermolecular interactions between the non-catalytic regulatory domains.* We have previously reported that the singly expressed Itk SH3 and SH2 domains interact in an intermolecular fashion (12). The interaction surfaces that mediate this binding event have been pinpointed through the analysis of NMR chemical shift perturbations. The regions involved in the interaction include the conserved binding cleft of the SH3 domain and a surface that only partially overlaps with the well-characterized phosphopeptide binding surface of the SH2 domain (Fig. 1a).

In an effort to first determine whether the intermolecular Itk SH3/SH2 interaction mediates self-association of the *full-length* Itk kinase, we generated differentially tagged versions of full-length, wild-type Itk for co-immunoprecipitation experiments in NIH 3T3 cells (Fig. 1b). The myc and V5 epitope tagged Itk constructs were both transiently transfected into NIH 3T3 cells and subjected to immunoprecipitation using anti-myc
antibody. The immunoprecipitated fraction was then resolved by SDS-PAGE and probed with an antibody to the V5 tag. As a control, NIH 3T3 cells were also transiently transfected with V5 tagged Itk and/or the empty vector alone. Consistent with intermolecular self-association of full-length wild-type Itk, V5 tagged full-length Itk is readily detected in anti-myc immunoprecipitates from co-transfected NIH 3T3 cells (Fig. 1b). This data is also consistent with previous in vivo data that indicates intermolecular association of full length Itk molecules (21).

To directly probe the contribution of the catalytic domain of Itk to the observed self-association, the Itk kinase domain alone was separately tagged with myc and V5, co-transfected into NIH 3T3 cells, and treated in the same manner as described above for full-length Itk. In contrast to the full-length protein, the co-immunoprecipitation experiment provides no evidence for self-association of the isolated kinase domain (Fig. 1c). This result suggests that the non-catalytic regulatory domains of Itk are the primary determinant of intermolecular self-association of the full-length protein. We therefore turned our attention to the structural features of the SH3/SH2 intermolecular interaction to identify sequence changes that might disrupt Itk self-association when incorporated into the full-length molecule.

Itk SH3 domain interacts specifically with the Itk SH2 domain. The regulatory SH2 and SH3 domains of Itk interact via an intermolecular association that does not fit the established paradigms for SH2 or SH3 recognition (12). The SH3/SH2 interaction is neither phosphotyrosine-dependent nor mediated by a polyproline motif. Given the non-canonical nature of this interaction, Itk presents an ideal target to specifically disrupt self-association by incorporating sequence changes into the SH3 and/or SH2 domains that do not affect binding
to canonical proline-rich or phosphotyrosine ligands. Mutations that selectively disrupt self-association would permit functional assays to compare full-length, wild-type Itk that interacts with itself in an intermolecular fashion (Fig. 1b) to a full-length Itk variant in which self-association is selectively disfavored.

*Molecular determinants of the SH3/SH2 interaction.* In an effort to identify sequence changes that will abrogate the interaction between the Itk SH3 and SH2 domains, we first considered a point mutation in the well characterized SH3 binding cleft that would affect the interaction between the singly expressed SH2 and SH3 domains. Mutation of the central residue in the SH3 binding cleft, Trp 208, has been previously shown to disrupt the interaction between the SH2 domain and SH3 domain of Itk (12) and therefore might also diminish the extent of full length Itk oligomerization. However, this conserved tryptophan also makes extensive contacts to cognate proline-rich ligands (26,27) and is therefore not an appealing target for mutation in our current study. Instead, we turned our attention to an approach that would disrupt Itk self-association but maintain the canonical ligand binding characteristics of the Itk regulatory domains.

