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Abstract
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Keywords
Itk, Btk, substrate docking, SH2 domain, XLA

Disciplines
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Comments
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SH2 dependent autophosphorylation within the Tec family kinase Itk

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Abstract

The Tec family kinase, Itk, undergoes an in cis autophosphorylation on Y180 within its SH3 domain. Autophosphorylation of the Itk SH3 domain by the Itk kinase domain is strictly dependent on the presence of the intervening SH2 domain. A direct docking interaction between the Itk kinase and SH2 domains brings the Itk SH3 domain into the active site where Y180 is then phosphorylated. We now identify the residues on the surface of the Itk SH2 domain responsible for substrate docking and show that this SH2 surface mediates autophosphorylation in the full length Itk molecule. The canonical phospholigand binding site on the SH2 domain is not involved in substrate docking, instead the docking site consists of side chains from three loop regions (AB, EF and BG) and part of the βD strand. These results are extended into Btk, a Tec family kinase linked to the B cell deficiency X-linked agammaglobulinemia (XLA). Our results suggest that some XLA causing mutations might impair Btk phosphorylation.

Keywords

Itk; Btk; Substrate docking; SH2 domain; XLA

INTRODUCTION

Tyrosine kinases are enzymes that catalyze the transfer of the gamma phosphate of ATP to a tyrosine residue within the substrate. Although a large number of tyrosine residues are accessible for phosphorylation within the cellular milieu, most tyrosine kinases phosphorylate a limited subset of potential substrates within the cell. Substrate selectivity is crucial for the normal functioning of the cell. However, the mechanisms by which substrate specificity is achieved by kinases is far from clear.

Specific docking mechanisms are emerging as a theme in kinase substrate recognition. A docking interaction is defined as a direct interaction between a site on the kinase domain (remote from the active site) and a complementary site on the substrate (remote from the phosphorylation site). Docking modules are well characterized for serine/threonine kinases and consist of short peptide motifs such as the D box of MAPK substrates and the DEF domain.
of ERK substrates. While substrate docking interactions are well known for serine/threonine kinases, they have been less well characterized for tyrosine kinases with only two specific examples described to date: C-terminal Src kinase (Csk) and Tec family kinases.

We have previously reported biochemical data that support a substrate docking mechanism for the Tec kinase family (Itk, Btk, Tec, Bmx and Txk) using Itk as a model system, we have shown that Itk autophosphorylation within the Itk SH3 domain (specifically on Y180) is dependent on the presence of the neighboring Itk SH2 domain. During autophosphorylation, the Itk SH2 domain directly binds the Itk kinase domain via an interaction that does not involve the phosphopeptide binding pocket of the Itk SH2 domain, positioning the substrate (Itk SH3 Y180) into the active site of the Itk kinase domain. This substrate docking mechanism is distinct from the processive phosphorylation mechanism that has been described previously for other kinases, where an SH2 domain located within a kinase molecule itself binds to a phosphotyrosine site on the substrate facilitating further substrate phosphorylation events.

We now identify the interaction surface on the Itk SH2 domain that binds directly to the Itk kinase domain to mediate autophosphorylation of Y180. The docking site on the Itk SH2 domain maps to a surface that involves residues from beta strand D, the AB, EF and BG loops and is distinct from the classical phosphopeptide binding surface on the Itk SH2 domain. We demonstrate that mutations within the Itk SH2 domain can disrupt autophosphorylation on Itk SH3 Y180 in the context of full-length Itk. These results identify an interaction surface on the Itk SH2 domain that can be added to the growing list of non-canonical interactions mediated by this multifunctional domain. In addition, we show that a related subset of mutations in the Btk SH2 domain that cause the immune disorder X-linked agammaglobulinemia (XLA) also disrupt Btk SH3 autophosphorylation. These mutations map to the same substrate-docking surface identified for Itk SH2 and suggest a possible mechanistic explanation for this subset of XLA causing mutations in the Btk SH2 domain.

RESULTS

The SH2 domain of each Tec kinase docks onto the kinase domain via a conserved SH2 surface

With the exception of Txk, each Tec family kinase contains four domains in addition to the catalytic kinase domain (PH, TH, SH3, SH2; Fig. 1A) and each Tec kinase autophosphorylates a tyrosine residue within its own SH3 domain (Y180 in Itk). Autophosphorylation occurs in an intramolecular fashion (in cis) and depends on the presence of the SH2 domain. Specifically, Y180 is a very poor substrate for Itk in the context of the isolated Itk SH3 domain, but the same tyrosine is readily phosphorylated by Itk in the context of the larger Itk SH3-SH2 dual domain substrate. This SH2 dependence is explained by a direct docking interaction between the SH2 domain and the kinase domain for each of the individual Tec kinases (Itk, Btk and Tec) resulting in proper positioning of the neighboring SH3 domain into the active site for efficient autophosphorylation.

To determine the residues on the surface of the Itk SH2 domain that are required for substrate docking, we first evaluated whether each of the Tec family kinase domains (Itk, Btk and Tec) can phosphorylate the SH3-SH2 fragments derived from the same three kinases (Itk, Btk and Tec). We expect that if Itk phosphorylates the SH3-SH2 domains of Btk and Tec in addition to its own SH3-SH2 substrate (and Btk and Tec also phosphorylate all three substrates), then
the residues on the Itk SH2 domain that mediate substrate docking are likely conserved within the Tec family.

