

# The Linker between SH2 and Kinase Domains Positively Regulates Catalysis of the Tec Family Kinases<sup>†</sup>

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**ABSTRACT:** Tec family nonreceptor tyrosine kinases are key immunological enzymes that control processes that range from T and B cell development to reorganization of the actin cytoskeleton. The full-length Tec kinases have been resistant to crystallization. This lack of structural data and the paucity of *in vitro* biochemical data for this kinase family leave a void in our understanding of Tec kinase regulation. In this report we have used interleukin-2 tyrosine kinase (Itk) as a model system to gain insight into the regulatory apparatus of the Tec kinases. Use of a quantitative *in vitro* kinase assay has uncovered an essential role for the short linker region flanked by the SH2 and kinase domains of Itk in positively regulating Itk catalytic activity. The precise residues that allosterically regulate Itk are conserved among Tec kinases, pointing to the conserved nature of this regulatory mechanism within the family. These findings indicate that Tec kinases are not regulated in the same manner as the Src kinases but rather share some of the regulatory features of Csk instead.

Protein kinases have evolved a variety of regulatory mechanisms that exploit the effects of site-specific phosphorylation and precise protein/domain interactions (1, 2). The largest family of nonreceptor protein tyrosine kinases consists of the eleven Src kinases (3) that each contain an N-terminal unique region, followed by an Src homology-3 (SH3<sup>1</sup>) domain, Src homology-2 (SH2) domain, catalytic domain, and a C-terminal tail with a conserved regulatory tyrosine. A significant leap in our understanding of kinase regulation has been provided by the three-dimensional structures of two Src family kinases (Hck and Src) in their inactive states (4–6). These structures reveal that the SH3 and SH2 domains are located on the “back” surface of the kinase domain mediating a network of intramolecular interactions that stabilize the kinase domain in an *inactive* conformation.

The C-terminal Src kinase (Csk) shares a similar overall domain architecture with Src yet lacks the C-terminal tail that contains the regulatory tyrosine residue of Src (7). The three-dimensional structure of full-length, active Csk shows that the regulatory SH3 and SH2 domains are placed on top of the N-terminal lobe of the kinase domain with the two linker regions (between SH3 and SH2 and between SH2 and the kinase domain) running almost parallel to each other and extensively contacting the N-terminal lobe of the kinase domain (7). These contacts serve to stabilize the orientation of the Csk C-helix that is required for full catalytic activity.

An interesting contrast exists between the Csk and Src kinases in that the noncatalytic SH3 and SH2 domains of Src serve a negative regulatory role (deletion or mutation of these domains results in increased activity of the neighboring kinase domain) while the same noncatalytic domains in Csk serve a positive regulatory role. Indeed, the isolated Csk kinase domain exhibits very poor catalytic activity while the isolated Src kinase domain is fully active (8, 9). Thus, in two protein tyrosine kinases that appear closely related in domain structure, the SH3 and SH2 domains play opposite regulatory roles. These opposing regulatory functions make simple predictions related to the regulation of other kinase families quite challenging. One such group of tyrosine kinases is the Tec family (Tec, Btk, Rlk/Txk, Bmx/Etk, and Itk), proteins that mediate signals downstream of a variety of receptors on multiple cell types including B and T cells (10–12).

The Tec family of nonreceptor tyrosine kinases contains the ubiquitous SH3 and SH2 domains as well as a pleckstrin homology (PH) and Tec homology (TH) domain (10). Activation of Tec kinases involves transphosphorylation of the activation loop of the kinase domain by Src family kinases, followed by autophosphorylation within the SH3 domain (11). For Itk, this corresponds to phosphorylation on Y511 within the activation loop of the kinase domain by the Src family kinase Lck, followed by autophosphorylation on Y180 within the SH3 domain (13, 14). *In vitro* kinase assays using immunoprecipitated enzymes have shown that while phosphorylation on Itk Y511 is critical for Itk activity, phosphorylation on Itk Y180 is dispensable for Itk catalytic activity (14). While high-resolution structures of the isolated domains derived from many of the Tec kinases have been solved (15–19), the current lack of structural information for the full-length kinases or even multidomain fragments of the Tec kinases creates a void in our understanding of