The Btk SH3 domain is 51% identical to that of Itk. Of the Itk SH3 domain surface residues that contact the Itk SH2 domain (Fig. 1a), there are eight residues that differ between Itk and Btk SH3 domains (Fig 2a). These sequence differences are enough to eliminate the intermolecular SH3/SH2 interaction; no chemical shift perturbations are observed in NMR spectra upon mixing the Btk SH3 domain with the Itk SH2 domain at concentrations required for NMR analysis (Fig. 2b). Canonical proline-rich binding was tested next. We compared the affinities of the Itk and Btk SH3 domains for a proline-rich peptide derived from S1p-76 (Q_{184}QPVPQPRMA_{195}) and previously shown to bind the
SH3 domain of Itk (28). Separate titrations of the QQPVPPQRPMA peptide into $^{15}$N labeled Itk or Btk SH3 domains induced chemical shift perturbations that were analyzed to determine the respective dissociation constants. The binding curves generated upon addition of increasing concentration of the Slp-76 peptide into the Btk and Itk SH3 domains (Fig. 2c) are indistinguishable. Thus, the Btk SH3 domain does not interact with the Itk SH2 domain yet maintains normal proline binding characteristics. This data provides a strategy for altering the full-length Itk sequence to disfavor the observed self-association of wild-type Itk.

A full-length chimeric Itk protein (designated Itk$_{(BtkSH3)}$) that contains the Btk sequence within the region that normally spans the Itk SH3 domain (Fig. 2d) was constructed. All other regions of Itk$_{(BtkSH3)}$ are identical to wild-type full-length Itk.

**Btk SH3 sequence limits Itk self-association.** In a manner identical to the wild-type Itk sequence, myc and V5 epitope tagged Itk$_{(BtkSH3)}$ constructs were transiently transfected into NIH 3T3 cells and subjected to co-immunoprecipitation. Consistent with the absence of an intermolecular interaction between the isolated Itk SH2 domain and the Btk SH3 domains in Figure 2b, co-immunoprecipitation data indicate that full-length Itk$_{(BtkSH3)}$ does not self-associate to a level that is detected in the co-IP experiment (Fig. 1d). Efforts to further characterize purified Itk and Itk$_{(BtkSH3)}$ by analytical ultracentrifugation were complicated by the presence of multiple self-associating species for both wild type Itk and Itk$_{(BtkSH3)}$ potentially due to the high concentrations required for ultracentrifugation (over 10-fold that of the co-IP experiment) and the known self-association of the PH domain (20). Regardless, the differences in the co-IP data for Itk and Itk$_{(BtkSH3)}$ (Fig. 1b & d) suggest that disruption of the non-canonical Itk SH3/SH2 interaction reduces intermolecular self-association of full-length Itk compared to wild type protein. These observations prompted us to proceed toward
an examination of the kinase activities of full length Itk and Itk(BtkSH3) to assess the functional significance of altering Itk intermolecular self-association.

Prior to functional characterization of the full-length chimeric Itk protein we set out to confirm that the sequence changes required to replace the SH3 domain in full-length Itk with that of Btk do not drastically modify the enzyme. We examined the phosphorylation status of both Tyr 511 in the Itk activation loop and Tyr 180 within the SH3 domain. Phosphorylation at both sites accompanies activation of Itk in T cells (9) and if disrupted could produce unwanted ancillary effects on Itk functional assays in cells. Phosphorylation assays using antibodies specific to the phosphotyrosines of interest reveal that both Itk and Itk(BtkSH3) are phosphorylated at the expected sites; Tyr 180 (Fig. 3a) and Tyr 511 (Fig. 3b). Thus, sequence changes in the full length Itk(BtkSH3) diminish intermolecular self-association but do not alter the normal phosphorylation patterns associated with wild type Itk activation.

*Intermolecular self-association negatively regulates Itk activity in vitro and in vivo.* Next, recombinant flag tagged wild-type Itk and Itk(BtkSH3) were separately purified from baculovirus-infected insect cells and used for *in vitro* kinase assays. In an *in vitro* kinase assay that we have described previously for wild-type Itk (22), a biotinylated peptide substrate (24) was used to measure the initial velocity of each protein over a range of enzyme concentrations (Fig. 3c). The concentration of peptide substrate in these assays is 400 μM (~five times $K_m$), to ensure that substrate is not limiting (22). Itk(BtkSH3) gives rise to a linear increase in enzyme activity as a function of increasing enzyme concentration (Fig. 3c). In contrast, the initial velocity of full-length wild-type Itk increases linearly at lower enzyme concentrations but begins to deviate from linearity at 1 μM and above (Fig. 3c). At these higher concentrations of wild-type Itk, the catalytic activity deviates from linearity in a
negative direction suggesting that increased concentration of the wild-type enzyme diminishes Itk kinase activity. The observed differences in the enzymatic activity of the wild-type and Itk(BtkSH3) and differences in the extent to which these proteins self-associate in the co-immunoprecipitation experiment (Fig. 1d) suggest that intermolecular interactions mediated by the SH3 and SH2 domains of Itk serve to negatively regulate Itk catalytic activity.