To test this idea, full-length, purified Itk was separately incubated with the SH3-SH2 domains of Itk, Btk or Tec in an in vitro kinase assay. The samples were resolved on an SDS-PAGE gel and western blotted with an anti-phospho Y223 Btk antibody (which has been used previously to recognize phosphorylation on Btk SH3 Y223, Itk SH3 Y180 and Tec SH3 Y187). Full-length Itk can readily phosphorylate the SH3-SH2 domains of each of the Tec kinases (Itk, Btk and Tec) (Fig. 1C, lanes 4–6). Likewise, full-length Btk and Tec also phosphorylate the SH3-SH2 domain fragments derived from Itk, Btk and Tec (Fig. 1C, lanes 7–9 and 10–12 respectively). This result indicates that the residues on the Itk SH2 domain that mediate substrate docking are conserved between Itk, Btk and Tec.

Mapping of the Itk SH2 domain substrate docking surface

In order to identify the conserved docking surface on the Itk SH2 domain, the primary sequence of this domain was aligned with the SH2 domains of other Tec kinases (Btk, Tec, Txk and Bmx) as well as two unrelated SH2 domains derived from PI3K and Grb2 which have been used previously as negative controls (Fig. 1D). Residues that are both surface exposed on the structure of the Itk SH2 domain (PDB 1LUN) and conserved within the Tec family SH2 domains, but not conserved in the SH2 domains of PI3K and Grb2 were targeted for mutation. A total of 13 residues were identified by this process that were then mutated to alanine in the context of the Itk SH3-SH2 domain substrate (boxed residues in Fig. 1D). Mutation of SH2 domain residues that mediate the substrate docking interaction between the SH2 domain and kinase domain were expected to diminish phosphorylation on Itk SH3 Y180.

As shown in Fig 2A, mutation of the residues E235, Y237, L252, K258, Y292, E308, K309, G328, L329, R332 and R334 within the Itk SH2 domain significantly diminished phosphorylation on Itk SH3 Y180. This indicates that these SH2 domain residues are involved in the substrate docking interaction between the Itk SH2 domain and the Itk kinase domain. Mutation of Itk Q323 and N325 did not affect Itk SH3 domain phosphorylation suggesting that these residues do not contribute to substrate docking (Fig. 2A, lanes 15 and 16). To rule out structural defects in the mutated SH2 domain, circular dichroism (CD) spectra for each of the mutant substrates was compared with that of the wild-type substrate. The CD spectra of all the Itk SH3-SH2 mutants are similar to that of the wild-type protein (Fig 2B). Hence, the lack of phosphorylation on the Itk SH3 domain is not due to general domain unfolding that could be induced by the Itk SH2 domain mutations but instead is likely due to specific disruption of the SH2/kinase docking interaction.

We carried out a second round of mutational screening to more broadly survey the Itk SH2 domain surface. Ten residues (indicated by open circles on Fig. 1C) were mutated to alanine in the context of the Itk SH3-SH2 domain and tested as substrates as before. The Itk SH2 domain residues mutated in the second round of the mutational screen (K242, R246, K251, E259, R268, T269, S314, N298, D299, and K280) exhibit little or no effect on the level of Itk SH3 domain phosphorylation when compared to wild type substrate (Fig. 2C). This indicates that these SH2 residues are not involved in Itk kinase docking. These results contrast the results from the first round of mutational screening, where almost 90% of the mutated Itk SH2 domain residues had a significant effect on Itk SH3 domain phosphorylation. The outcome of these two rounds of mutational screening is consistent with the underlying strategy employed during the two screens: the more targeted approach in the first screen identified more residues involved in docking, compared to the more random approach employed in the second round of screening. Taken together, we have identified a set of residues on the Itk SH2 domain that are required for docking of the Itk SH2 domain onto the Itk kinase domain and mediating Itk SH3 domain phosphorylation.
The Itk SH2 domain substrate-docking surface is distinct from the canonical Itk SH2 domain ligand binding surface

Mapping the residues that disrupt Itk SH3 domain autophosphorylation (E235, Y237, L252, Y292, K258, E308, K309, G328, L329, R332 and R334) onto the structure of the unliganded Itk SH2 domain (PDB ID 1LUN) reveals a mostly contiguous surface involving beta strand D (βD), the AB, EF and BG loops (red residues in Figs. 3A&B). Three residues (E235, Y237 and L252) lie outside of the contiguous surface defined by the other eight residues yet clearly have an effect on substrate phosphorylation when mutated (Fig. 2A). Whether the structure of the isolated SH2 domain is insufficient to judge the location of these residues (E235 and Y237 are located on the flexible N terminus) or whether loss of these side chains can allosterically affect the SH2 docking surface remains to be determined. The residues that are not involved in substrate docking, based on the mutational data shown in Figure 2C, are scattered over the surface of the SH2 domain (colored tan in Figs. 3A&B).

The substrate-docking surface on the Itk SH2 domain is largely separate from the canonical phospholigand binding surface. In Figure 3C, a bound phosphotyrosine containing peptide is shown on the Itk SH2 structure. It is evident that the phosphotyrosine (pY) itself contacts the SH2 domain in a region that is removed from the surface that mediates docking onto the Itk kinase domain. This is consistent with our earlier published results that show that disruption of phosphopeptide ligand interactions (R265K mutation in the pY binding pocket of the Itk SH2 domain) has no effect on autophosphorylation of Y180. The pY+3 ligand-binding pocket of the Itk SH2 domain overlaps slightly with the mapped docking surface (Fig. 3C). While most of the docking surface lies well outside of the pY+3 pocket, Y292 lines the bottom of the pocket and so it is not entirely clear whether substrate docking and phospholigand binding can occur simultaneously. Our efforts to directly test the effect of SH2/phosphopeptide binding on Itk autophosphorylation were thwarted due to toxicity effects of the phosphopeptide on kinase activity (data not shown).