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<sup>1</sup> Abbreviations: Itk, interleukin-2 tyrosine kinase; SH3, Src homology-3; SH2, Src homology-2; Csk, C-terminal Src kinase.

their regulation. Biochemical analyses probing the regulatory mechanisms at work within the Tec family kinases have been limited to *in vivo* experiments and *in vitro* assays using immunoprecipitated proteins (20–24). These experiments have identified the *in vivo* requirements for the different domains of the Tec kinase, yet it has been difficult to delineate the contribution of specific amino acids to intrinsic activity. *In vitro* activity measurements using immunoprecipitated enzymes are difficult to quantify accurately and only detect changes in overall activity, without distinguishing between effects on substrate affinity versus catalytic turnover.

In this report we have examined Itk as a model system to understand the regulation of Tec family kinases by the use of quantitative kinetic analysis. This approach allows a direct measure of the extent to which specific amino acid mutations or domain deletions affect both substrate affinity ( $K_m$ ) and catalytic activity ( $k_{cat}$ ). We have identified the 17-residue linker sequence between the SH2 and kinase domains (the SH2–kinase linker) as a critical component that maintains Itk kinase activity ( $k_{cat}/K_m$ ). Furthermore, we identify several specific residues that mediate interactions between this regulatory region and the catalytic domain of Itk and extend these observations to other Tec family members highlighting the conserved nature of the regulation. Taken together with the insights provided by structures of other tyrosine kinases, our results provide a framework to understand the regulation of the Tec kinases at the molecular level.

## EXPERIMENTAL PROCEDURES

**Baculoviral and Bacterial Constructs.** Full-length and N-terminal deletion mutants of wild-type (WT) and kinase dead (K390R) Itk (mouse sequence) were PCR amplified using a reverse primer which encoded a FLAG epitope tag. The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. Full-length Btk and full-length Tec IV were PCR amplified and cloned into the same vector. Point mutations in the full-length constructs were created by using the site-directed mutagenesis (SDM) kit (Stratagene). The bacterial expression construct for the Itk SH3–SH2 fragment has been described previously (25). All constructs were verified by sequencing at the Iowa State University DNA synthesis and sequencing facility.

**Baculovirus Production.** The pENTR vectors with various inserts were recombined *in vitro* with BaculoDirect C-Term Linear DNA using the LR Clonase II enzyme (Invitrogen). The DNA was transfected into *Sf9* cells using Effectene (Qiagen) followed by three rounds of viral selection and amplification. For protein production, the cells were infected with a 1:1 ratio of Itk:Lck baculovirus. A 1:1 ratio of Itk:Lck baculovirus was chosen as it achieved maximal phosphorylation on Itk Y511 (data not shown). The cells were harvested 72 h postinfection and stored at  $-80^\circ\text{C}$ .

**Protein Purification.** Bacterial protein purification of Itk SH3SH2 domain was carried out as described previously (25). All other proteins used in this report were purified from *Sf9* cells. Protein purification from *Sf9* cells was carried as previously described with minor modifications (26). Briefly, the cell pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 2 mM EDTA, 1 mM PMSF) and lysed by dounce homogenization. The homogenate was spun at 16K for 1 h at  $4^\circ\text{C}$ . Glycerol was added to the supernatant

to a final volume of 10%, and then incubated with anti-FLAG M2 affinity resin (Sigma) at  $4^\circ\text{C}$ . The resin was rinsed in wash buffer (50 mM Tris pH 8.0, 500 mM NaCl, 1 mM PMSF, 10% glycerol), and the protein was eluted in elution buffer (wash buffer with 200  $\mu\text{g}/\text{mL}$  FLAG peptide). The purified protein was quantified by measuring absorbance at 280 nm. All proteins were greater than 95% pure by Coomassie staining. Purified proteins were monitored for phosphorylation on Itk Y511 by Western blotting using a Btk pY551 specific antibody. Phosphorylation on Itk Y180 was monitored using a Btk pY223 specific antibody (14). All Western blots were developed by chemiluminescence followed by autoradiography. The ratio of intensities of the phosphorylated (pY511) band to that of the total protein (from the anti-FLAG blot) was used to compare the phosphorylation levels of the various proteins.