To test whether the findings from the in vitro kinase assay are borne out in a more physiological context, we first co-infected Sf9 insect cells with the Itk substrate, PLC-γ, and either wild-type Itk or Itk(BtkSH3). Tyrosine phosphorylation of PLCγ1 on Tyr783 was more robustly induced by Itk(BtkSH3) than by wild-type Itk, consistent with enhanced kinase activity for Itk(BtkSH3) on its native substrate (Fig 4a).

Wild-type Itk and Itk(BtkSH3) were then introduced into Itk−/− primary CD4+ T cells by retrovirus transduction; using this retrovirus vector, GFP is translated from the same mRNA transcript as Itk. Importantly, Itk and Itk(BtkSH3) were expressed at comparable levels in Itk−/− deficient cells, as indicated by GFP fluorescence (Fig. 4b). Previous studies have shown that optimal activation and phosphorylation of the p42/44 ERK Map-kinases following T cell activation are dependent on Itk (8,29). To compare wild type Itk and Itk(BtkSH3) function in primary T cells, transduced T cells were stimulated by T cell receptor (TCR) engagement, and ERK phosphorylation was assessed. Itk+/− T cells expressing wild-type Itk were ~20% positive for ERK phosphorylation, in contrast to Itk−/− T cells transduced with the retrovirus vector alone (~10% phospho-ERK+). Expression of Itk(BtkSH3) further enhanced ERK activation, leading to ~30% phospho-ERK+ cells. Average data from three experiments are shown in Figure 4c. Of note, the level of Itk(BtkSH3) in retrovirus-infected Itk−/− T cells is
lower than the normal level of endogenous Itk in wild-type T cells (Fig 4d). Nonetheless, even this low level of Itk_{(BtkSH3)} induces ERK phosphorylation that is similar if not greater than that seen in wild-type T cells following TCR stimulation (Fig 4d). These data indicate that Itk_{(BtkSH3)} is able to function in the TCR signaling pathway of primary T cells and compared to wild type Itk expressed at the same level, the non-clustering Itk mutant exhibits enhanced TCR signaling.

It is clear that intermolecular self-association of Itk is a concentration dependent phenomenon and it therefore would be useful to have some measure of Itk concentration in T cells. Using quantitated immunoblots of SDS-PAGE-separated cellular lysates derived from a known number of Jurkat E6.1 cells (not shown), purified Itk as a concentration standard, and information from microscopy studies of Jurkat E6.1 cells estimating the cytosolic volume to be 2.4 picoliters (Jon Houtman, personal communication), we estimate that Itk concentration in Jurkat T cells is between 0.2 and 0.4 mM. The difference between the concentration range for which we observe activity changes in vitro and this rough estimate of Itk concentration in T cells is a factor of 2.5 to a factor of five; less than one order of magnitude. Studies aimed at measuring global and local concentrations of cellular proteins indicate that global cytoplasmic concentrations can range from tens of nM to mM while local accumulation of certain proteins can result in several orders of magnitude increase in protein concentration (tens of mM to mM) (30,31). Thus, even without taking local increases in Itk concentration into account, we can surmise that the concentration of Itk in the cytosol of the T cell could allow for intermolecular clustering and concomitant down regulation of catalytic activity.
Discussion

Strength and duration of TCR signaling are important in regulating T cell development and lineage commitment. As a key component of the TCR signaling pathway, Itk has been found to modulate several aspects of thymocyte maturation, including positive selection, negative selection, and conventional versus innate CD8+ T cell development (32,33). These observations suggest that Itk activity is involved in fine-tuning TCR signaling, and that this, in turn, regulates T cell fate decisions. Further investigation of this process would be greatly aided by a mouse model in which Itk kinase activity is increased relative to wild-type Itk.