Mutations in the Itk SH2 domain docking-site disrupt autophosphorylation in full-length Itk

To determine whether Itk SH2 domain mutations disrupt Itk SH3 domain autophosphorylation in the context of full-length Itk, a subset of the mutations that reduce Y180 phosphorylation in the context of the isolated Itk SH3-SH2 domain substrate were introduced into the SH2 domain of full-length Itk. These full-length Itk mutants were co-expressed with Lck in Sf9 cells since Lck, by phosphorylating Y511 in the Itk activation loop, is required to activate Itk. Full length Itk, wild type and mutants, were then purified and incubated in an in vitro kinase assay and assessed for intramolecular autophosphorylation on Y180 in the SH3 domain by western blotting.

The three different full-length Itk mutants tested (E308A/K309A, G328A/L329A and R332A/R334A) all showed decreased phosphorylation on Itk SH3 Y180 compared to wild-type full-length Itk (Fig. 4A). These results should be interpreted cautiously since reduced autophosphorylation on Y180 in the context of full-length Itk could be due to several factors including: (1) disruption of Itk SH2 domain substrate docking, (2) reduced phosphorylation on the kinase domain activation loop tyrosine (Y511) which is required for Itk kinase activity, and/or (3) reduced/ altered kinase activity of the full-length Itk mutant. These properties were assessed for each of the full-length Itk mutants to establish whether substrate docking is indeed mediated by the identified surface residues in the context of full length Itk.

The Itk G328A/L329A and R332A/R334A double mutants showed reduced autophosphorylation on Y180, yet they both also exhibited reduced phosphorylation on Y511 (Fig. 4A). Since a reduction in the phosphorylation level of Y511 reduces Itk kinase activity, the loss of Y180 autophosphorylation for these two mutants cannot be fully...
attributed to disruption of SH2 mediated docking. Thus, while both the G328A/L329A and the R332A/R334A double mutants are important for substrate docking and autophosphorylation on Y180 in the context of the Itk SH3-SH2 domain fragment, in the context of full-length Itk these mutations have additional effects that account for at least part of the observed reduction in autophosphorylation. Indeed, the effect of certain Itk SH2 domain mutations on Y511 phosphorylation indicates an involvement of the Itk SH2 domain in mediating Y511 phosphorylation by Lck (Joseph & Andreotti, unpublished data).

The E308A/K309A double mutant exhibits reduced autophosphorylation on Y180 compared to wild-type full-length Itk, without loss of phosphorylation on Y511 (Fig. 4A) indicating that these mutations selectively affect SH2 docking. To further confirm that the reduced autophosphorylation levels are not due to impaired kinase activity of the mutant, the kinetic parameters of the full length Itk E308A/K309A double mutant were compared to those of wild-type full-length Itk. The $K_m$ and $k_{cat}$ values of Itk E308A/K309A double mutant are indistinguishable from those of wild-type Itk (Fig. 4C, D and F). We additionally tested whether the E308A/K309A double mutation has an effect on the Itk SH2 domain ligand binding function. Phosphopeptide ligand binding affinity of the Itk SH2 E308A/K309A double mutant was measured using NMR spectroscopy and compared with that of wild-type Itk SH2 domain. As shown in Fig. 4E, the affinity of the Itk SH2 E308A/K309A double mutant for the SLP76 phospho-peptide ligand is comparable to that of the wild-type Itk SH2 domain (Dissociation constant, ($K_d$) of 0.48 ± 0.06 mM and 0.38 ± 0.04 mM, respectively). Thus, the E308A/K309A mutation does not affect either $K_m$ or $k_{cat}$ for the kinase domain nor does it alter phospholigand binding behavior of the SH2 domain and so the loss of autophosphorylation on Y180 in the full-length Itk E308A/K309A double mutant can be interpreted as causing a specific disruption of the direct SH2 domain docking that mediates Y180 autophosphorylation. Taken together, these data provide evidence for a specific substrate docking role for the Itk SH2 domain during autophosphorylation of Y180 in the SH3 domain of full-length Itk.

The Btk SH2 domain XLA mutations affect SH2 mediated substrate docking

Genetic mutations in the Tec family kinase, Btk, are correlated with a specific B cell immunodeficiency in humans: X-linked agammaglobulinemia (XLA)\textsuperscript{24, 25}. XLA causing mutations have been identified throughout the entire Btk molecule including the Btk SH2 domain\textsuperscript{24}. To date, XLA causing mutations have been identified at 43 unique positions in the 97 amino acid Btk SH2 domain\textsuperscript{24}. Examination of these XLA causing mutations in the Btk SH2 domain suggests classification into several categories: (1) stop codon mutations resulting in premature termination of translation and giving rise to truncated protein products, (2) mutations causing an amino acid change in the classical phosphopeptide ligand binding pocket of the SH2 domain that alter ligand binding in a deleterious fashion, (3) mutations causing an amino acid change in the core of the SH2 domain that disrupt the structural integrity of the SH2 domain and (4) mutations causing an amino acid change on the surface of the SH2 domain that are not close to the phospholigand binding site and therefore are not readily explained by the known functions of the Btk SH2 domain. We focused on this last category of XLA causing mutations (14 in total) and evaluated whether these SH2 mutations might disrupt autophosphorylation on Btk SH3 Y223 (corresponds to Y180 in Itk) by altering SH2 mediated substrate docking to the Btk kinase domain.