**Kinase Assay.** Following procedures outlined previously (26),  $K_m$  determinations for ATP or Peptide B [(aminohexanoyl biotin-EQEDEPEGIYGVLF-NH<sub>2</sub>) (Anaspec Inc.)] were carried out by incubating purified enzyme in reaction buffer (50 mM Hepes pH 7.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mg/mL BSA, and 1 mM Pefabloc) and 5  $\mu\text{Ci}$  of [<sup>32</sup>P] ATP (Amersham Biosciences) at room temperature. Peptide B concentration was varied between 0 and 400  $\mu\text{M}$ . ATP concentration was varied between 0 and 320  $\mu\text{M}$ . The enzyme concentrations used for comparison ranged from 50 nM to 2  $\mu\text{M}$ . Each assay was done in duplicate. Data was fit with the Michaelis–Menten equation using GraphFit 5 software.

**In Vivo Kinase Assay and Western Blotting.** *Sf9* cells were infected with the respective Itk virus. Three days postinfection, the cells were lysed in radioimmunoprecipitation (RIPA) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM NaF, 1 mM DTT, 1 mM PMSF). The lysate was spun at 10000g for 10 min at  $4^\circ\text{C}$ . The supernatants were normalized for protein content using the Bradford assay, separated by SDS–PAGE, and transferred onto a PVDF membrane. The membranes were then blotted with either an anti-phosphotyrosine antibody (4G10 from Upstate) or anti-FLAG antibody (Sigma).

## RESULTS

**The SH2–Kinase Linker Region Is Critical for Itk Activity.** It has been noted previously that the isolated kinase domain of Itk exhibits poor catalytic activity when compared to the full-length enzyme (17, 26). This finding implies a positive regulatory role for the noncatalytic domains adjacent to the Itk kinase domain. To determine the precise contribution of each regulatory domain to the kinase activity of Itk, we compared the *in vitro* activities of a series of N-terminal deletion constructs (Figure 1A). Kinase assays were carried out using peptide B (EQEDEPEGIYGVLF), which has been shown to be a substrate for full-length Itk (26). The kinase inactive (K390R) versions of each deletion construct show negligible activity when compared to the wild-type (WT) full-length enzyme, indicating that the measured activity corresponds to Itk and not to impurities that may copurify with Itk (Supporting Information Figure 1a). The substrate curves for WT full-length Itk and the panel of deletion constructs are shown in Figure 1B,C. The fit of these data yielded values for  $k_{cat}$  and  $K_m$  for each construct. The ratio

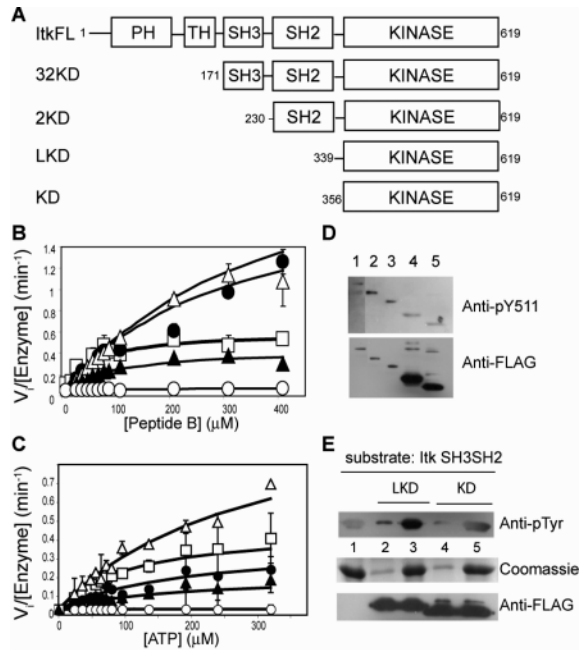


FIGURE 1: The SH2–kinase linker of Itk is critical for kinase activity. (A) Itk deletion fragments. (B and C) The *in vitro* kinase activities of the deletion fragments of Itk were compared. Substrate (peptide B and ATP) curves of ItkFL (open squares), 32KD (filled circles), 2KD, (open triangles), LKD (filled triangles), and KD (open circles) were fit to the Michaelis–Menten equation to obtain the kinetic parameters reported in Table 1. (D) Western blot showing the pY511 levels of various FLAG-tagged Itk mutants. Lanes 1–5: Itk FL, 32KD, 2KD, LKD, and KD. (E) Comparison of the *in vitro* kinase activities of LKD with that of KD using a protein substrate. 2  $\mu$ M purified FLAG-tagged Itk LKD or Itk KD was incubated with 1  $\mu$ M (lanes 2 and 4) or 10  $\mu$ M (lanes 3 and 5) Itk SH3SH2 domain in an *in vitro* kinase reaction. Lane 1 is 10  $\mu$ M Itk SH3SH2 domain alone with no enzyme. The samples were Western blotted with the Btk anti-pY223 antibody. Coomassie stain and an anti-FLAG blot show protein levels.

