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Mechanistic insight into the regulation of Tec family kinase activity – substrate selection and conformational preferences

by

Qian Xie

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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# TABLE OF CONTENTS

## CHAPTER 1. GENERAL INTRODUCTION
- Literature Review
- Thesis Organization
- Motivation
- References

## CHAPTER 2. SUBSTRATE RECOGNITION OF PLC\(\gamma\)1 VIA A SPECIFIC DOCKING SURFACE ON ITK
- Abstract
- Introduction
- Results
- Discussion
- Materials and Methods
- Acknowledgements
- References
- Figure Captions
- Figures

## CHAPTER 3. INTRODUCING NMR PROBES TO MONITOR THE CONFORMATIONAL CHANGES ASSOCIATED WITH PROTEIN KINASE ACTIVATION
- Abstract
- Main Body
- Materials and Methods
- Acknowledgements
- References
- Figure Captions
- Figures

## CHAPTER 4. CHARACTERIZATION OF A CURIOUS MUTATION IN BTK KINASE DOMAIN: THE IMPLICATION
CHAPTER 1. GENERAL INTRODUCTION

Literature review

1. Protein kinases

The subject of scientific investigation in this thesis is protein kinases, a class of enzymes that catalyze the transfer of the $\gamma$-phosphate group from ATP to a specific amino acid side chain (most often that of Ser, Thr, Tyr or His) in their protein substrates. Protein kinases are ubiquitously expressed and involved in various cellular signaling pathways. Currently, more than 500 protein kinases have been identified in the human proteome, encoded by about 2% of the human genome (1). It is estimated that about 30% of the cellular proteins are phosphorylated on at least one residue (1), making protein phosphorylation one of the most prevalent post-translational modifications inside the cell. The phosphate group appended by protein kinases serves as a signal to coordinate cellular response to the environmental cues, as seen in both the fundamental cellular activities like proliferation, migration and metabolism and in the specialized functions like muscle contraction and immune response. Because of the key role of protein kinases in different cellular processes, precise regulation of their activity is crucial for the vitality of any cell types. Dysregulation of kinase activity, as expected, causes many serious human diseases such as cancers, diabetes and cardiovascular diseases (2-4). To design effective therapeutics to control protein kinase functionality, detailed knowledge of the structure and function of the kinase of interest is desired.

My research on protein kinases primarily focuses on the kinase domain, the domain shared among all protein kinases where the catalysis takes place. The kinase domains of all
protein kinases adopt the same overall fold (Fig. 1), a bilobal, bean-shaped structure. It has a smaller N-terminal lobe consisting of mostly β strands and a larger C-terminal lobe consisting of mostly α helices, connected by a hinge at the backside of the kinase domain. Phosphate transfer occurs in the cleft in between the N- and C-lobes, termed the active site. A number of structural motifs in the kinase domain have to be deployed to ensure effective processing of their substrates. For instance, the αC-helix in the N-lobe has to rotate inward such that an internal structural motif called the regulatory spine (cyan), consisting of primarily hydrophobic residues, is aligned and a conserved glutamate (red) on this helix forms a salt bridge with a conserved lysine (blue) on the β3 strand. The activation loop in the C-lobe has to adopt the ‘DFG-in’ conformation, with the aspartate (magenta) in the DFG motif pointing into the active site to coordinate Mg$^{2+}$ for proper positioning of ATP. The activation loop also has to flip open to allow accessibility to the active site. Even though with a common conformational destination to achieve full functionality, protein kinases take different routes to reach the active state and this is because each protein kinase employs distinct mechanism to hold its activity at the resting level.
2. **Tec family kinases**

Our lab studies the regulatory mechanisms of a specific class of protein kinases called Tec family kinases. Tec family kinases are non-receptor protein tyrosine kinases, indicating that they are not constitutively associated with the cellular membrane and they phosphorylate specific tyrosine residue in their substrates. The Tec kinase family consists of six members, Tec, the founding member of the family, Btk, Itk/Emt/Tsk, Bmx/Etk, Rlk/Txk and Dsrc29 (5). They share similar domain architecture, from the N-terminus to the C-terminus, including a pleckstrin homology (PH) domain, which typically binds to the product of phosphoinositide 3-kinase (PI3K) and recruits Tec kinases close to the plasma membrane; a Btk homology (BH) domain, which binds a zinc ion; a proline rich region; a Src homology 3 (SH3) domain, which typically binds to the left-handed polyproline helix; a Src homology 2 (SH2) domain, which typically binds to the phosphotyrosine containing sequences and a kinase domain, which harbors the
transferase activity (Fig. 2). Two exceptions of this domain architecture in the Tec kinase family are Rlk that has a cysteine string motif instead of a PH domain at the N-terminus and Bmx that lacks any proline rich region. Tec family kinases are expressed primarily in hematopoietic cells and are recognized as important regulators of antigen receptor signaling pathways. The physiological significance of Tec family kinases was first underscored by the discovery that loss-of-function mutations in Btk were responsible for X-linked agammaglobulinemia (XLA), a rare human genetic disorder characterized by reduced level of immunoglobins in the serum and defective B cell development. It is known to date that dysregulation of Tec family kinases is associated with many other disease states such as asthma and rheumatoid arthritis and Tec kinases are proposed drug targets for multiple lymphomas and even HIV infection (6, 7). In fact, several Tec family kinases have drawn significant input from pharmaceutical companies to screen for small molecule modulators. This thesis focuses on the basic science side and we strive to understand the molecular details of specific protein-protein interactions and the concomitant conformational changes that occur in Tec family kinases upon activation. Our focus is on two Tec kinases, Interleukin-2 inducible T cell kinase (Itk) and Bruton’s tyrosine kinase (Btk).

**Figure 2. Domain architecture of Tec family kinases**
3. *Itk and Btk*

Itk was first discovered and cloned independently in Stephen Desiderio’s group, Leslie Berg’s group and Toshiaki Kawakami’s group in the early 1990s (8). Itk is the predominant Tec kinase expressed in T cells when T cells are activated and is an important modulator of the T cell response. When the peptide-bound major histocompatibility complex (MHC) on the surface of the antigen presenting cell (APC) engages the T cell receptor (TCR), it also interacts with the coreceptor, CD4 on the helper T cells or CD8 on the cytotoxic T cells. The interaction between MHC and CD4/CD8 dissociates CD45, a protein tyrosine phosphatase, from the coreceptor. CD45 dephosphorylates the phosphorylated tail tyrosine (Y505) of the membrane associated Src family kinase Lck and activates it. Active Lck phosphorylates a conserved motif in the cytoplasmic domain of TCR, termed ITAM (Immune receptor Tyrosine based Activation Motif), which then recruits the Syk family kinase Zap70 close to the plasma membrane. Zap70 is also activated by Lck phosphorylation and in turn phosphorylates the adaptor protein LAT, which serves as anchor points for many other cytoplasmic proteins to nucleate into a signaling complex, including Itk and PLCγ1. Migration of Itk close to the plasma membrane is also responsive to PI3K activity, which generates phosphatidylinositol-1, 4, 5-trisphosphate (PIP3) that the PH domain of Itk binds to. Membrane localized Itk is activated by Lck phosphorylation on its activation loop tyrosine Y511 and then phosphorylates PLCγ1 on Y783. This latter phosphorylation event is essential for full phospholipase activity of PLCγ1, which cleaves the phosphodiester bond at the sn-3 position in phosphatidylinositol-4, 5-bisphosphate (PIP2), generating two secondary messengers, inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses in the cytosol and binds to the IP3 gated calcium channel on the membrane.
of endoplasmic reticulum (ER), eliciting calcium efflux from the ER storage into the cytoplasm. Decrease of ER luminal $[\text{Ca}^{2+}]$ activates STIM (stromal interaction molecule), which in turn triggers the opening of the plasma membrane Ca$^{2+}$ channel, Orai, leading to calcium influx from the extracellular space. The increased cytoplasmic $[\text{Ca}^{2+}]$ is sensed by calmodulin, and the Ca$^{2+}$-calmodulin complex binds to and activates calcineurin, the phosphatase that dephosphorylates the transcription factor NFAT and induces its translocation from the cytoplasm into the nucleus. The other secondary messenger, DAG, activates both PKC\(\theta\) and RasGRP and leads to translocation of transcription factors NFkB and AP-1 into the nucleus respectively. Together, they result in the expression of genes essential for T cell-mediated immunity. The functional significance of Itk in this T cell receptor signaling pathway has been demonstrated in T cells isolated from Itk deficient mice, with the cellular phenotype of impaired PLC\(\gamma\)1 activation, deficient actin reorganization and defective immune response when TCR is stimulated. Therefore, Itk is suggested to function as a rheostat in this pathway, fine-tuning the level of T cell response.

Btk is an Itk counterpart expressed in B cells, mast cells and macrophages but not T cells. Btk and Itk share 66% primary sequence homology and a very similar domain architecture. Btk plays an analogous role as Itk under the B cell receptor. In B cell receptor signaling, Btk is activated by another Src family kinase Lyn and in turn phosphorylates PLC\(\gamma\)2 on Y753. Many regulatory mechanisms of Itk and Btk, as we shall see, are comparable.
4. Review on the regulatory mechanism of Itk and Btk

The activity level of Itk and Btk needs to be buffered within a normal range. Defective Itk and Btk cause immunodeficiency while excessive Itk and Btk activity causes autoimmune disorders and lymphomas. Like many other protein kinases, regulation of Itk and Btk activity is implemented at multiple levels. Empirically, it is insightful to think of kinase regulation as fine-tuning the parameters in the Michaelis-Menten equation, which describes the enzyme kinetics at the steady state. In this subsection, we shall review on what has been known about the regulatory mechanism of Itk and Btk.

4.1 Regulation on the effective kinase concentration ([E]_{t})
Inside the cell, the level of kinase activity is dependent on both the total amount of kinase expressed and its cellular distribution. The temporal and spatial control determines the effective concentration of kinase at a specific cellular location. For instance, Itk is expressed at a low basal level and is located in the cytoplasm in the resting T cells. When the T cells are stimulated, Itk is recruited into the signaling complex close to the T cell membrane via the interaction between its PH domain and phosphoinositides embedded in the cellular membrane as well as between its SH3 and SH2 domains and the adaptor protein SLP76. The activity of Itk contributes to the signal relay from the T cell membrane into the nucleus, which up-regulates the expression level of interleukin-2. Interleukin-2 in turn induces the expression of Itk (hence its name) (9). Such positive feedback loop T cells exploit sensitizes the production of Itk in response to external stimuli. Localization close to the plasma membrane is another essential factor for cellular function of Itk and Btk. This is well illustrated by a number of XLA-causing mutations in Btk PH domain that prevent the binding to D3-phosphorylated phosphoinositides, and a E41K mutation in the same PH domain that constitutively targets Btk to the membrane and imparts transforming potential (10).

4.2 Regulation on substrate recognition ($K_m$)

Protein kinases are generally specific in substrate selection. In the case of Itk, only a small number of substrates have been characterized, including Y783 of PLCγ1, Y180 of Itk and Y173 of SLP76 (11-13). Peptide sequence derived from PLCγ1 containing Y783 and that derived from Itk containing Y180, however, are both very poor substrates of Itk in vitro. This observation prompted us to look for structural motifs outside the active site in Itk and distant from the phosphoacceptor in PLCγ1 and Itk that contribute to the specificity of the two kinase-
substrate pairs. Previously, our lab identified a remote docking interaction between the kinase domain of Itk and the C-terminal SH2 domain of PLCγ1 that promotes the specificity of Itk towards PLCγ1 Y783. A similar docking interaction between Itk kinase domain and Itk SH2 domain was found responsible for the specificity in its autophosphorylation on Y180 (12)(Fig.4). In both cases, the docking interaction is found to be independent of the canonical function of SH2 domain to bind phosphotyrosine. This is entirely consistent with the docking sites subsequently mapped on PLCγ1 C-terminal SH2 domain and Itk SH2 domain. Several basic residues located on the CD and BG loops in PLCγ1 C-terminal SH2 domain were found essential in the recognition of PLCγ1 Y783 as Itk substrate (14). They form a basic patch distinct from the canonical pY binding pocket in the SH2 domain. It has been demonstrated that mutating this basic patch has no effect on pY containing peptide binding by PLCγ1 C-terminal SH2 domain and the occupancy of the pY binding pocket has no effect on the docking interaction with Itk kinase domain. In Itk SH2 domain, the AB, EF and BG loops as well as part of the βD strand were mapped to be the docking site, also distinct from the canonical pY binding pocket (15). Hence, we observed two cases where specific interaction between Itk kinase domain and the SH2 domain in its substrate was leveraged to recruit the phosphoacceptor tyrosine close to its active site, thus making the phosphorylation more efficient.
4.3 Regulation on intrinsic catalytic activity \( (k_{cat}) \)

Itk and Btk share a domain cassette (SH3-SH2-kinase domain) with Src family kinases but differ significantly in their regulatory mechanism of the catalytic activity. In Src family kinases, it has been very well characterized that the kinase domain was locked into an inactive conformation by autoinhibitory intramolecular interactions between its SH2 domain and the phosphorylated tyrosine in its tail as well as between its SH3 domain and a proline rich sequence in the SH2-kinase linker. In the absence of such intramolecular interactions, for instance the isolated kinase domain of Src family kinases is intrinsically active \( (16) \). On the contrary, Itk and Btk do not have a tail sequence containing a phosphorylatable tyrosine, and the catalytic activity of the isolated Itk and Btk kinase domains are both very poor in vitro. Biochemical characterization of Itk and Btk constructs revealed that the non-catalytic domains, namely the SH3 domain, the SH2 domain and the SH2-kinase linker, activate kinase activity of Itk and Btk \( (17) \). Currently, we only have access to the structures of individual domains of Itk and Btk, while their full-length structures or any multi-domain structures containing the kinase domain are not available. Hence, the molecular details on how these non-catalytic domains impact on kinase activity are not clear.
activity have yet been elucidated. But accumulated biochemical characterization in these systems highlights several sequence/structural motifs in Itk and Btk essential for full catalytic activity. One site is a tryptophan (W355 of Itk and W395 of Btk) in the conserved ‘WEI’ motif in SH2-kinase linker sequence. Alanine mutation at this site completely abrogates the positive effect of the non-catalytic domains on kinase activity (17). We now know that this tryptophan exerts its effect via stabilizing the ‘αC-helix in’ conformation and an assembled regulatory spine in the kinase core. Another structural motif is the activation loop in the kinase C-lobe, which contains a tyrosine to be phosphorylated by upstream kinase to attain full activity. Difference in the flexibility of the activation loop has been demonstrated to directly relate to the difference in the intrinsic catalytic activity observed between Itk and Btk. Simply by swapping their activation loop, we can change the dynamics of this loop and interconvert the activity between Itk and Btk (18).

**Thesis organization**

This thesis includes five chapters: a general introduction chapter (Chapter 1), followed by three chapters describing the scientific results (Chapter 2-4) and a general conclusion chapter (Chapter 5). Introduction in Chapter 1 provides the necessary background information on protein kinases, specifically on Tec family kinases, as well as the cellular signaling pathway of two Tec family kinases, Itk and Btk. It also reviews the literature on the regulatory mechanisms of Tec family kinases. Chapter 2, which has been published in a peer-reviewed journal, describes the biochemical and biophysical mapping of a substrate recognition site in Itk kinase domain. Chapter 3, which is an ongoing work and will be submitted for publication upon identifying the reductive methylation condition that maintains the kinase activity, describes a novel method to
examine the conformational change of protein kinases in their activation pathway by NMR spectroscopy. Chapter 4, which is a manuscript in preparation for publication, describes the discovery of a solubilizing mutation in Btk kinase domain and its interesting activating effect and shows biochemical and computational data suggesting a possible activation mechanism. The general conclusion chapter (Chapter 5) summarizes all the scientific findings made and discusses on the future directions.

Motivation

The chapters in this thesis dissertation aim to contribute to our knowledge on the regulation of Tec family kinases at the molecular level. The work described in Chapter 2 is a follow-up to the previous discovery of the remote docking interaction, which Itk utilizes to select its substrates. In this work, I identified the surface on Itk kinase domain that is involved in the recognition of PLCγ1 as Itk substrate. Elucidation of this substrate recognition surface in Itk provides an alternative site to target Itk. The work in Chapter 3 describes a novel method, taking advantage of the simple reductive methylation chemistry and the power of NMR spectroscopy, to monitor the activation status of protein kinases in solution. The easy applicability of this method to different protein kinase systems, including Tec family kinases, makes examination of their conformational ensemble in solution feasible and potentially is another way to screen small molecule regulators for those protein kinases. The work detailed in Chapter 4 highlights an activating mutation found in Btk, which sheds light onto how allostERIC mechanism may impact on the kinase active site and make catalysis more efficient.

References


CHAPTER 2. SUBSTRATE RECOGNITION OF PLCγ1 VIA A SPECIFIC DOCKING SURFACE ON ITK


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Abstract

IL-2 inducible T-cell kinase (Itk) is a non-receptor protein tyrosine kinase expressed primarily in T cells. Itk catalyzes phosphorylation on tyrosine residues within a number of its natural substrates, including the well-characterized Y783 of PLCγ1. However, the molecular mechanisms Itk exploits to recognize its substrates are not completely understood. We have previously identified a specific docking interaction between the kinase domain of Itk and the C-terminal SH2 (SH2C) domain of PLCγ1 that promotes substrate specificity for this enzyme/substrate pair. In the current study, we identify and map the interaction surface on the Itk kinase domain as an acidic patch centered on the G helix. Mutation of the residues on and adjacent to the G helix within the Itk kinase domain impairs the catalytic efficacy of PLCγ1 substrate phosphorylation by specifically altering the protein-protein interaction interface and not the inherent catalytic activity of Itk. NMR titration experiments using a Btk kinase domain as a
surrogate for the Itk kinase domain provide further support for an Itk/PLCγ1 SH2C interaction surrounding the G helix of the kinase domain. The work presented here provides structural insight into how the Itk kinase uses the G helix to single out Y783 of PLCγ1 for specific phosphorylation. Comparing these results to other well-characterized kinase/substrate systems suggests that the G helix is a general structural feature used by kinases for substrate recognition during signaling.

KEYWORDS
Tec family kinase Itk; Substrate recognition; PLCγ1 phosphorylation; Kinase domain; G helix

Introduction

Protein phosphorylation, catalyzed by protein kinases, is one of the most prevalent post-translational modifications in cells and its specificity is critical for normal cell function \(^{(1, 2)}\). Improper regulation of kinases has been linked with multiple disease states, such as cancers, diabetes and neurodegenerative disorders \(^{(3-5)}\). Precise phosphoryl transfer requires kinases to preferentially select their native substrates over other potential targets. In cells, this is accomplished in part by fine-tuning the timing of protein expression and co-localization of enzyme and substrate. It is also becoming evident that molecular determinants for substrate specificity are present in most kinases, as substrate preferences are often observed \textit{in vitro} \(^{(6)}\).

Molecular mechanisms that drive substrate discrimination have been described for several kinase systems. In the kinase active site, the shape of the cavity and the distribution of adjacent charge and hydrophobicity can determine the amino acid preferences flanking the
phosphorylation site. For instance, insulin receptor kinase shows a strong preference for methionine at both the P+1 and P+3 positions, due to two hydrophobic pockets near the active site\(^7\). However, for many kinases, the native substrate is far better than an optimized peptide substrate derived from library screening, indicating the presence of substrate specificity determinants outside of the active site. For example, PKR and Csk exploit a specific substrate-docking surface on the kinase domain, distinct from the kinase active site, that interacts with a region on the substrate remote from the phosphorylation site\(^8, 9\). Such docking interactions tether the enzyme and the substrate in a regiospecific manner leading to a productive enzyme/substrate complex and enhanced catalytic efficiency.

Itk is a non-receptor protein tyrosine kinase that belongs to the Tec kinase family. Itk is primarily expressed in T cells and plays an important regulatory role in T cell signaling and development\(^10-12\). Itk contains, from amino- to carboxy-terminus, a Pleckstrin Homology (PH) domain, a Tec Homology (TH) domain, a Src Homology 3 (SH3) domain, a Src Homology 2 (SH2) domain and a kinase domain responsible for the phosphotransferase activity\(^12\). A number of specific tyrosines targeted by Itk have been identified, including Y783 of PLC\(\gamma\)1, Y180 of Itk, Y173 of SLP-76 and recently Y210 as well as Y222 of DEF6\(^13-17\). Among these Itk-mediated phosphorylation on Y783 of PLC\(\gamma\)1 is the best studied and most important in the context of T cell signaling. Phosphorylation on PLC\(\gamma\)1 Y783 activates the lipase activity of PLC\(\gamma\)1, which generates two essential secondary messengers, diacylglycerol (DAG) and inositol trisphosphate (IP\(\gamma\)), by hydrolyzing phosphatidylinositol 4,5-bisphosphate\(^18, 19\). DAG and IP\(\gamma\) lead to activation of Protein Kinase C and release of calcium ions from the endoplasmic reticulum, respectively.
We have previously described a specific docking interaction between Itk and PLCγ1 that is required for phosphorylation on Y783 and subsequent downstream signaling in T cells\textsuperscript{(14)}. The specific site on PLCγ1 that is required for efficient phosphorylation by Itk has been delineated and consists of a largely basic cluster of surface exposed residues on the carboxy-terminal SH2 domain (SH2C) of PLCγ1\textsuperscript{(20)}. This recognition site is remote from the site of tyrosine phosphorylation, Y783 which resides 29 amino acids beyond the carboxy-terminus of the PLCγ1 SH2C domain in what appears, based on NMR data, to be a flexible linker region (data not shown). The PLCγ1 SH2C domain interacts directly with the kinase domain of Itk in a manner that is completely independent of the normal SH2 mediated phosphotyrosine interaction\textsuperscript{(14)}. Mutation of this recognition site within full length PLCγ1 leads to loss of phosphorylation on Y783 and significantly reduced calcium flux in T cells\textsuperscript{(20)}.

We now define the complementary interaction surface on the Itk kinase domain that serves as the specific substrate-docking surface for PLCγ1. Coupling mutagenesis and functional screening, we narrowed the potential interaction area to an acidic patch surrounding the G helix in the C-terminal lobe of the Itk kinase domain. Kinetic parameters support the importance of the mapped residues in the phosphorylation efficiency towards PLCγ1 and mutation of the docking surface on Itk leads to diminished binding of the kinase domain to PLCγ1 SH2C. Analysis of the enzyme/substrate interaction using NMR spectroscopy provides further support for the location of the docking site centered around the G-helix. These data contribute to our understanding of the specificity determining elements that control Itk function.
Results

Mapping the PLCγ1 docking surface on the Itk kinase domain

The preponderance of basic residues within the previously defined recognition site on the PLCγ1 SH2C domain (Fig. 1A) suggests the likely involvement of acidic residues within the complementary surface on the Itk kinase domain. Surface exposed glutamate and aspartate residues that might mediate the PLCγ1 docking interaction are shown on the structure of the Itk kinase domain in Figure 1B. Given the large number and wide distribution of glutamate and aspartate residues across the Itk kinase domain structure, we first examined geometrical requirements of the PLCγ1 substrate in an effort to reduce the complexity of the problem.

The site of phosphorylation within PLCγ1 (Y783) is separated from the Itk recognition site on the PLCγ1 SH2C domain by 29 amino acids (Fig. 2A & B). Truncation of portions of the 29 amino acid linker to generate constructs with shorter linker sequences between SH2C and Y783 was carried out to ascertain the minimal linker length required to maintain docking-specific phosphorylation of Y783 in PLCγ1 (Fig. 2A & B). Wild type PLCγ1 substrate (L29), the PLCγ1 substrate truncated by 20 amino acids (L9) and the PLCγ1 substrate truncated by 25 amino acids (L4) were subjected to phosphorylation by Itk (Fig. 2B & C). We find that the PLCγ1 L9 construct maintains phosphorylation on Y783 comparable to wild type PLCγ1 while the PLCγ1 L4 construct does not (Fig. 2C). The PLCγ1 substrate concentrations of 1 and 5 mM were chosen for this experiment because our previous work on this system demonstrated that a docking-incompetent substrate containing the same Y783 phosphorylation site is not phosphorylated at detectable levels at these concentrations (14). Thus, by keeping substrate concentration within this range, we ensure that the docking interaction between PLCγ1 SH2C
and the Itk kinase domain remains intact for substrate variants that get phosphorylated by Itk in this assay.

Next, we extended the linker length of the PLCγ1 L4 construct by insertion of a single glycine or a glycine-serine-glycine tripeptide spacer to produce a substrate with a linker length of five amino acids (PLCγ1 L5) or seven amino acids (PLCγ1 L7) (Fig. 2B). Phosphorylation of PLCγ1 L5 remains poor but the slightly longer linker in PLCγ1 L7 restores Y783 phosphorylation to wild type levels. We conclude that the minimal linker length between the SH2C domain and Y783 that is necessary to satisfy both the SH2C/kinase docking interaction and phosphorylation of Y783 within the Itk kinase domain active site is seven amino acids.

The geometrical restraint imposed by a linker length of seven amino acids between the PLCγ1 SH2C domain and Y783 (whether the docking interaction is a dynamic event that increases local concentration of Y783 or is a more rigid binding event that ‘locks’ the substrate in place) suggests that the substrate docking site on the Itk kinase domain likely resides at a maximum radius of approximately 28 Å from the acceptor tyrosine bound in the kinase active site. This estimate assumes a distance between alpha carbons of 3.8 Å in an extended conformation. Using this estimate as a guide (as well as structural information from other kinases showing the location of the acceptor tyrosine in the active site), we narrowed the possible glutamate and aspartate docking residues to 12 candidates: E367, E394, E399, E400, D401, E404, D444, E559, E565, E568, D569 and E596 (the location of each of these side chains is shown on the Itk structure in Figure 1B).
Alanine scan of the acidic Itk kinase residues identifies the G helix as docking site

Following previous work that successfully mapped the substrate-docking site on the surface of the Csk kinase\(^{(21)}\), we mutated each of the twelve Itk kinase domain residues identified by the analysis described above to alanine. The activated forms of wild type Itk and the Itk alanine mutants were produced from insect cells by co-expression of Lck as described previously\(^{(22)}\). Each Itk enzyme (wild type or alanine mutant) was purified and assessed using equalized amount of enzyme in *in vitro* kinase assays for phosphorylation of two separate substrates (Peptide B alone and Peptide B fused to the PLC\(\gamma\)1 SH2C domain) (Fig. 3A). Peptide B has been previously characterized as a docking-independent Itk substrate \((K_m \approx 80 \mu\text{M})\) while Itk phosphorylation of Peptide B fused to PLC\(\gamma\)1 SH2C is enhanced \((K_m \approx 5 \mu\text{M})\) by virtue of the PLC\(\gamma\)1 SH2C/Itk kinase domain docking interaction\(^{(14)}\). Comparison of the two substrates (Peptide B and PLC\(\gamma\)1 SH2C-Peptide B) allows us to evaluate the extent to which alanine mutations in Itk alter the intrinsic kinase activity (Peptide B phosphorylation will decrease) versus the extent to which alanine mutation disrupts the substrate-docking site (PLC\(\gamma\)1 SH2C-Peptide B phosphorylation will decrease).