Fourteen specific Btk SH2 XLA causing mutations (indicated in Fig. 1D) were individually introduced into the Btk SH3-SH2 substrate to test the extent to which these SH2 residues mediate Y223 phosphorylation in an \textit{in vitro} kinase assay using full-length Btk as the enzyme. Of the 14 Btk SH2 residues targeted for mutation, three mutants (G302R, Y361D and H362R) resulted in expression of insoluble protein in bacteria and were therefore not tested further. As shown in Fig. 5A, six Btk SH2 XLA mutations tested (Y334S, L358F, H364D, S371P, R372G

\textcite{Joseph et al. J Mol Biol. Author manuscript; available in PMC 2010 August 7.}
and K374N; lanes 7–8, 11–12, 13–14, 23–24, 25–26, 27–28) decrease Btk SH3 Y223 phosphorylation compared to the wild type Btk SH3-SH2 substrate. To ensure that the Btk SH2 domain XLA mutations do not disrupt the structural integrity of the Btk SH2 domain, we compared the CD spectra of the mutants that showed the most significant decrease in Btk SH3 domain phosphorylation (H364D, S371P and R372G) with that of the wild-type Btk SH3-SH2 protein. The CD spectra of all three mutants are similar to that of the wild-type protein (Fig. 5B), suggesting that the mutations do not cause major structural rearrangements in the protein. Thus, in analogy to the Itk results shown in Figure 2A, the decrease in Btk SH3 domain phosphorylation in the Btk SH2 H364D, S371P and R372G mutants is likely due to the disruption of Btk SH2 domain mediated kinase docking.

To further characterize the XLA causing mutations in the Btk SH2 domain, we tested the effect of these mutations on phospho-peptide ligand binding by NMR. The R372G mutant was not stable at concentrations required for NMR spectroscopy and hence its phosphopeptide ligand binding affinity was not measured. The phosphopeptide ligand affinities of Btk H364D and S371P mutants are nearly identical to each other (Dissociation constant, $K_d$ of $0.50 \pm 0.20$ mM and $0.60 \pm 0.11$ mM respectively) and to that of the wild-type protein ($K_d$ of $0.45 \pm 0.04$ mM) (Fig. 5C). Hence, the Btk H364D and S371P mutations do not significantly affect the phospho-peptide ligand binding property of the Btk SH2 domain (Fig. 5C). Notably, the Btk SH2 XLA mutations that disrupt Btk Y223 phosphorylation map to the base of the Btk SH2 domain BG loop, which corresponds to the same region of the Itk SH2 domain that mediates substrate docking and Itk autophosphorylation (Fig. 5D). While the observed connection between the Btk XLA causing mutations and disruption of substrate docking is compelling, further experiments are clearly needed to confirm a link between a protein interaction interface and disease.

**Structural model of Itk during autophosphorylation**

While the docking surface on the Itk SH2 domain has been identified in this study, the complimentary docking surface on the kinase domain remains unknown. Introducing mutations into the Itk kinase domain to identify the docking site for the SH2 domain will require careful characterization since point mutations in the kinase domain may drastically affect catalytic activity without affecting SH2/kinase docking. In the meantime, we have generated a preliminary model (Fig. 6A) for substrate docking in the context of Itk autophosphorylation that provides a structural context to consider the results presented here and provides guidance as we move toward mutagenesis of the Itk kinase domain itself. In developing the model we took into consideration two previously published observations: (1) autophosphorylation of Y180 in the Itk SH3 domain occurs via an intramolecular interaction (in cis), where the Itk kinase domain phosphorylates the SH3 domain within the same polypeptide chain and (2) contact between W355 in the Itk SH2-kinase linker and M410 in the C-helix of the Itk kinase domain is critical for Itk kinase activity. We have previously reported a model of the Itk SH3 domain bound to the Itk kinase domain during SH3 Y180 autophosphorylation. To generate this model, the Itk kinase domain was aligned with the Insulin receptor kinase (Irk) domain within the Irk:peptide substrate complex, and Y180 within the Itk SH3 domain was aligned with the tyrosine of the Irk peptide substrate in the active site (Fig. 6A). Once aligned, the Itk SH3 domain fits well into the Itk kinase active site and no further structural rearrangements are necessary.

In the primary sequence of full length Itk, the SH2 domain is tethered to the Itk SH3 domain at its N-terminus and to the SH2-kinase linker at its C-terminus (Fig. 6A). The Itk SH2 domain was therefore docked onto the Itk SH3/Itk kinase complex to a position that satisfies the length and steric requirements of the covalent linkers between domains. In the model, the linkers were assumed to adopt extended conformations and W355 in the SH2-kinase linker is maintained.
in a position that is proximate to M410 in the kinase domain (Fig. 6A). The orientation of the Itk SH2 domain was determined by maximizing contacts between the Itk SH2 residues identified in this study (Fig. 3) and the kinase domain. The resulting model shown in Figure 6A represents one plausible location for the Itk SH2 domain on the Itk kinase domain, the details of which remains to be verified experimentally. In this model, the Itk SH2 domain can contact residues from both the N- and C-terminal lobes of the Itk kinase domain. As well, a phosphopeptide may be placed into the ligand-binding pocket of the SH2 domain in this model without steric hindrance suggesting that the ligand-binding surface of the Itk SH2 domain remains largely accessible during autophosphorylation.

In addition to the location of SH2 docking on the Itk kinase domain, the model predicts a relative orientation between the Itk SH3 and SH2 domains during autophosphorylation. To examine the dual domain SH3-SH2 fragment further, we extracted this region from the modeled structure and, as expected, find that the SH2 side chains that mediate contacts to the kinase domain (Fig. 3) are located on the same side as Y180 within the SH3 domain (Fig. 6B). Notably, we find that the Itk SH3-SH2 domain fragment in the modeled structure is remarkably similar to the previously reported crystal structure of the corresponding Lck SH3-SH2 dual domain fragment (Fig 6B). Specifically, the relative orientations between the SH3 and SH2 domains in the experimentally solved Lck SH3-SH2 structure and in the modeled Itk arrangement of these domains are almost the same. While the model generated here is certainly useful to visualize how SH2 docking might be mediating Itk autophosphorylation, future mapping experiments to identify the precise binding site on the kinase domain, NMR solution studies to examine domain orientations and crystal structures of the Itk kinase/substrate complex will ultimately be needed to reveal the details of this interaction.