of  $k_{cat}/K_m$  was determined for each Itk fragment and compared to that of the full-length enzyme (Table 1). Hereafter, kinase activity refers to the ratio of  $k_{cat}/K_m$ , unless specified otherwise.

Several features emerge on consideration of the kinetic data for full-length Itk and related deletions. Most notably, the only construct that exhibits negligible kinase activity is the isolated kinase domain (KD). The slightly larger fragment that contains the 17-residue SH2–kinase linker sequence plus the kinase domain (LKD) retains nearly half of the activity of the full-length enzyme (Figure 1, Table 1); this is in spite of the fact that the overall level of phosphorylation on Y511 (a parameter previously linked to Itk activity (13)) is significantly lower for LKD than full-length enzyme (Figure 1D; lanes 1 and 4)). However, Y511 phosphorylation levels are identical between KD and LKD, as are the  $K_m$  values for peptide B and ATP, which are, within error, unchanged from that of full-length Itk. Thus, the short 17 amino acid region located just N-terminal to the Itk kinase domain appears to significantly stabilize the active conformation of the Itk kinase domain as evidenced by an increase in  $k_{cat}$  for LKD compared to that of KD. The difference in the catalytic properties of LKD and KD can also be observed in the phosphorylation levels of a protein substrate (Figure 1E). Using the Itk SH3–SH2 fragment that contains the auto-phosphorylation site of Itk (Y180 in the SH3 domain) as a

Table 1: Kinetic Parameters of Itk Deletion Constructs and Point Mutants

enzyme	$K_m(\text{peptideB}) \mu\text{M}$	$k_{cat} \text{ min}^{-1}$	$k_{cat}/K_m \text{ min}^{-1} \mu\text{M}^{-1}$
ItkFL	46 ± 13	0.54 ± 0.05	0.01
KD	84 ± 19	0.02 ± 0.002	0.0002
LKD	87 ± 19	0.39 ± 0.03	0.004
2KD	290 ± 49	1.94 ± 0.18	0.007
32KD	388 ± 61	2.56 ± 0.20	0.007
W355A	115 ± 25	0.02 ± 0.001	0.0002
M410A	43 ± 4.1	0.05 ± 0.001	0.001
Y421A	51 ± 6.7	0.25 ± 0.01	0.005
Y421F	73 ± 5.0	0.74 ± 0.02	0.01
L350A	55 ± 11	0.40 ± 0.03	0.007
E394A	54 ± 5.8	0.27 ± 0.01	0.005

enzyme	$K_m(\text{ATP}) \mu\text{M}$	$k_{cat} \text{ min}^{-1}$	$k_{cat}/K_m \text{ min}^{-1} \mu\text{M}^{-1}$
ItkFL	107 ± 35	0.43 ± 0.05	0.004
KD	45 ± 27	0.003 ± 0.001	0.0001
LKD	169 ± 28	0.18 ± 0.01	0.001
2KD	297 ± 56	1.13 ± 0.13	0.004
32KD	194 ± 66	0.35 ± 0.06	0.002
W355A	93 ± 17	0.01 ± 0.001	0.0001
M410A	166 ± 31	0.02 ± 0.002	0.0001
Y421A	155 ± 42	0.32 ± 0.04	0.002
Y421F	132 ± 33	0.56 ± 0.07	0.004
L350A	189 ± 25	0.69 ± 0.05	0.004
E394A	136 ± 45	0.26 ± 0.04	0.002

substrate (14), it is apparent that the Itk LKD fragment exhibits greater activity when compared to the isolated Itk KD. This qualitative assessment of activities is consistent with the kinetic parameters that suggest a role for the 17 residue linker in increasing the kinase activity of Itk.