For each Itk enzyme (wild type and alanine mutants) the initial velocity was measured for the Peptide B substrate and the docking-dependent substrate, PLC\(\gamma\)1 SH2C-Peptide B. The initial velocity values of wild type Itk for the Peptide B and PLC\(\gamma\)1 SH2C-Peptide B substrates are each normalized to 1 so that the activity of each mutant Itk is expressed relative to wild type Itk (Figure 3B & D). We expect that if an Itk mutation disrupts the docking interaction between the Itk kinase domain and PLC\(\gamma\)1 SH2C, the initial velocity toward the PLC\(\gamma\)1 SH2C-Peptide B substrate will decrease relative to the initial velocity for the same mutant toward the Peptide B.
substrate. If the alanine mutation in the Itk kinase domain adversely affects Itk catalytic activity, a decrease in the initial velocity toward Peptide B will be observed. This latter point is important as point mutations in kinases are well known to cause allosteric effects on catalytic activity.

Initial velocity measurements for 10 glutamate/aspartate to alanine Itk mutants revealed that three mutants, E565A, E568A/D569A and E596A, result in decreased activity toward the docking substrate (PLCγ1 SH2C-Peptide B) compared to the generic Peptide B substrate (Fig. 3B). Examining the location of these acidic residues on the structure of the Itk kinase domain shows that E565, E568 and D569 cluster on the G helix, while E596 is located on the adjacent αH-αI loop (Fig. 3C). A second round of mutagenesis was then carried out to assess the contribution of 16 additional residues surrounding the G helix and αH-αI loop (Fig. 3D). The results indicate that F529, S530, S571, T572, Y577, K595 and K597 are also part of the substrate-docking site (Fig. 3D & E) while the other nine residues examined by mutation to alanine do not contribute to PLCγ1 docking on the Itk kinase domain (Fig. 3D). S571 and T572 reside on the G helix further supporting a role for this region of the Itk kinase domain in substrate docking, F529 and S530 are located on the short helix in between the P+1 loop and F helix, and like E596 identified in the initial screen, K595 and K597 are located on the αH-αI loop (Fig. 3E). All of these residues cluster to a contiguous region on the C-terminal lobe of the Itk kinase domain (Fig. 3F). Y577 on the αG-αH loop is the exception as it is somewhat removed from the other residues identified to be important for docking (Fig. 3F). We speculate that mutation of Y577 to alanine might indirectly perturb the docking interaction surface on the Itk kinase domain by altering the structure or position of the G helix.
It is worth noting that certain mutations in the Itk kinase domain appear to enhance the initial velocity toward the PLCγ1 SH2C-Peptide B substrate compared to the Peptide B substrate alone. The most notable example of this is the R561A/S562A mutant (Fig. 3D). R561 and S562 are located at the amino-terminus of the G-helix and are therefore quite close to the SH2C binding site. The observed increase in initial velocity for this mutant may reflect an overall reduction of positive charge that stabilizes binding to the net positively charged SH2C binding partner. That said, the K595A/K597A mutant, located on the opposite side of the G-helix, also reduces the net positive charge of the docking site but has a deleterious effect on substrate docking. It is possible that the side chain of K595 and/or K597 form favorable contacts to the acidic side chain of PLCγ1 E709, previously identified as a component of the PLCγ1 SH2C docking surface.

*Mutation of the substrate-docking site on Itk increases $K_m$ for the PLCγ1 SH2C-Peptide B substrate but not Peptide B alone.*

To more quantitatively assess how mutation of the substrate-docking site on the Itk kinase domain affects phosphorylation of PLCγ1, we next determined the $K_m$ and $k_{cat}$ values for the Peptide B and PLCγ1 SH2C-Peptide B substrates for both wild type Itk kinase domain and the Itk E565A mutant (Fig. 4). For the Peptide B substrate, both wild type Itk (Fig. 4A) and Itk E565A (Fig. 4C) have similar $K_m$ and $k_{cat}$ values (Fig. 4E). In contrast, for the docking competent PLCγ1 SH2C-Peptide B substrate, the Itk E565A mutant exhibits an increased $K_m$ value (Fig. 4D) compared to wild type Itk for the same substrate (Fig. 4B). As a result, enzyme efficiency ($k_{cat}/K_m$) for phosphorylation of PLCγ1 SH2C-Peptide B is lower for the Itk E565A mutant than wild type Itk (Fig. 4E) whereas efficiency of phosphorylation of Peptide B alone is not affected...
by the mutation of E565 on the G helix further supporting a role for this residue in substrate docking (Fig. 4E). Attempts to combine kinase domain mutations in this assay were not successful as triple and quadruple Itk mutations had deleterious effects on $k_{cat}$.

*Itk mutations diminish protein-protein interaction between Itk kinase domain and PLCγ1 SH2C.*

To complement the functional assays used to elucidate and characterize the PLCγ1 docking site on the Itk kinase domain, we next assessed the extent to which mutations in the Itk kinase domain affect the direct interaction between PLCγ1 SH2C and Itk. Purified GST-PLCγ1 SH2C fusion protein (or GST alone) was immobilized on glutathione beads and incubated with purified Itk kinase domain (wild type or mutants). Consistent with previously published data(14), wild type Itk kinase domain binds to the GST-fusion of PLCγ1 SH2C domain and not GST alone (Fig. 5A, lane 3). Mutations of the Itk kinase domain in the newly described PLCγ1 docking site, Itk E568A/D569A and Itk F529A/S530A, result in diminished binding to the immobilized GST-PLCγ1 SH2C domain (Fig. 5A, lanes 6 & 9). This observation provides additional evidence that the Itk kinase domain, in the region including and adjacent to the G helix, mediates a direct interaction with the PLCγ1 SH2C domain.

*NMR analysis of the kinase/SH2C domain interaction.*

We previously reported the use of NMR spectroscopy to monitor the Itk kinase/PLCγ1 SH2C domain interaction with a focus on mapping the PLCγ1 SH2C residues involved in the docking interaction(20). Incorporating NMR active isotopes into the Itk kinase domain rather than the PLCγ1 SH2 domain would allow a similar strategy for identifying/confirming amino acids at the Itk/PLCγ1 interface on Itk. Producing sufficient quantities of isotopically labeled kinase
domain is generally quite challenging and we have not to date been successful in producing a labeled NMR sample of the Itk kinase domain for broad NMR applications. Instead, we have successfully identified a mutant of the related Btk kinase domain that expresses at high levels in bacteria and yields excellent NMR spectra (Fig. 5B). We have therefore moved forward using this Btk kinase domain mutant as a surrogate for the Itk kinase domain in our NMR studies. This approach is supported by the in vitro observation that the Btk kinase domain phosphorylates Y783 of PLCγ1 more efficiently when the adjacent PLCγ1 SH2C domain is present (Supplementary Fig. 1a). Moreover, the docking residues we have biochemically mapped on the Itk kinase domain (Fig. 3) are largely conserved between Itk and Btk (Supplementary Fig. 1b). This suggests that the molecular details of a docking interaction between Btk and the PLCγ1 SH2C domain are similar to the Itk/PLCγ1 interaction.

Initial experiments revealed that the NMR sample containing the Btk kinase domain and the larger PLCγ1 fragment consisting of both SH2 domains (SH2N-SH2C) is more stable than samples containing the kinase domain plus PLCγ1 SH2C alone and so we proceeded with the larger PLCγ1 fragment. Addition of unlabeled PLCγ1 SH2N-SH2C into the sample of 15N-labeled Btk kinase domain mutant leads to selective line broadening for a subset of resonances in the [15N, 1H]-TROSY spectrum of the Btk kinase domain mutant due to exchange between PLCγ1-bound and unbound kinase domain (Fig. 5C). Given a protein of this size, complete resonance assignments are a major undertaking and so as a first step, we have used amino acid specific isotopic incorporation to further characterize the kinase/SH2C interaction.
Taking advantage of the excellent expression characteristics of the Btk kinase domain mutant, we produced protein that is selectively $^{15}$N-labeled on tyrosine. There are many amino acids types that are good candidates for specific labeling strategies and our choice of tyrosine reflects a combination of ease of synthesis, limited scrambling of the isotope label, as well as the observation that two tyrosine residues (Y571 and Y598) flank the G helix in the Btk structure (Fig. 5D). Focusing on Btk Y571 and Y598, we were able to assign each specific resonance (Supplemental Fig. 2) and then examine NMR spectral changes as a result of addition of PLCγ1 (Fig. 5E). We observe significant exchange broadening of the resonance corresponding to Y598 that is consistent with binding of PLCγ1 to the nearby G helix of the kinase domain (Fig. 5D & E). The resonance corresponding to Y571 broadens only slightly over the course of the titration and does not change to the same extent as Y598 perhaps due to its distance from the G helix. The more modest spectral changes observed for Y571 could indicate that this residue is located at the periphery of the PLCγ1 binding site or could arise simply due to the longer tumbling time of the higher molecular weight PLCγ1/kinase complex, i.e., non-residue-specific line broadening. These data (in particular the dramatic linewidth changes observed for Y598) provide additional evidence for the substrate-docking site centered around the G helix.

*The substrate-docking surface on the C-terminal lobe of the Itk kinase domain also mediates phosphorylation of PLCγ1 in full length Itk.*

In the experiments carried out to this point we have focused on the Itk kinase domain rather than the multi-domain full-length protein. For the purposes of mapping the Itk/PLCγ1 interaction, use of the active Itk catalytic domain by itself is justified but does not answer the question of how the substrate-docking surface behaves within full length Itk. To address this
question, we examined PLCγ1 phosphorylation by wild type, full-length Itk and the full-length Itk mutants: E565A and F529A/S530A (Fig. 6). All three full-length Itk enzymes were expressed, purified and subjected to the same activity assay as described above for the Itk kinase domain (Fig 3). For both the Itk E565A and Itk F529A/S530A mutants, phosphorylation of the PLCγ1 SH2C-peptide B substrate is selectively compromised compared to phosphorylation of Peptide B (Fig. 6A). This is consistent with the results obtained using the truncated Itk kinase domain and suggests that the substrate-docking surface on the C-terminal lobe of the Itk kinase domain is an important specificity determinant in phosphorylation of PLCγ1 by full length Itk.

Discussion

*G helix is a recurring substrate docking site on protein kinases.*

Direct interaction of PLCγ1 SH2C with the Itk kinase domain leads to specific and efficient phosphorylation of the PLCγ1 Y783 target site. The data presented here point to a largely acidic surface centered at the G helix of the Itk kinase domain (Fig. 6B) that serves as a recognition site for the previously identified basic region of the PLCγ1 SH2C domain\(^{(2)}\). Comparison of the model that emerges for Itk mediated phosphorylation of PLCγ1 (Fig. 7A) with substrate docking surfaces that have been characterized for other kinase domains reveals interesting similarities (Fig. 7). Compiling the kinase structures for which docking sites have been mapped and those for which enzyme/substrate co-crystal structures are available, shows that the large C-terminal kinase lobe is the primary site for substrate recognition. Moreover, the G helix, in particular, plays a central role in mediating remote substrate recognition. The structures of the double-stranded RNA dependent protein kinase (PKR) in complex with eIF2α and the Rho-associated protein kinase I (ROCKI) in complex with RhoE are strikingly similar to
the Itk/PLCγ1 interaction (Fig. 7B, C)\(^{8, 23}\). The substrate proteins, RhoE, eIF2α and PLCγ1 all interact with their cognate kinase domain at and around the G helix. This structural similarity occurs despite differences in the chemical nature of the complementary interaction surfaces. In yet another example of the role of the G helix in mediating phosphorylation, the serine/threonine kinase, p21-activated kinase 1/2 (PAK1/2), appears to dimerize and autophosphorylate via an interface that includes the G helix\(^{24}\). The similarities in these different systems suggest that the exposure and flexibility of the G helix, as well as its location with respect to the active site, make this structural sub-element an ideal platform for substrate-docking interactions.

The G helix is not the only site for substrate docking on the C-terminal kinase lobe. ERK1/2 is considered a paradigm for modular docking interactions; this well-studied MAP kinase contains two distinct sites on the kinase domain functioning independently to recruit different substrates\(^{25}\). One of the docking sites, which binds to the DEF (docking site for ERK, FXFP) motif or F-site, maps to part of the activation segment, the N-terminal tip of the F helix, the MAPK insert and the G helix\(^{26}\). It is clear that this docking site resembles the Itk substrate-docking site (Fig. 7D,E) despite the structural differences between these two kinases in this region due to the MAPK insert. The other Erk2 docking site, which binds to the DEJL (docking site for ERK and JNK, LXL) motif or D-site, is located at the β7-β8 and αD-αE loops on the C-terminal lobe (Fig. 7E)\(^{27}\). This surface is reminiscent of the surface the C-terminal Src kinase (Csk) exploits to engage in the docking interaction with its Src family kinase substrate (Fig. 7F)\(^{9}\). Thus, while the G helix is certainly a recurring site for substrate docking interactions, kinase domains also use other motifs to mediate substrate recognition.
For Itk, we have previously characterized an intramolecular docking interaction with its own SH2 domain that controls autophosphorylation of Y180 on the Itk SH3 domain\(^{(14)}\). Our data for that system suggest a docking interaction surface on the back side of the Itk kinase domain, including both N-lobe and C-lobe residues\(^{(28)}\). Thus, like Erk2, Itk appears to exploit at least two distinct surfaces on the kinase domain to recognize different substrates. Consistent with non-overlapping substrate-docking surfaces, we find that addition of the isolated PLC\(\gamma\)1 SH2C effectively competes with Itk mediated phosphorylation of PLC\(\gamma\)1 Y783\(^{(14)}\), while addition of the other substrate recognition element, Itk SH2, to the same Itk kinase assay has no effect on phosphorylation of PLC\(\gamma\)1 Y783 (Ruo Xu, data not shown). The presence of multiple docking interaction surfaces explains, at least in part, how Itk and other kinases distinguish between distinct substrates.

**Does post-translational modification enhance substrate docking?**

The acidic docking site on the Itk G helix is adjacent to a serine residue (S564) that has been identified as a novel phosphorylation site in a proteomic analysis of protein kinases from stimulated Jurkat T cells (Fig. 6B)\(^{(29)}\). Given the chemical nature of the Itk/PLC\(\gamma\)1 interaction, it is possible that introduction of additional negative charge via phosphorylation at S564 following T cell stimulation might enhance docking to the basic PLC\(\gamma\)1 SH2C domain. In contrast to the well-studied tyrosine phosphorylation sites in Itk (Y180 and Y551)\(^{(16, 30)}\), there is little known about the role of S564 phosphorylation in regulating Itk function. Our attempts to mimic this phosphorylation site using a serine to aspartate mutation led to an inactive Itk kinase domain. Thus, with the appropriate tools it will be interesting to probe the precise role of S564
phosphorylation in future experiments with the possibility that Itk substrate recognition might be modulated by the phosphorylation status of S564.

**Does the docking interaction with PLCγ1 SH2C domain regulate Itk activity?**

Docking interactions in general, apart from acting as a passive substrate specificity determinant, are also known to be capable of directly regulating the kinase activity for enzymes such as ERK2 and PDK1\(^\text{27, 31}\). To assess whether the docking interaction between the PLCγ1 SH2C domain and the Itk kinase domain modulates Itk activity, isolated PLCγ1 SH2C domain was titrated into the *in vitro* Itk kinase assay and the initial velocity for Peptide B phosphorylation by Itk was determined. Since we have not yet directly measured the affinity between PLCγ1 SH2C domain and Itk kinase domain, \(K_m\) of PLCγ1 SH2C-Peptide B substrate (~5 \(\mu\)M) was used as an estimate. PLCγ1 SH2C domain was added from 0 to 10 fold \(K_m\) of PLCγ1 SH2C-Peptide B substrate (50 \(\mu\)M) to ensure saturation of binding. Within this range, the initial velocity of Peptide B phosphorylation remained largely unchanged (data not shown), indicating that the docking interaction between PLCγ1 SH2C domain and Itk kinase domain by itself has no direct effect on the intrinsic activity of Itk. This is consistent with the \(k_{cat}\) value measured for PLCγ1 SH2C-Peptide B substrate being similar to that measured for Peptide B substrate\(^\text{14}\). Our *in vitro* assay is a minimal system however, and it is likely that in the signaling complex that contains full length Itk and PLCγ1 peripheral to the T cell membrane, the interactions between Itk, PLCγ1 and other signaling proteins as well as transient posttranslational modifications might indeed serve to enhance Itk catalytic efficiency.

*The docking surface on Itk expands target sites for small molecules*
Interest in small molecule inhibitors for Itk has increased in recent years as drug leads are sought for treatment of inflammatory disorders such as asthma and rheumatoid arthritis. Currently, the reported small molecule Itk inhibitors target the ATP binding pocket, a binding site that promotes high affinity binding but generally lacks the unique features required to achieve selectivity over other kinases. Our current findings suggest an alternative strategy to target Itk kinase activity in a pathway-specific manner. Small molecules that block the PLCγ1 substrate-docking surface on Itk, or bidentate inhibitors that bind to both the Itk active site and at least a portion of the substrate-docking surface, are likely to exhibit greater specificity than active site inhibitors alone. As the Itk/PLCγ1 and other kinase/substrate recognition motifs are further characterized, it will be interesting to determine the extent to which substrate-docking mechanisms can be exploited in inhibitor design.

Materials and Methods

Constructs

The baculoviral expression constructs for full-length Itk and Itk kinase domain (342-619) (previously referred to as Linker-Kinase) and the bacterial expression constructs for PLCγ1 derived fragments have been described previously. To improve protein solubility, sample stability and protein dynamics, the Btk kinase domain (residues 396-659) used for the NMR experiments contains the following mutations: Y617P, L542M, S543T, V555T, R562K, S564A and P565S. All mutations were introduced by using the site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing at Iowa State University DNA Synthesis and Sequencing facility. Mouse numbering is used for Itk and Btk sequences and bovine numbering is used for PLCγ1 sequences throughout the text.
Protein expression and purification

Baculoviruses were produced for Itk fragments containing the kinase domain as previously described (22). Full-length Itk, Itk kinase domain and the corresponding mutants were expressed in High Five cells (Invitrogen). High Five cells were cultured in suspension in spinner flasks using Express Five serum free medium (Invitrogen) supplemented with glutamine and gentamycin and infected with 1:1 ratio of Itk:Lck baculovirus when the density of the cells reached 0.4-1.25×10^6 cells/ml. Infected cells were harvested 24 h post-infection and the cell pellets were stored at -80°C. Protein purification from High Five cells followed the procedure described previously (22). Purified full-length Itk or Itk kinase domain and the corresponding mutants were resolved on SDS-PAGE gel to verify purity above 95% and to equalize enzyme level for the phosphorylation assay. Bacterial expression and purification of all PLCγ1 fragments were previously described (14).

15N-uniformly labeled Btk kinase domain was expressed in BL21 (DE3) cells in minimal medium containing 15N-NH4Cl (Cambridge Isotope Laboratories) as the sole source of nitrogen. When the optical density at 600 nm reached 0.6-0.8, 1 mM IPTG was added to induce protein expression at 17°C for 24 h. 15N-Tyr selectively labeled Btk kinase domain was expressed in minimal medium containing 14N-NH4Cl. When the optical density at 600 nm reached 0.6-0.7, 0.1 g 15N-Tyr (Cambridge Isotope Laboratories) and 0.1 g of the other 19 unlabeled amino acids were supplemented to each 500 ml culture and grown at 37°C for 30 minutes. Standard induction at 17°C for 24 h was then commenced with the addition of 1mM IPTG. The harvested cell pellets were resuspended in lysis buffer (0.5 mg/ml lysozyme, 50 mM Tris pH 7.8, 75 mM
NaCl, 20 mM imidazole) and stored overnight at -80°C. Cells were lysed at room temperature with addition of 3000 Units DNaseI (Sigma) and 1 mM PMSF. The lysate was clarified by centrifugation at 16K for 45 min at 4°C and the supernatant was incubated with Ni-NTA resin (Qiagen) pre-equilibrated with lysis buffer. The resin was washed with wash buffer (50 mM Tris pH 7.8, 75 mM NaCl, 40 mM imidazole) and then eluted with elution buffer (50 mM Tris pH 7.8, 75 mM NaCl, 250 mM imidazole, 10% glycerol). The eluent was concentrated and dialyzed overnight against NMR buffer (50 mM bicine pH 8.0, 75 mM NaCl, 2 mM DTT, 0.02% NaN₃, 5% glycerol).

**Kinase assays and western blotting**

For the *in vitro* kinase assay, Itk kinase domain was incubated with the indicated substrates in reaction buffer (50 mM HEPES pH 7.0, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 µM ATP) at room temperature for 1 hour. The samples were boiled, separated by SDS-PAGE and transferred onto PVDF membrane. Y783 phosphorylation was monitored using anti-pY783 antibody (Biosource) as described previously.¹⁴

For determination of kinase activity, a previously described procedure was followed with minor modifications. Briefly, 100 nM full-length Itk or 500 nM Itk kinase domain was incubated with either biotinylated Peptide B (aminohexanoyl biotin-EQEDEPEGIYGVLF-NH₂ (Anaspec)) or biotinylated PLCγ1 SH2C-Peptide B at a concentration 4 times the previously determined $K_m$ ¹⁴ in reaction buffer (50 mM HEPES pH 7.0, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 µM ATP and 5 µCi of [³²P]-ATP (PerkinElmer)). To determine the initial velocity of phosphorylation, 10 µl of the reaction mixture was removed and mixed with 5 µl 8 M
guanidine hydrochloride to terminate the reaction, after a 7.5 min and a 15 min reaction time, respectively. 10 µl of this mixture was spotted onto the biotin capture membrane (Promega), which was washed 2 times with 1 M NaCl, 2 times with 1 M NaCl with 0.1% phosphoric acid, 1 time with H₂O and 1 time with 95% ethanol. The radioactivity incorporated on biotinylated Peptide B or biotinylated PLCγ₁ SH2C-Peptide B was quantified by scintillation counting. Each assay was performed in duplicate and the experiment was repeated at least twice to ensure reproducibility.

For determination of $k_{cat}$ and $K_m$, 1 μM Itk kinase domain or kinase mutant was used. Peptide B concentration was varied between 0 and 400 μM and the concentration of PLCγ₁ SH2C-Peptide B was varied between 0 and 75 μM. Each assay was performed in duplicate. Data were fit to the Michaelis-Menten equation using GraFit 5 (Erithacus) to obtain the kinetic parameters.

**NMR Spectroscopy**

All NMR spectra were acquired at 30°C on a Bruker AVII700 spectrometer with a 5 mm HCN z-gradient cryoprobe operating at ¹H frequency of 700.13 MHz using standard experimental protocols (Bruker pulse program trosyf3gpphsi19). For the NMR titration experiment, increasing amounts of PLCγ₁ SH2N-SH2C were added stepwise to 160 µM of the ¹⁵N labeled Btk kinase domain (a kinase-inactive version of Btk (K430R) was used to ensure sample homogeneity). The molar ratio of labeled kinase domain to unlabeled SH2N-SH2C in each of the four titration points was 1:0, 1:0.75, 1:2 and 1:4. [¹⁵N, ¹H]-TROSY HSQC spectra were collected at each titration point. To assign the tyrosine peaks of interest on the kinase
domain spectrum, the tyrosine was mutated to either alanine or phenylalanine. [$^{15}$N, $^1$H]-TROSY HSQC spectra were acquired for the $^{15}$N-Tyr labeled Btk kinase domain (400 μM) as well as the $^{15}$N-Tyr labeled Btk kinase domain carrying either the Y571A or Y598F mutations. Comparison of two spectra permitted unequivocal assignment of the tyrosine in question (either Y571 or Y598).

**Binding assay**

50 nM Itk kinase domain or the corresponding mutant was incubated with 0.5 μM purified GST or 0.5 μM purified GST-PLCγ1 SH2C immobilized on glutathione beads in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM PMSF, 1% NP40, 1 mM EDTA and 1 mM NaF) at 4°C overnight. The samples were washed, boiled, separated by SDS-PAGE and transferred onto PVDF membrane. The amount of kinase domain that interacts with the protein-bound beads was monitored by blotting with anti-FLAG antibody (Sigma).

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**References**


**Figure captions**

Figure 1. Structures of the SH2C domain of PLCγ1 and the kinase domain of Itk. (A) NMR structure of *Bos taurus* PLCγ1 SH2C domain (PDB code: 2PLD). Previously identified docking residues in PLCγ1 SH2C are highlighted in blue and labeled. (B) Surface-exposed acidic residues (with the exception of D508 and D509 in the activation loop, not visible) are shown in
red on the Itk kinase domain X-ray crystal structure (PDB code: 1SNU). Residues labeled with italics and underlined were targeted for mutagenesis and functional screening.

Figure 2. (A) Cartoon illustration of the strategy used to estimate the distance between the substrate-docking surface and the active site on the Itk kinase domain. (B) Truncation constructs of PLCγ1 SH2C-linker used as substrates in the Itk kinase assay. Constructs vary with respect to the length of the linker between R753 in SH2C and the phosphorylation site, Y783. (C) In lanes 1-12, 1 μM and 5 μM of the truncated constructs of PLCγ1 SH2C-linker, wild type PLCγ1 SH2C-linker and PLCγ1 SH2C-linker (Y783F) mutant were subjected to phosphorylation by 0.25 μM FLAG-tagged Itk. Lanes 13 and 14 contain the substrate alone control and enzyme alone control, respectively. Anti-pY783 detects substrate phosphorylation (pY783 in PLCγ1 SH2C-linker), anti-FLAG detects level of Itk enzyme and Coomassie stain detects substrate level in each lane.

Figure 3. (A) Comparison of catalytic efficiency and specificity of Itk kinase domain mutants was made with respect to the two substrates in this study. (B) The initial velocity of the active form of the wild type Itk kinase domain (WT-Itk) toward each of the two substrates shown in (A) was measured (see Methods) and normalized to 1. The initial velocity value for each mutant Itk enzyme (for both the Peptide B and PLCγ1 SH2C-Peptide B substrate) is then divided by the initial velocity of wild type Itk and expressed as the ratio: \( V_i/V_i (WT-Itk) \). Normalized initial velocity values for Peptide B substrate are the dark bars and for PLCγ1 SH2C-Peptide B substrate are the light bars for wild type and each mutant Itk. (C) Itk kinase domain residues mutated in this study are shown in ball and stick, those side chains that, upon mutation to
alanine, lead to disruption in docking based on initial velocity measurements are shown in red and labeled. (D) Secondary alanine scan results acquired and presented as described for (B). (E) Results of the alanine scan shown on the ribbon structure of the Itk kinase domain. Red side chains are the same as in (C) and the labeled, orange side chains are those derived from the secondary screen shown in (D). (F) Surface representation of the Itk kinase domain showing the amino acids identified to be involved in the docking interaction with PLCγ1 SH2C (red and orange as in (C) and (E)). This view of the kinase domain is rotated to show the ‘bottom’ of the C-terminal lobe.

Figure 4. (A)-(D) Substrate (Peptide B (A & C) and PLCγ1 SH2C-Peptide B (B & D)) curves for wild type Itk (A & B) and Itk E565A (C & D) were fit to the Michaelis–Menten equation using GraFit 5 to obtain the kinetic parameters reported in (E). (E) Kinetic parameters for wild type Itk and Itk E565A.