**DISCUSSION**

SH2 domains are present in hundreds of different signaling proteins and have been studied extensively since they were first recognized as phosphotyrosine-binding domains. Typically, the role of a specific SH2 domain in a given signaling cascade is evaluated by a standard mutation that renders the phosphotyrosine binding pocket of the SH2 domain inactive. In this vein, it has been demonstrated previously that the SH2 domains of the Tec kinases are required for proper localization and functioning of this family of kinases during immune cell signaling. For example, binding of Itk via its SH2 domain to the phospho-SLP76/LAT complex is essential for the propagation of T cell receptor signaling. Mutation of the conserved arginine (R265) in the Itk SH2 domain leads to reduced association of full length Itk with LAT and reduced transphosphorylation of Itk, in turn leading to severely reduced kinase activity. Classical phosphotyrosine binding by SH2 domains is clearly a critical component of SH2 mediated signaling.

It is evident that there are also various signal transduction pathways that are mediated by non-phosphotyrosine dependent SH2 interactions. The Itk SH2 substrate-docking surface identified in this study contains residues that are distinct from those that mediate classical phosphotyrosine recognition (Fig. 3C) adding to the growing list of alternative SH2 mediated interactions. Whether some SH2 domains, such as that of Itk, might simultaneously mediate more than one protein-protein interaction remains to be determined. In fact, it should be noted that the autophosphorylation event under investigation here is likely a transient conformational state rather than a stable configuration.

Our model of Itk autophosphorylation (Fig. 6A) suggests that the SH2 domain can mediate Y180 phosphorylation while also retaining binding to at least the phosphotyrosine of a phospholigand in the signaling complex. Hence, it is possible that Itk autophosphorylation occurs while Itk is bound to the phospho-SLP76/LAT complex or alternatively,
autophosphorylation might facilitate displacement of Itk from the SLP76/LAT complex by competing with binding to the pY+3 pocket. Ultimately, understanding the precise order in which all of these signaling steps take place and the significance of each in terms of propagating or dampening signals that emanate from the T cell receptor will require more investigation.

Substrate docking within the Tec kinase family can be compared to another non-receptor tyrosine kinase for which substrate docking has been described. Csk phosphorylates a tyrosine in the C-terminal tail of Src via a direct substrate docking interaction[5; 6; 7]. Mutational studies, as well as a recent crystal structure of the Csk/Src enzyme/substrate complex, have revealed the precise surfaces on both enzyme (Csk) and substrate (Src) that mediate this interaction[5; 6; 7]. There is no involvement of an SH2 domain; rather the Csk kinase domain phosphorylates the Src tail via direct interaction with a subset of residues presented on the C-terminal lobe of the Src kinase domain surface[5; 6; 7]. Regardless of the accuracy of our modeled docking interface (Fig. 6A), it appears that the Csk/Src docking interface is distinct from the Itk SH2/kinase interface both in the nature of the substrate domain required for docking and in the location of the docking site on the kinase domain itself. The intramolecular nature of the Itk docking interaction and the covalent constraints imposed by the linkers between domains mean that the Itk SH2 domain is not likely to occupy the same position on the Itk kinase domain as the Src substrate does on the Csk kinase domain.

For decades the phosphotyrosine binding function of SH2 domains has been recognized as a critical component of many signaling pathways. What then is the role of non-classical interactions such as that described here for the Itk SH2 domain? An immune dysregulation syndrome following Epstein-Barr virus infection in girls has recently been linked to a single amino acid change in the Itk SH2 domain that lies outside of the classical phospholigand binding site[47] but is central to the SH2 docking surface defined here. Specifically, a missense mutation that results in substitution of R334 (mouse numbering) with tryptophan leads to a phenotype consistent with that observed in Itk deficient mouse models[47]. As illustrated in Figure 2 and Figure 4, we have shown that loss of the R334 sidechain in the Itk SH2 domain reduces the phosphorylation levels of both Y511 and Y180, which would render Itk inactive. Interestingly, mutation of precisely the same site in the SH2 domain of SAP causes X-linked lymphoproliferative disease[48] and we report here that a subset of XLA causing mutations in the Btk SH2 domain (including K374N, the site corresponding to R334 in Itk) significantly alter Btk autophosphorylation efficiency (Fig. 5).

While it is tempting to speculate that these mutations disrupt important non-canonical interactions of the SH2 domains required for activation and signaling, specific studies that examined the disease-causing SH2 mutations in Itk and SAP (R334W for Itk and Q99P in SAP) conclude that these particular mutations lead to protein instability and resulting deficiencies in enzyme levels are the cause of the severe phenotype[47; 48]. In contrast, protein instability has not been observed for the corresponding site in Btk that carries the XLA causing mutation, K374N[49]. In fact, consistent with our finding that this XLA causing mutation reduces Btk autophosphorylation in vitro (Fig. 5), these authors report that the K374N mutation in the SH2 domain of full length Btk shows significantly reduced phosphorylation on Y223 and fails to trigger calcium flux in response to IgM crosslinking[49]. The SH2 mediated docking interaction defined here for Itk and Btk begins to provide mechanistic insight into why particular mutations in the SH2 domain that appear remote from the phospholigand binding site can nevertheless adversely affect the signaling function of these immune kinases. Thus, while acknowledging that the links between specific mutations and specific diseases likely arise from multiple phenomena, it is nevertheless interesting to consider the importance of non-classical domain functions and the potential for mutations in these less well characterized regions to cause disease.
EXPERIMENTAL PROCEDURES

Materials

The Itk SH2 phospho-peptide ligand (phospho-SLP76 peptide, ADpYEPP) and the Btk SH2 phospho-peptide ligand (GDGpYELEEISPLLL) were obtained from GenScript. Biotin labeled Peptide-B (Aminohexanoyl biotin-EQEDEPEGIYGVLF-NH₂) was obtained from AnaSpec Inc. The peptide concentration and sequences were confirmed by amino acid analysis (Keck Biotechnology Resource Laboratory, Yale) and MALDI-TOF MS (Iowa State University Protein Facility) respectively. The anti-pY223 Btk antibody (corresponding to Itk pY180 and Tec pY187) and anti-pY551 Btk antibody (corresponding to Itk pY511) were kind gifts from Dr Owen Witte.