Next we examined the kinetic parameters corresponding to the larger Itk fragments that include the SH2 domain (2KD) and the SH3–SH2 region (32KD). These two fragments are active and show similar kinetic behavior to one another, suggesting that the SH3 domain is dispensable for this level of Itk kinase activity (Figure 1B,C, Table 1). The  $K_m$  values for peptide B binding to the 2KD and 32KD fragments indicate a 6- to 8-fold drop in substrate affinity (increased  $K_m$ ) when compared to full-length enzyme. The  $K_m$  values measured for ATP binding to the 32KD and 2KD constructs are significantly closer to full-length enzyme. The peptide B binding data therefore suggest that in the context of the full-length enzyme, the PH–TH region of Itk may play a role in sequestering the SH2 region in a manner that facilitates substrate binding to the active site. As indicated above, further removal of the SH2 domain (LKD and KD) restores affinity for the substrates to near wild-type levels (Table 1).

Despite the increase in  $K_m$  values observed for 2KD and 32KD, the overall activity ( $k_{cat}/K_m$ ) of these fragments drops less than 2-fold compared to full-length enzyme. This is explained by the concomitant increase in  $k_{cat}$  that balances the decrease in substrate affinity of the 32KD and 2KD fragments. Specifically, deletion of the PH–TH or PH–TH–SH3 region of Itk leads to a 4- to 5-fold increase in  $k_{cat}$  (Table 1), indicating that the positive regulatory interactions between the SH2 domain or the SH2–kinase linker and the catalytic domain may be enhanced in the absence of the PH–TH domains. These data suggest that it will be interesting to explore the precise balance of the regulatory role(s) of the N-terminal PH–TH domains especially in light of their established role in membrane association during T cell



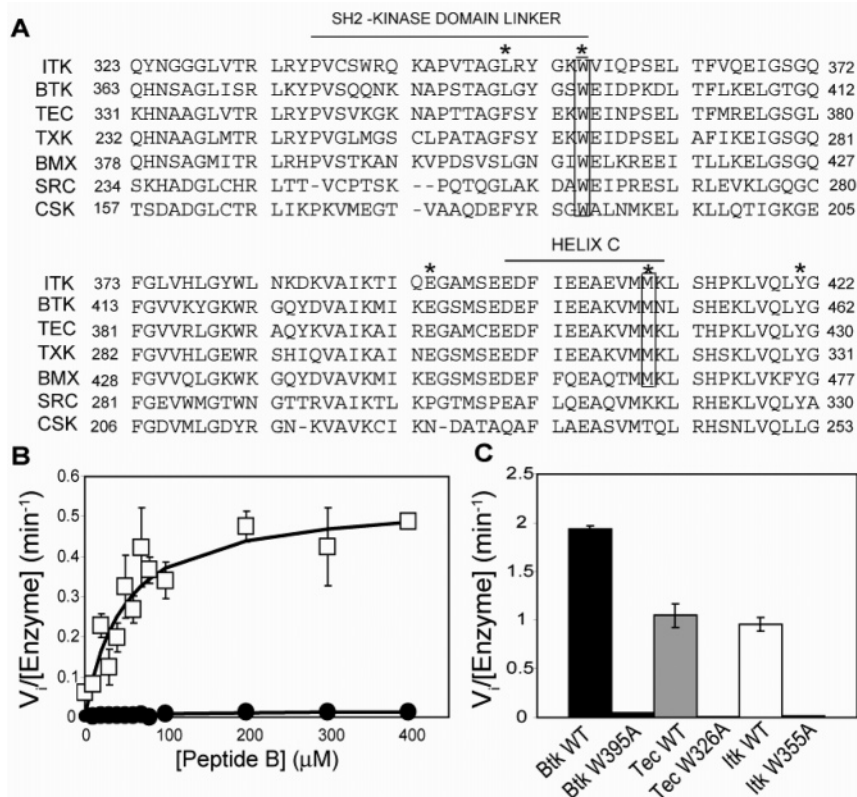


FIGURE 2: The conserved Trp in the SH2–kinase linker regulates the activity of Tec family kinases. (A) Sequence alignment of Tec family kinases with Src and Csk. The region depicted begins at the C-terminus of the SH2 domain and extends beyond the C-helix of the N-terminal lobe of the kinase domain. The SH2–kinase linker and the C-helix regions are indicated above the sequences. The conserved Trp in the SH2–kinase linker and the Met residue that is conserved among the Tec kinases are boxed. Asterisks indicate the amino acid residues that are evaluated in this work. (B) *In vitro* kinase activity of full-length wild-type Itk (open squares) and the W355A (filled circles) mutant of Itk using peptide B as a substrate. (C) Comparison of wild-type full-length activity of Btk, Tec, and Itk to the corresponding Trp mutants of Btk, Tec, and Itk. All data shown are the average of at least two independent experiments.