Figure 5. Amino acid residues centered around the G helix are involved in the direct interaction with the PLCγ1 SH2C domain. (A) Purified FLAG-tagged, wild type Itk kinase domain and Itk kinase domain mutants E568A/D569A and F529A/S530A (lane 1, 4 and 7) were incubated with either GST (lane 2, 5 and 8) or the GST-SH2C fusion protein (lane 3, 6 and 9) immobilized on glutathione beads. Following extensive washing, the extent to which the FLAG-tagged Itk kinase domains (wild type and mutants) bind to PLCγ1 SH2C was assessed by blotting with an anti-FLAG antibody (top panel). The Itk kinase domain mutants, E568A/D569A and F529A/S530A bind less efficiently to SH2C than wild type Itk kinase domain. Band intensities were integrated using the Image Lab software along with the ChemiDoc XRS+ System (BioRad).
and normalized values are shown above each band for bound Itk kinase. The bottom panel shows uniform levels of GST and GST-SH2C visualized by Ponceau S staining. (B) $^{[15N, ^1H]}$-TROSY HSQC spectra of uniformly $^{15}$N labeled Btk kinase domain. (C) Addition of PLCγ1 SH2N-SH2C to uniformly $^{15}$N labeled Btk kinase domain results in significant broadening of selected peaks (top two panels) in the $^{[15N, ^1H]}$-TROSY HSQC spectrum. A large subset of kinase domain resonances does not change over the course of the titration (lower panel). (D) Two tyrosines, Y571 and Y598, are adjacent to the G helix in the Btk kinase domain and were targeted for specific resonance assignment. (E) Four $^{[15N, ^1H]}$-TROSY HSQC spectra of $^{15}$N labeled Btk kinase domain with increasing amount of PLCγ1 SH2N-SH2C added. The peak corresponding to Y598 shows dramatic exchange broadening with the addition of 0.75 molar ratio PLCγ1 dual SH2 domain and broadens beyond detection with further addition of SH2 domain. The peak corresponding to Y571 broadens only slightly with increasing addition of PLCγ1 SH2N-SH2C.

Figure 6. (A) The initial velocity for full-length wild type and two Itk kinases (E565A and F529A/S530A) is measured and presented for the Peptide B and PLCγ1 SH2C-Peptide B substrates in a manner identical to that described in Figure 3. (B) Structure of the C-terminal lobe of the Itk kinase domain showing the location of E565, F529 and S530 within the substrate-docking surface. Superimposed on the ribbon structure is the electrostatic surface showing the acidic nature of the Itk G helix in red (basic regions are blue). The putative phosphorylation site on the G helix, serine 564 (S564), is circled and labeled on the Itk structure.
Figure 7. Comparison of Itk/PLCγ1 docking interaction with other enzyme/substrate pairs. (A) Individual structures of the PLCγ1 SH2C domain (2PLD) and the Itk kinase domain (1SNX) are oriented in a manner that brings the two docking surfaces (side chains indicated in red ball and stick) together. The amino-terminal kinase lobe (N-lobe) and carboxy-terminal kinase lobe (C-lobe) are labeled as is the G helix and the active site of the Itk kinase domain. (B, C) Co-crystal structures of the ROCKI/RhoE and PKR/eIF2a enzyme/substrate pairs (2V55 and 2A1A, respectively). The ROCKI and PKR kinase domains are shown in the same orientation as the Itk kinase domain in (A). The G helix and the active site of ROCKI and PKR are labeled. (D) The Itk kinase domain shown in a different orientation than that shown in (A) with the substrate-docking surface on the C-lobe identified in this study highlighted in red and labeled (PLCγ1 docking site). (E, F) Structures of ERK2 (E) and Csk (F), 2GPH and 3D7T, respectively, are shown with various docking sites highlighted and labeled. The DEJL-motif docking site on ERK2 (E) and the Src docking site on Csk (F) are both orange. The DEF-motif docking site on ERK2 (E) is coincident with the PLCγ1 docking site on Itk.

Supplemental Figure 1. (a) Initial velocity measurements of Btk and Itk kinase activity toward the Peptide B substrate (at a concentration of 400 mM) and the PLCγ1 SH2C-Peptide substrate (at a significantly lower concentration of 20 mM). Btk and Itk kinase domains (Btk residues 396-659 and Itk residues 342-619) are both at a concentration of 500 nM. The PLCγ1 SH2C-Peptide substrate is a better substrate for both kinases due to the docking interaction between the kinase domain and SH2C. (b) Crystal structures of the Itk kinase domain (1SNU) and the Btk kinase domain (3GEN) showing the G helix in the C-terminal lobe. Acidic docking residues on the G helix of Itk that were biochemically mapped in this study are shown in red and labeled. The
corresponding residues on the Btk kinase domain G helix are also shown in red and labeled; three of the four residues are conserved. The tyrosine residues that have been assigned in the $[^{15}\text{N}, ^1\text{H}]-\text{TROSY HSQC}$ spectrum of the Btk kinase domain are shown in cyan and labeled. The corresponding residues are shown on the Itk kinase domain structure on the left. Overall, the docking region of Itk is largely conserved on the Btk kinase domain.

Supplemental Figure 2. Assignment of the resonances corresponding to Y571 and Y598 was carried out by expressing the Btk kinase domain in media that incorporates the NMR active isotope, $^{15}\text{N}$, specifically at Tyr residues. Two different mutant Btk kinase domains (either (a) Y571A or (b) Y598F) were expressed in the same manner as the non-mutated kinase. The superimposed $[^{15}\text{N}, ^1\text{H}]-\text{TROSY HSQC}$ spectra of (a) $^{15}\text{N}$-Tyr labeled Btk kinase domain (red) and the Y571A mutant (blue) or (b) $^{15}\text{N}$-Tyr labeled Btk kinase domain (red) and the Y598F mutant (blue) show that a single resonance is missing (even at lower contour levels) from the spectra of $^{15}\text{N}$-Tyr labeled Y571 or Y598 mutant. The resonance in the red spectrum at the position of the missing peak in the blue spectrum can then be assigned to the specific tyrosine that is mutated in the corresponding sample. Using this approach we determined that (a) the resonance corresponding to Y571 is located at $^1\text{H}$ frequency of 6.53 ppm and $^{15}\text{N}$ frequency of 113.25 ppm. Similarly, for (b) Y598 is assigned to a $^1\text{H}$ frequency of 6.22 ppm and $^{15}\text{N}$ frequency of 118.14 ppm. These assignments are shown in the context of the HSQC spectrum of the uniformly labeled Btk kinase domain in Fig. 5B.
Figures

Figure 1
Figure 2

A

B

PLCγ1 wt (L29)  

PLCγ1 L9  

PLCγ1 L4  

PLCγ1 L5  

PLCγ1 L7  

PLCγ1 Y783F  

29 amino acids

C

anti-pY783

anti-FLAG

Coomassie

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Figure 3
Figure 4

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<th>Activity with PLCy1 SH2C-Peptide B</th>
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<td>$k_{cat}$ (min$^{-1}$)</td>
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Figure 5
Figure 6

A

- Peptide B
- PLCγ1
- SH2C-Peptide B

\[ \frac{Y}{N} \text{(WT-full length Iκκ)} \]

<table>
<thead>
<tr>
<th></th>
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B

E565

S564

F529

G-helix

S530

Figure 7

A

active site

N-lobe

C-lobe

PLCγ1 SH2C

Itk

B

active site

G-helix

ROCKI

C

active site

G-helix

PKR

D

active site

DEIL-motif docking site

N-lobe

C-lobe

Itk

E

active site

Src docking site

Erk2

F

active site

DEF-motif docking site

Csk
Supplementary Figure 1

(a) Vi (nmol/min/ul)

- Peptide B (400 μM)
- PLCγ1 SH2C-Peptide B (20 μM)

(b) Itk and Btk structures

Supplementary Figure 2

(a) Red spectrum: no mutations in the kinase docking site
   Blue spectrum: single mutation of Y571 to alanine

(b) Red spectrum: no mutations in the kinase docking site
   Blue spectrum: single mutation of Y598 to phenylalanine
CHAPTER 3. INTRODUCING NMR PROBES TO MONITOR THE
CONFORMATIONAL CHANGES ASSOCIATED WITH PROTEIN
KINASE ACTIVATION

A paper to be submitted to ACS Chemical Biology

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Abstract

Protein kinases are conformationally dynamic systems and so monitoring their conformational changes in solution is a challenging experimental problem. In this paper, we apply \(^{13}\)C reductive methylation to chemically introduce NMR active nuclei into unlabeled protein kinases. Then using the Src kinase as our model system, we demonstrate that solution NMR spectroscopy can be used to monitor the change in the chemical environment of structurally important lysines in the \(^{13}\)C methylated protein kinase when it shifts from the inactive state to the active state. Hence, we develop a structural method, easily applicable to almost any protein kinases, to examine the changes in the conformational equilibrium between the inactive state and the active state under a range of experimental conditions.
Protein kinases catalyze the transfer of the \( \gamma \)-phosphate from ATP to the hydroxyl group of Ser, Thr or Tyr in their substrates. Phosphorylation is an essential part of different cellular processes and deregulation of protein kinase activity is the known cause of multiple diseases, including various types of cancers, diabetes and developmental defects (1-3), making them the subject of active research in both basic science and the pharmaceutical industry. Prompted by the success of Imatinib in targeting Bcr-Abl to cure chronic myelogenous leukemia (4), much effort has been dedicated to design protein kinase inhibitors with high efficacy and high specificity. Detailed structural understanding of protein kinases is desired in this process. To date, we have learned a great deal about the structures of protein kinases from the X-ray diffraction pattern of their crystals. However, protein kinases are dynamic in solution while a crystal structure captures only a snapshot of the multiple conformations that protein kinases can sample. Hence, solution structural techniques such as NMR spectroscopy are in need to acquire information on the entire conformational ensemble of protein kinases. Currently, few NMR studies on protein kinases have been reported, mainly because most protein kinases require expression in eukaryotic cells to fold correctly, where isotopic labeling is very costly and laborious and the yield is usually too low for NMR. Here, we present a novel approach, which makes feasible the NMR study of the conformational states of almost any protein kinases.

This approach uses \([^{13}\text{C}]\) formaldehyde in reductive methylation to introduce NMR active nuclei on the primary amines in the purified unlabeled protein kinase. The reaction, when driven to completion, introduces two \([^{13}\text{C}]\) methyl groups onto the \(\varepsilon\)-NH\(_2\) group of the lysine side chains as well as the N-terminus of the protein (Fig. 1). Importantly, reductive methylation does not perturb salt bridge formation by lysine residues, some of which are known to be important for
the structure and function of protein kinases, as the methylated lysines still can be protonated at the substituted ε-amine. This approach is inspired by recently published work, where this labeling method was applied to a G-protein coupled receptor (GPCR) to examine the chemical environment change of a specific Lys-Asp salt bridge in GPCR induced by ligand binding (5). In the context of protein kinases, an important salt bridge exists between a conserved β3 strand Lys and a conserved Glu on the αC helix in the active state and breaks apart when protein kinases shift to the inactive state. We therefore propose that [13C] reductive methylation coupled with NMR spectroscopy can be adapted to examine the conformational transition of protein kinases along the course of their activation.

We tested the applicability of the proposed approach using the protein tyrosine kinase Src as our model system, because the regulatory mechanism of Src has been well understood at the molecular level (6). The domain structure of Src includes, from the N-terminus to the C-terminus, an SH3 domain, an SH2 domain, the catalytic kinase domain and a C-terminal tail containing a tyrosine (Y527) that is phosphorylated in the down-regulated state. When the tail Y527 is phosphorylated, it is bound by Src SH2 domain in an intramolecular manner and Src SH3 domain interacts with a stretch of proline rich sequence in the SH2-kinase linker. Thus, Src is clamped into an autoinhibitory conformation where Src SH3 and SH2 domains are located on the backside of the kinase domain. When Y527 is dephosphorylated or exogenous SH3 and SH2 ligands compete for binding to Src SH3 and SH2 domains, the kinase domain is freed from the restraint imposed by SH3 and SH2 domains and shifts towards the active state (Fig. 2). For the same reason, the isolated kinase domain of Src is regarded to be constitutively active.
We purified from bacteria the isolated kinase domain (KD) of Src, which represents the kinase active state, and the SH3-SH2-kinase domain (3K) of Src, which represents the kinase inactive state, and performed $[^{13}\text{C}]$ reductive methylation on both proteins. Success of the chemical modification is indicated by slower migration of the protein bands corresponding to the methylated samples contrasted with the unmethylated proteins on SDS-PAGE gel (Fig. 3A). This is consistent with an increase of molecular weight due to addition of two methyl groups onto each primary amine. Also, no other protein bands with molecular weight higher than that of monomeric Src protein were observed, indicating minimal intermolecular crosslinking in this process. Solely based on SDS-PAGE gel, we cannot completely rule out the possibility of any intramolecular crosslinking within Src proteins, but close examination of Src kinase domain crystal structure does not reveal the $\epsilon$-NH$_2$ groups of any pair of lysines within a distance of 4 Å, making intramolecular crosslinking via a methylene group very unlikely. To further characterize methylated proteins, we analyzed the peptides of the methylated Src samples after trypsin digestion using MALDI-TOF (Fig. 3B). We were able to identify the peptide containing the $\beta$3 strand Lys of Src (K295) in both Src KD and Src 3K samples, with a mass to charge ratio consistent with all the lysines in this peptide being dimethylated. Dimethylation on K295 was further confirmed by MS/MS analysis of the same peptide. Although reductive methylation has been demonstrated to cause little structural perturbation in many cases (7, 8), we compared the CD spectra of Src protein before and after this chemical modification. Their CD traces are very similar, indicating that the overall protein fold of Src was not changed by reductive methylation (Fig. 3C).
We then used \([^{1}H, ^{13}C]\) Heteronuclear Single Quantum Coherence (HSQC) pulse sequence to monitor the chemical environment of the lysine side chains in Src KD and 3K by NMR spectroscopy. The full HSQC spectrum can be generally divided into three regions (Fig. 4A). All the buffer peaks cluster in the low field, with \(^{1}H\) chemical shift 3.0-4.0 ppm and \(^{13}C\) chemical shift 50-75 ppm. The dimethyl groups resonate at a \(^{13}C\) chemical shift of ~45 ppm and the monomethyl groups resonate at a \(^{13}C\) chemical shift of ~35 ppm (9). The peaks corresponding to the dimethylated lysines clearly dominate over the monomethylated lysine peaks in the spectrum, indicating that the reductive methylation almost reaches completion. Hence, we only focus on the dimethyls region in our analysis. In this region, the methyl groups attached to the solvent exposed lysines experience similar chemical environment, except for minor differences in local primary sequence, and therefore they show almost degenerate \(^{1}H\) chemical shift values. These peak resonances are difficult to study due to poor resolution. Methyl peak resonances of the functionally important lysines, on the other hand, are expected to resolve from those of the solvent exposed lysines, because they interact with other parts of the kinase, making their chemical environment distinct. These methyl peaks are therefore amenable to further analysis.

We noticed that the resolved dimethyl region is drastically different between the spectra of Src KD and Src 3K (Fig. 4B). For instance, the peak resonance with \(^{1}H\) chemical shift ~2.37 ppm in Src KD spectrum is not found in Src 3K spectrum. We wondered whether such spectral differences reflect the conformational differences between the active state and inactive state of Src. To examine this potential correlation, we first assigned peak resonances in the resolved region on both spectra. In making assignment of a specific peak resonance, we mutated the
lysine candidate in Src to another amino acid type that is inert to reductive methylation. The same chemical modification was then applied to the mutant and we examined whether the peak of interest is missing in the mutant spectrum compared with the wild type spectrum. Using this approach, we were able to assign two peaks in Src 3K spectrum, one with $^1$H chemical shift ~2.49 ppm and the other with $^1$H chemical shift ~2.22 ppm, to the $\beta$3 strand Lys (K295) and a Lys located on the $\alpha$C helix (K315) respectively (Fig. 4C). Both peaks are potential probes on the HSQC spectrum reporting on the conformational change along the course of Src activation.

Previous biochemical studies have demonstrated that addition of Src SH3 or SH2 peptide ligands to Src 3K activates its kinase activity (10). To monitor the conformational transition of Src 3K in this process, we first titrated an Src SH3 ligand peptide with sub-micromolar affinity, VSLARRPLPPLP, into the [13C] reductively methylated Src 3K NMR sample (Fig. 5A). As SH3 ligand was added stepwise, the intensity of the K295 methyl peak diminished until it completely disappeared. Because the K295 methyl peak is adjacent to another major peak resonance, it is difficult to differentiate whether this peak disappearance was due to a line broadening effect or a chemical shift change that results in overlap with its neighboring peak. The K315 methyl peak, on the other hand, clearly shows slow exchange in response to SH3 ligand titration. We observed that its peak intensity in the spectrum of Src 3K in the absence of peptide ($^1$H chemical shift ~2.22 ppm) decreases and increases at a new resonance frequency ($^1$H chemical shift ~2.28 ppm) during the course of the peptide titration. Upon saturation of Src 3K with the SH3 ligand peptide, the K315 methyl peak shifted completely to the new $^1$H frequency. That high molar equivalent of SH3 ligand was required to reach saturation is most likely due to the need to compete against an intramolecular interaction.
Comparing the crystal structure between the active and inactive Src, we speculate that the spectral change for the K295 methyl peak reflects a more frequent sampling of the salt bridge formation with E310. The same trend was observed in studying a Tec kinase Btk using this approach, where the more active the kinase construct, the weaker intensity of the β3 strand lysine (K430) methyl peak. For the most active Btk construct tested, K430 methyl peak completely disappears in the [1H, 13C] HSQC spectrum. We also suggest that the shift of Src K315 peak is due to change in the relative side chain orientation between K315 and the W260 of the WEI motif in the SH2-kinase linker. K315 side chain is packed against W260 side chain in the inactive state while in the active state W260 side chain moves further away from K315 (Fig. 5B).

We continued to titrate a high affinity Src SH2 ligand peptide, Caffeic acid-pYEEIE, into the Src 3K sample saturated with SH3 ligand, to observe additional spectral changes as the SH2 domain binds its ligand (Fig. 5C). In this case, K315 methyl peak shifts further, in the same slow exchange manner, to a 1H chemical shift value of ~2.37 ppm. Upon saturation of Src 3K with both SH3 and SH2 ligands, the kinase domain in Src 3K is expected to be free from the conformational constraint imposed by the SH3 and SH2 domains and be able to adopt the active state like the isolated Src kinase domain. Hence, we compared the Src 3K spectrum at the endpoint of the titration with the Src KD spectrum, and remarkably, there was a very good overlap between them, except for one major peak resonance present only in Src 3K spectrum, which we suspect corresponds to a lysine residue in the SH3 or SH2 domain and not present in the Src KD construct (Fig. 5D). Hence, we demonstrate that introducing the [13C] methyls to
specific lysine side chains within Src using reductive methylation chemistry allows us to follow the activation pathway of the Src kinase by NMR spectroscopy (Fig. 5E).

To examine whether reductive methylation affects the catalytic activity of the protein kinases, we measured the activity of Src KD and Src 3K before and after [13C] reductive methylation toward a generic peptide substrate (Supplemental Figure). In activity assay using the unmethylated Src proteins, we find as expected that Src KD is more active than Src 3K. In our assay, Src KD phosphorylated the peptide substrate ~3 fold faster than Src 3K. The fold of activity difference between Src KD and Src 3K is smaller compared with over an order of magnitude difference reported in the literature (11). The discrepancy might arise from the different phosphorylation level on the tail tyrosine Y527 of Src 3K purified from bacteria and insect cells or simply different peptide substrates used in the two assays. However, when we tested the activity of both Src KD and Src 3K after [13C] reductive methylation, there was a significant decrease compared with the activity of the unmodified enzyme. In retrospect, this is not too surprising. In addition to forming the salt bridge with E310 on the αC helix, the β3 strand Lys (K295) also coordinates α- and β- phosphates of ATP in the active site. Therefore, addition of two methyl groups at the terminus of this lysine side chain may cause steric hindrance and preclude ATP binding. Currently, we are trying several methods to control the extent of methylation on K295, such as applying this chemistry under the condition selective for mono-methylation (12), to examine whether decreasing the steric bulk introduced to the active site can rescue the kinase activity. As well we are carrying out reductive methylation chemistry under conditions where the active site is saturated with ATP to test whether we can disfavor
methylation at K295. This approach would then rely on the methylated K315 resonance to monitor the conformational transition between inactive and active Src.

To the best of our knowledge, the approach presented here provides the first experimental tool to examine the activation pathway of protein kinases. It is complemented by the computational methods, which together provide structural information of the intermediates along the course of kinase activation. For instance, we observed in the titration experiment that there existed an intermediate state in which the SH3 domain of Src 3K was released from the SH2-kinase linker but the SH2 domain was still engaged by the tail tyrosine sequence. This is consistent with the previous finding on Hck, another Src family kinase, that while the HIV Nef protein binds to Hck SH3 domain, the interaction between Hck SH2 domain and the phosphorylated tail sequence remains in place (13, 14). However, it is also known from the literature that minor differences exist in the activation pathway between the two subgroups of Src family kinases, represented by Src and Hck (15, 16). Hence, it would be interesting to test using the method we propose here whether it will show a different trend in spectral changes when SH3 and SH2 ligands are titrated into Hck 3K. This method can be readily applied to study many other protein kinases. As the chemical modification is performed on the purified unlabeled kinases, it bypasses the requirement for isotopic labeling during expression, which allows for great flexibility in protein production. Moreover, the favorable relaxation property of the methyl group gives rise to sharp NMR peak resonances, so the approach requires much lower concentration of the protein sample for acquiring an NMR spectrum. Indeed, [1H, 13C] HSQC spectra have been reported using [13C] reductively methylated protein samples with low micromolar or even sub-micromolar concentration (17, 18). Collectively, we predict that this
method will allow us to begin to gain some structural insight on the conformation of a large number of pharmaceutically important protein kinases in solution, which may have to rely on eukaryotic expression systems for production and show limited yield. Hence, we have provided a tool for researchers interested in protein kinases to address such questions as what conformational state do small molecules stabilize when they bind a protein kinase and whether an interaction between a protein kinase and its binding partner, be it a substrate, a regulator or an adaptor protein, drives its conformation towards kinase active state or kinase inactive state. Such information is instrumental not only in understanding the molecular basis of how those protein kinases are regulated but also in screening and classifying novel kinase inhibitors.

Materials and Methods

Human Src KD and 3K constructs, the C-terminal tail of which is modified into the high affinity SH2 ligand sequence (YEEIP), also containing the full-length YopH phosphatase gene from Yersinia for co-expression, are kind gifts from Dr. Thomas Smithgall’s group. All the mutations were introduced using site-directed mutagenesis kit (Strategene). All constructs were verified by sequencing at Iowa State University DNA Synthesis and Sequencing Facility. In protein production, the plasmids were transformed into Escherichia coli BL21(DE3) cells and expressed as described previously (19). The cell pellet was harvested by 10 min centrifugation at 6,300g at 4°C, resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl and 20 mM imidazole) supplemented with 0.5 mg/ml lysozyme and stored at -80°C overnight. In purification, cells were lysed at room temperature with addition of 3000 Units DNaseI (Sigma) and 1 mM PMSF. The lysate was centrifuged at 16K for 45 min at 4°C and the supernatant was incubated with Ni-NTA resin (Qiagen) in lysis buffer supplemented with 10% glycerol at 4°C.
The resin was then washed before eluting with elution buffer (50 mM Tris pH 8.0, 150 mM NaCl, 250 mM imidazole and 10% glycerol). The eluents were concentrated and overnight dialyzed to remove the imidazole, before loading onto Source Q anion exchange column pre-equilibrated with buffer A (50 mM Tris pH 8.0, 2 mM DTT and 10% glycerol). The column was washed with five column volumes of buffer A and then eluted with a linear gradient of 0-100% buffer B (50 mM Tris pH 8.0, 2 mM DTT, 1M NaCl and 10% glycerol). The majority of YopH co-purified from Ni column was separated in this process. After SDS-PAGE analysis, the fractions containing Src proteins were pooled, concentrated and loaded onto a size exclusion column equilibrated with 50 mM HEPES pH 8.0, 100 mM NaCl, 2 mM DTT, 10% glycerol. Fractions corresponding to the monomeric form were pooled and concentrated to ~6 mg/ml for lysine reductive methylation.

Lysine reductive methylation procedure followed previous work, with minor modifications (20). Briefly, for each 1 ml protein, 20 µl 1 M NH₃-BH₃ complex (reducing agent) and 40 µl 1 M [¹³C] formaldehyde were added and incubated at 4°C for 2 h. This step was repeated and then finally 10 µl 1 M NH₃-BH₃ complex was added and incubated at 4°C overnight. The reaction was stopped by dialysis into NMR buffer (50 mM bicine pH 8.0, 100 mM NaCl, 2 mM DTT, 5% glycerol and 0.02% NaN₃).

In trypsin digestion, 15 µl digestion buffer (50 mM NH₄HCO₃) and 1.5 µl reducing buffer (100 mM DTT) were added to 10 µl 0.8 mg/ml kinase sample and the volume was adjusted to 27 µl with epure H₂O. The mixture was incubated at 95°C for 5 min before 3 µl freshly prepared alkylation buffer (100 mM iodoacetamide) was added. This mixture was incubated in dark at
room temperature for 20 min and then 1 ul of sequencing grade modified trypsin (Promega) at 0.1 µg/µl concentration was added and incubated for another 3 hr at 37°C. The trypsin digested samples were analyzed by MALDI-TOF and MS/MS using Q-Star XL quadrupole-TOF tandem mass spectrometer (ABI) in the Protein Facility at Iowa State University.

All NMR spectra were acquired at 25°C on a Bruker AVII700 spectrometer with a 5 mm HCN z-gradient cryoprobe operating at ^1H frequency of 700.13 MHz using standard experiment protocols (Bruker pulse program hsqctgsp.2). For the NMR titration experiment, Src SH3 ligand peptide (VSLARRPLPPLP, GenScript) was added stepwise to 135 µM [¹³C] reductively methylated Src 3K. [¹H, ¹³C] HSQC spectra were collected at the molar ratio of 1:0, 1:0.5, 1:1, 1:2, 1:3, 1:5.5, 1:10 between Src 3K and SH3 ligand peptide. Likewise, Src SH2 ligand peptide (Caffeic acid-pYEEIE, Tocris Bioscience) was then added stepwise to the [¹³C] reductively methylated Src 3K sample saturated with 10 molar equivalent SH3 ligand peptide. [¹H, ¹³C] HSQC spectra were collected at the molar ratio of 1:0.5, 1:1 and 1:6 between Src 3K and SH2 ligand peptide.

For determination of Src kinase activity, we measured the initial velocity of Src KD and Src 3K toward a generic peptide substrate. In this assay, 50 nM Src KD or 500 nM Src 3K was incubated with poly (Glu, Tyr) substrate at 2 mg/ml concentration in reaction buffer (20 mM HEPES pH 7.0, 10 mM MgCl2, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc, 200 µM ATP and 5 µCi γ [³²P]-ATP (Perkin Elmer)) at room temperature. 10 µl of the reaction mixture was removed and mixed with 5 µl 8 M guanidine hydrochloride to terminate the reaction, after 5 min, 10 min and 20 min reaction time respectively. 10 µl of this mixture was spotted onto Whatman™ grade
P81 paper disc (GE healthcare) and was washed 3 times with 0.1% phosphoric acid and 1 time with 95% ethanol. The radioactivity incorporated on poly (Glu, Tyr) was quantified by scintillation counting. Each assay was performed in duplicate.