Constructs

The baculoviral expression constructs for full-length Itk, Btk and Tec have been described previously. The full-length baculoviral construct for Lck was a kind gift from Dr. Leslie Berg. The bacterial expression constructs for the Btk and Tec SH3-SH2 domain constructs have been described elsewhere. The C-terminally Myc-His tagged Itk SH3-SH2 domain was created by PCR amplifying the Itk SH3-SH2 domain (residues 171–338) and cloning it into the pTrcHis2-TOPO vector (Invitrogen). All constructs were verified by sequencing at the Iowa State DNA synthesis and sequencing facility.

Protein expression and purification

Baculoviral constructs were expressed and purified from Sf9 cells as described previously. The bacterial expression constructs for the His-tagged Itk, Btk and Tec SH3-SH2 domains were transformed into BL21 cells (Novagen). The cells were grown in LB media to an OD₆₀₀nm of 0.7 and induced with 1 mM IPTG at 16 °C overnight. The cells were harvested, re-suspended in lysis buffer (50 mM KH₂PO₄, pH 8.0, 150 mM NaCl, 20 mM imidazole, 0.5 mg/ml lysozyme) and frozen overnight at −80°C. The cell pellets were thawed and incubated on ice for 15 min with 1 mM PMSF and 5000 Units of DNase I (Sigma). The lysate was spun at 16K for 1 hour at 4°C and the proteins were loaded onto the Nickel-NTA resin (Qiagen), washed with wash buffer I (50 mM KH₂PO₄, pH 8.0, 150 mM NaCl, 40 mM imidazole), followed by wash buffer II (50 mM Hepes, pH 7.5, 150 mM NaCl). The proteins were then eluted in elution buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 250 mM imidazole).

Kinase assays and western blotting

In vitro kinase assays were performed by incubating full-length Itk, Btk or Tec with their respective substrate 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 µM ATP) at 25 °C for one hour. The samples were boiled, separated by SDS-PAGE and western blotted with the Anti Btk phosphoY223 antibody, anti-FLAG (Sigma) antibody or anti-Myc (Invitrogen) antibody as described previously. Kinetic parameters for the full-length wild-type and mutant Itk are derived using radioactive assays that have been described previously.

Circular dichroism (CD) Spectra

CD measurements of the Itk SH3-SH2 domains and Btk SH3-SH2 domains were performed on a Jasco J-715 CD spectropolarimeter (Jasco Inc.) in the far UV region (190–260 nm) at 25 °C. Samples were prepared in 10 mM KH₂PO₄, pH 7.4, at a concentration of 8 µM. CD spectra were recorded at a scanning rate of 50 nm / min with a spectral bandwidth of 2 nm and response times of 2 msec. Ten accumulations were acquired and the results were averaged.

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background subtraction, all the CD data were converted from mdegree into molar residue ellipticity (deg cm$^2$ dmol$^{-1}$).

**NMR titrations**

NMR Spectra were acquired using a Bruker DRX500 spectrometer operating at $^1$H frequency of 499.867 MHz. All spectra were obtained at 298K. $^1$H and $^{15}$N chemical shifts were externally referenced to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) in identical buffer. NMR titrations were carried out as described $^{13; 14; 15; 16}$. All protein samples were prepared in 50 mM sodium phosphate (pH 7.2), 75 mM NaCl, 2 mM DTT, 0.02% (w/v) NaN$_3$. Unlabeled phospho-peptide ligand was added stepwise to $^{15}$N-labeled protein and each step was monitored by acquiring a 2D $^1$H-$^{15}$N HSQC spectrum $^{17}$. The changes in $^1$H and $^{15}$N chemical shifts were quantified using the formula $^{18}$:

$$\Delta \delta_{\text{ave}} = \left[ \frac{1}{2} \left( (\Delta \delta_h)^2 + (0.2 \Delta \delta_n)^2 \right) \right]^{1/2}$$

The dissociation constants were derived from binding curves using the Matlab (version 5.3.1, The Mathworks Inc.) suite of programs by plotting $\Delta \delta_{\text{ave}}$ versus ligand concentration.

**Abbreviations**

Itk, Interleukin-2 tyrosine kinase; Btk, Bruton’s tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; XLA, X-linked agammaglobulinemia.

**ACKNOWLEDGMENTS**

We thank Dr. Leslie Berg for providing the Lck baculovirus, Dr. Owen Witte for providing the anti-pY223 and anti-pY551 Btk antibodies as well as the Btk cDNA template, and Dr. Hiroyuki Mano for the Tec IV cDNA template. This work is supported by a grant from the National Institutes of Health (National Institute of Allergy and Infectious Diseases, AI43957) to A.H.A.