signaling (27, 28). Moreover, Y511 phosphorylation levels for 32KD and 2KD appear slightly higher than that of full-length Itk, and this difference could also account for the increase in  $k_{cat}$  values for the 32KD and 2KD fragments. Indeed, the differences in Y511 phosphorylation levels across the panel of deletion mutants suggest that more targeted point mutants might provide clearer insights into the regulatory role of the noncatalytic region of Itk.

*W355 in the SH2–Kinase Linker Region Is Critical for the Activity of Tec Family Kinases.* The large difference in  $k_{cat}$  between the LKD and KD fragments, combined with similar  $K_m$  values and identical levels of Y511 phosphorylation for LKD and KD, clearly suggests that the 17 amino acid linker is a critical component of the Itk regulatory apparatus that merits further exploration. Sequence alignment of the SH2–kinase linker region of Tec family kinases with those of Src and Csk shows the presence of a conserved Trp (Figure 2A). This conserved Trp mediates key regulatory interactions between the noncatalytic and catalytic domains of both the Src and Csk kinases (29, 30) and in turn exerts opposing effects on the catalytic activity of Src and Csk. Specifically, mutation of this Trp to Ala in the Src family kinase Hck (W260A) activates Hck, whereas the analogous mutation in Csk (W188A) leads to inactivation of Csk.

To determine the extent to which this residue plays a regulatory role in Itk, W355 was mutated to Ala within the full-length protein and the kinetic parameters of the resulting mutant were measured. Mutation of this single residue in

full-length Itk leads to a precipitous drop in activity to the level of activity associated with the isolated kinase domain (Figure 2B, Table 1). The decrease in activity of the W355A mutant is not due to decreased stability of this protein, as the urea denaturation curve of the Itk W355A mutant is indistinguishable from that of the WT protein (Supporting Information Figure 1b). Since the  $K_m$  for peptide B and ATP for the W355A mutant is similar to that of the WT enzyme, the drop in  $k_{cat}/K_m$  is not due to changes in the ability of the kinase domain to interact with the substrate, but rather to a complete loss of positive regulation by the noncatalytic domains. This finding extends to other Tec family members, as mutation of the corresponding Trp in both Btk (W395A) and Tec (W362A) also shows a sharp decrease in kinase activity (Figure 2C). Indeed, this observation is interesting in light of a recent report that assessed the effect of disease causing mutations in Btk that included the W395 mutation (31). Immunoprecipitated Btk W395A mutant exhibited very poor activity *in vitro* and *in vivo*. Thus, this Trp is a critical residue within the regulatory apparatus of the Tec family kinases.

*Probing the Role of Specific Amino Acids in Maintaining Itk Activity.* In contrast to the abundant structural data on full-length Src and Csk family kinases, there is currently no structural information available for any of the full-length Tec kinases, thwarting efforts aimed at gaining molecular level insight into Tec kinase regulation. Given the positive regulatory role played by the noncatalytic domains of both