Previous efforts to increase Itk activity by overexpression have uniformly been unsuccessful (28). The data presented in this report provide a biochemical explanation for these failures, and further suggest that a knock-in mouse expressing \text{Itk}_{(BkSH3)} (or a second generation mutant that further skews the equilibrium away from clustered Itk) in place of wild-type Itk could provide a system for examining T cell development and lineage commitment in cells with increased Itk kinase activity. To our knowledge, \text{Itk}_{(BkSH3)} is the first Itk variant to show activity greater than that of the wild type enzyme in T cells.

The quantitative effects of modulating Itk self-association on T cell receptor signaling events (Fig. 4) are similar to those seen in studies of the Src family kinase, Lck. Src kinases, including Lck, are negatively-regulated by an intramolecular association between the SH2 domain and a phosphorylated tyrosine in the C terminal tail that is absent in the Tec kinases. A comprehensive examination of Lck autoinhibition was recently reported in which this intramolecular interaction is either disrupted or strengthened by sequence changes close to the phosphotyrosine (34). Lck-deficient Jurkat T cells were reconstituted with wild-type Lck
or Lck mutants, and T cell receptor signaling leading to Erk activation was examined. Similar to our findings for wild-type Itk and Itk\(_{Btk\text{SH}3}\), modulation of the Lck autoinhibitory interaction led to detectable, but modest changes in Erk activation. Together these studies indicate that shifting the equilibrium between active and inactive kinase conformations alters the strength of TCR mediated signaling but does so within a limited range of outcomes. This is in contrast to mutations located in active sites that have pronounced, all or nothing, effects on downstream signaling.

Specific intermolecular self-association has been characterized for a number of protein systems (35). Within the kinase superfamily this mechanism activates receptor kinases by promoting trans auto-phosphorylation both within and outside of the protein kinase domain (36). In another example of activation by intermolecular association, the antiviral protein kinase PKR dimerizes via phosphotyrosine-dependent binding to double-stranded RNA (37,38). Alternatively, inhibition by dimerization occurs for the receptor-like protein tyrosine phosphatase-\(\alpha\) where an inhibitory ‘wedge’ on one molecule inserts into the catalytic site of another molecule (39). A crystal structure of the kinase domain from yeast Snf1 also reveals a dimeric arrangement that putatively impedes catalytic activity by steric means (40). Likewise, a crystal structure of Ca\(_2+\)/calmodulin-dependent protein kinase II (CaMKII) reveals a regulatory segment that sterically blocks substrate binding to the catalytic site (41). The CaMKII kinase domain itself is intrinsically active and dimer formation brings the regulatory segment into position to inhibit activity.

In light of these examples, a simple mechanistic model for Itk autoinhibition by self-association would invoke steric blockage of the catalytic site. However, it has been well documented that the isolated Itk kinase domain exhibits little or no catalytic activity (22-24)
suggesting the possibility of an alternative autoinhibition mechanism. We have recently reported a kinetic analysis of a series of Itk fragments and have shown that the SH2 domain and linker between SH2 and kinase domains are required to achieve wild-type levels of activity (22). Like the Csk kinase (42), the SH2 and SH2-kinase linker region make direct contact with the kinase domain to stabilize the active conformation of Itk. Thus, inhibition of Itk catalytic activity by SH3/SH2 mediated self-association could be explained if the intermolecular interactions between Itk regulatory domains (particularly those involving the SH2 domain) compete with activating interactions between the SH2 and kinase domains.

T cell activation itself produces signals that compete with the self-association equilibrium and would shift the population of Itk in the cell toward a monomeric state. Specifically, exogenous binding partners such as transiently produced phospholigands would compete directly with Itk self-association by interfering with the SH2/SV3 interaction interface (9,12,16,28,43,44). If self-association is disfavored, the regulatory domains could then adopt the catalytically competent conformation (22). Conversely, in the absence of such activating factors, Itk might remain self-associated and autoinhibited.

It is also possible to envision a role for Itk autoinhibition following activation of Itk by TCR engagement; in this case, self-association would be one mechanism that could terminate Itk signaling. In detailed binding studies of the Itk SH3 and SH2 domains we found that a mimic of Y180 phosphorylation in the SH3 domain enhances affinity for the Itk SH2 domain (45). Together with the observations that autophosphorylation at Y180 in the SH3 domain has no effect on Itk kinase activity and occurs in cis (45) it is reasonable to suggest that autophosphorylation may be a first step toward turning off Itk mediated signaling, by promoting intermolecular self-association leading to a drop in kinase activity.
Indeed, a previous report suggests that Itk clustering in T cells occurs following membrane association (21).