Acknowledgements

We would like to thank Dr. Thomas E. Smithgall for the generosity of sharing the Src constructs. This work is supported by grants from the National Institutes of Health (National Institute of Allergy and Infectious Diseases, AI043957 and AI075150) to A.H.A.

References


**Figure captions**

Figure 1. Chemical reaction of $^{13}$C reductive methylation on lysine side chains, in which NH$_3$-BH$_3$ serves as the reducing agent.

Figure 2. Cartoon illustration of the conformational difference between the inactive state and the active state of Src kinase.

Figure 3. (A) Unmethylated and $^{13}$C reductively methylated Src kinase domain (Src KD) and Src SH3-SH2-kinase domain (Src 3K) were resolved on SDS-PAGE gel. In each case, the $^{13}$C methylated sample migrated slightly slower than the unmethylated sample, indicating an increase of the molecular weight. Src KD construct contains 16 lysines and Src 3K construct contains 27 lysines. Together with the N-terminus which can also be modified by reductive methylation procedure, it translates into a molecular weight increase of 510 Da and 840 Da for Src KD and Src 3K respectively, provided that all lysines are fully dimethylated. (B) $^{13}$C methylated Src KD and Src 3K were analyzed by MALDI-MS and MS/MS after trypsin digestion. Shown is the MS of a representative tryptic peptide (VAIKTLKPGTMSPEAFLQEAQVMKKLR) with expected m/z = 3134.8431 for all four lysines in this peptide $^{13}$C dimethylated. MS/MS confirmed the identity of this peptide. (C) Superimposed CD spectra of Src KD and Src 3K before and after $^{13}$C reductive methylation.
Figure 4. (A) Full view of \([^{1}H, ^{13}C]\) HSQC spectrum of \([^{13}C]\) methylated Src 3K. NMR signals arisen from the buffer are boxed and designated. The monomethylated lysine region is boxed and designated as MM and the dimethylated lysine region is boxed and designated as DM. Based on the intensity of the peak resonances, the majority of lysine residues are dimethylated. The dimethylated region can also be generally classified into two groups, one with degenerate \(^1H\) chemical shift representing dimethyls on the solvent exposed lysines and the other with better resolved peaks representing dimethyls on lysines with distinct chemical environment. Structurally and functionally important lysines are expected to give rise to dimethyl peaks in the resolved region. (B) Superimposed DM region in the \([^{1}H, ^{13}C]\) HSQC spectra of \([^{13}C]\) methylated Src 3K and \([^{13}C]\) methylated Src KD. (C) Assignment of methyl peaks on the \([^{1}H, ^{13}C]\) HSQC spectrum is achieved by mutating the lysine candidate into other amino acid residue inert to reductive methylation chemistry and then comparing the mutant spectrum with the wild type spectrum to examine whether the methyl peak of interest is missing. Using this approach, we managed to assign two methyl peaks, with \(^1H\) chemical shift \(\approx 2.49\) ppm and \(\approx 2.22\) ppm, to K295 and K315 in Src respectively.

Figure 5. (A) SH3 ligand peptide (VSLARRPLPPLP) was stepwise titrated into 135 \(\mu\)M \([^{13}C]\) methylated Src 3K NMR sample and \([^{1}H, ^{13}C]\) HSQC spectra were collected at each titration point. Spectral changes on the K295 methyl peak and K315 methyl peak were monitored. (B) The difference in the chemical environment of K295 and K315 between the inactive Src (PDB: 2SRC) and the active Src (PDB: 1Y57). (C) Upon saturating the \([^{13}C]\) methylated Src 3K with SH3 ligand peptide, SH2 ligand peptide (Caffeic acid-pYEEIE) was further titrated into the same NMR sample and additional spectral changes were monitored. (D) When the \([^{13}C]\) methylated
Src 3K is saturated with both SH3 ligand peptide and SH2 ligand peptide, judged by no more visible change on the NMR spectrum upon addition of excess ligands, the spectrum at the endpoint of the titration was found perfectly superimposed with that of the [\(^{13}\)C] methylated Src KD. (E) Src 3K with a phosphorylated tail is expected to be in the autoinhibitory conformation (left). Upon binding of SH3 ligand to Src SH3 domain (red) and SH2 ligand to Src SH2 domain (magenta), Src SH3 domain and Src SH2 domain are released from the backside of the kinase domain (gray) and the kinase domain is free to adopt the kinase active conformation (middle and right). The trend of spectral changes we observed in the titration experiment perfectly correlates with the expected conformational changes associated with Src kinase activation.

Supplemental Figure: The activity of unmethylated and [\(^{13}\)C] methylated Src KD and Src 3K toward a generic peptide substrate, poly (Glu, Tyr), was measured in an in vitro kinase assay, following the procedure outlined in the Material and Methods section.
Figures

Figure 1

Figure 2
Figure 3

(A) Gel image showing unmethylated and F-Cl methylated Src KD and Src 3K.

(B) Mass spectrometry data with peaks at m/z 3135.8723, 3136.8623, 3134.8727, and 3137.8110.

(C) UV-Vis spectra comparing unmethylated and F-Cl methylated Src KD and Src 3K.
Figure 5.
Figure 5 (cont.)
Supplemental Figure
CHAPTER 4. CHARACTERIZATION OF A CURIOUS MUTATION IN
BTK KINASE DOMAIN: THE IMPLICATION ON ALLOSTERIC
REGULATION

A paper to be submitted to the Journal of Molecular Biology

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Abstract

Allostery in enzymes is an intriguing phenomenon that is not yet fully understood in all systems. In this paper, we report the finding of a single mutation, Y617P, in the Btk kinase domain, that not only improves the solubility of Btk constructs expressed in E. coli, but also enhances Btk kinase domain activity in a cooperative manner with another mutation at the gatekeeper position. Our biochemical analysis, coupled with computational methods, favors the hypothesis that Y617P mutation activates Btk by providing extra stabilization to the regulatory spine in the kinase domain. This work therefore reveals a potential allosteric site in Btk kinase domain that small molecules may target to modulate Btk activity.

Introduction
Protein kinases function to add a phosphate group onto a specific residue in their substrates, which serves as a signal to coordinate cellular response to the external stimuli. The temporal and spatial control of protein kinase activity is crucial for cellular viability. Faulty activation of protein kinases is the known cause of multiple disease states, including cancers, developmental defects, immunological and neurological disorders (1-3), and many protein kinases are therefore compelling therapeutic targets. One therapeutic approach currently undertaken is to administer a small molecule, which binds to the protein kinase of interest with high specificity and perturbs its catalytic function. For instance, Imatinib is a very selective inhibitor of Abl tyrosine kinase and is successfully used to treat chronic myelogenous leukemia (4). However, rational design of such protein kinase inhibitors as Imatinib still remains a challenging task and requires an in-depth knowledge of the conformational preferences of a protein kinase and then discovery of a druggable site. The majority of the kinase inhibitors now available target the active site, a deep pocket in between the N- and C- lobes of protein kinases where ATP binds. One potential caveat associated with the active site inhibitors is that specificity of kinase inhibition is difficult to achieve, because all protein kinases share structurally very similar active sites, in order to accomplish catalysis of the same phosphoryl group transfer reaction. An alternative solution to this problem is to identify and target an allosteric site of a protein kinase, which remotely regulates the kinase activity. Small molecules targeting such allosteric sites on protein kinases promises higher selectivity, since the allosteric site does not have as much evolutionary pressure as the active site to maintain kinase functionality and therefore usually more structural diversity can be exploited (5). Also, studying how the allosteric site communicates with the active site of protein kinases would advance our knowledge on the molecular mechanism of protein kinase regulation in general.
Bruton’s tyrosine kinase (Btk) is a Tec family protein tyrosine kinase expressed in B cells and phosphorylates PLCγ2 in the B cell antigen receptor signaling pathway. Btk is physiologically essential for antigen production by B cells. Loss-of-function mutations in Btk lead to a human disorder called X-linked agammaglobulinemia (XLA) and a similar but milder disease called X-linked immunodeficiency (xid) in mice, both with a common phenotype of lack of mature antibodies in circulating bloodstream and the increased susceptibility of host to pathogen infection (6, 7). Because of the crucial role of Btk in the B cell receptor signaling, pharmaceutical companies have put much effort into seeking potent Btk inhibitors to cure diseases such as B lymphocyte malignancy. An irreversible inhibitor of Btk, Ibrutinib, showed promising outcomes in clinical trials and was recently approved by FDA as an anti-cancer drug to treat mantle cell lymphoma and chronic lymphocytic lymphoma (8).

In this paper, we report the discovery of a novel allosteric regulatory site, Y617, in the C-terminal lobe of Btk kinase domain. A proline substitution of Y617 in Btk enhances the catalytic activity of the isolated kinase domain. Interestingly, the activating effect of Y617P mutation is more pronounced when combined with a previously characterized activating gatekeeper mutation T474M. Our biochemical assays and computational simulations comparing Btk kinase domain with and without Y617P mutation suggest that the activation mechanism of Y617P is most likely via stabilization of the regulatory spine, a signature structural motif conserved in the active conformation of all protein kinases. Collectively, our data implicate the C-terminal half of the αG-αH loop, where Y617 of Btk is located, together with the C-terminus of the αF helix and the
N-terminus of the αF-αG loop, forming an allosteric pocket with the potential for small molecules to target and tune Btk activity.

**Results**

*Y617P is a solubilizing mutation of Btk*

Wild type Btk kinase domain, like the kinase domain of other Tec family kinases, suffers from low yield and poor solution behavior when expressed and purified from bacteria (9). Previously reported structural studies on the Btk kinase domain all rely on insect cells for expression (10-12). While working with a set of Btk kinase domain mutants, in which specific residues were mutated to the corresponding residues of Hck, we noticed that one particular mutant, harboring E599P, L614Y and Y617P mutations, showed much higher yield than the wild type Btk kinase domain in a side-by-side batch purification (Fig. 1A). Testing the role of each mutation in the increased solubility of Btk kinase domain, we found that Y617P contributed the most. On an SDS-PAGE gel, it was observed that more than 80% of the Btk kinase domain Y617P mutant that is over-expressed in *E. coli* remained soluble (Fig. 1B), contrasted with only ~10% solubility for the wild type Btk kinase domain (data not shown). With this solubilizing mutation, we now have a robust system to produce sufficient amount of Btk kinase domain and multi-domain Btk constructs containing the kinase domain for structural characterization. For instance, we were able to uniformly 15N label Btk kinase domain expressed in E. coli for NMR study. Shown in the figure is the [\(^1\text{H}, \text{15N}\)] HSQC spectrum of the purified Btk kinase domain Y617 mutant. Peaks on this [\(^1\text{H}, \text{15N}\)] HSQC spectrum show good dispersion on both \(^1\text{H}\) and \(^{15}\text{N}\) dimensions, indicating that this mutant is indeed well folded in solution (Fig. 1C).
**Y617P mutation activates the catalytic activity of Btk kinase domain**

In characterizing the activity of the Btk kinase domain Y617P mutant, we measured the extent of autophosphorylation on its activation loop tyrosine (Y551) by western immunoblotting and its phosphorylation of a generic peptide substrate in a radioactive assay. We found that with the presence of Y617P mutation, Btk kinase domain is more active than the wild type, and interestingly, the activating effect is more pronounced when Y617P is coupled with a previously reported activating mutation T474M at the gatekeeper position (13)(Fig. 2A and 2B). Similar activation was also observed in the kinase domain of Interleukin-2 inducible T cell kinase (Itk), a Btk homolog expressed in T cells, when Y577, the tyrosine corresponding to Y617 of Btk, was mutated to proline in the background of the activating gatekeeper mutation F434M (Fig. 2C and 2D). Our data suggest the cooperativity between a mutation at the Y617 site of Btk and a mutation at the gatekeeper position in enhancing kinase activity. This phenomenon is rather intriguing, considering that Y617 of Btk is located at the bottom of the kinase C-lobe, not only distant from the active site but also over 30 Å away from the active site and the gatekeeper residue (Fig. 2E).

Y617 in Btk has been previously identified as a phosphorylation site in B cells (14). Phosphorylation on Btk Y617 site was reported to selectively suppress the capability of Btk to phosphorylate PLCγ2 and accordingly dampen the downstream calcium flux. With our finding that Y617P increases the activity of Btk kinase domain, it would be a sound hypothesis that the activation effect arises from removal of a putative down-regulatory phosphorylation site. To test this hypothesis, we mutated Y617 to a phosphomimetic glutamate or a non-phosphorylatable phenylalanine in the isolated Btk kinase domain in the context of T474M mutation. Then, their
activity was measured and compared with that of T474M/Y617P double mutant (Fig. 2A and 2B). Surprisingly, T474M/Y617E mutant shows a similar level of phosphorylation on both Y551 in the activation loop and the peptide substrate as T474M/Y617P double mutant, while Y617F mutation shows no activating effect. This suggests that phosphorylation on Y617, at least in the context of the isolated Btk kinase domain, does not adversely affect its catalytic activity. The discrepancy with the observation reported in the literature is presumably attributed to the different role of this tyrosine in full-length Btk from that in the isolated Btk kinase domain and the different substrates used to test Btk activity in the assay, full length PLCγ2 in the cellular context versus the activation loop tyrosine of Btk and a peptide substrate in in vitro assay.

The activating effect of Y617P is not due to improved substrate binding

To better understand the molecular mechanism how Y617P activates Btk kinase domain, we measured and compared the kinetic parameters of Btk kinase domain T474M mutant and T474M/Y617P double mutant toward the generic peptide substrate and ATP (Fig. 3A-E). $K_m$ toward the peptide substrate does not change with Y617P mutation present, indicating that this mutation does not alter the capability of Btk to bind its substrate for phosphorylation. Accordingly, the conformation of the substrate binding loop is most likely unperturbed. However, it is interesting to notice that Y617P mutation increases $K_m$ toward ATP, which is counter-intuitive for an activating mutation. The activating effect of Y617P is solely reflected in a ~4 fold increase of $k_{cat}$, toward both ATP and the peptide substrate.

The activating effect of Y617P is not dependent on the activation loop tyrosine phosphorylation
We next examined whether the Y617P mutation changes known structural features essential for kinase catalytic activity. Phosphorylation on the activation loop is known as a prerequisite for many protein kinases to achieve their fully active state (15-17). In fact, a previous study suggests that the activity difference between Btk kinase domain and full-length Btk is mainly due to the difference in the phosphorylation level on the activation loop (18). Hence, we speculate that the activating Y617P mutation may enhance the flexibility of the activation loop of Btk and results in a higher extent of phosphorylation on Y551. To test this hypothesis, we created a panel of kinase-dead Btk kinase domains, with or without Y617P mutation in both the wild type and the T474M background, and examined the flexibility of their activation loop by subjecting them to an upstream kinase Lck phosphorylation (Fig. 4A). As the activation loop of Btk has to be in the open conformation to be accessible to the active site of Lck, the phosphorylation level on the activation loop Y551 correlates with the frequency of the activation loop sampling the open conformation. On the western blot, little variation in the Y551 phosphorylation level was observed, if any, between the Btk constructs with and without Y617P. This suggests that enhancing the activation loop flexibility is unlikely to be the dominant mechanism by which Y617P activates Btk kinase domain. Then we proceeded to directly test whether the activation loop tyrosine phosphorylation is necessary for the activating effect of Y617P. Here, we mutated Y551 into phenylalanine in the Btk kinase domain T474M/Y617P double mutant and measured its activity toward the peptide substrate (Fig. 4B). Removal of the phosphorylation site from the activation loop in this Btk kinase domain mutant had almost no effect on kinase activity, indicating that similar extent of activation can be achieved by Y617P in the absence of the activation loop phosphorylation. The independence of activation loop tyrosine phosphorylation for kinase activity is not entirely surprising here because it has been reported in
the Btk kinase domain with lone T474M mutation (13). Yet, this experiment supports the notion that the activating effect of Y617P is not caused by increasing the extent of phosphorylation on the activation loop.

The activating effect of Y617P requires an intact regulatory spine

An intact regulatory spine is another hallmark structural feature of active protein kinases. Regulatory spine residues have been previously characterized in Btk and mutation of these spine residues significantly impairs the kinase activity (13). Here, we tested whether this activating Y617P mutation could override the requirement of an intact regulatory spine for maintaining functionality. Btk regulatory spine residues M449, L460, H519, F540 and D579 were individually mutated into alanine in the Btk kinase domain T474M/Y617P double mutant, and their activity toward the peptide substrate was measured in a radioactive assay (Fig. 4C). Consistent with the trend previously observed in the Btk kinase domain with lone T474M mutation, alanine mutation on each of the spine residues except for L460 impairs the kinase catalytic activity. Circular dichroism spectra of all the regulatory spine mutants superimpose well with Btk kinase domain T474M/Y617P double mutant, confirming that the loss of kinase activity is not due to unfolding of the kinase (Supplemental Fig. 1). Hence, activation of Btk kinase domain by Y617P still requires an intact regulatory spine in place.

Y617P and the SH2 domain of Btk are functionally redundant in activating the kinase activity

Btk is a multi-domain protein tyrosine kinase in which its kinase domain is at the very C-terminus. Isolated wild type Btk kinase domain is catalytically inactive and non-catalytic domains in the N-terminal half of the enzyme have been shown to positively regulate kinase
activity. For instance, the SH2-kinase linker significantly enhances the activity of Btk mainly because W395 in this linker packs on top of the regulatory spine in the isolated kinase domain and stabilizes the C-helix in its active ‘αC-in’ conformation (13). We are curious about whether the activating effect of Y617P mutation persists in the Btk constructs including these non-catalytic domains. So we compared the activity of Btk linker-kinase domain (LKD), SH2-kinase domain (2KD), SH3-SH2-kinase domain (32KD) and full length Btk toward the peptide substrate, with and without Y617P mutation in the T474M background (Fig. 4D and 4E). We found that the Y617P mutation in Btk LKD construct achieves the same extent of activation on kinase activity as it does in the isolated kinase domain. On the contrary, as the construct is extended to include the Btk SH2 domain, the activating effect of Y617P mutation was completely lost, as observed in Btk 2KD, 32KD and full length Btk constructs. This suggests that Y617P mutation and the SH2 domain are functionally redundant in activating Btk kinase activity. How the SH2 domain of Btk enhances catalytic activity has yet been elucidated, but it was suggested that SH2 domain might participate in properly positioning the SH2-kinase linker to better stabilize the regulatory spine (19). Therefore, we propose that Y617P mutation activates Btk kinase domain, at least in part, by stabilization of the regulatory spine.

*Molecular dynamics simulation indicates that Y617P mutation stabilizes the regulatory spine*

With no crystal structures of Btk kinase domain T474M mutant and T474M/Y617P double mutant currently available, we sought to gain insight into the mutational effect of Y617P on the structure and dynamics of Btk kinase domain using computational methods. Molecular dynamic simulation was initiated on both Btk kinase domain T474M mutant and T474M/Y617P double mutant, starting from the active conformation of Btk kinase domain. As the two systems
evolve, it is observed that the T474M/Y617P double mutant is comparatively more stable in the starting kinase active conformation. On comparison of the RMSD (time dependent root mean square deviation, which is a measure of the structural change from the original state) of the hallmarks of an active kinase such as the C-helix and the K430-E445 salt-bridge, we found that the T474M/Y617P double mutant shows a much smaller RMSD throughout the course of the simulation than T474M mutant (Fig. 5A and 5C). This is consistent with our observation that T474M/Y617P double mutant samples conformations close to the αC-in state more frequently in the frames of the simulation. We also compared the RMSF (root mean square fluctuation), which is a measure of the time-averaged mobility, of all the residues in the kinase domain between T474M mutant and T474M/Y617P double mutant (Fig. 5B and 5C). One region with major difference locates at the activation segment (residues 543-559), suggesting that the activation segment of T474M/Y617P double mutant is more flexible than that of T474M mutant. In the experimental setting, we observed little, if any, increase in the activation loop flexibility when Y617P is present. As our data clearly indicate that the activating effect of Y617P is not dependent on the activation loop phosphorylation, the flexibility of the activation loop is unlikely to account for the activating effect of Y617P. Most residues in Btk kinase domain share similar extent of mobility between the two mutants, but we noticed that RMSF value of several regulatory spine residues, specifically F540, H519 and L460, is smaller in T474M/Y617P double mutant than in T474M mutant, suggesting less fluctuation of this part of the regulatory spine from the kinase active conformation when Y617P is present. It supports our hypothesis that Y617P mutation stabilizes the regulatory spine of Btk kinase domain. In sum, molecular dynamic simulation comparing the T474M/Y617P double mutant and the T474M mutant of Btk kinase domain successfully captures several structural features indicating that the double mutant
is catalytically more active. Analysis of the simulation data supports the proposed mechanism that Y617P activates Btk kinase domain via stabilization of the regulatory spine.

Discussion

The cooperativity effect in activation of Tec family kinases

We described an interesting cooperativity effect in activating Btk kinase domain between two mutations, Y617P characterized in this paper and the previously documented gatekeeper mutation T474M. The gatekeeper residue T474 is located on the β5 strand in the N-lobe of Btk kinase domain and it controls the accessibility to the hydrophobic pocket in the kinase active site. The gatekeeper residue, in general, is a hotspot for oncogenic mutations in protein kinases, where a small hydrophilic residue such as threonine in the wild type is mutated to a hydrophobic residue such as methionine or isoleucine. Mechanistically, it is suggested that the substitution by a hydrophobic residue at this position promotes the stabilization of an internal structural motif termed the ‘regulatory spine’ close by. Because the perfect alignment of the regulatory spine, which consists of mostly hydrophobic residues in the kinase core, is a hallmark of any active kinase conformation, this mutation at the gatekeeper position makes protein kinases constitutively active. Y617, on the other hand, is located on the αG-αH loop in the C-lobe of the kinase domain and is over 30 Å distant from the gatekeeper residue T474 in Btk. Hence, it is rather intriguing that mutation at Y617 site cooperates with mutation at the gatekeeper residue in activating Btk kinase domain. Such allosteric crosstalk between the Y617 site and the gatekeeper residue is also observed in other Tec family kinases. Proline mutation at Y577 in Itk, the corresponding site to Y617 in Btk, and the gatekeeper mutation F434M in the kinase domain of Itk also manifest cooperativity in activating Itk activity. In addition, unpublished data from our
group suggest a third structural motif, the activation loop, is allosterically coupled to both the
gatekeeper and the Y577 site in Itk. It has been demonstrated that swapping the Itk activation
loop with that of Btk activates the Itk kinase domain (20). A similar cooperative effect in kinase
activation was observed when the Y577P mutation or the gatekeeper mutation F434M was
introduced into this activation loop chimera of Itk (Raji Joseph, unpublished data). Hence, our
work reveals a long-distance communication between the N-lobe and the C-lobe in the kinase
domain of Btk and Itk and these sites orchestrate to impact on kinase catalytic activity. Such
allosteric cooperativity also has been reported in other protein kinases, such as protein kinase A,
where either ATP or peptide substrate binding to PKA has been shown to enhance the affinity for
the other (21). Understanding the dynamics of protein kinases appears to be critical in
elucidating their allosteric regulatory mechanism.

Y617 is a native phosphorylation site in Btk

Y617 has been previously characterized as a down-regulatory phosphorylation site in Btk
in the cellular context. Whereas, in our in vitro assays, mimicking the phosphorylation on Y617
by a glutamate mutation activates the isolated Btk kinase domain. The apparent contradiction on
the role of Y617 phosphorylation in Btk from these two experiments possibly reflects a dual role
of Y617 of Btk. We have previously shown in Itk that the docking interaction surface Itk exploits
to recognize PLCγ1 locates in the G helix region and mutation of Y577 on the αG-αH loop
preferentially decreases phosphorylation of PLCγ1, possibly via altering the position of the G
helix (22). Our data also suggest that the corresponding surface in Btk is highly likely to be
important for its phosphorylation of PLCγ2. Hence, in the cellular context, it is observed that
mutation of Y617 in Btk, the corresponding site of Y577 in Itk, to a glutamate specifically
compromises phosphorylation of PLCγ2 while mutation to a chemically analogous phenylalanine has no effect on cellular signaling. In in vitro assays, we demonstrate that Y617 in the isolated Btk kinase domain, when mutated to proline or glutamate, enhances the kinase activity likely via stabilization of the regulatory spine. How the mutation at Y617 site effects on the regulatory spine remains unclear. It is worth noticing that in the extended Btk constructs containing the SH2 domain, we no longer observed the activating effect of Y617P mutation, implying that the Y617P mutation and the SH2 domain of Btk might activate kinase activity via the same molecular mechanism and therefore be functionally redundant.

Targeting the allosteric site is promising for small molecules to selectively regulate kinase activity

Btk is an appealing pharmaceutical target for curing B cell lymphoma. Recently, an irreversible Btk inhibitor, Ibrutinib, has been approved by FDA as an anti-cancer drug (23). Yet, the majority of kinase inhibitors, including Ibrutinib, target the ATP binding site. Limitations associated with these active site inhibitors include the difficulty to avoid off-target inhibition and the necessity to compete with high level of ATP inside the cell. Targeting the allosteric site in protein kinases then becomes a promising alternative to solve these problems. However, to date, only a few allosteric regulatory sites have been characterized in a limited number of protein kinases. This includes the PIF pocket in protein kinases of the AGC superfamily and S108 site in the β subunit of AMP-activated protein kinase (24, 25). As for Tec family kinases, an SPR based approach was recently applied to Itk and identified several small molecules that showed binding to Itk other than the active site (26). However, where these potential allosteric regulators bind on Itk remains to be answered. Here, our finding that Y617 of Btk, when mutated, could activate
Btk kinase activity suggests that Y617 is likely to represent an allosteric regulatory site. Examination of the surface around Y617 in Btk kinase domain crystal structure reveals a shallow pocket that constitutes of the C-terminal half of the αG-αH loop, the C-terminus of the αF helix and the N-terminus of the αF-αG loop as a potential allosteric site for designing small molecule modulators of Btk (Fig. 6).

**Materials and methods**

*Constructs*

The bacterial expression constructs of Btk kinase domain (residues 396-659), Btk linker-kinase domain (residues 382-659) and Itk kinase domain (residues 356-619) were cloned into pET28a expression vector with a C-terminal hexahistidine tag. The bacterial expression constructs of Btk SH2-kinase domain (residues 270-659) and Btk SH3-SH2-kinase domain (residues 212-659) were cloned into pET20b expression vector with a C-terminal hexahistidine tag. The baculoviral expression construct of full length Btk has been previously described (27). All mutations were introduced by using site-directed mutagenesis kit (Stratagene) and the sequence of all constructs was verified at Iowa State University DNA Synthesis and Sequencing facility. Mouse numbering is used for Itk and Btk throughout the text.

*Protein expression and purification*

All constructs of the His-tagged Btk kinase domain, linker-kinase domain and Itk kinase domain were expressed and purified from ArcticExpress DE3 cells (Stratagene) as described previously (9). All constructs of the His-tagged Btk SH2-kinase domain and SH3-SH2-kinase domain were co-transformed with pCDFDuet vector expressing the phosphatase YopH gene
from *Yersinia* into ArcticExpress DE3 cells. Their expression and purification were the same as that of His-tagged Btk kinase domain. Expression and purification of full length Btk from Sf9 cells have been described elsewhere (27). All purified proteins were aliquoted, flash frozen and stored at -80 °C. All protein preparations were greater than 90% purity as determined by Coomassie Blue staining of the SDS-PAGE gel.