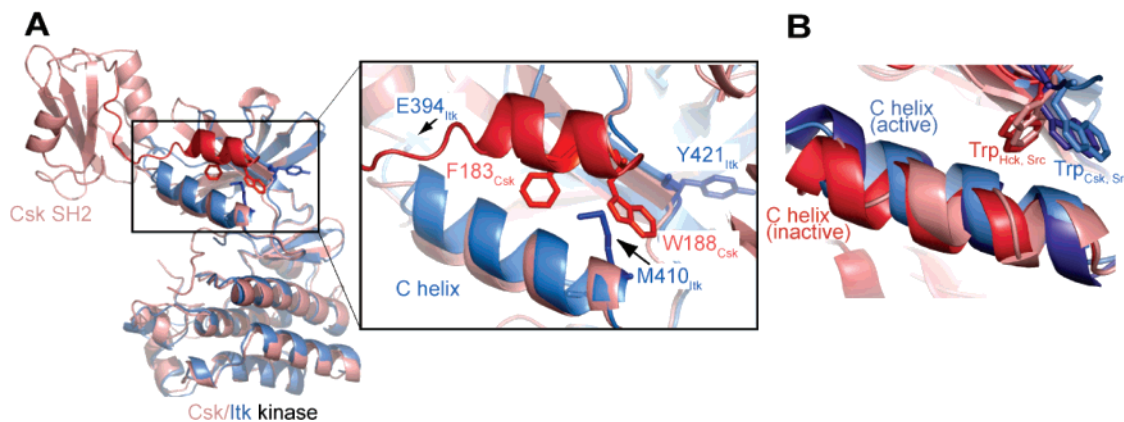


FIGURE 3: Comparison of kinase domain structures. (A) Superposition of the structure of the Itk kinase domain (1SM2, blue) with the structure of full-length Csk (1K9A, salmon). The SH3 domain of Csk is not included in the superposition. The boxed region is enlarged to show the residues mutated in this study. The SH2–kinase linker of Csk is in red. The side chains of E394, M410, and Y421 of Itk are depicted in blue. The Csk residues W188 (corresponds to Itk L350) and F183 (corresponds to Itk L350) are in red. (B) Overlay of the structures of inactive Src (2PTK, red), inactive Hck (1QCF, salmon), active Src (1Y57, dark blue), and active Csk (1K9A, light blue). The region shown is the C-helix and the conserved Trp. Conversion from the inactive to the active state in Src kinase is accompanied by movement in the C-helix and the conserved Trp. Structural superpositions were generated using the alignment program within PyMOL (41). The entire sequence of each kinase domain was used for alignment.

Itk and Csk, we overlaid the available crystal structure of the isolated Itk kinase domain (17) with the structure of full-length Csk (7) in an effort to identify candidate residues within the SH2–kinase linker stretch and the Itk kinase domain itself that are likely to be involved in mediating positive regulatory contacts in Itk (Figure 3A).

*Mutations in the Conserved Trp Pocket Deregulate Itk Activity.* As already demonstrated, the conserved Trp located in the SH2–kinase linker serves an important role in maintaining Csk (30) and Itk (this work) in their active conformations. Indeed, structures of the inactive and active configurations of tyrosine kinases (7, 32–34) can be characterized by the position of this conserved Trp residue. In superimposing the structures of two active tyrosine kinases (Csk and Src) with two inactive tyrosine kinases (Hck and Src) we find that the bulky Trp side chain either acts as a wedge that prevents the inward movement of the C-helix in the inactive state or switches to a conformation that stabilizes the C-helix in the conformation characteristic of active kinases (Figure 3B). For example, in the structure of full-length active Csk, W188 (corresponding to W355 in Itk) lies parallel to the C-helix rather than in a perpendicular position that would maintain the C-helix in its inactive conformation (Figure 3A,B).

Given the correlation between the position of the Trp side chain and kinase activation (Figure 3B), as well as our finding that mutation of W355 in Itk is inactivating (Figure 2), we hypothesize that W355 of Itk adopts a position similar to that of Csk (Figure 3). The model of the Csk/Itk superposition therefore allows identification of additional Itk residues that play a putative role in stabilizing the active conformation of the C-helix via contacts to W355 in the full-length kinase. Excluded from our mutational analysis are amino acids that directly contact ATP in the Itk kinase domain structure (17) in order to selectively probe allosteric regulation of Itk by the noncatalytic regions. With these criteria in mind, three residues comprising a hydrophobic patch can be readily identified that likely surround the conserved W355 in Itk (Figure 3A, inset). Two of the side chains, M410 and Y421, are present in the fragment of Itk

that has been crystallized while the third, L350, resides in the SH2–kinase linker region and is not part of the published Itk structure. This latter residue corresponds to F183 in Csk and to L255 in Src. Mutation of L255 in Src or F183 in Csk has been previously implicated in regulation of these kinases, providing further support for an important role for this position in Itk (35–37).