While the precise mechanistic details of when and how intermolecular association of Itk modulates its activity remain a question, this mode of autoinhibition for a non-receptor tyrosine kinase suggests that reagents (or mutations) that shift the equilibrium toward the self-associated state could dampen T cell activation while disfavoring self-association will increase Itk activity and concomitant T cell activation.

References


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Figure Legends

Figure 1. The Itk SH3/SH2 interaction is specific and mediates intermolecular self-association of full-length Itk. (a) Structures of both the Itk SH2 and SH3 domains have been solved (11,13) and chemical shift mapping has been used to determine the residues at the interface of the SH3/SH2 complex (light shading) (12). The classical pY and pY+3 binding pockets are labeled on the SH2 domain. (b) Myc and V5 tagged full-length Itk were transiently transfected into NIH 3T3 cells (lane 1). As controls, V5 tagged protein or pcDNA3 vector alone (lanes 2 & 3, respectively) were transiently transfected into NIH 3T3 cells. In all three lanes, anti-myc antibody was used to precipitate myc tagged protein from cell lysates; V5 antibody was used to detect V5 tagged protein that co-immunoprecipitates with myc tagged protein (top panel). The presence of V5 and myc-tagged proteins is confirmed in the center and bottom panels. (c,d) Same experiment as in (b) using V5- and myc-tagged kinase domain alone (c) or V5- and myc-tagged full-length Itk(BtkSH3) (d).

Figure 2. Specific disruption of the intermolecular SH3/SH2 interaction. (a) The Itk SH3 and Btk SH3 domain structures are superimposed (only labeled side chains from the Btk SH3
domain are shown for clarity). The Itk SH3/SH2 interaction surface is indicated in orange on the SH3 structure (12). Within this binding surface, only eight residues are not conserved between Itk and Btk. Each of the non-conserved pairs of residues are labeled and indicated on the structure (orange are Itk side chains and red are Btk side chains). (b) Select region of heteronuclear single quantum coherence (HSQC) spectra of $^{15}$N-labeled SH3 domains (from either Itk (top) or Btk (bottom)) indicating the chemical shift perturbations associated with binding to unlabeled Itk SH2 domain. The black spectrum is $^{15}$N labeled protein alone and the red spectrum has been acquired following addition of equimolar unlabeled Itk SH2 domain in each case. In the top panel the arrow indicates the change in resonance frequency for a representative peak upon addition of Itk SH2 to $^{15}$N labeled Itk SH3 domain. In the bottom panel a representative region of the HSQC spectrum that results from addition of unlabeled Itk SH2 domain to $^{15}$N labeled Btk SH3 domain show no chemical shift change indicative of the absence of an interaction between Itk SH2 and Btk SH3. (c) Concentration dependence of the normalized $^1$H chemical shift ($Dd/Dd_{max}$) for representative residues of $^{15}$N labeled Btk SH3 domain (top) and $^{15}$N labeled Itk SH3 domain (bottom) upon titration of the Slp76 derived proline-rich peptide. The titration data were acquired and analyzed as previously described (15). (d) Design of the Itk$^{(BtkSH3)}$ chimeric protein. The Itk SH3 sequence within full-length Itk is replaced with the SH3 sequence of Btk. The sequence of the Btk SH3 domain is shown in bold below the domain map of Itk and the surrounding Itk residues within the Itk$^{(BtkSH3)}$ chimeric construct are non-bold and underlined.