**Kinase assays and western blotting**

*In vitro* kinase assays were performed by incubating purified Btk or Itk proteins in a kinase assay buffer (20 mM HEPES pH 7.0, 10 mM MgCl2, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 µM ATP) at room temperature for 1 hr. The samples were boiled, separated by SDS-PAGE and then Western blotted by anti-Btk pY551 antibody (BD Bioscience) or anti-His (Upstate) as previously described (28). Anti-Btk pY551 antibody detects the phosphorylation on both Btk Y551 and Itk Y511 on the activation segment. Quantitative determination of kinase activity was made by measuring the initial velocity of their phosphorylation on a generic peptide substrate, biotinylated Peptide B (aminohexanoyl biotin-EQEDEPEGIYGVLF-NH2 (Anaspec)) of saturating concentration 400 µM following a radioactive assay procedure previously described (27), with one minor modification that 1 mM Na3VO3 is included in the reaction buffer to inhibit any residual YopH phosphatase activity, which be carried with Btk SH2-kinase domain and Btk SH3-SH2-kinase domain preparation.

**Molecular dynamics simulation**

Btk kinase domain crystal structure (PDB ID: 3K54, cutoff: 396-659), which represents the kinase active conformation, was used as the starting frame. The missing regions in the
electron density map of 3K54 were modeled as follows: residues Lck (PDB ID: 3LCK) and Csk (PDB ID: 1K9A, chain B) were used as templates for residues 435-441 in 3K54 structure, Lck (PDB ID: 3LCK) and Btk (PDB ID: 1K2P) for residues 542-558 using MODELLER. In the simulations, the activation loop Y551 was phosphorylated using the TP2 patch to mimic the physiological state of active kinase state. The T474M and T474M/Y617P mutants were generated using the mutate_model option in MODELLER.

The system was prepared for equilibrium simulation setup as follows: The structures were solvated in a periodic water box with 15 Å buffering distance between protein surface and the box. 150 mM concentration of Na⁺ and Cl⁻ ions were used to charge neutralize the system. Particle Mesh Ewald was used to treat long-range electrostatics and 12 Å was used as cutoff for the short-range electrostatics and Van der Waal interactions. The hydrogen bonds were kept rigid using ShakeH algorithm. The time step used was 2 fs. The water and ions were first minimized for 20 ps and equilibrated for 50 ps, holding the protein rigid. The modeled loops were then minimized to remove any steric clashes. The entire system was then minimized releasing harmonic constraints on the protein heavy atoms and the temperature was raised to 310 K. The system was then equilibrated for 1 ns. All atom unrestrained simulations for 20 ns were run for each mutant kinase domain.

Acknowledgements

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References


**Figure captions**

Figure 1. (A) SDS-PAGE gel comparing the yield of the wild type and the E599PL614YY617P mutant of Btk kinase domain (BtkKD) in a side-by-side batch purification. (B) SDS-PAGE gel of the fractions of Btk kinase domain Y617P mutant in test expression. The majority of proteins over-expressed in E. coli remain soluble. (C) \[^{1}H, {^{15}}N\] HSQC spectrum of 200 \(\mu\)M uniformly {^{15}}N labeled Btk kinase domain Y617P mutant.

Figure 2. (A) The activity of Btk kinase domain wild type and mutants was correlated with the extent of autophosphorylation on their activation loop tyrosine Y551, detected by anti-Btk pY551 antibody using western immunoblotting. The presence of Y617P mutation enhances the activity of Btk kinase domain in both the wild type and the T474M gatekeeper mutation background. Mutation of Y617 to glutamate but not phenylalanine shows a similar activating effect. Anti-His antibody is used to confirm equal amount of enzymes loaded on the SDS-PAGE gel. (B) Initial velocity of Btk kinase domain wild type and mutants toward a generic peptide substrate, peptide B, was measured in an in vitro kinase assay as previously
described, as another indicator of kinase intrinsic activity. (C) and (D) The corresponding
mutation in Itk, Y577P, also activates the activity of Itk kinase domain, as indicated both in
the activation loop tyrosine Y511 phosphorylation on the western blot and in their
phosphorylation on peptide B substrate in \textit{in vitro} kinase assay. (E) Y617 locates in the $\alpha$G-
$\alpha$H loop at the base of the C-lobe in Btk kinase domain (PDB: 3K54) and it is distant from
both the active site and the gatekeeper residue T474M.

Figure 3. (A)-(D) Substrate (ATP (A & B) and peptide B (C & D)) curves of Btk kinase
domain T474M mutant (A & C) and T474M/Y617P double mutant (B & D) were fit to the
Michaelis-Menten equation using GraFit 5. E) Kinetic parameters of Btk kinase domain
T474M mutant and T474M/Y617P double mutant.

Figure 4. (A) Kinase dead version of Btk kinase domain wild type and mutants were
subjected to phosphorylation by upstream kinase Lck in \textit{in vitro} kinase assay. The
phosphorylation level on Y551 was detected by anti-Btk pY551 antibody as a measure of the
flexibility of their activation loop. (B) The activity toward the peptide B substrate was
compared between Btk kinase domain T474M/Y617P double mutant with a native activation
loop and with Y551 mutated to phenylalanine. (C) The activity toward the peptide B
substrate was compared between Btk kinase domain T474M/Y617P double mutant with a
native regulatory spine and with each regulatory spine residue (highlighted in cyan)
individually mutated to alanine. (D) and (E) The activating effect of Y617P mutation was
examined by comparing the activity toward the peptide B substrate between T474M mutant
and T474M/Y617P double mutant in the context of Btk kinase domain (BtkKD, residues
396-659), Btk linker-kinase domain (BtkLKD, residues 382-659), Btk SH2-kinase domain (Btk2KD, residues 270-659), Btk SH3-SH2-kinase domain (Btk3KD, residues 212-659) and full-length Btk.

Figure 5. (A) RMSD value comparison of the structural motifs between Btk kinase domain T474M mutant (red) and T474M/Y617P double mutant (black) along the course of MD simulation. (B) RMSF value comparison of each individual residue in the kinase domain between Btk kinase domain T474M mutant (blue) and T474M/Y617P double mutant (black). (C) Structural motifs examined in the MD simulation are highlighted in Btk kinase domain structure (PDB: 3K54): the N-lobe (residues 396-494 in raspberry), the C-lobe (residues 495-538 and 568-658 in green), the activation loop (A-loop, residues 539-567 in magenta), the regulatory spine (R-spine, residue 449, 460, 519, 540 and 579 in space-filling model in cyan) and the C-helix (residues 439-451 in orange).

Figure 6. Examination of Btk kinase domain surface adjacent to Y617 site reveals a potential binding pocket for allosteric modulators, which consists of the C-terminal half of the $\alpha$G-$\alpha$H loop, the C-terminus of the $\alpha$F helix and the N-terminus of $\alpha$F-$\alpha$G loop.

Supplementary Figure: The CD spectrum of Btk kinase domain T474M/Y617P double mutant superimposed with the spectra of the corresponding regulatory spine mutants (M449A, L460A, H519A, F540A and D579A). The procedure of the CD measurement has been described.
Figures

Figure 1

![Figure 1](image1)

Figure 2

![Figure 2](image2)
Figure 3

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Supplementary Figure

Supplementary Figure

ΔAbsorbance

Wavelength (nm)
CHAPTER 5. GENERAL CONCLUSIONS

Summary

Tec family kinases have been identified since early 1990s (1-3), but the molecular mechanisms that regulate the activity of Tec family kinases have not yet been very well understood. This is partly due to the lack of a crystal structure of any full length Tec family kinase to date. Alternatively, using a combination of biochemical, biophysical and computational approaches in the lab, we begin to understand the molecular details of the protein-protein interactions as well as the conformational changes in Tec family kinases associated with their functionality. This thesis dissertation describes my contribution in this process.

Chapter 2 of this thesis focuses on one important aspect of protein kinase regulation, how the substrate specificity is achieved by protein kinases. Protein kinases are generally exquisitely specific in substrate selection and this fidelity in phosphorylation is essential for cellular viability. Itk, a Tec family kinase expressed in T cells, for instance, specifically phosphorylates PLCγ1 on Y783 in the T cell receptor signaling pathway (4). Our lab previously identified a remote docking interaction between the kinase domain of Itk and the C-terminal SH2 domain of PLCγ1, contributing to a ~20 fold enhancement in the recognition of PLCγ1 Y783 as Itk substrate (5). This docking interaction utilizes a non-canonical mode of interaction mediated by the PLCγ1 SH2 domain, independent of its phosphotyrosine binding capability. Subsequent mapping on PLCγ1 side further confirmed that the docking site, which consists of primarily basic residues on the CD loop and the BG loop of the SH2 domain, is non-overlapping with the pY binding pocket (6). My work
followed up these findings and mapped the docking interaction surface on the Itk kinase domain to the $\alpha$G helix region. Mutation of the residues on this docking interaction surface of Itk compromises the phosphorylation of PLC$\gamma$1 substrate. Thus, the substrate recognition site delineated here provides a novel interface that may be targeted using small molecules to affect Itk function. This substrate docking site is distinct from the kinase active site that is most often exploited for kinase inhibition. It is conceivable that targeting the substrate recognition site of Itk (rather than the active site) is advantageous due to the structural diversity of this site among protein kinases. Targeting a unique site outside of the active site is a strategy to avoid off-targets (i.e. inhibiting other protein kinases). As well, the promise of specifically inhibiting PLC$\gamma$1 phosphorylation while maintaining Itk-mediated phosphorylation of other substrates is appealing. For instance, previous data suggest that in phosphorylating Y180 in Itk SH3 domain, Itk kinase domain utilizes a distinct surface other than the G helix region for the docking interaction (7). Hence, targeting the recognition site on Itk for PLC$\gamma$1 substrate may not affect its autophosphorylation on Itk Y180. This mode of inhibition is desired when only the specific pathway mediated by phosphorylation of a substrate, rather than the full spectrum of the kinase activity, needs to be turned off.

Chapter 3 of this thesis dissertation tackles a challenging experimental problem, how to monitor the conformations of the dynamic protein kinases in solution, by combining an easily applicable protein modification method and solution NMR spectroscopy. Using $[^{13}\text{C}]$ reductive methylation, $[^{13}\text{C}]$ methyl groups can be covalently attached to the primary amines in the unlabeled protein kinases as NMR probes. We demonstrated the applicability of this approach with a prototypical protein kinase Src and showed that the $[^{13}\text{C}]$ methyl groups
attached to the structurally important lysines successfully report on the conformational changes associated with the activation process of Src kinase. This approach has several advantages. Firstly, it provides a rapid approach to examine the conformational preferences of a given protein kinase under different circumstances such as binding to small molecules or allosteric regulators. Moreover, because this approach requires lower protein concentration and no isotopic labeling during protein production, compared with the conventional solution NMR, it can be easily adapted to study both bacterially expressed protein kinases and protein kinases dependent on eukaryotic cells for expression.

Chapter 4 of this thesis dissertation identifies a mutation at an allosteric site, Y617P, in Btk kinase domain that improves the solubility of the Btk construct and enhances its catalytic activity. The mutation has proven its utility in the structural characterization of Btk currently undergoing in the lab. This chapter focuses on dissecting the molecular mechanism of its activating effect. Our biochemical characterization of this mutation led us to a hypothesis that the mutation enhances the kinase activity via stabilization of a structural motif in the kinase core termed the ‘regulatory spine’. Gratifyingly, the molecular dynamic simulation performed independently on this system supported this hypothesis. The discovery of an activating mutation at this allosteric site suggests the possibility of targeting the pocket where Y617 is located to modulate Btk activity. Moreover, the location of this allosteric site Y617 in the kinase C-lobe is interesting as the majority of allosteric site in kinases are located in the smaller N-lobe.
Overall, the work presented in this thesis dissertation adds to our knowledge of the molecular mechanism Tec family kinases exploit to attain substrate specificity and catalytic activity. It also provides an experimental tool with potential to facilitate the structure-function study on not only Tec family kinases but other therapeutically important protein kinases as well.

**Future directions**

*Substrate recognition by Tec family kinases*

Our current knowledge on the docking interaction surface on both PLCγ1 and Itk sets the stage to study the structure of this enzyme-substrate complex in details. Several questions about this complex remain. What is the affinity of the Itk kinase domain and PLCγ1 C-terminal SH2 domain interaction? Our NMR analysis to date did not allow for an unambiguous measurement of affinity since addition of binding partner led to line broadening. Measurement of the affinity of the complex is a prerequisite for the effort in structural characterization. Surface Plasmon Resonance (SPR) is a good technique to use for this purpose because of the small amount of samples required in the measurement. Another unanswered question relates to the relative orientation and the stoichiometry of the Itk kinase domain/ PLCγ1 C-terminal SH2 domain complex. Crystallization of the enzyme-substrate complex still remains a challenge but will be very informative about the molecular details of the docking interaction, while carefully designed biochemical assays will be approachable to gain initial insight on the relative orientation and the stoichiometry of this complex. For instance, we now know that the docking site on PLCγ1 being primarily basic and the complementary surface on Itk being largely acidic. Swapping the charges presented on the
two surfaces by mutagenesis and examining the corresponding effect on the affinity of the complex will help us determine the salt bridge pair, as swapping only the pair that forms a salt bridge in the native complex will have no effect on the affinity. Figuring out several salt bridge pairs in the interface would be helpful in determining the relative orientation of the complex. In terms of the stoichiometry of the enzyme-substrate complex, apart from Itk:PLCγ1 equals 1:1, an alternative model was proposed where the docking interaction with PLCγ1 occurs on one molecule of Itk and Y783 phosphorylation occurs in the active site of another molecule of Itk. Now that we have the docking defective mutant of Itk available, a mixing experiment can be performed to test this latter model. In this experiment, the kinase dead Itk and the docking defective Itk will be mixed and the efficiency of their phosphorylation on PLCγ1 will be measured. If the docking interaction and the phosphorylation event occur \textit{in cis} (i.e. on the same Itk molecule), there will be little phosphorylation detected as both types of Itk mutants are defective in phosphorylating PLCγ1. However, if the docking interaction and the phosphorylation event occur \textit{in trans} (i.e. on two Itk molecules separately), we expect that the kinase dead Itk and the docking defective Itk can compensate the defect in each other and shows detectable level of PLCγ1 phosphorylation.

\textit{[13C] reductive methylation coupled with NMR to examine the conformation of protein kinases}

We demonstrated using Src kinase that the conformational changes associated with protein kinase activation can be monitored by NMR applied to a [13C] reductively methylated sample. The approach allows detection of the different conformations of protein kinases as
they transition between inactive and active states. The method we have developed has multiple applications. For instance, small molecule inhibitors of protein kinases have been classified into different groups. Type I inhibitors are ATP competitive as represented by staurosporin and Type II inhibitors are ATP non-competitive, which could be divided into a subgroup stabilizing the ‘DFG-out’ conformation, represented by Abl inhibitor Imatinib (8), and a subgroup stabilizing the ‘αC-out’ conformation, represented by MEK inhibitor PD334581 (9). It will be interesting to use the approach we proposed to examine whether the [$^{13}$C] methyl groups on the lysine side chains could report on the difference in the conformational preferences imposed by these groups of kinase inhibitors in different kinase systems.

**Y617 as an allosteric regulatory site in Btk**

Based on the biochemical characterization and the molecular dynamic simulation results, we hypothesized that Y617P mutation in Btk activates the kinase via stabilization of the regulatory spine. This hypothesis will be directly tested in a detailed structural analysis, comparing the degree of regulatory spine assembly in the crystal structures and comparing the flexibility of the regulatory spine residue by heteronuclear NOE measurement between Btk kinase domain with and without Y617P mutation. Moreover, our data suggest that the pocket where Y617 is located is potentially an allosteric site regulating Btk activity. Currently, small molecule libraries are screened *in silico* to find initial hits that target this site. Discovery of the small molecule Btk allosteric modulators may have potential in the clinical treatment.
In summary, my thesis work has advanced our knowledge on both the molecular details of substrate recognition in Tec family kinases and the conformational changes associated with activation of Tec family kinases. It also provides valuable tools for detailed structural characterization of Tec family kinases, a solubilizing mutation in Btk that allows our ongoing investigation on the structure of full-length Btk and an NMR method applicable to almost any protein kinases to examine their conformational ensemble in solution.

References


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The appendix includes two pieces of the published work that I was involved in. In the work presented in Appendix A, we defined the residues in the regulatory spine, a conserved structural motif assembled in the interior of any active protein kinases, of Tec family kinases Itk and Btk. We showed that disruption of this regulatory spine by mutagenesis significantly impairs the catalytic activity of Itk and Btk. Moreover, mutation of the gatekeeper residue (Phe434 in Itk and Thr474 in Btk) to a hydrophobic residue (Met) stabilizes the regulatory spine and therefore activates the kinase domain. We found that because the gatekeeper mutation facilitates the pre-assembly of the regulatory spine, this mutant does not depend on phosphorylation of the activation loop tyrosine to be fully active. An electrostatic network switch has been previously established in other kinase systems, which accompanies phosphorylation of the activation loop tyrosine and drives the kinase toward the active conformation. In this electrostatic network switch, the phosphate attached to the activation loop tyrosine forms a salt bridge with a conserved arginine at the N-terminus of the activation loop, with concomitant break up of the interaction between the arginine and a conserved glutamate on the αC helix. Thus, it allows the αC helix to adopt the ‘αC-in’ conformation and a new salt bridge to be formed between the conserved glutamate on the αC helix and a conserved lysine on the β3 strand. Here, I proposed and we demonstrated in Btk that disruption of this electrostatic network by mutating the arginine (R544) in the activation loop facilitates the inward movement of the αC helix and the assembly of the regulatory spine, therefore increasing the activity of Btk kinase domain.
In the work presented in Appendix B, we identified that the activity of Itk and Btk is interchangeable simply by swapping their activation loops. The difference in the dynamics of the activation loop region accounts for the activity differences between Itk and Btk. I contributed to this work in making assignment of several tyrosine peak resonances on the $^{[\text{1}H, \text{15}N]}$ TROSY (Transverse Relaxation-Optimized Spectroscopy) HSQC spectrum of the $^{15}$N-Tyr selectively labeled Btk kinase domain for interpreting the backbone $^{1}H-^{15}N$ Heteronuclear NOE data.
APPENDIX A. IDENTIFICATION OF AN ALLOSTERIC SIGNALING NETWORK WITHIN TEC FAMILY KINASES


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ABSTRACT

The Tec family kinases are tyrosine kinases that function primarily in hematopoietic cells. The catalytic activity of the Tec kinases is positively influenced by the regulatory domains outside of the kinase domain. The current lack of a full-length Tec kinase structure leaves a void in our understanding of how these positive regulatory signals are transmitted to the kinase domain. Recently, a conserved structure within kinases, the ‘regulatory spine’, has been identified that assembles and disassembles as a kinase switches between its active and inactive states. Here we define the residues that comprise the regulatory spine within Tec kinases. Compared to previously characterized systems, the Tec kinases contain an extended regulatory spine that includes a conserved methionine within the C-helix and a conserved tryptophan within the SH2-kinase linker of Tec kinases. This extended regulatory spine forms a conduit for transmitting the presence of the regulatory domains of Tec kinases.
to the catalytic domain. We further show that mutation of the gatekeeper residue at the edge of the regulatory spine stabilizes the regulatory spine resulting in a constitutively active kinase domain. Importantly, the regulatory spine is preassembled in this gatekeeper mutant rendering phosphorylation on the activation loop unnecessary for its activity. Moreover, we show that the disruption of the conserved electrostatic interaction between Btk R544 on the activation loop and Btk E445 on the C-helix also aids in the assembly of the regulatory spine. Thus, the extended regulatory spine is a key structure that is critical for maintaining the activity of Tec kinases.

**Keywords:** Itk and Btk; regulatory spine; kinase activation; gatekeeper residue; phosphorylation

**INTRODUCTION**

Protein kinases catalyze the transfer of a phosphate group from ATP to a hydroxyl containing amino acid side chain; either Ser/Thr for serine-threonine kinases or Tyr for tyrosine kinases\[^1\]. The activity of protein kinases is exquisitely regulated within the cell\[^1; 2; 3; 4\]. The switch from the catalytically inactive state of a kinase to the active state is often accompanied by the phosphorylation of a key residue within the activation loop, a large flexible loop that lies between the two lobes of the kinase domain structure\[^1\]. Phosphorylation of the activation loop residue can trigger concerted movements in other mobile elements within the kinase domain such as the C-helix and the DFG motif that brings about the assembly of the catalytically critical residues in the kinase active state\[^1\]. The active state conformations associated with different kinases are nearly identical across the many kinases for which high resolution structures have been solved\[^2; 3; 5; 6; 7\]. It is not
surprising that the active states of distinct kinases are very similar since the phospho-transfer chemistry carried out by different kinases is the same. In contrast, it is becoming clear that the structural features associated with the inactive state of various kinases differ widely.

The search for features that are conserved within the structures of active kinases has recently led to the identification of a structure termed the ‘regulatory spine’\(^8; 9\). The regulatory spine was first identified by Local Spatial Patterns alignment analysis using a set of serine-threonine and tyrosine kinase structures that included PKA as a model kinase\(^8; 9\). The regulatory spine defines a stretch of amino acid residues that is assembled only in the active state of kinases. Unlike consensus sequences that consist of a continuous stretch of amino acids in the primary structure, the regulatory spine consists of disparate residues that span the N- and C-terminal lobes of the kinase domain. Assembly of the regulatory spine has been proposed to be a crucial step in the activation of protein kinases and this structure is disrupted in structures of kinases in the inactive state. Moreover, the regulatory spine has been proposed as the mechanism by which allosteric effects can be propagated to the kinase active site\(^8; 9\).

The Tec kinases are immunologically related tyrosine kinases which consist of five mammalian members: Itk, Btk, Tec, Txk and Bmx\(^10; 11\). Tec kinases share similar domain architecture as Src, Abl and Csk family of kinases, in that they have the SH3-SH2-kinase domain cassette\(^10\). Despite domain similarities, we and others have shown that the regulation of Tec kinases is distinct from Src and Abl kinases\(^10; 12; 13; 14\). Unlike the Src and Abl kinases, the N-terminal regulatory domains of Tec kinases positively regulate the activity of the kinase domain\(^14\). While the isolated kinase domains of Src and Abl are active, the isolated kinase domains of the Tec family exhibit poor catalytic activity\(^13; 14; 15\). Regulation
of the Tec kinases is in fact more similar to the Csk family of enzymes, whose N-terminal regulatory domains are essential for the catalytic activity of the kinase domain\cite{16}. Moreover, while the structures of full-length Src and Csk kinases are available, the structure of a full-length Tec kinase remains elusive\cite{2, 17, 18}. This leaves a significant gap in our mechanistic understanding of the regulation of the Tec family kinases.

In this manuscript, we define the regulatory spine within the Tec family kinases and show that mutation of the spine residues leads to different effects in the context of the isolated kinase domain versus full-length Itk. Moreover, we show that the assembly of a stable regulatory spine within members of the Tec kinase family is critically dependent on the presence of the SH2-kinase linker region. The residues that comprise the regulatory spine within PKA are insufficient to promote the assembly of a stable regulatory spine within the Tec kinases. We extend the Tec regulatory spine to include a conserved methionine within the C-helix and a conserved tryptophan residue within the SH2-kinase linker of Tec kinases. Together with the spine residues originally identified within the PKA kinase domain, the conserved methionine and tryptophan residues form a continuous structure that links the kinase active site to the N-terminal regulatory domains of Tec kinases. Furthermore, we show that the stabilization of the regulatory spine by the gatekeeper threonine to methionine mutation eliminates the need for phosphorylation on the activation loop for Btk kinase activity. Thus, the extended regulatory spine is a structure that is critical for the regulation of Tec kinases and if stabilized appropriately, is sufficient for activation in the absence of activation loop tyrosine phosphorylation and in the absence of the non-catalytic Tec regulatory domains.
RESULTS

The regulatory spine controls Tec kinase activity.

The regulatory spine within PKA consists of five residues: L95, L106, Y164, F185, and D220\[^9\]. These residues are spread throughout the primary sequence of the PKA kinase domain: L95 is located on the C-helix, L106 is on the N-terminus of the b4 strand, Y164 is part of the ‘HRD’ motif, F185 is from the ‘DFG’ motif and D220 is on the F-helix within the C-terminal lobe\[^9\]. Alignment of the structure of the Btk and Itk kinase domains with that of PKA shows that the corresponding regulatory spine residues within the Btk and Itk kinase domains should consist of: Btk M449, L460, H519, F540 and D579 and Itk M409, L420, H479, F500 and D539 respectively (Fig. 1a).

To test the role of the predicted spine residues in the Tec kinases, we wished to take advantage of the rapid bacterial expression and purification system that has been developed for the isolated kinase domains of Itk and Btk\[^19\]. One issue that arises, however, is the fact that the isolated kinase domains of the Tec kinases exhibit poor catalytic activity and so the expected loss of function mutations in the regulatory spine would be difficult to characterize given the already low activity of the wild type kinase domains. In order to take advantage of the ease of the bacterial expression system, we needed an isolated Btk and Itk kinase domain with higher catalytic activity. It has been demonstrated previously that introduction of a bulky hydrophobic residue such as isoleucine or methionine at the gatekeeper position activates multiple kinases\[^20\]. Indeed, we find that the Itk F434M and Btk T474M isolated kinase domains are more active when compared to wild-type isolated kinase domain (manuscript in preparation). We therefore probed the importance of the predicted regulatory spine residues in Itk and Btk by mutating them individually to alanine in the context of the
Itk F434M and Btk T474M isolated kinase domain mutants. Activity measurements are carried out by monitoring the phosphorylation levels of a peptide substrate in a radioactive assay or by detecting the level of autophosphorylation on the activation loop tyrosine (Y511 in Itk and Y551 in Btk) by western immunoblotting. While phosphorylation of the activation loop tyrosine is achieved by the activity of the Src family kinases Lck and Lyn respectively in vivo, under in vitro conditions Tec kinases autophosphorylate on the activation loop tyrosine[21; 22; 23].

Disruption of the regulatory spine residues by mutation to alanine is predicted to disrupt kinase activity. Indeed, mutation of Btk M449, H519, F540 and D579 and Itk M409, H479, F500 and D539 to alanine within the isolated kinase domain of Itk F434M and Btk T474M leads to decreased phosphorylation on the activation loop tyrosine and drastically reduces the catalytic activity (Fig. 1b, c, d and e). Itk kinase residues such as M409, F500 and H479 are highly conserved with other kinases with well-defined roles in catalysis[8]. Itk M409 is involved in binding the substrate ATP[12]. Itk F500 is part of the ‘DFG’ motif at the start of the activation loop segment and is responsible for stabilizing the conformation of the preceding aspartate, and the C-helix[8]. Itk H479 is part of the ‘HRD’ motif where it serves as a scaffold for D499 and F500[8]. It is therefore not surprising that mutation at these sites leads to a loss in activity. However, Itk D539 is not part of any previously characterized regulatory motif and therefore the drastic reduction in Itk activity upon mutation of this residue to alanine highlights the importance of the regulatory spine in regulating Itk activity. Moreover, since the regulatory spine residues are conserved within the Tec kinase family, this structure would be predicted to be critical for regulating the activity of all Tec kinase family members.
Unexpectedly, mutation of Itk L420 and Btk L460 to alanine, which based on the previous PKA work is predicted to be part of the regulatory spine, failed to inactivate the isolated kinase domains of Itk F434M or Btk T474M (Fig. 1b, c, d and e). In fact, the isolated kinase domains of both Itk L420A/F434M and Btk L460A/T474M double mutants showed an increase in activity when compared to the isolated kinase domain of Itk F434M and Btk T474M, respectively. To ensure that the activating effect of the Leu to Ala mutation was not an artifact of working with the activated (gatekeeper mutant) isolated kinase domains of Itk and Btk, the regulatory spine residues were mutated to alanine in the context of wild-type isolated kinase domains of Itk and Btk. Since the catalytic activity of the isolated kinase domain of Tec kinases is generally poor, the detection of activation loop phosphorylation levels by western immunoblotting is difficult and for Itk, in particular, phosphorylation Y511 is below the detection limit by western immunoblotting. Hence, the activity of the wild-type and regulatory spine mutants of the isolated kinase domains of Itk and Btk are monitored by their ability to phosphorylate a peptide substrate in the radioactive assay.