**REFERENCES**


Figure 1. Tec kinases mediate SH3 domain autophosphorylation via a conserved surface on the SH2 domain
(A) Domain architecture of full-length Itk (residues 1–619), with a Pleckstrin homology (PH), Tec homology (TH), SH3, SH2 and kinase domain. The autophosphorylation site (Y180) is located within the Itk SH3 domain. (B) Schematic representation of Itk SH3 domain autophosphorylation. Autophosphorylation on Y180 in the SH3 domain occurs ‘in cis’ and a direct binding interaction between the Itk SH2 domain and the Itk kinase domain is necessary for Itk SH3 domain phosphorylation. It should be noted that full length Itk or single polypeptide fragments of Itk that contain both the kinase domain and the Y180 containing SH3 domain (SH3-SH2-kinase) exclusively autophosphorylate in cis. Nevertheless, the smaller Itk...
SH3-SH2 domain fragment can be phosphorylated by Itk in trans as shown in (C). (C) Itk, Btk and Tec can each phosphorylate the SH3 domain of Itk, Btk or Tec. Full-length Itk (lanes 4, 5 and 6), full-length Btk (lanes 7, 8 and 9) or full-length Tec (lanes 10, 11 and 12) were incubated with the substrate SH3-SH2 domain construct of Itk (lanes 4, 7 and 10), Btk (lanes 5, 8 and 11) or Tec (lanes 6, 9 and 12) in an in vitro kinase assay buffer at RT for one hour. The samples were separated by SDS-PAGE and western blotted with an anti-Btk phospho-Y223 antibody (used to detect phosphorylation on Itk Y180, and Tec Y187) or an anti-FLAG antibody to detect enzyme levels. Ponceau S stain of the membrane in the bottom panel indicates substrate levels. Lanes 1, 2 and 3 are the no enzyme controls for the SH3-SH2 domains of Itk, Btk and Tec, respectively. The data shown is representative of at least three independent experiments. (D) Sequence alignment of the Itk SH2 domain with the SH2 domains of other Tec family members Btk, Tec, Txk and Bmx, and two unrelated SH2 domains: phosphoinositide 3-kinase (PI3K) and Grb2. The secondary structure elements derived from the structure of the Itk SH2 domain are indicated by arrows (β strand) and boxes (α helix) above the sequence. All SH2 domain residues that have been mutated in this study are boxed or indicated by open circles above the sequence. The Btk XLA mutations tested in this study are indicated by asterisks.
Figure 2. Mutations in the Itk SH2 domain disrupt phosphorylation on Itk SH3 Y180

(A) Surface residues on the Itk SH2 domain that are conserved within the Tec family (boxed residues in Fig. 1D) but different from the corresponding residues in the SH2 domains of PI3K and Grb2 were mutated in the context of Myc-tagged Itk SH3-SH2 domain. These mutants (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19: one µM substrate; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20: five µM substrate) were incubated with full-length FLAG-tagged Itk in an in vitro kinase assay at room temperature for one hour. The samples were separated by SDS-PAGE and western blotted with an anti-Btk phospho-Y223 antibody, anti-Myc antibody (to detect substrate levels) or an anti-FLAG antibody (to detect enzyme levels). Lanes 1 and 2 are the no enzyme controls. The data shown is representative of at least three independent experiments.
The phosphorylation level of each mutant SH3-SH2 substrate (indicated by arrow) is compared to that of wild type substrate. Reduced Y180 phosphorylation for a mutant compared to wild type suggests a disruption of the SH2 mediated docking interaction. (B) The Itk SH2 domain docking mutations do not cause major structural changes in the Itk SH2 domain. Circular Dichroism (CD) spectra of Itk mutant substrates overlaid with that of the wild-type protein show no major changes in secondary structure. (C) Additional Itk SH2 domain surface residues were mutated in a second round of screening (indicated by open circles in Fig. 1D) and tested as in Fig. 2A. Lanes 1–12 were probed using the anti-Btk phospho-Y223 antibody from Dr. Owen Witte while lanes 13–20 were probed using the anti-Btk phospho-Y223 antibody from Invitrogen.
Figure 3. Mapping of the Itk SH2 domain residues involved in Itk kinase docking onto the domain structure
(A) The Itk SH2 domain residues that are mutated in this study are mapped onto the structure of the Itk SH2 domain (ribbon structure shown in cyan, PDB ID 1LUN). Based on the data shown in Fig. 2, residues that, upon mutation, disrupt autophosphorylation on SH3 Y180 are shown in red. The residues that, upon mutation, have no effect on Y180 phosphorylation are in tan. The docking interface on the Itk SH2 domain clusters primarily on one side of the molecule (two views are shown) and involves residues from the AB, BG and EF loops as well as the base of the βD strand. (B) Surface representation of the same structural information shown in (A). The red surface area maps the residues that, when mutated to alanine, diminish Y180 phosphorylation in the context of the SH3-SH2 substrate and the tan color indicates mutations that have no effect on Y180 phosphorylation. (C) Surface rendering of the Itk SH2 domain bound to its classical phosphotyrosine containing peptide. The phosphopeptide is...
shown in orange with the phosphotyrosine (pY) and proline residue in the pY+3 position included as sticks and labeled. The red surface indicates those residues mapped by mutation that mediate phosphorylation of Y180. This substrate docking surface is circled on both structures and the views of the structure differ by 90°. Y292 (labeled on structure) is the only docking residue that is within the pY+3 binding pocket. All structure figures were generated using PyMol.50.
Figure 4. Mutations within the Itk SH2 domain disrupt autophosphorylation on Itk SH3 Y180 within full-length Itk

(A) FLAG-tagged Itk full-length wild-type, Y180F (negative control), E308A/K309A, G328A/L329A, or R332A/R334A mutants are expressed in Sf9 cells along with full-length wild-type Lck. Itk is purified using an anti-FLAG resin and the proteins are then incubated at room temperature in an in vitro kinase assay buffer and western blotted with an anti-Btk phosphoY223 antibody, anti-pY511 antibody and anti-Itk antibody. Y180 phosphorylation levels are reduced for all four mutant substrates compared to wild type substrate (top panel).

(B) Initial velocity ($V_i$) of wild type, full length Itk (lane 1, WT), G328A/L329A (lane 2) and R332A/R334A (lane 3) mutants are measured using Peptide B as substrate. Lane 4 is the no enzyme control.