**Figure 3. In vitro comparison of Itk$^{(BtkSH3)}$ and wild-type Itk.** Western blot analysis using phosphotyrosine specific antibodies to the autophosphorylation site in the SH3 domain (a)
and the activation loop tyrosine (b). (a) Lane 1: wild-type full-length Itk, lane 2: full-length, mutant Itk(Tyr180Phe) and lane 3: Itk(BtkSH3) were expressed as fusions with the flag peptide and purified from insect cells, resolved by SDS-PAGE, transferred to PDVF membrane and blotted with the Btk antibody to pY223 (Btk numbering; this tyrosine corresponds to Y180 in Itk) to detect the autophosphorylation status in the SH3 domain (top panel). Cross reactivity of the Btk pY223 antibody for Itk pY180 has been previously demonstrated (9). (b) Lane 1: wild type full length Itk, lane 2: Itk(BtkSH3) were prepared as in (a) and blotted with the anti-pY551 antibody to detect the activation loop phosphorylation status (top panel). Itk protein levels are indicated in the bottom panel for both (a) and (b). (c) Wild-type Itk activity deviates from linearity at increased Itk concentrations. Concentration dependence of initial velocity, $V_i$ (mM/min) for wild-type full-length Itk (closed squares) and Itk(BtkSH3) (open diamonds). The linear increase in initial velocity as a function of concentration for Itk(BtkSH3) is indicated by the solid line. Experiment was carried out in duplicate and the data shown are representative of three independent experiments.

**Figure 4. Increased activity of Itk(BtkSH3) versus wild-type Itk in insect cells and T cells.** (a) Full-length wild-type Itk (lane 1) or Itk(BtkSH3) (lane 2) were co-expressed with PLC[Δ] in Sf9 insect cells. After three days, cell lysates were prepared, and immunoblotted for phospho-PLC[Δ] (top panel), total PLC[Δ] (middle panel) or Itk (bottom panel). (b) Itk−/− primary CD4+ T cells were infected with the retrovirus vector alone (MSCV, solid line), a retrovirus expressing wild-type Itk (Itk, long dashed line) or a retrovirus expressing Itk(BtkSH3) (short dashed line); GFP+ cells were sorted on day 4 post-infection. Histograms of GFP fluorescence are shown compared to unstained cells (filled histogram). Cells were stimulated
with anti-CD3 antibody for 5 minutes, fixed, permeabilized, and stained with anti-phospho-ERK antibody. The percentages of phospho-ERK+ cells are indicated on each histogram. (c) The graph shows data from three experiments in which Itk-/- primary CD4+ T cells were infected with retrovirus vector alone (MSCV), retrovirus expressing wild-type Itk (Itk), or retrovirus expressing Itk(BtkSH3). GFP+ cells were sorted, stimulated with anti-CD3 antibody as in (b), and analyzed for ERK activation. Bars indicate mean±S.D. of the percentage of phospho-ERK+ T cells. *, p<0.0005; **, p<0.0006; ***, p<0.004. (d) Itk-/- primary CD4+ T cells were infected with the retrovirus vector alone (lane 3), a retrovirus expressing wild-type Itk (lane 4) or a retrovirus expressing Itk(BtkSH3) (lane 5); GFP+ cells were sorted on day 4 post-infection. Cells were stimulated with anti-CD3 antibody for 5 minutes, lysed, and immunoblotted for Itk (top panel), phospho-ERK (middle panel) and total ERK (bottom panel). Lanes 1 and 2 represent primary Itk+/+ CD4+ T cells stimulated with anti-CD3 antibody for 0 and 5 minutes, respectively.
Figure 2
Figure 3

(a) 64.2 kD

1

2

3

WB:

anti-pY223

anti-Flag

(b) 64.2 kD

1

2

WB:

anti-pY551

anti-Flag

(c) $V_i$ (μM/min) vs. [Kinase] (μM)
CHAPTER 6. GENERAL CONCLUSIONS

Summary

Itk is a tyrosine kinase that is implicated in the T cell signal transduction and cytoskeleton reorganization after TCR engagement (1). It is well established that Itk needs to be recruited to the plasma membrane and transphosphorylated by Lck to be activated (2, 3). However, the detailed mechanisms that regulate Itk kinase activity are still elusive. Itk consists of a PH domain, proline rich region, and a SH3, SH2 and kinase domain. Our group previously reported that the Itk SH3 domain can interact with Itk SH2 domain leading to self-association of the large fragment that includes both the SH3 and SH2 domains (4). In this thesis, I have extended this observation to the full-length Itk molecule. A full-length Itk-SH3 mutant that exhibits decreased self-association was constructed by replacing the Itk SH3 domain with the Btk SH3 domain. In vitro kinase assays indicate that the wild type full-length Itk kinase activity decreases at higher protein concentration while kinase activity of the Itk-SH3 mutant increased linearly with increasing enzyme concentration. When coexpressed with PLC⁠[\textsuperscript{1}] in insect cells, the full-length Itk-SH3 mutant phosphorylates PLC⁠[\textsuperscript{1}] to a greater extent than does wild type full-length Itk. Furthermore, expression of the full-length Itk-SH3 mutant in primary T cells leads to higher ERK phosphorylation following TCR stimulation than wild-type Itk. Our data suggest that the tyrosine kinase Itk can be down regulated by intermolecular self-association.