The results of the regulatory spine mutations in the context of the wild type kinase domains mirror the results for the activating gatekeeper mutants described above. Mutation of Btk M449, H519, F540 and D579 to alanine within the isolated wild type kinase domain of Btk leads to decreased catalytic activity (Fig. 1f). Circular dichroism spectra of the Btk mutants overlay well with that of wild-type Btk showing that the decreased activity of the Btk mutants are not due to unfolding of the kinase (Supp. Fig. 1). Since the activity of the isolated Itk wild-type kinase domain is not significantly above background levels, the resulting activity of the Itk spine mutants: Itk M409A, H479A, F500A and D539A can not be interpreted except to say activity is no greater than wild type (Fig. 1g). However, consistent
with our earlier results with the gatekeeper mutant, the Itk L420A and Btk L460A mutants are both more active than wild-type isolated kinase domain of Itk and Btk, respectively (Fig. 1f and g). Interestingly, previous studies on the regulatory spine within the Abl kinase have shown that mutation of Abl L320 to glycine (corresponding to Itk L420 and Btk L460), leads to only a slight decrease in kinase activity of full length Abl\textsuperscript{[20]}. This is also consistent with modeling studies of Abl that have shown that Abl L320 has a modest impact on regulatory spine formation\textsuperscript{[20]}. Thus, Itk L420 and Btk L460 do not play a major role in the assembly of the regulatory spine within the context of the isolated kinase domain of Itk and Btk.

**The regulatory spine is not assembled in the structure of phosphorylated Itk kinase domain.**

Activation of Tec kinases requires the phosphorylation of a conserved tyrosine within the activation loop\textsuperscript{[12; 21; 23]}. Separate high-resolution structures of the Btk kinase domain with Y551 either phosphorylated (active) or unphosphorylated (inactive) have recently been reported\textsuperscript{[24]}. A comparison of the inactive and active states of Btk kinase domain shows clear differences in the region of the regulatory spine (Fig. 2a). The regulatory spine residues assemble into a linear arrangement in the structure of the active Btk kinase, while interactions between the regulatory spine residues (in particular L460, M449 and F540) are disrupted in the structure of inactive Btk kinase. The structural differences in this region between active and inactive Btk are consistent with the role of the regulatory spine as defined previously for PKA (Fig. 2a).

Crystal structures of the isolated kinase domain of Itk that is either unphosphorylated or phosphorylated on Y511 in the activation loop are also available\textsuperscript{[12]}. Surprisingly, the
available Itk kinase domain structures overlay quite well, with little or no conformational differences between them regardless of the phosphorylation state of the activation loop. Inspection of the Itk kinase domain structures shows that the regulatory spine residues in the Itk kinase domain structures adopt a disrupted configuration similar to the inactive Btk kinase domain (Fig. 2b). An additional hallmark of an active kinase domain structure is the formation of a crucial ion pair between a conserved lysine positioned within the b3 strand of the kinase domain and a conserved glutamate on the C-helix\[^1\]. The distance between the corresponding residues (Itk K390 and E405) within the structures of both phosphorylated and unphosphorylated Itk kinase domain is on the order of 8.0 Å which is significantly greater than the 3 to 4 Å distance that is observed in the structures of other active kinases (Fig. 2b)\[^12\]. Indeed, the distance between the same conserved ion pair within Btk, (Btk K430 and E445) changes from 14.6 to 3.8 Å upon activation of the kinase (Fig. 2a). These observations suggest that both the phosphorylated and unphosphorylated Itk kinase domains adopt a conformation consistent with the ‘inactive’ enzyme. The inability of the Itk kinase domain to fully assemble into an active conformation, despite being phosphorylated on Y511 in the Itk activation loop, points to additional requirements for activation.

A notable difference between the Itk and Btk crystal structures is that the construct used for the crystallization of the Btk kinase domain included the SH2-kinase linker, while that of the Itk kinase did not (Fig. 2a & b)\[^12; 24\]. We hypothesize that the absence of the SH2-kinase linker within the Itk construct used for crystallography might prevent the assembly of a stable regulatory spine in the kinase domain despite phosphorylation on the activation loop Y511.
Extension of the Tec kinase regulatory spine.

We have previously shown that the SH2-kinase linker, the 17 amino acids between the SH2 domain and the kinase domain, is critical for the activity of both Btk and Itk, exerting a positive effect on the catalytic function of the kinase domain[14]. More specifically, a conserved tryptophan (Itk W355, Btk W395) in the SH2-kinase linker, as well as a methionine residue in the C helix (Itk M410, Btk M450) are crucial residues in the Tec family kinase regulatory apparatus (Fig. 3a)[14]. Like the regulatory spine mutants described in Figure 1, point mutations of Itk W355, Btk W395 or Itk M410 to alanine all result in a significant drop in catalytic activity (Fig. 3b). Examination of the crystal structures of Btk shows that Btk W395 and Btk M450 are located at the ‘top’ of the regulatory spine and, in the active Btk structure, serve to extend the hydrophobic packing of the spine residues well into the N-terminus of the kinase domain (Fig. 3c & d). In the inactive Btk structure, repositioning of the W395 and M450 side chains disrupts the extended regulatory spine (Fig. 3c & d) in much the same manner that repositioning of M449 in the inactive Btk structure disrupts the core of the spine (Fig. 2a). The active and inactive Btk structures consist of the same amino acids (residues 382-659) and differ with respect to the activation loop tyrosine (Y551). In the active structure the activation loop tyrosine is mutated to glutamate (Y551E) to mimic phosphorylated Btk, and for the inactive structure wild type Btk in the unphosphorylated form was used for crystallization[24].

Compared to the regulatory spine that has been defined for PKA[9], the Tec kinase family requires at least two additional residues (Itk W355 & M410) to fully assemble the regulatory spine. The tryptophan residue is located in the SH2-kinase linker region providing an explanation for the positive regulatory role of this non-catalytic region[14]. The available
Itk kinase domain structures are entirely consistent with this finding; without the contribution of the SH2-linker region, the regulatory spine of Itk does not assemble into the active conformation. As a result, the structures of the phosphorylated and unphosphorlated Itk kinase domain fragments are very similar to each other and neither resembles an active kinase. Future structures of Tec kinases with and without the SH2-kinase linker in various activation (phosphorylation) states will be required to fully probe this hypothesis.

**Itk L420 and Btk L460 are essential for the formation of the extended regulatory spine within full-length Tec kinases.**

The conserved tryptophan in the SH2-kinase linker is critical for the activity of full-length Tec kinases\(^{[14]}\). Upon activation, the tryptophan side-chain forms part of a hydrophobic pocket that is lined by the conserved methionine on the C-helix and leucine on the b4 strand (Itk M410, L420 and Btk M450, L460) (Fig. 3d). Although Itk L420 and Btk L460 were shown to not be a part of the regulatory spine in the context of the isolated kinase domain (Fig. 1), the structure of the Btk kinase domain (which contains the SH2-kinase linker region) suggests that this leucine would be critical for the formation of the extended regulatory spine in the context of the full-length kinase. We therefore tested the role of this leucine in the context of full-length Itk.

As shown in Figure 3e, mutation of Itk L420 to alanine within the context of full-length Itk disrupted the activity of Itk. Consistent with our earlier results, mutation of Itk M409, F500 and D539 to alanine within full length Itk also inactivated the kinase. Problems with cloning prevented us from testing the activity of the full-length Itk H479A mutant. Together, Itk M409, L420, H479, F500, D539, M410 and W355 form a conserved allosteric
signaling network that is absolutely required for the activity of Itk. Based on sequence conservation, the extended regulatory spine defined here likely controls the activity of the entire Tec kinase family.

**Phosphorylation on the activation loop tyrosine is not required for the activity of the Btk T474M mutant.**

It is well established that activation loop phosphorylation is required for activation of tyrosine kinases\(^1\)\(^-8\) and specifically for the Tec kinases, phosphorylation on the activation loop tyrosine results in at least a ten-fold increase in activity\(^21\)\(^-25\). Structures of active and inactive kinase domains from a number of different kinases demonstrate a conserved electrostatic network that switches between the active and inactive conformations (Fig. 4a)\(^1\)\(^-8\). In the inactive state of Btk, the conserved glutamate (Btk E445) on the C-helix (of the Lys-Glu ion pair) is associated with a conserved arginine (Btk R544) on the activation loop\(^24\)\(^-26\). Activation of Btk by phosphorylation on Btk Y551 (the activation loop tyrosine) leads to a specific interaction between pY551 and R544, and a concomitant loss of the association between the R544 and E445\(^24\)\(^-26\). Btk E445 on the C-helix then swings toward the kinase active site and associates with the conserved lysine side chain (Btk K430) to bring about the assembly of the active state of the kinase\(^24\)\(^-26\). The conformational adjustment of the C-helix brings M449 and M450 in line with the other residues of the regulatory spine (Fig. 4a). Thus, phosphorylation on the activation loop tyrosine is an initiating step in the process of regulatory spine assembly.

Since mutation of the gatekeeper residue to methioine appears to stabilize the regulatory spine within Btk, we next probed the requirement of activation loop
phosphorylation in the context of the T474M gatekeeper mutation. The activity of the BtkT474M/Y551F double mutant was compared to that of the Btk T474M mutant by monitoring phosphorylation of an exogenous substrate. We find that mutation of the activation loop Btk Y551 to phenylalanine has no effect on the activity of the Btk T474M mutant (Fig. 4b & c). The Btk T474M/Y551F double mutant and the Btk T474M single mutant are both active, whereas the wild type Btk kinase requires activation loop phosphorylation; the Btk Y551F single mutant exhibits poor activity (Fig. 4b). These data are consistent with the idea that phosphorylation on the activation loop tyrosine initiates the assembly of the regulatory spine by disrupting the association between Btk E445 and Btk R544. Once pY551 competes with E445 for association with R544, the E445/K430 salt bridge is formed, bringing the C-helix (and importantly M449) into the regulatory spine structure. Under conditions that pre-organize the regulatory spine structure (as in the Btk T474M mutant), phosphorylation on the activation loop Y551 is no longer required to trigger the conformational changes that accompany Btk activation.

We further tested the importance of this switched electrostatic network by mutating Btk R544. We reasoned that mutation of Btk R544 will activate the kinase as inward movement of the C-helix is restrained by the E445:R544 interaction and loss of this electrostatic interaction should facilitate movement of the C-helix. Indeed, mutation of Btk R544 to serine in the context of wild-type isolated kinase domain of Btk leads to a two-fold increase in activity (Fig. 4d & e). In contrast, mutation of Btk R544 to serine in the context of Btk T474M isolated kinase domain does not further activate the kinase (Fig. 4d & e). These results again suggest that pre-assembly of the kinase regulatory spine (by mutation) can overcome the regulatory interactions that normally control kinase activity. Thus, for the
wild-type kinase under physiological conditions, phosphorylation on the activation loop sets into motion a cascade of events: (1) formation of the pY551:R544 interaction with concomitant disruption of the Btk E445:R544 interaction, (2) inward movement of the C-helix and formation of the E445:K430 interaction and (3) assembly of the regulatory spine, that culminates in the formation of an active kinase (Fig. 4a).

DISCUSSION

The structures of the isolated kinase domain of Btk in both the active and inactive states have provided significant insight into Tec kinase activation[24; 26]. However, the lack of a full-length structure of any Tec kinase leaves numerous unanswered questions regarding the regulation of Tec kinases. While the SH2 domain and the SH2-kinase linker region of Tec kinases have been shown to be required for the activity of Tec kinases[12; 13; 14], the mechanism by which these regulatory domains positively influence catalytic activity has not been clear. Here we identify an intramolecular allosteric signaling network that extends from the SH2-kinase linker into the kinase domain of Tec kinases. This intramolecular connectivity defines how the non-catalytic Tec regulatory domains, through the SH2-kinase linker region, impinge on the kinase domain and stabilize the regulatory spine. In the absence of the Tec regulatory domains, whether achieved by deletion or conformational changes within the full-length molecule, the regulatory spine is disrupted and kinase activity is inhibited.

Comparing our results to those previously published for PKA shows that the Tec kinases require an extended spine structure and it is this longer regulatory spine that couples kinase activity to regions outside of the kinase domain. Specifically, a conserved methionine
in the C-helix and a conserved tryptophan in the SH2-linker swing in to ‘cap’ the spine in the active Btk structure and are critical for the activity of the Tec kinases. Mutation of these extended spine residues disables kinase activity even if the ‘core’ residues defined for PKA are intact.

Further support for the importance of the entire regulatory spine comes from examination of the genetic mutations in Btk that are associated with X-linked agammaglobulinemia (XLA) in humans, a disease that is characterized by impaired B cell development[27]. Several of the extended regulatory spine residues identified in this study are mutated in XLA patients[27]. In addition to nonsense mutations at Btk M449, M450 and W395, which cause premature truncation of the protein, there are also missense mutations at Btk M450, F450 and D579 (M450I, F450S and D579V). These mutations likely destabilize the regulatory spine and prevent Btk kinase activation.

Activation loop tyrosine phosphorylation has long been known to influence kinase activity. In addition to priming the kinase domain for the assembly of the regulatory spine, phosphorylation on the conserved Tyr of the activation loop is also thought to stabilize the substrate-binding site of the activation loop[1]. However, our work has shown that in the context of the activating gatekeeper Btk T474M mutation, loss of Y551 phosphorylation (by mutation to phenylalanine) has no affect on substrate phosphorylation. Pre-assembly of the regulatory spine (mutation of T474M) removes any requirement for activation loop phosphorylation. These results are consistent with earlier studies that focused on phosphorylation of Y551 in Btk[25]. That earlier work did show that phosphorylation on Btk Y551 significantly alters catalytic activity of Btk; $k_{cat}$ of the Btk Y551F mutant is greatly diminished compared to wild-type Btk presumably due to loss of the trigger for regulatory
spine assembly. However, the affinity for a peptide substrate is unaffected by Y551 phosphorylation; $K_m$ of a peptide substrate for Btk Y551F mutant is the same as wild-type Btk. As well, an alternative direct substrate docking mechanism has been identified for the Tec kinases, in which an SH2 domain within the substrate docks onto the kinase domain outside of the active site and facilitates substrate phosphorylation\cite{28, 29, 30}. It is therefore possible that the peptide substrate-binding region on the activation loop may only play a minor role in substrate recognition in Tec kinases and that activation loop phosphorylation plays one significant role: initiation of spine assembly by altering electrostatic interactions in and around the active site.

We have previously shown that the regulation of Tec family kinases is similar to that of Csk (C-terminal Src kinase)\cite{14}. Unlike the Src and Abl family of kinases, the SH3 and SH2 regulatory domains of Csk and Tec kinases positively influence the catalytic activity of the kinase domain\cite{14, 16}. Like Itk and Btk, the isolated kinase domain of Csk has poor catalytic activity\cite{16, 31, 32}. In order to define the elements within the Csk kinase domain that are responsible for the poor catalytic activity, a mutagenesis study was carried out in which Src:Csk kinase domain chimeras were created\cite{33}. A Src:Csk chimeric kinase domain where the N-terminal lobe of Csk was replaced with the N-terminal lobe of Src was shown to exhibit activity comparable to that of full-length Csk\cite{33}. Further mutagenesis of the N-terminal lobe, identified the C-helix and the b turn between the b4 and b5 strands (the ‘top’ of the regulatory spine) as key structural elements that are required for the activity of the isolated kinase domain of Csk\cite{33}.

There is remarkable correlation between the elements that are identified in the Csk mutagenesis study and the extended regulatory spine of Tec kinases identified in this study.
Since the Csk and Tec family kinases are both positively regulated by domains outside of the kinase domain, it is not surprising that they would share the key determinants of catalytic activity. Indeed, as suggested in the original identification of the kinase regulatory spine\cite{9}, this feature likely plays a critical role in all active kinases. Our work confirms this notion for the Tec family kinases, and illustrates that extension of the regulatory spine by two residues couples this regulatory feature to allosteric events occurring outside of the kinase domain. Moreover, our data suggest that despite a high degree of sequence conservation, the five core regulatory spine residues do not assemble into the active conformation in the context of the isolated Itk or Btk kinase domains. The energetics of spine assembly seem to be tailored for each specific regulatory environment; the Tec kinase domains are maintained in their inactive state (spine assembly does not occur even when the activation loop is phosphorylated) until additional regions of the protein (in this case the SH2-linker) are present to drive complete spine assembly forward and fully activate the kinase.

**MATERIALS and METHODS**

**Constructs**

The baculoviral expression constructs for full-length Itk has been described previously\cite{14}. The bacterial expression constructs for the Itk kinase domain has been described elsewhere\cite{19}. The mouse wild-type Btk kinase domain (396-659) was PCR amplified and cloned into the pET 28b (Novagen) vector to create the His-tagged Btk kinase domain. All mutations were introduced by using the site directed mutagenesis (SDM) kit (Stratagene). All constructs were verified by sequencing at the Iowa State DNA synthesis
and sequencing facility. Mouse numbering is used throughout for both the Itk and Btk sequences.

**Protein expression and purification**

Baculoviral constructs were expressed and purified from Sf9 cells as described previously[14]. The bacterial expression constructs for the His-tagged Itk, Btk kinase domains were expressed and purified from ArcticExpress cells (Stratagene) as described previously[19]. Briefly, the Itk or Btk kinase domains were expressed in ArcticExpress bacteria at 12°C for 23 hours. The cell pellets were re-resuspended in lysis buffer (0.5 mg/ml lysozyme, 50 mM KH₂PO₄ pH 8.0, 150 mM NaCl, 20 mM imidazole) and stored overnight at −80°C. The cell pellets were thawed after the addition of 1 mM PMSF and 3000 Units DNase I (Sigma). The lysate was spun at 14K for 1 hour at 4°C. The supernatant was incubated with Nickel NTA resin (Qiagen). The resin was washed with wash buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 40 mM imidazole) and then eluted with elution buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 250 mM imidazole, 10% glycerol). The proteins were aliquoted, flash frozen with liquid nitrogen and stored at -80°C. All protein preparations were greater than 95% pure as assessed by Coomassie Blue staining of the gel.

**Kinase assays and western blotting.**

*In vitro* kinase assays were performed by incubating either full-length or the isolated kinase domain of Itk or Btk in a kinase assay buffer (50 mM Hepes pH 7.0, 10 mM MgCl₂, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 mM ATP) at RT for one hour. The samples were boiled, separated by SDS-PAGE and western blotted with the Anti Btk
phosphoY551 antibody (BD Biosciences), or anti-His (Upstate) antibody as described previously[29]. Anti Btk pY551 is used throughout to detect both phosphorylation on Btk Y551 and Itk Y511. Kinetic parameters for the full-length wild-type and mutant Itk are derived using radioactive assays that have been described previously[14].

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REFERENCES


FIGURE CAPTIONS

Figure 1: Identification of the regulatory spine in Tec kinases. (a) The regulatory spine is assembled in the active Btk kinase domain (PDB ID 3GEN). The predicted regulatory spine residues, D579, H519, F540, L460, are shown in orange (sticks plus space filling model) with M449 on the C-helix shown in grey. The gatekeeper residue, T474, is shown in blue. The conserved ion pair, K430 and E545, in the active Btk conformation are shown using red sticks but not labeled. The C-helix and the amino (N) and carboxy (C)-lobes of the kinase domain are labeled. All structures in this and other figures were generated using PyMOL[34]. (b, c) The activity of the Tec kinase mutants correlate with the level of phosphorylation on the activation loop of the kinase. The purified isolated kinase domains
(residues 396-659 for Btk and residues 356-619 for Itk) were concentrated to 500 nM and incubated in a kinase assay buffer at RT for one hour, separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane, and probed with either an anti-Btk pY551 or anti-His antibody. The regulatory spine residues: Btk M449, L460, H519, F540 and D579 and Itk M409, L420, H479, F500 and D539 were separately mutated to Ala in the context of Btk T474M isolated kinase domain (b) or Itk F434M isolated kinase domain (c). (d, e, f & g) The regulatory spine residues were mutated to Ala in the context of either Btk T474M kinase domain (d) or wild-type Btk kinase domain (f), Itk F434M kinase domain (e) or wild-type Itk kinase domain (g). Activity (specifically initial velocity, \( V_i \)) is measured in an \textit{in vitro} kinase activity using Peptide B as a substrate as described previously\[^{14}\]. For (d) and (e) the data for the activating Leu mutation in Btk and Itk are shown in a separate panel for clarity.

Figure 2: \textbf{The regulatory spine is assembled only in the active state of kinases.}

Comparison of the regulatory spine of (a) inactive and active Btk structures (PDB IDs 3GEN and 3K54 respectively) and (b) Y511 unphosphorylated Itk (PDB ID 1SNU) and Y511 phosphorylated Itk (PDB ID 1SM2). As in Figure 1, the regulatory spine residues are orange the gatekeeper residue is shown in blue. The assembled regulatory spine in active Btk in indicated with a dotted line. The distances between the critical conserved ion pair (red sticks) that is a hallmark of kinase activation are indicated for each structure. A schematic of the constructs used for crystallization of the Btk and Itk kinase domains are indicated below the structures. The Btk construct includes the SH2-linker region preceding the kinase domain and extends to the C-terminus while the Itk construct was limited to the isolated kinase domain.
Figure 3: **Extension of the regulatory spine within Tec kinases to include the SH2-kinase linker.** (a) Schematic of the domain architecture of full length Itk with the SH2-kinase linker region and additional spine residues indicated. (b) Btk W395, Itk W355 and Itk M410 were mutated to alanine within full-length Btk or Itk respectively and tested for their *in vitro* kinase activity using Peptide B as a substrate. (c and d) The Btk M450 (M410 in Itk) and Btk W395 (W355 in Itk) form an extension of the regulatory spine to create an allostERIC network connecting the SH2-linker region to the kinase domain. The crystal structures of inactive and active Btk are shown with the location of the E/K salt bridge (red sticks unlabeled), regulatory spine residues (D579, H519, F540, L460: orange, M449: grey), gatekeeper residue T474 (blue), and M450 on the C-helix and W395 in the SH2-kinase linker (brown). The boxed regions in (c) are enlarged in (d). (e) The regulatory spine residues: Itk M409, L420, F500 and D539 were mutated to alanine in the context of full-length Itk and tested for activity by monitoring Peptide B phosphorylation as in Figure 1.

Figure 4: **The regulatory spine is preassembled in the Btk T474M gatekeeper mutant.** (a) Cartoon showing the key components of the allostERIC network within Tec kinases. Tec kinases have an extended regulatory spine, which includes residues beyond the isolated kinase domain: specifically the conserved Trp in the SH2-kinase linker. The residues that make up the regulatory spine (orange disks) are not assembled in the inactive conformation of the kinase. Phosphorylation on the activation loop triggers the assembly of the regulatory spine. Phosphorylation on the activation loop engages Btk R544, which then releases Btk E445 on the C-helix to swing inwards and form the conserved salt bridge with Btk K430. (b)
The Btk T474M mutant does not require phosphorylation on the activation loop for its activity. The Btk T474M, Btk Y551F or Btk T474M/Y551F isolated kinase domain were incubated with a substrate (the SH3SH2 domain of Itk) in a kinase assay buffer for one hour at RT. Phosphorylation on the substrate was monitored by western blotting with a Btk pY223 antibody which has been used previously to recognize phosphorylation on Itk Y180\(^{[29]}\). (c) The Btk T474M/Y551F isolated kinase domain mutant is as active as Btk T474M isolated kinase domain. Btk T474M or Btk T474M/Y551F were tested for their \textit{in vitro} kinase activity using Peptide B as a substrate as described previously\(^{[14]}\). (d & e) Disruption of the conserved electrostatic network by mutation of Btk R544 to Ser activates wild type Btk. Btk R544 was mutated to Ser in the context of wild-type isolated kinase domain of Btk or Btk T474M isolated kinase domain and tested for its activity as in Figure 1. In (d) autophosphorylation on the activation loop tyrosine is monitored and in (e) Peptide B phosphorylation is measured. The use of serine instead of alanine in this case is based on the location of this mutation in the exposed activation loop. We expected that the activation loop could accommodate a hydrophilic side chain more readily than a hydrophobic side chain.
Figures

Figure 1
Figure 2

(a) Inactive Btk

 inactive Btk

14.6 Å

T474

K438

M445

E445

F545

D579

(b) Active Btk

3.8 Å

Y511

Y511 unphosphorylated
Itk

7.7 Å

K308

L420

F434

K445

F500

D539

Y511 phosphorylated
Itk

8.0 Å

SH2-kinase linker

BTK KINASE

ITK KINASE
Figure 3

(a) Structure of Itk showing its domains.

(b) Enzyme activity comparison.

(c) Comparative images of inactive and active Btk.

(d) Structural details of Btk in different states.

(e) Additional enzyme activity data.
Figure 4

(a) Diagram showing the phosphorylation on activation loop, leading to the transition from an inactive to an active kinase.

(b) Western blot analysis showing the expression of anti-pY180 and anti-His antibodies for BK T47AM, BK T47AM Y551F, and BK T47AM Y551F.

(c) Bar graph comparing the $V_{max}/[E]_{max}$ values for BK T47AM and BK T47AM Y551F.

(d) Western blot analysis showing the expression of anti-pY551 and anti-His antibodies for BK WT, BK R544S, BK T47AM, and BK T47AM R544S.

(e) Bar graph comparing the $V_{max}/[E]_{max}$ values for No Enzyme, BK WT, BK R544S, BK T47AM, and BK T47AM R544S.
Supplementary Figure
APPENDIX B. ACTIVATION LOOP DYNAMICS DETERMINE THE DIFFERENT CATALYTIC EFFICIENCIES OF B CELL- AND T CELL-SPECIFIC TEC KINASES


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Abstract

Itk and Btk are nonreceptor tyrosine kinases of the Tec family that signal downstream of the T cell receptor (TCR) and B cell receptor (BCR), respectively. Despite their high sequence similarity and related signaling roles, Btk is a substantially more active kinase than Itk. We showed that substitution of six of the 619 amino acid residues of Itk with those of
Btk was sufficient to completely switch the activities of Itk and Btk. The substitutions responsible for the swap in activity are all localized to the activation segment of the kinase domain. Nuclear magnetic resonance and hydrogen-deuterium exchange mass spectrometry analyses revealed that Itk and Btk had distinct protein dynamics in this region, which could explain the observed differences in catalytic efficiency between these kinases. Introducing Itk with enhanced activity into T cells led to enhanced and prolonged TCR signaling compared to that in cells with wild-type Itk. These findings imply that evolutionary pressures have led to Tec kinases having distinct enzymatic properties depending on the cellular context. We suggest that the weaker catalytic activities observed for T cell–specific kinases is one mechanism to regulate cellular activation and prevent aberrant immune responses.