(C and D) The E308A/K309A double mutation has no significant effect on full-length Itk kinase activity. Itk substrate (Peptide B and ATP respectively) curves of WT full-length Itk (filled triangles) and the full length Itk E308A/K309A mutant (open squares) were fit to the Michaelis-Menten equation using GraphFit to obtain the kinetic parameters reported in (F). (E) Itk SH2 E308A/K309A double mutation has no effect on phospho-peptide

<table>
<thead>
<tr>
<th>Peptide B</th>
<th>ATP</th>
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<tbody>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>$k_{cat}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>FL WT</td>
<td>46.0 +/- 13.4</td>
</tr>
<tr>
<td>FL E308AK309A</td>
<td>46.5 +/- 9.2</td>
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binding. An initial concentration of 300 µM of either \( ^{15}\text{N}\)-labeled WT Itk SH2 (open circles) or \( ^{15}\text{N}\)-labeled SH2 E308A/K309A mutant (filled circles) was titrated with increasing concentration of phospho-SLP76 peptide (ADpYEPP). \( ^{1}\text{H},^{15}\text{N}\) HSQC spectra were obtained upon addition of each aliquot of ligand. Binding curves were generated by plotting the normalized concentration dependence of amide \( ^{1}\text{H}\) chemical shifts for resonances corresponding to Itk K242, V275, C288, K290, Y292, I294, K295, E296, Y305 and A307. (F) The kinetic parameters for wild-type Itk and Itk E308AK309A. The kinetic parameters for wild-type Itk have been reported previously\(^{12}\).
Figure 5. XLA causing mutations within the Btk SH2 domain disrupt autophosphorylation on Y223 in the Btk SH3 domain

(A) A subset of the XLA causing mutations that have been identified within the Btk SH2 domain were introduced into the Btk SH3-SH2 domain fragment. These mutants (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27: one µM SH3-SH2 substrate; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28: five µM SH3-SH2 substrate) were incubated with full-length FLAG-tagged Btk in an in vitro kinase assay at room temperature for one hour. The samples were separated by SDS-PAGE and western blotted with an anti-Btk phospho-Y223 antibody or an anti-FLAG antibody (to detect enzyme levels). Coomassie staining of the gel shows the substrate levels. Lanes 1 and 2 are the no enzyme controls. The data shown is representative of at least three independent experiments. A subset of these XLA causing mutations result in a reduction of Y223 phosphorylation (lanes 7, 8, 11–14, 23–28). (B) The CD spectra of wild-type Btk SH3-SH2 superimposed with spectra of the Btk SH2 XLA mutants (H364D, S371P and R372G). (C) Btk SH2 XLA H364D and S371P mutations do not affect

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phospho-peptide binding. An initial concentration of 250 µM of either $^{15}$N-labeled WT Btk SH2 (open circles), Btk SH2 H364D (filled circles) or Btk SH2 S371P (open squares) were titrated with increasing concentration of phospho-peptide (GDGpYEEISPLL). $^1$H-$^{15}$N HSQC spectra were obtained upon addition of each aliquot of ligand. Binding curves were generated by plotting the normalized concentration dependence of amide $^1$H chemical shifts for resonances corresponding to Btk Y279, R288, E292, R307, T316, Y334, C337, S338, A347, K349, L373 and V377. (D) The Btk SH2 XLA mutations that disrupt Btk SH3 domain autophosphorylation map to the same surface as that identified on the Itk SH2 domain. Btk SH2 XLA causing mutations were mapped onto the Btk SH2 domain (shown in grey on left, PDB ID 2GE9). Mutations that disrupt Btk autophosphorylation are represented as sticks in green and labeled and those residues that do not diminish phosphorylation are shown as tan sticks. The color scheme highlighting the docking interface on the Itk SH2 domain is the same as that described in Figure 3. The substrate docking surfaces on both Btk and Itk SH2 domains are circled and the Btk and Itk SH2 domains are shown in the same orientation.
Figure 6. Model of the Itk kinase domain docked with the Itk SH2 domain

(A) Two views of the structural model showing a possible docking configuration of the Itk SH2 domain with the Itk kinase domain during SH3 autophosphorylation. The domain structure of the SH3-SH2-Kinase fragment of Itk is shown as a schematic. The Itk kinase domain structure (shown in blue, PDB ID 1SNX) was aligned with the structure of the substrate-bound insulin receptor kinase domain (PDB ID 1IR3). For clarity, the insulin receptor kinase substrate bound structure is not shown. In the view on the left, the kinase domain is oriented with the active site facing the viewer and the N- and C-lobes are labeled. The Itk SH3 domain (black ribbon) was docked into the Itk kinase domain active site by superimposing Itk Y180 (represented by sticks in red) with the tyrosine residue of the insulin receptor kinase substrate as previously described. The docked SH2 domain is orange and the residues identified by mutagenesis are depicted in green. The SH2-kinase linker is labeled and shown with a broken brown line and the SH3-SH2 domain linker is labeled and shown as a broken yellow line. W355 and M410 (residues required for Itk kinase activity) are labeled. The red arc with the double-headed arrows is to indicate that this model represents one location within a range of possible locations for the Itk SH2 domain on the Itk kinase domain. To illustrate the accessibility of the SH2 phospholigand binding site during autophosphorylation, a bound phosphopeptide is added (based on PDB ID 2ETZ) to the model and depicted in cyan; the pY and pY+3 residues are labeled. In the view on the right, the side chains of the Itk SH2 domain that, upon mutation, disrupt phosphorylation of Y180 are labeled.

(B) Structural comparison of the Lck SH3-SH2

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dual domain structure (shown in tan, PDB ID 1LCK) solved by crystallography (right) \(^\text{28}\), with the Itk SH3-SH2 dual domain fragment (left) extracted from the model of autophosphorylation shown in (A). The Itk SH2 residues that mediate Y180 phosphorylation are depicted in green and Y180 is red. The standard secondary structural elements for the SH3 and SH2 domains are indicated to illustrate the similarity in the relative domain orientations.