PLC⁠[\textsuperscript{1}] is the substrate for Itk in T cells (2). After the TCR engagement, Itk is activated and phosphorylates its substrate PLC⁠[\textsuperscript{1}]. Activated PLC⁠[\textsuperscript{1}] hydrolyzes PIP₂ into IP₃ and DAG, which mobilize Ca²⁺ release and activate PKC respectively (5-7).
Phosphorylation is required for PLC\(_1\) activation. However, the mechanism by which PLC\(_1\) is specifically phosphorylated by Itk is not clear. In this thesis, I discover that a docking interaction is necessary for efficient phosphorylation of PLC\(_1\) by Itk (8). Interestingly, a similar docking interaction is required for autophosphorylation in the Itk SH3 domain (8). Further studies indicate that the docking interaction is mediated by the Itk kinase domain and a portion of the substrate SH2 domain. This docking interaction is different from typical SH2 domain mediated interactions as it does not involve phosphor-tyrosine binding pockets of SH2 domain and nor does it involve phosphorylation of the Itk kinase domain. The docking interaction enhances Itk mediated phosphorylation by \(~7\) fold by increasing the affinity between the Itk kinase domain and its substrates. Furthermore, the Tec family kinases are conserved in exploiting this docking interaction to phosphorylate their substrates.

The docking interaction provides a possibility to selectively target the enzyme-substrate interaction surface instead of the enzyme catalytic site to modulate enzyme activity. The structural basis for the docking interaction is not yet completely understood. Site-directed-mutagenesis was used to map out the docking site on the PLC\(_1\) SH2C. We found that the docking site on the PLC\(_1\) SH2C is composed of a largely basic surface from the BG loop and CD loop of the SH2C. Consistent with the location of the docking site on the SH2C, the presence of phosphor-tyrosine ligand does not interfere with the interaction between the Itk kinase domain and the PLC\(_1\) SH2C. Furthermore the docking site that is mapped out in the SH2C domain fragment can be extended to full-length PLC\(_1\). The presence of excess wild type PLC\(_1\) SH2C domain in an \textit{in vitro} kinase assay, but not the SH2C docking mutants, can decrease the phosphorylation of full-length PLC\(_1\). Mutation of
the residues that are involved in the docking interaction in the full-length PLC\[\text{g}\] substrate diminishes the phosphorylation by Itk. Our data indicate that the docking interaction is required for the full-length PLC\[\text{g}\] phosphorylation by Itk.

We also found an intramolecular interaction between the PLC\[\text{g}\] SH2C and the adjacent linker that can modulate the docking interaction between SH2C and Itk kinase domain. Biochemical and NMR spectroscopic analyses indicate that the intramolecular interaction is different from the canonical SH2/phosphor-tyrosine interaction. Residues in the SH2C domain that mediate linker binding form a hydrophobic groove that overlaps with the canonical pY+3 binding pocket. Our data suggest that the intramolecular interaction might be one of the mechanisms that keep PLC\[\text{g}\] in its inactive conformation before its phosphorylation and activation. Overall this thesis provides significant molecular level insight into mechanisms of the substrate recognition by Itk and regulation of TCR signaling.

**Future directions**

Substrate docking and its regulation by PLC\[\text{g}\] intramolecular interaction