Introduction

Finely tuned enzymatic activity controls cellular function at all levels. Of the numerous cellular activities controlled by enzymes, kinase-mediated phosphotransfer reactions are a key feature of intracellular signaling cascades, directing information flow and ultimately cellular responses to various stimuli. Kinase activity can be controlled by specific regulatory mechanisms, the amount of enzyme expressed in the cell, the localization pattern of the enzyme, or the intrinsic activity of the kinase in question (1). It is well known that dysregulation of kinase activity by mechanisms such as increasing kinase abundance or mutations that alter regulatory control is associated with numerous disease states (2-4).

Detailed knowledge of kinase structures and their associated regulatory mechanisms can lead to an understanding of disease at a molecular level, and can be harnessed to
intentionally modulate enzymatic activity by mutation of specific amino acid residues. Indeed, hyperactivated or constitutively activated kinases serve as valuable tools to dissect specific signaling pathways, increasing our ability to fully understand cellular communication at the molecular level. High-resolution crystal structures of many protein kinases have been solved (5-7), and they reveal a common kinase domain fold consisting of two lobes (Fig. 1A); the smaller N-terminal lobe (N-lobe) is made up of five b strands and one helix referred to as the C-helix, whereas the larger C-terminal lobe (C-lobe) consists primarily of a-helices. The substrate adenosine triphosphate (ATP) binds in the catalytic cleft that is formed between the two lobes, whereas the protein or peptide substrate interacts primarily with the C-lobe. A large flexible loop lies between the N-lobe and C-lobe, and it is referred to as the activation segment. This activation segment is not always visible in electron density maps, which has made this region of the kinase domain seem somewhat enigmatic. Most, but not all, kinases have one or more residues in the activation segment that undergo phosphorylation as a prerequisite for activation (8). Phosphorylation on the activation loop switches the enzyme from an inactive to an active state by triggering concerted movements in different regions of the kinase to form a catalytically competent active site.

Kinases that share high sequence similarity and belong to the same family are often assumed to share similar activity and regulatory features; however, there are indications that important differences exist between closely related kinases. One interesting example of such a difference emerges from early studies of Syk (spleen tyrosine kinase) and ZAP-70 (ζ chain–associated protein kinase of 70 kD), related tyrosine kinases that exhibit very different
levels of regulation (9, 10). ZAP-70, which is found in mature T cells, exhibits much stricter control over its catalytic activity that does Syk, which is found in B cells (11).

Like ZAP-70 and Syk, the Tec family kinases interleukin-2 (IL-2)–inducible T cell kinase (Itk) and Bruton’s tyrosine kinase (Btk) are nonreceptor tyrosine kinases that function downstream of the T cell receptor (TCR) and B cell receptor (BCR), respectively (12)(13). We performed a direct comparison of the catalytic activities of Itk and Btk and found that Btk was the more active kinase, a finding that mirrors previous findings from analyses of ZAP-70 and Syk. In an effort to better understand how and why highly related kinase sequences exhibit such different levels of catalytic activity, we determined the molecular basis for the catalytic differences between Itk and Btk. We found that specific amino acids within the activation loop segments of Itk and Btk were wholly responsible for the observed differences in catalytic activity. Our findings demonstrate that activation loop dynamics, which are determined by just a few amino acids within the activation segment, directly control the catalytic efficiency of the kinase. Our findings led to construction of the first hyperactivated form of Itk, and incorporating this mutant into T cells enabled us to speculate on why specific kinase activities, or enzymatic activities in general, have evolved precise activity levels that suit the cellular environment in which they are found.

Results

Btk is a more active kinase than Itk

In our previous work on the Tec family kinases, we noted that the purified full-length kinase Btk has greater activity than full-length Itk in activity assays in vitro (14, 15). As a
first step to identify the molecular basis for this unexpected difference in activity, we directly compared the catalytic activities of the kinase domains of Itk and Btk, and we found that the activity difference between these kinases was pronounced and persisted even for the isolated catalytic kinase domains (Fig. 1, B and C).

We turned our attention to the activation segment of the kinase domain, which is defined as the long flexible loop between a conserved “DFG” motif at the N-terminal end and an “APE” motif at the C-terminal end (Fig. 1, A and D). The “APE” motif is partially conserved in the Tec kinases and consists of an “SPE” and a “PPE” motif in Itk and Btk, respectively (Fig. 1D). Crystal structures of Btk show that the activation segment can undergo a major conformational change from a collapsed inactive conformation (Fig. 1A, left) to an extended conformation similar to that found in the active tyrosine kinase Lck (Fig. 1A, right) (16, 17).

**Substitution of the activation segment of Itk with that of Btk activates Itk**

To determine whether the activation segment was responsible for the activity differences between Btk and Itk, we swapped the activation segment sequences between the two proteins. The kinase domain of Itk containing the Btk activation segment sequence (the Itk_Btk activation loop mutant) and the kinase domain of Btk with the Itk activation segment (the Btk_Itk activation loop mutant) were expressed and purified from bacteria, and their activities were measured either by comparing the extents of phosphorylation of residues in the activation loops of each kinase [Tyr<sup>511</sup> (Y511) for Itk and Tyr<sup>551</sup> (Y551) for Btk] (Fig. 1B), or by monitoring the kinetics of phosphorylation (initial velocity, V<sub>i</sub>) of a general
tyrosine kinase peptide substrate, Peptide B (Fig. 1C) (15, 18). Comparison of the activities of these two swapped mutants with those of the kinase domains of wild-type Itk and Btk demonstrated that substitution of the activation segment of Itk with that of Btk activated the Itk kinase domain such that it had a level of activity similar to that of wild-type Btk, whereas substitution of the activation segment of Btk with that of Itk resulted in a Btk kinase domain that had poor activity, similar to that of wild-type Itk (Fig. 1, B and C). Thus, the activity difference between Itk and Btk is completely determined by the amino acid sequences of the activation segments of these enzymes.

Comparison of the sequences of the kinase activation segments showed that there are nine amino acids that differ between Itk and Btk (Fig. 1D). To determine the relative contribution of each of these nine amino acids to the observed activity difference between Itk and Btk, we mutated each of these amino acids in the Itk_Btk activation loop mutant, individually or in pairs, to the corresponding Itk residues (Fig. 2, A and B). Mutation of the activation segment residues Tyr^{505} (Y505), Glu^{510} (E510), and Ser^{517} (S517) to the corresponding residues of Itk had no effect on the activity of Itk_Btk activation loop mutant (Fig. 2, A and B). However, mutation of the Btk activation segment residues at positions 502, 503, 515, 522, 524, and 525 to the corresponding residues in Itk reduced the kinase activity of the Itk_Btk activation loop mutant to less than half that of the original Itk_Btk activation loop protein (Fig. 2, A and B). In a complementary fashion, mutation of the Btk activation segment residues Leu^{542} (L542), Ser^{543} (S543), Val^{555} (V555), Arg^{562} (R562), Ser^{564} (S564), and Pro^{565} (P565) to the corresponding residues of Itk decreased the activity of Btk (fig. S1). Together, these findings indicate that swapping six residues between the activation segments
of Itk and Btk is sufficient to completely switch the activity of these two kinases. Five of these six residues are located at the N-terminal and C-terminal anchor points of the activation segment of the kinase domain, whereas only one residue is located in the middle of the activation segment (Fig. 2C).

**Activation of Itk by substituting its residues with those of Btk only partially depends on phosphorylation at Tyr^511**

Activation loop phosphorylation is a well-studied activation mechanism for many kinases, including those of the Tec family (19, 20). To determine the extent to which enhancement of the activation of Itk by substitution of its activation segment residues with those of Btk required phosphorylation on the activation loop of Itk, we mutated Tyr^511 to phenylalanine and assessed the kinase activity of the mutant protein by monitoring phosphorylation of the Peptide B substrate. The Y511F mutation of Itk in the context of activating Btk residues resulted in decreased kinase activity, but not to the level of activity of wild-type Itk (Fig. 2D). This finding suggests that the activating effect of the activation segment residues of Btk extended beyond increasing the accessibility of Tyr^511 for phosphorylation. Based on our previous work with various Itk mutations (14), we suggest that the activation segment residues of Btk might serve to stabilize the active conformation of the kinase, either by stabilizing the conserved regulatory spine (21), the C helix, or both to a greater extent than do the corresponding activation segment residues of Itk.

**Introduction of the activation segment residues of Btk into full-length Itk activates Itk**
We next introduced the six activation segment residues of Btk that we identified in the earlier kinase domain studies (M502L, T503S, T515V, K522R, A524S, and S525P) into full-length Itk to determine whether these mutations also activated the full-length enzyme (Fig. 3A). This full-length Itk mutant is hereafter referred to as the Itk_Btk loop-6 mutant to indicate the minimum set of activation loop mutations required to activate the kinase activity of Itk. We expressed the Itk_Btk loop-6 mutant protein and purified it from *Sf9* cells, and then we compared its activity to that of full-length, wild-type Itk. The Peptide B and ATP substrate curves indicated that the Itk_Btk loop-6 protein was more active than wild-type Itk (Fig. 3B). The $K_m$ values (an indicator of substrate affinity) of the hyperactive Itk_Btk loop-6 mutant protein for Peptide B and ATP were similar to those of wild-type Itk (Fig. 3C). The increased activity of the Itk_Btk loop-6 protein came entirely from an increase in $k_{cat}$ (a measure of catalytic rate); the value of $k_{cat}$ for the hyperactive Itk mutant was ~3.5- to 4-fold greater than that of wild-type Itk, and was the same as that of full-length Btk (15, 22). Thus, introduction of six activation segment residues of Btk into full-length Itk activated the mutant Itk similarly to full-length Btk by altering the catalytic rate of the Itk_Btk loop-6 protein, and not by increasing its affinity for its substrates. To our knowledge, Itk_Btk loop-6 is the first hyperactive form of full-length Itk.

**Hyperactive Itk is more active than wild-type Itk in T cells**

To determine whether the Itk_Btk loop-6 mutant was also more active than wild-type Itk in a cellular setting, we transfected Jurkat cells with plasmids encoding either FLAG-tagged wild-type Itk or the Itk_Btk loop-6 mutant. We stimulated the cells with anti-CD3 antibody to activate the TCR, and then monitored Itk activity by Western blotting analysis of
the abundance of phosphorylated Tyr$^{511}$ (pY511), or by means of an in vitro kinase assay with immunoprecipitated Itk enzyme and phospholipase Cγ1 (PLC-γ1) as the substrate. Cells expressing the full-length Itk_Btk loop-6 mutant exhibited enhanced phosphorylation of the activation loop residue Tyr$^{511}$ compared to that in cells expressing wild-type Itk (Fig. 4A). In addition, immunoprecipitated Itk_Btk loop-6 from stimulated Jurkat T cells generated more phosphorylated PLC-g1 than did wild-type Itk (Fig. 4B). Interestingly, cells expressing Itk_Btk loop-6 mutant had higher basal activity as compared to cells expressing wild-type Itk. In the absence of TCR stimulation, Itk_Btk loop-6 had higher phosphorylation on the activation loop residue Tyr$^{511}$ and phosphorylated PLC-g1 to a greater extent as compared to wild-type Itk (Fig. 4, A and B). Thus, substitution of just six activation segment residues among the 619 amino acids of full-length Itk with the corresponding residues of Btk was sufficient to activate Itk in Jurkat cells.

To test whether the Itk_Btk loop-6 mutant led to enhanced signaling in the physiological setting of primary T cells, we used retroviral transduction to introduce Itk constructs into murine Itk-deficient (Itk$^{-/-}$)CD4$^+$ T cells. To ensure the production of equivalent amounts of the various Itk constructs, we used a retroviral vector that expresses enhanced green fluorescent protein (eGFP) from an internal ribosome entry site (IRES) on the same mRNA as that encoding Itk; in all experiments, only GFP$^+$ cells with comparable magnitudes of GFP fluorescence were included in the analysis. Furthermore, to confirm the equivalent abundances of the various Itk constructs, we sorted GFP$^+$ cells by flow cytometry and determined Itk protein abundance by Western blotting analysis (fig. S2A). Transduced cells were stimulated by crosslinking TCR-associated CD3 with an activating antibody, and
then were analyzed for the phosphorylation (and thus activation) of the mitogen-activated protein kinases (MAPKs) extracellular signal–regulated kinase 1 (ERK1) and ERK2 (collectively known as ERK1/2), which are downstream of PLC-g1 activation in T cells. In this system, cells with the Itk_Btk loop-6 mutant exhibited enhanced ERK1/2 phosphorylation compared to that in cells with wild-type Itk (Fig. 4C and fig. S2B). In addition to the enhanced magnitude of phosphorylation of ERK1/2 at the peak time of the response (2 to 3 min after stimulation), cells expressing Itk_Btk loop-6 also exhibited prolonged signaling that lasted up to 15 min after stimulation. We also observed that the Itk_Btk loop-6 mutant enhanced TCR signaling in wild-type CD4+ T cells, even in the presence of endogenous Itk (Fig. 4C). In a long-term assay of TCR signaling, the measurement of IL-2 production 5 to 6 hours after stimulation, we detected no enhancement in the response of Itk−/− CD4+ T cells expressing the Itk_Btk loop-6 mutant compared to that in cells expressing wild-type Itk (Fig. 4, D and E). This latter result may be accounted for by the low abundance of Itk protein in the retrovirally transduced Itk−/− T cells (fig. S2A).

**Tyr511 is more readily phosphorylated in Itk containing the Btk activation segment**

Having successfully swapped the catalytic activities of Itk and Btk by swapping specific activation segment residues between the two enzymes, we next wished to determine how a small subset of the total amino acid residues in these kinases could confer such different activity profiles. We reasoned that the increased flexibility, accessibility, or both of the activation segment could lead to increased phosphorylation of Btk Tyr551 (or Itk Tyr511), providing one mechanism to explain the enhanced activity of Btk and the Itk_Btk activation loop mutant compared to that of wild-type Itk. To test this, we purified catalytically inactive
(K390R) versions of the kinase domains of wild-type Itk and of the Itk_Btk activation loop mutant, and subjected them to in vitro phosphorylation by purified Lck, the upstream Src family kinase member that phosphorylates Itk on Tyr$^{511}$ in T cells (19). The abundance of pTyr$^{511}$ in reactions containing the Itk_Btk activation loop mutant (K390R) was greater than that in reactions containing Itk (K390R) (Fig. 5A), consistent with the hypothesis that the Btk activation loop, particularly Tyr$^{511}$, is more accessible to Lck, whereas in the Itk loop, this residue is more occluded.

The activation loop segment of Btk is more solvent-accessible than that of Itk

Based on the differential extents of tyrosine phosphorylation within the Itk and Btk activation loops (Fig. 5A), we next directly tested whether the activation segment of Btk was more solvent-accessible than that of Itk in experiments with hydrogen-deuterium exchange mass spectrometry (HDX-MS). To avoid unwanted heterogeneity in the sample because of Btk autophosphorylation of the activation segment, we performed the HDX-MS analysis on kinase-inactivated Btk mutants (Btk K430R, which corresponds to position 390 in Itk). In addition, because of the sample demands of biophysical techniques such as HDX-MS and nuclear magnetic resonance (NMR) spectroscopy, we used the Btk kinase domain and the mutant Btk kinase domain containing residues from the activation loop segment of Itk (Btk_Itk loop-6) for ease of protein production (see the Materials and Methods for details). The lack of a bacterial expression system to produce large amounts of soluble Itk kinase domain prevented us from performing similar biophysical analysis on the Itk system.
The complete peptide map, as well as the raw deuterium uptake curves, for all of the peptides derived from Btk kinase domain and the Btk_Itk loop-6 mutant were determined (fig. S3, A to E). Comparison of the deuterium uptake curves of peptides derived from the activation segment of wild-type Btk or the Btk_Itk loop-6 mutant (Fig. 5, B to F) showed that the activation segment of wild-type Btk incorporated deuterium more readily than did that of the Btk_Itk loop-6 mutant. This suggests that the activation segment of wild-type Btk is more flexible and therefore more easily deuterated than is the corresponding segment of the Btk_Itk loop-6 mutant. These results are consistent with our observation that the activation loop tyrosine (Tyr\textsuperscript{511} for Itk and Tyr\textsuperscript{551} for Btk) was more readily phosphorylated by Lck in the context of the Btk activation segment residues (Fig. 5A). Peptides derived from the C-terminal third of the activation segments of Btk and Btk_Itk loop-6 (Fig. 5, D to G) showed the most marked differences in deuterium uptake. This finding is consistent with biochemical results that showed that mutation of residues at the C-terminal end of the activation segment of Itk or Btk resulted in the most substantial changes in kinase activity (Fig. 2 and fig. S1). Peptides derived from the N-terminal third as well as the middle third of the activation segment (Fig. 5, C and D) showed minor differences in deuterium uptake.

**Distinct dynamics characterize the activation segments of Itk and Btk**

To complement the dynamic picture that emerged from HDX-MS analysis, we next analyzed the two kinase domains (Btk and Btk_Itk loop-6) by NMR spectroscopy. The resonance frequencies of every hydrogen (\textsuperscript{1}H) that is directly attached to a nitrogen (\textsuperscript{15}N) is measured using the Heteronuclear Single Quantum Correlation (HSQC) spectrum, allowing a direct measure of each amide N-H group in the protein. Given the large size of the kinase
domain, we used a TROSY (Transverse Relaxation-Optimized Spectroscopy) version of the HSQC to improve spectral quality. Comparison of the $^1$H-$^{15}$N TROSY HSQC spectrum of the uniformly labeled kinase domain of Btk (Fig. 6A) with that of the Btk_Itk loop-6 mutant (Fig. 6B) showed differences in both chemical shifts and linewidths. The linewidths observed for the Btk_Itk loop-6 sample were quite uniform compared to those of Btk. In contrast, the linewidths for the Btk sample are variable across the spectrum. The linewidth differences among the Btk amide resonances indicate variations in dynamics throughout the kinase domain of Btk that depended on the amino acid sequence of the activation segment. We pursued a residue-selective isotopic labeling approach to obtain a partial set of sequence-specific chemical shift assignments for the kinase domain of Btk. Phenylalanine and tyrosine were selected for specific labeling based on their lower propensity for isotope scrambling (metabolic interconversion to other amino acids during protein expression in bacteria), as well as because of the relatively low number of these residues in the kinase domain. Comparison of the activation segments of wild-type Btk and the Btk_Itk loop-6 mutant showed that the residues Phe$^{450}$ (F540), Tyr$^{545}$ (Y545), Tyr$^{551}$ (Y551), and Phe$^{559}$ (F559) of Btk were common to both proteins, and hence could serve as effective probes to monitor the dynamics of the activation segment in wild-type Btk and the Btk_Itk loop mutant (Fig. 6, C and D). Chemical shift assignments for the specifically $^{15}$N-labeled residues in Btk and Btk_Itk loop-6 (Fig. 6, E and F) were obtained by site-specific alanine mutagenesis (figs. S4 and S5).

We next measured the steady-state $^1$H-$^{15}$N heteronuclear NOE for the backbone amides of each of the phenylalanine and tyrosine resonances. The backbone $^1$H-$^{15}$N
heteronuclear NOE (Nuclear Overhauser Effect) provides information about the motion of individual N-H bond vectors. Amide bonds undergoing motions that are faster than the overall tumbling of the molecule show a decreased NOE while those undergoing slower motions are characterized by higher NOE values. In spectra of the selectively labeled proteins, nine resonances were identified as corresponding to phenylalanine or tyrosine, but were not specifically assigned. The NOE values for these resonances did not differ between wild-type Btk and the Btk_Itk loop-6 mutant (Fig. 6G). Tyr$^{571}$ (Y571) and Tyr$^{598}$ (Y598) were assigned previously (23), and also showed no difference within error between the two samples (Fig. 6G). Focusing our attention on activation segment dynamics, the NOE values measured for Phe$^{540}$ (F540), Tyr$^{545}$ (Y545), Tyr$^{551}$ (Y551), and Phe$^{559}$ (F559) within the kinase domain of the Btk_Itk loop-6 mutant were all between 0.6 and 0.7 (Fig. 6G), consistent with uniform flexibility across the activation segment containing the six Itk residues. These NOE values were consistent with the range of NOE values expected for residues in loop regions of folded proteins (24).

The activation segment of the kinase domain of Btk exhibited distinct dynamics. The NOE value measured for Phe$^{540}$ (F540) at the N-terminus of the activation loop was ~0.9, indicating rigidity on the nanosecond timescale compared to the corresponding residue within the activation loop of the Btk_Itk loop-6 mutant (Fig. 6G). Phe$^{540}$ is one of five kinase regulatory spine residues (14), and the greater rigidity observed for this residue at the N-terminal end of the activation segment of Btk may be indicative of a higher degree of pre-organization in the regulatory spine of the more active Btk compared to that of the less active Btk_Itk loop-6 mutant. There was no difference in the flexibility of Tyr$^{545}$ between Btk and
the Btk_Itk loop-6 mutant, and Tyr$^{551}$ (the site of phosphorylation) was only slightly more flexible in Btk than in the Btk_Itk loop-6 mutant. In contrast to all of the other resonances examined by selective labeling, the peak corresponding to Phe$^{559}$ (F559) at the C-terminal end of the activation segment in Btk showed extensive line-broadening in the HSQC spectrum (compare to the peak for the corresponding residue in the Itk loop, Fig. 6H) prohibiting NOE measurement for this residue. Nevertheless, the extent of line broadening clearly indicates that Phe$^{559}$ of Btk underwent conformational exchange on the microsecond, or greater, timescale (25). Thus, the N-terminal and C-terminal regions of the activation loop in Btk are characterized by very different types of motions. The N-terminus of the Btk loop (Phe$^{540}$) showed restricted motion on the nanosecond timescale, whereas the C-terminus of the same loop (Phe$^{559}$) underwent motions extending to the microsecond timescale. The slower conformational fluctuations on the microsecond timescale for the C-terminal end of the Btk loop sequence may facilitate phosphorylation of the activation segment tyrosine by providing greater access to Tyr$^{551}$ or, as has been suggested for a number of other enzyme systems (26, 27), the microsecond timescale motions observed for the Btk activation segment may contribute to catalysis.

The extent of loop dynamics based on measured NOE values and linewidth comparisons for the activation segments of Btk and Btk-Itk loop-6 were visualized on the structure of Btk (Fig. 6I). The variation in flexibility from the N-terminus to the C-terminus of the activation loop of Btk (Fig. 6I, right) was more substantial than that of the Itk loop (Fig. 6I, left). The flexibility of the C-terminus of the Btk activation loop, in particular, was consistent with the biochemical and HDX-MS analyses, which pointed to the C-terminal end
of the activation segment as being the primary region responsible for the differences in activity and dynamics between Btk and Itk.

**Discussion**

Despite similarities in sequence and structure between closely related kinases, it is becoming increasingly clear that there are functional and regulatory differences between kinases even within the same family. For the Tec family kinases Itk and Btk, which share 58% sequence identity and 76% similarity in the isolated kinase domains, we have observed distinct extents of activity and identified a specific and small set of amino acid residues that control the catalytic efficiencies of each kinase. Swapping just six residues in the activation segments of Itk and Btk completely switched the kinetic profile of these kinases; the Itk_Btk loop-6 mutant was kinetically indistinguishable from wild-type Btk, whereas the Btk_Itk loop-6 mutant exhibited the poor kinetics of wild-type Itk (Fig. 1, B and C). We found that the basis for the switch in kinase activity derives from differences between the dynamics of the activation loop segments of Itk and Btk.

The sequence differences between Itk and Btk in their activation segments are subtle (Fig. 1D). The one exception is Pro$^{565}$ (P565) at the C-terminus of the activation segment of Btk; the corresponding residue in Itk is a serine. The nature of the prolyl amino acid does not intuitively point to the greater flexibility that we have observed for the C-terminus of the Btk activation loop. The role of this proline in stabilizing the active conformation of Btk may stem from improved packing of the side chain of Pro$^{565}$ of Btk with the F-helix kinase core, which enables better coupling and coordinated movements of the activation segment with
other regulatory regions of the kinase. In contrast, the side chain of Ser^{525} of Itk is less likely to contribute to the hydrophobic interactions that stabilize the kinase core.

The localization of the functional differences between Itk and Btk to the dynamically variant activation segment is reminiscent of the tendency for kinase activation segments to be hot spots for oncogenic mutations (28). HDX studies of a mutant form of the tyrosine kinase c-Kit that is associated with gastrointestinal stromal tumors indicate that the D816H mutation, at the N-terminus of the activation segment, increases solvent-accessibility at the C-terminal end of the activation segment (29). Similar to the sequence changes that we introduced into Itk to produce the activated Itk_Btk loop-6 mutant, a change in activation loop dynamics induced by the D816H mutation of c-Kit correlates with a higher rate of autophosphorylation compared to that of the wild-type kinase (29). Indeed, it is likely that many disease-causing mutations fundamentally alter protein dynamics, which leads to functional deregulation. Several mutations that cause X-linked agammaglobulinemia (XLA) have been identified in the activation segment of Btk (30). Additionally, the COSMIC (catalogue of somatic mutations in cancer) database lists several mutations in the activation segments of Btk and Itk. Future studies will test the effects of these mutations on kinase dynamics. A clear understanding of how conformational dynamics in specific regions within the kinase scaffold controls catalytic function should lend itself to development strategies for new inhibitors.

The C-terminal end of the activation segment is important for the binding, alignment, or both of the substrate in other systems (31). Activation loop swapping between Itk and Btk
altered the amino acid residues at the C-terminal end of the activation segment but they affected only $k_{\text{cat}}$; the $K_m$ values measured for each enzyme and substrate were the same. We previously demonstrated that protein substrate selection in the Tec kinases occurs through a docking interaction that is mediated by helix G of the kinase domain, which is a region separate from, but adjacent to, the C-terminal end of the activation segment (23, 32). This docking interaction may be the primary determinant for substrate selection in the Tec kinases, or else the docking of substrate onto the G helix may allosterically alter the flexibility of the activation segment in a manner that promotes catalytic activity. It is also possible that the sequence of the activation segment might affect $K_m$ for substrates other than the model peptide substrate used here. Regardless, the amino acid differences between the activation segments of Itk and Btk have a clear effect on $k_{\text{cat}}$, which strongly suggests that evolutionary pressures have resulted in sequence differences that altered loop dynamics and reduced the catalytic activity of Itk, which is found in T cells, compared to that of the kinase Btk, which is found in B cells.

T cells and B cells have remarkably similar signaling machinery downstream of the TCR and BCR, respectively (33, 34). Stimulation of TCRs and BCRs leads to the activation of Src family kinases, which phosphorylate receptor-associated immunoreceptor tyrosine-based activation motifs (ITAMs). The phosphorylated ITAM sites then recruit ZAP-70 in T cells or Syk in B cells, which are also activated by Src family kinases. The kinases ZAP-70 and Syk then phosphorylate the adaptor protein Src homology 2 (SH2) domain–containing leukocyte phosphoprotein of 76 kD (SLP-76) in T cells and SLP-65 in B cells, which in turn recruit the Tec kinases Itk in T cells and Btk in B cells, respectively. Our finding that Btk is a
more efficient kinase than Itk parallels previous findings that Syk outperforms ZAP-70 and is under less regulatory control (9-11). We speculate that the weaker catalytic activity and more stringent regulation of the T cell kinases (ZAP-70 and Itk) compared to their B cell counterparts (Syk and Btk) might serve a regulatory role in T cell signaling that is not needed in B cells.