The novel substrate docking interaction interface on the SH2 domain has been mapped, but the interaction surface on the Itk kinase domain is still unknown. The nature of the residues on the surface of SH2C (basic) suggested an acidic patch in the Itk kinase domain could be responsible for the binding. Inspection of the Itk kinase domain structure reveals a potential acidic patch in the small lobe of the kinase that will be the target of future mutational analysis. The structural information about the interaction surface could provide clues for designing specific inhibitors for the substrate docking interaction.
In my research here, I also discovered a novel intramolecular interaction between the PLC\[\text{g}1\] SH2C and the adjacent linker. This intramolecular interaction likely occurs prior to the phosphorylation of PLC\[\text{g}1\] and is not dependent on phosphotyrosine. We propose that this intramolecular interaction is one of the mechanisms that keep PLC\[\text{g}1\] in its inactive conformation before its phosphorylation and activation. To date, our investigations of PLC\[\text{g}1\] regulation have focused on protein fragments. One line of future studies will include identifying mutations that break up the intramolecular interaction in the SH2C-linker fragment, introducing these mutations into the full length PLC\[\text{g}1\], and then measuring the phosphorylation level of Y783 and the phospholipase activity of the full length PLC\[\text{g}1\].

Based on my fragment studies, the intramolecular interaction between the PLC\[\text{g}1\] SH2C and the adjacent linker can also regulate the substrate docking interaction based on a pull-down assay. Quantitatively studying the affinity differences between SH2C/Itk kinase domain and SH2C-linker/Itk kinase domain will help us understand the extent of this regulation. Because of the challenges associated with obtaining sufficient amounts of the Itk kinase domain, we have not yet been successful in using Isothermal Titration Calorimetry (ITC) to measure the affinity between the PLC\[\text{g}1\] SH2C and the Itk kinase domain. Surface Plasmon Resonance (SPR) is another technique that requires less protein and therefore might be a good alternative technique to study these protein-protein interactions.

Solving the structure of the intramolecular SH2C-linker complex will provide a structural basis for understanding the regulatory role of this non-classical interaction. We have tried to solve this complex structure by NMR spectroscopy. $^{13}\text{C}$, $^{15}\text{N}$ double-labeled SH2C-linker has been made and a series of spectra have been acquired for this protein: $^{15}\text{N}$-edited NOESY, $^{15}\text{N}$-edited TOCSY, $^{13}\text{C}$-HCCH-TOCSY, HNCA, HNCOCA, HNCA CB
and CACBCONH. But because of exchange broadening (likely due to the dynamics of the SH2C/linker association), many of these spectra are incomplete. By comparing NOESY spectra of isolated SH2C and SH2C-linker, I am able to assign the backbone resonances of most residues of the SH2C portion in the context of SH2C-linker. Complete assignment will be needed for structure determination. The following strategies will be tried to improve the quality of the NMR spectra. We will try a different pulse sequence to suppress relaxation during transfer. As well, we are designing mutants to enhance the affinity between the linker region and the SH2C domain so that the dynamic exchange rate might be modified. Based on our truncation and mutation data, we know that the last four residues in the linker region A786NPM789 are associating with the residues from the SH2C domain, which form a large hydrophobic groove. A series of mutants will be made to screen for a mutant that has higher affinity between the SH2C domain and the linker. GST pull-down assays will be used for this screen. For the mutants that have higher affinity for the SH2C, we are expecting to see diminished interaction with the Itk kinase domain. For the promising mutants, 15N-labeled protein will be made and 15N edited TOCSY will be acquired. Chemical shift difference between the mutant and the isolated SH2C will be analyzed to make sure the SH2C residues in the mutant are associating with the linker in the similar manner. When an appropriate mutant is available, a series of NMR spectra can be acquired for assigning resonances and then the structural determination of the mutant will be feasible.

Itk self-association and its regulation of Itk kinase activity

In the Chapter 5, I found an Itk-SH3 mutant that diminishes the self-association of Itk downregulates the Itk kinase activity. Significant effort has been spent on biophysical
characterization of the wild type Itk and Itk-SH3 mutant. Unfortunately it has been difficult to achieve sufficient concentrations of these proteins for detailed studies. Both proteins self-associate and form higher order aggregates based on native gels. Recently, it is reported that the Itk PH domain could dimerize or self-associate (9, 10), which provides another direction to further control the aggregation status of this protein. Along these lines, if we can identify some residues in the Itk PH domain that are mediating PH domain self-association, and combine the mutation of these residues and Itk-SH3 mutations, it will be possible to further shift the equilibrium toward monomeric full-length Itk.

This thesis provides significant insight into the mechanism of substrate docking and negative regulation of Itk function. It also provides a foundation for continued effort to understand the regulation of TCR signaling and structural basis for both the substrate recognition and inhibition of Itk catalytic activity.

References


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