T cell activation is a tightly controlled process, which is necessary to avoid autoimmunity by preventing aberrant responses to weakly recognized self-antigens. Our data indicate that primary T cells expressing even a small amount of the Itk_Btk loop-6 mutant have enhanced and prolonged short-term responses to TCR stimulation. One outcome of this hyperactive Itk may be to lower the threshold amount of TCR stimulation required to promote T cell activation, thereby predisposing the system to autoimmune disease. Thus, a poorly active Itk in wild-type T cells may constitute an important mechanism for dampening responses to low abundance or weakly-recognized self-antigens. Indeed, in unrelated work aimed at developing an inhibitor screen, Douhan et al. showed that DT40-\textit{Btk}^{-/-} cells expressing a full-length Btk that contains the kinase domain of Itk in place of the kinase domain of Btk requires a four- to six-fold higher concentration of agonist antibody to elicit a calcium signaling response compared to the same cells expressing wild-type Btk (35). Moreover, the magnitude of the calcium response is lower in cells expressing the chimeric Btk molecule as compared to wild-type Btk (35), further supporting our notion that the weakly catalytic kinase domain of wild-type Itk is fine-tuned to maintain appropriate T cell responses.
It is also likely that additional mechanisms regulate the magnitude and duration of T cell responses. In spite of there being enhanced short-term signaling in T cells expressing the Itk_Btk loop-6 mutant, we did not observe an overall increase in IL-2 production. This latter response requires a complex series of events, including the activation and de novo synthesis of transcription factors, changes in chromatin structure, and reorganization of the actin cytoskeleton. Given this complexity, there are undoubtedly multiple checkpoints in place to regulate the throughput of this pathway. Nonetheless, it is evident that a small number of amino acid changes in the activation segment of the kinase domain of Itk are capable of altering the dynamic behavior of this loop, with consequences for enzyme activity in contexts ranging from the isolated protein to intact primary T lymphocytes.

Materials and Methods

Constructs

The baculoviral constructs for full-length mouse Itk and Btk have been described previously (15). The bacterial constructs for the isolated kinase domains (without the SH2-kinase linker) of mouse Lck (amino acid residues 230 to 509), Itk (356 to 619), and Btk (396 to 659) have been described elsewhere (14, 36). Full-length wild-type, kinase inactive (K390R), and hyperactive Itk (Itk_Btk loop-6) were also cloned into the pIRES2-EGFP vector (Clontech) for mammalian cell expression. The full length Itk_Btk loop-6 construct contains six mutations in the activation segment: M502L, T503S, T515V, K522R, A524S, and S525P. The bacterial construct for the C-terminal, SH2-linker construct of PLC-γ1 has been described elsewhere (37). All mutations were introduced with a site-directed
mutagenesis (SDM) kit (Stratagene). All constructs were verified by sequencing at the Iowa State University DNA synthesis and sequencing facility.

**Protein expression and purification**

Baculoviral constructs were expressed and purified from Sf9 or High five cells as described previously (15). The bacterial constructs encoding the His-tagged kinase domains of Itk and Btk were expressed and purified from ArcticExpress cells (Stratagene) as described previously (36). None of the kinases was co-expressed with the Src family kinase Lck to avoid differential extents of phosphorylation of the activation loop in the in vitro kinase assays. For the HDX-MS analysis, the proteins were further purified by size exclusion chromatography (Hiload Superdex 26/60 75 pg, GE Healthcare). The final buffer consisted of 20 mM Tris (pH 8.0), 150 mM sodium chloride, 10% glycerol. The expression and purification of the C-terminal SH2-linker fragment of PLC-γ1 has been described elsewhere (37).

**Kinase assays and Western blotting**

In vitro kinase assays were performed by incubating the isolated kinase domain of Itk or Btk in kinase assay buffer [50 mM Hepes (pH 7.0), 10 mM MgCl₂, 1 mM dithiothreitol (DTT), bovine serum albumin (BSA, 1 mg/ml), 1 mM Pefabloc, 200 μM ATP] at room temperature for 1 hour. The samples were boiled, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting with an antibody against Btk phosphorylated at Tyr⁵⁵¹ (anti-Btk pY551, BD Biosciences), anti-FLAG antibody (Sigma), or anti-His antibody (Upstate), as described previously (15). The anti-Btk pY551 antibody was
also used to detect the phosphorylation of Tyr^{511} of Itk. Kinetic parameters for full-length wild-type and mutant Itk proteins were derived from radioactive assays using \(^{32}\)P-ATP and a biotinylated Peptide B substrate that were described previously\(^{15}\). The substrate Peptide B (aminohexanoyl biotin-EQDEPEGIYGVLF-NH\(_2\)) is an artificial tyrosine kinase substrate that was generated by combining sequences that are derived from hematopoetic-lineage cell-specific protein and a peptide library of optimized Lck peptide substrates \(^{38}\). The tyrosine residue targeted by Itk and Btk is in bold.

**Jurkat cell experiments**

Jurkat E6-1 cells were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and penicillin and streptomycin (each at 100 U/ml, Invitrogen). The cells were electroporated at 975 mF and 260 V with a Biorad Gene Pulser II with 10 mg of the appropriate DNA. Twenty-four hours after transfection, the cells were rinsed once with RPMI-1640 medium. Then, \(1 \times 10^6\) cells were incubated at 37°C for 10 min and stimulated with an Anti-T-cell receptor (clone C305) antibody (6.5 mg/ml, Millipore) for 0.5, 1, and 2 min. The cells were lysed on ice in RIPA buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with 1× EDTA-free Halt Protease and Phosphatase Inhibitor cocktail (ThermoScientific). For the unstimulated controls, C305 antibody (6.5 mg/ml) was added after cell lysis. The cell lysate was centrifuged and the supernatant was incubated with 20 ml of anti-FLAG resin (Sigma) for 2 hours at 4°C. The resin was rinsed and boiled in SDS-PAGE sample buffer. The extent of phosphorylation of Itk Tyr^{511} was monitored by Western blotting analysis with anti-Btk pY511 antibody, as described earlier. The in vitro kinase assay was performed by incubating the
immunoprecipitated Itk enzyme with the purified C-terminal SH2-linker fragment of PLC-γ1 at a final concentration of 10 mM in the kinase assay buffer for 1 hour at room temperature. The reaction mixture was boiled in SDS-PAGE sample buffer. Kinase activity was monitored by Western blotting analysis with an anti-PLC-γ1 pY783 antibody, as described previously (37).

**Mice**

*Rag1*−/− OT-II transgenic C57BL/6 mice (model 4234-F/M) were purchased from Taconic and were crossed with *Itk*−/− C57BL/6 mice (39). Animals were housed at the University of Massachusetts Medical School animal facility in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines.

**Retrovirus production**

Complementary DNAs (cDNAs) encoding wild-type, kinase-deficient (K390R) (19), and hyperactive Itk were cloned into the pMiT vector (40). Human embryonic kidney (HEK) 293T cells were co-transfected with the X-tremeGENE 9 DNA transfection reagent (Roche), 1 μg of the pCL-Eco packaging vector, 0.1 μg of pmaxGFP reporter (Amaxa), and 2 μg of each of the pMiT2 Itk-derivatives or 2 μg of empty pMiT-v5 vector. Cell supernatants were harvested two to four times during the 48 to 72 hours after infection and were stored at -80°C before being used for infections.

**T cell purification and retroviral infection**
Spleen and lymph node cells were isolated from wild-type and Itk<sup>−/−</sup> OT-II transgenic mice. CD4<sup>+</sup> T cells were purified and stimulated on plates coated with anti-CD3 antibody (aCD3, clone 145-2C11, 0.8 μg/ml) and anti-CD28 antibody (aCD28, clone 37.51, 2 μg/ml). After two days, cells were mixed with 1 ml of retrovirus-containing supernatant with polybrene (8 μg/ml) and were centrifuged at 1600g for 2.5 hours at room temperature. Cells were then cultured in medium containing IL-2, IL-4, and IL-7 (each at 10 ng/ml) for 2 to 5 days. For the pERK1/2 assay, infected Thy1.1-expressing cells were enriched with aCD90.1(Thy1.1) microbeads before being cultured in cytokine-containing medium.

**T cell stimulation assays**

For IL-2 stimulation assays, cells were stimulated for 5 to 6 hours on plates coated with aCD3 and αCD28 antibodies in the presence of GolgiStop (Life Technologies) and GolgiPlug (Life Technologies). Cells were incubated with fluorescently tagged antibodies specific for Thy1.1, CD4, and TCRb, fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Biosciences), and then were incubated with anti-IL-2 antibody according to the manufacturer’s protocol. For pERK analysis, Thy1.1<sup>+</sup> cells were incubated with biotinylated anti-CD3 antibody for 5 min, and then were stimulated for the indicated times with streptavidin at 37°C. Reactions were stopped by the addition of 4% paraformaldehyde warmed to 37°C. After fixation, cells were permeabilized with 90% methanol at -20°C, and cells were stained with anti-pERK1/2 antibody (BD Biosciences), as previously described (41). For positive controls, cells were stimulated for 4 min with PMA (75 ng/ml, Sigma-Aldrich) and ionomycin (600 ng/ml, Calbiochem). Samples were analyzed with a BD LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo.
(TreeStar Inc.) software. Statistical analysis was performed with the student’s t test in Graphpad Prism 5 software.

**Western blotting analysis**

Cells were lysed in RIPA buffer, and proteins were resolved by SDS-PAGE and then transferred to membranes. Membranes were incubated with anti-Itk antibody (clone 6k237; US Biological) or an anti-ERK antibody (Cell Signaling Technology) as a loading control. Band intensities were analyzed with a Versadoc Imager and Quantity One software (Bio-Rad).

**Deuterium exchange reactions**

Kinase-deficient (K430R) forms of Btk and Btk_Itk loop-6 mutant (L542M, S543T, V555T, R562K, S564A, and P565S) proteins carrying a Y617P mutation (23) were purified from bacteria. Deuterium labeling was initiated with an 18-fold dilution of an aliquot (80 pmoles) of Btk and Btk_Itk loop-6 protein into a buffer containing 99.9 % D₂O, 20 mM Tris, 150 mM NaCl, 10% glycerol, pD 8.01 (pD = pH + 0.4). At specific time points, the labeling reaction was quenched with the addition of an equal volume of quench buffer [150 mM potassium phosphate (pH 2.47),]. Quenched samples were immediately frozen on dry ice until required for liquid chromatography-mass spectrometry (LC-MS) analysis. Quenched samples were rapidly thawed and injected immediately into a Waters nanoACQUITY with HDX technology for online pepsin digestion and ultra performance liquid chromatography (UPLC) separation of the resulting peptic peptides, and were analyzed as reported previously (42). All mass spectra were acquired with a WATERS SYNAPT HDMS² mass spectrometer.
The data were analyzed with DynamX software. Relative deuterium amounts for each peptide were calculated by subtracting the average mass of the undeuterated control sample from that of the deuterium-labeled sample for isotopic distributions corresponding to the +1, +2, or +3 charge state of each peptide. The data were not corrected for back exchange and are therefore reported as relative (43, 44). Calculations of the percentage deuterium incorporation by each peptide also took into account the lack of an exchangeable amide in proline residues.

**NMR analysis**

Uniformly $^{15}$N-labeled isolated kinase domains of Btk or of Btk containing the Itk activation segment (Btk_Itk loop-6) were produced in *E. coli* BL21(DE3) cells, as described previously (23). Kinase domain samples for NMR analysis contained a Y617P mutation that increases protein production in bacteria (23). The Btk_Itk loop-6 construct contains six mutations in the activation segment (L542M, S543T, V555T, R562K, S564A, and P565S). $^{15}$N-Phenylalanine and $^{15}$N-tyrosine selectively labeled samples were produced by growing the *E. coli* BL21(DE3) cells in modified minimal media as described earlier (23). The purified proteins were concentrated and dialyzed into NMR buffer, which consisted of 50 mM Bicine (pH 8.0), 75 mM NaCl, 2 mM DTT, 5% glycerol and 0.02% NaN$_3$. All NMR spectra were collected at 298 K on a Bruker AVII 700 spectrometer equipped with a 5-mm HCN z-gradient cryoprobe operating at a $^1$H frequency of 700.13 MHz. Selective amino acid labeling was pursued because of difficulties in preparing a uniformly labeled sample of this protein required for conventional assignment methods. Chemical shift assignments for F540, F559, Y545, and Y551 residues in Btk and Btk_Itk loop-6 were determined by comparing the
tyrosine or phenylalanine selectively labeled $^1$H-$^{15}$N TROSY-HSQC spectra of the wild-type protein to that of the respective alanine mutant in both the Btk and Btk_Itk loop-6 backgrounds (figs. S4 and S5). All data were analyzed with NMRView J software (45). $^1$H-$^{15}$N NOEs were obtained from the ratio of the volumes of TROSY-based experiments recorded with and without $^1$H saturation. NOE = $V_{\text{sat}}/V_{\text{nosat}}$, where $V_{\text{sat}}$ and $V_{\text{nosat}}$ are peak volumes measured with and without proton saturation, respectively (46). The uncertainties in the NOEs were obtained from the standard deviation between the intensities of duplicate experiments.

**Supplementary Materials**

Fig. S1. Mutational analysis of the kinase activation segment of Btk.

Fig. S2. Analysis of pERK1/2 in primary T cells transduced with retrovirus encoding Itk.

Fig. S3. HDX-MS analysis of Btk and Btk_Itk loop-6.

Fig. S4. Chemical shift assignment of Btk_Itk loop-6 kinase residues.

Fig. S5. Chemical shift assignment of the kinase residues of Btk.

**References**


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**Figure Captions**

Fig. 1. Swapping the activation segments of Itk and Btk switches the catalytic activity profiles of these two related kinases. (A) Structures of the kinase domain of Btk with the activation segment in multiple conformations [Protein Data Bank (PDB): 3GEN, 1K2P]. The N-terminal and C-terminal lobes of the kinase domain, the C-helix, and Tyr^{551} (Y551) on the activation loop are labeled, and the activation segment in each structure is highlighted in red
and labeled. (B and C) The in vitro kinase activities of the indicated proteins were monitored by (B) Western blotting analysis for autophosphorylation on the activation loop at Tyr\textsuperscript{551} (Y551) or (C) phosphorylation of a peptide substrate (Peptide B) with \textsuperscript{32}P-ATP and determination of initial velocity ($V_i$). Pooled Western blotting data were quantified by normalizing the intensity of the band corresponding to pY551 of wild-type (WT) Btk (lane 4) to 100\% and reporting the extent of phosphorylation of Y551 for the other proteins accordingly. Lane 1: negative control (no enzyme); lane 2: WT Itk; lane 3: the Itk_Btk activation loop; lane 4: WT Btk; lane 5: the Btk_Itk activation loop. Data in (B) and (C) are mean values $\pm$ SD from three independent experiments. The Western blot in (B) is representative of three independent experiments. (D) Amino acid sequences of the activation segments of Itk and Btk. The phosphorylation site, Y511 in Itk and Y551 in Btk is shown in red, and asterisks indicate sequence differences between the two kinases. Residues in cyan indicate the minimal sequence changes required to swap the activities of Itk and Btk [determined in (Fig. 2, A and B)].

Fig. 2. Detailed mutational analysis of the kinase activation segment of Itk. (A) The Itk_Btk activation loop mutant containing the entire activation segment of Btk was mutated back to Itk by modifying the indicated residues. The numbering used to indicate mutations is that of Itk. Proteins were then used in at least two experiments to determine $V_i$ by monitoring phosphorylation of a peptide substrate (Peptide B) with \textsuperscript{32}P-ATP. Those single or double mutants whose $V_i$ values were below half that of the Itk_Btk activation loop mutant were considered important for conferring Itk with an activity similar to that of Btk. (B) An extended set of mutants including those examined in (A) were analyzed by Western blotting
to determine the extent of phosphorylation of Tyr$^{551}$ (pY551) as a measurement of their overall activity. Quantification of the relative abundance of pY551 from two experiments is shown in the bar graph. The abundance of pY551 for the Itk_Btk activation loop mutant was set to 100%, and the abundances of pY551 for all of the other proteins were calculated as percentages of that of the Itk_Btk activation loop mutant. Four mutants (Itk_Btk activation loop S517T single mutant, Y505F/E510Q double mutant, Y505F/S517T double mutant and Y505F/E510Q/S517T triple mutant) were tested only once by Western blotting and were not pursued further because the difference in activity between these mutants and wild-type Btk were small when determined both by Western blotting and measurement of $V_i$ (see A). Western blots are representative of two experiments. (C) The structure of the activation segment of Btk (PDB: 1K2P) illustrates the location of the minimal set of residues that must be swapped between Itk and Btk to confer Itk with the activity of Btk. As in (A) and (B), residue numbering is that of Itk. The phosphorylation sites in the activation segments of Itk (Y511) and Btk (Y551) are shown. The N-terminal and C-terminal ends of the activation segment (shown in red) are labeled, and the C, F, and G helices are included for reference. (D) Phosphorylation of Itk Tyr$^{511}$ is required for the full activity of the Itk_Btk activation loop. The recombinant proteins WT Itk, Itk_Btk activation loop, and the same Itk_Btk activation loop construct with an additional Y511F mutation were used in three experiments to measure $V_i$ by monitoring phosphorylation of a peptide substrate (Peptide B) with $^{32}$P-ATP. Mean $V_i$ values ± SD were determined from three independent experiments.

**Fig. 3. Kinetic analysis of full-length Itk and the full-length Itk_Btk loop-6 protein. (A)** Substitution of the indicated six amino acid residues in the activation segment of Itk with
those of Btk was required to create the full-length Itk_Btk loop-6 mutant. The N-terminal regulatory domains of full-length Itk or Itk_Btk loop-6 proteins consists of an N-terminal pleckstrin homology (PH) domain, followed by a Tec homology (TH) domain, and Src homology (SH) domains SH3 and SH2. (B) Peptide B and ATP substrate curves comparing the in vitro phosphorylation kinetics of wild-type Itk (open squares) and the Itk_Btk loop-6 mutant (filled circles). The magnitude of the activity increase for full-length Itk_Btk loop-6 compared to that of full-length WT Itk was less than that observed for the corresponding WT and mutant isolated kinase domain fragments (Fig. 1B), whereas the overall activity ($V_i$ value) was higher for the full-length proteins as compared to the $V_i$ of the isolated kinase domain fragments. (C) Kinetic constants for the WT Itk and Itk_Btk loop-6 proteins were derived from two independent experiments. Error bars represent SD from the mean.

**Fig. 4. Hyperactive Itk enhances phosphorylation of the activation loop and PLC-γ1 in Jurkat cells, and enhances and prolongs TCR signaling.** (A) Jurkat cells were transfected with plasmids encoding FLAG-tagged WT Itk or the Itk_Btk loop-6 mutant. Twenty four hours after transfection, cells were left unstimulated or were stimulated with C305 (anti-TCR) antibody for the indicated times. Cells were lysed, subjected to immunoprecipitation with anti-FLAG antibody, and the extent of phosphorylation on the activation loop was determined by Western blotting analysis of samples for pY511 (top blots). Data are representative of two independent experiments. Band intensities from both experiments were quantified by densitometric analysis. The abundance of pY511 in the Itk_Btk loop-6 mutant after 1 min of TCR stimulation was normalized to 100%, and the relative amounts of pY511 for the other proteins at all time points were determined. (B) WT Itk or the Itk_Btk loop
mutant were immunoprecipitated from unstimulated or TCR-stimulated Jurkat cells and their activities were monitored by an in vitro kinase assay with the SH2-linker region of PLC-γ1 as a substrate. The abundance of PLC-γ1 pY783 was determined by Western blotting analysis with a specific antibody. Data are representative of two independent experiments. Band intensities from both experiments were quantified by densitometric analysis. The abundance of PLC-γ1 pY783 in the reaction containing the Itk_Btk loop-6 mutant (normalized for total Itk) after TCR stimulation was normalized to 100%, and the relative amounts of PLC-γ1 pY783 from the reactions with the other proteins were determined. (C) *Itk*⁻⁻ or WT OT-II TCR transgenic CD4⁺ T cells were infected with control retrovirus (no Itk) or with retroviruses expressing the indicated Itk constructs. Thy1.1⁺ cells were isolated and were left untreated or were incubated with anti-CD3 antibody (aCD3) to stimulate the TCR for 2, 3, 5, 7, or 15 min. Cells were fixed and permeabilized, and incubated with antibodies specific for CD4, Thy1.1, and pERK1/2. Graphs show the normalized mean fluorescence intensities (MFIs) ± SEM for pERK1/2 in gated CD4⁺ Thy1.1⁺ cells (see fig. S2B). Values were normalized within each experiment to the average MFI of pERK1/2 staining for all samples in that experiment, and data are means ± SEM from 4 to 6 independent experiments. Statistically significant differences between cells expressing hyperactive Itk_Btk loop-6 compared to cells expressing WT Itk are indicated: *P < 0.05; **P < 0.01; ***P < 0.001. Non-stimulated cells (no stim) were not incubated with aCD3. As a positive control for the activation of ERK1/2, cells were stimulated with PMA and ionomycin (P+I) for 4 min. (D and E) The Itk_Btk loop-6 construct does not promote increased IL-2 production after TCR stimulation of primary T cells. *Itk*⁻⁻ OT-II TCR transgenic CD4⁺ T cells were infected with control retrovirus (no Itk) or with retroviruses expressing the indicated Itk proteins. Infected
cells were left untreated or were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies (αCD3/CD28) for 5 to 6 hours before being incubated with fluorescently tagged antibodies specific for CD4, TCRβ, Thy1.1, and IL-2. (D) Histograms show representative flow cytometry plots of IL-2 staining in gated CD4+, TCRβ+, Thy1.1+ cells. (E) The graph shows a compilation of flow cytometry data from six independent experiments showing the percentages of cells under each condition that were positive for IL-2. Dashed horizontal lines indicate the means and bars represent the SEM. The percentages of cells expressing WT Itk or the Itk_Btk loop-6 mutant that produced IL-2 were significantly greater than those of cells expressing kinase-deficient Itk or those that had no Itk (P < 0.05). There was no statistically significant difference in the amounts of IL-2 produced by cells expressing WT Itk and those expressing they hyperactive Itk_Btk loop-6 mutant.

**Fig. 5. Tyr511 of Itk is more accessible for phosphorylation by Lck when in the context of the Itk_Btk activation loop.** (A) Kinase-deficient (K390R) mutants of Itk or the hyperactive Itk_Btk activation loop proteins were subjected to phosphorylation by Lck in an in vitro kinase assay. Phosphorylation of Itk Tyr511 was monitored at three time points (10, 20, and 40 min) by Western blotting analysis with an anti-pY511 antibody. The Western blot is representative of three independent experiments. Densitometric data from all three experiments were quantified and are presented in the bar graph on the right. To normalize for exposure times between Western blots from different experiments, we set the abundance of pY551 in the Itk_Btk activation loop (K390R) mutant after 40 min of stimulation to 100% in each independent experiment. The abundances of the phosphorylated Itk (K390R) protein (all three time points) and Itk_Btk activation loop (K390R) protein (at the 10- and 20-min time
points) are shown relative to that for the Itk_Btk activation loop (K390R) protein at the 40-min time point. Data summarized from three independent experiments with error bars representing SD from the mean. (B) Pepsin digestions of the WT Btk and Btk_Itk loop-6 mutant proteins produced coincident peptides, which are indicated under the sequences that covered the entire activation segments of both proteins. (C to F) Deuterium exchange was measured for the WT Btk and Btk_Itk loop-6 mutant proteins (see the full dataset in fig. S3) and data were plotted as the relative percentage of deuterium incorporation versus time for the peptides identified in (B) for Btk (blue diamonds) and Btk_Itk loop-6 (red squares). (G) The locations of the individual peptides, color-coded as in (B), are shown on the structure of the kinase domain of Btk, with the activation segment in the collapsed inactive conformation (PDB: 3GEN).

**Fig. 6. The C-terminus of the kinase activation segment of Btk is more dynamic than that of Itk.** (A and B) Comparison of the $^{1}$H-$^{15}$N TROSY HSQC spectra of the uniformly $^{15}$N labeled (A) kinase domain of WT Btk and (B) kinase domain of the Btk_Itk loop-6 mutant. Data is representative of at least three independent experiments. (C) Comparison of the sequences of the activation segments of WT Btk and the Btk_Itk loop-6 mutant. (D) Structure of the kinase domain of Btk (PDB: 1K2P) showing the tyrosine and phenylalanine residues throughout the protein. Six tyrosine and phenylalanine backbone amide resonances have been assigned (shown in red), whereas the remainder of the tyrosines and phenylalanines (in blue) have not been assigned. (E and F) $^{1}$H-$^{15}$N TROSY HSQC spectra for the kinase domains of WT Btk and the Btk_Itk loop-6 mutant specifically labeled with (E) $^{15}$N-Phe and (F) $^{15}$N-Tyr. Peaks that were assigned are labeled, and the unassigned peaks in
the spectra that correspond to either phenylalanine or tyrosine resonances are numbered. (G) Steady-state \(^{1}H\)-\(^{15}N\) heteronuclear NOE data for WT Btk (blue circles) and the Btk_Itk loop-6 mutant (red squares) are plotted for assigned and unassigned tyrosines and phenylalanines. F540, Y545, Y551, and F559 reside in the activation loop segment of each kinase. (H) Btk F559 shows extensive line broadening in the context of WT Btk as compared to the Btk_Itk loop-6 mutant. (I) Dynamical differences based on Het-NOE values for the activation loop segments of Itk (left) and Btk (right) are plotted onto the structure of WT Btk (PDB: 1K2P). Increasing flexibility is indicated by the color and width of the structural trace.
Figures

Figure 1
Figure 3

A

Itk_Btk loop-6

PH  TH  SH3  SH2  KINASE


B

$V_\text{f}/[\text{Enzyme}]$ (min$^{-1}$)

Peptide B (µM)

0  100  200  300  400

0.2  0.4  0.6  0.8  1.0  1.2  1.4  1.6

C

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<th>Peptide</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>ATP $K_m$ (µM)</th>
<th>ATP $k_{cat}$ (min$^{-1}$)</th>
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<tr>
<td>Wild-type Itk</td>
<td>46 ± 13</td>
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<td>107 ± 35</td>
<td>0.43 ± 0.05</td>
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<tr>
<td>Itk_Btk loop-6</td>
<td>42 ± 10</td>
<td>1.75 ± 0.13</td>
<td>102 ± 19</td>
<td>1.56 ± 0.12</td>
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</tbody>
</table>
Figure 4
Figure 5

A

Btk

Btk loop-6 539  DFGERYVLDDEYTSSTGSKFPVWASPEVL
Btk 539  DFGLSRYVLDDEYTSVGSKFPVWSPPEVL

B

C

D

E

F

G

Relative % Deuterium Level

Relative % Deuterium Level

Relative % Deuterium Level
Figure 6

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

I.