As anticipated, the  $K_m$  values for ATP binding to each Itk point mutant are unchanged from wild-type full-length enzyme (Table 1). Inspection of Y511 phosphorylation for each point mutant (including the already discussed W355 site) reveals levels that are significantly more uniform than those observed for the deletion constructs (Figure 4A). With the exception of L350 (discussed below) the variation in Y511 phosphorylation among the point mutants reflects, at most, a 2-fold decrease for the W355A mutant when compared to the WT enzyme, much less than the greater than 10-fold decrease in Y511 phosphorylation observed earlier for LKD when compared to WT protein. Since the LKD fragment is about 40% as active as the WT enzyme, the 2-fold difference in Y511 phosphorylation of the W355A mutant cannot solely account for the greater than 90% drop in activity of this mutant. Thus, the differences in the measured catalytic activities of the full-length mutants can be interpreted in terms of the regulatory roles played by the amino acid sites in question.

*M410 in Itk C-Helix Is Critical for Activity.* Within the putative active form of full-length Itk, the location of M410 in the C-helix of Itk seems well positioned to stabilize W355 in a configuration that is parallel to the C-helix and may therefore be a critical residue in the Itk regulatory apparatus (Figure 3A). Indeed, sequence alignment of Itk from different organisms shows that this Met is conserved throughout evolution. Moreover, this residue is unique to the Tec family kinases where it is also conserved (Figure 2A). Mutation of this Met to Ala in full-length Itk leads to a significant drop in activity, with no change in  $K_m$  for either the peptide B substrate or ATP (Figure 4B,C, Table 1). The decrease in activity of the M410A mutant is also not due to decreased stability of this protein, as the urea denaturation curve of

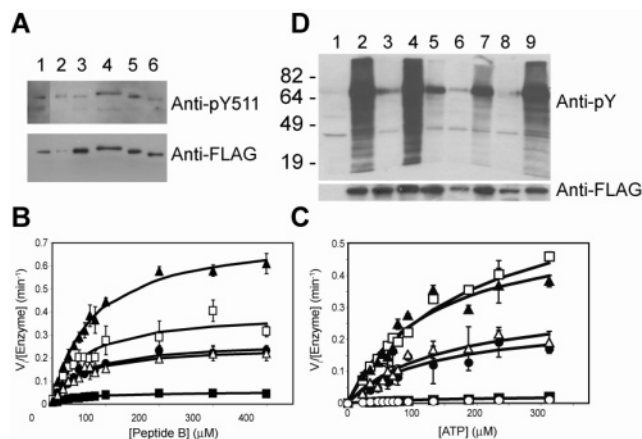


FIGURE 4: Mutations around the conserved Trp in Itk influence kinase activity. (A) Western blot showing the pY511 levels of various FLAG-tagged Itk mutants. Lanes 1–6: Itk FL, L350A, W355A, E395A, M410A, and Y421A. (B and C) Substrate (peptide B and ATP) curves of the Itk full-length point mutants: L350A (open squares), E394A (filled circles), M410A (filled squares), Y421A (open triangles), Y421F (filled triangles), and W355A (open circles). For reference the Y421F mutant (filled triangles) exhibits activity that is nearly identical to that of full-length wild-type Itk. Kinetic parameters for the mutants are reported in Table 1. (D) *In vivo* activity of Itk full-length mutants correlates with *in vitro* kinase activity. *Sf9* cells were infected with baculoviruses for the full-length FLAG-tagged Itk mutants. The cell lysates were then probed by Western blotting with an anti-pTyr or anti-FLAG antibody to detect total Itk levels. Lanes 1–9: uninfected cells, WT, kinase dead (K390R), L350A, W355A, E394A, M410A, Y421A, and Y421F. Data shown are representative of two independent experiments.

the Itk M410A mutant is identical to that of the WT protein (Supporting Information Figure 1b). These data suggest that Itk M410 is a critical residue within the small kinase lobe that likely stabilizes positive regulatory interactions to the SH2–kinase linker via W355.

**Probing the Regulatory Role of L350 and Y421.** Further perturbations to the hypothetical hydrophobic pocket surrounding W355 in Itk (Figure 3A) involved mutation of Y421 to Ala, Asp, or Phe and L350 to Ala. The Itk Y421A mutant maintains approximately half of the activity of wild-type enzyme while mutation of this same residue to phenylalanine Y421F had no effect on activity (Figure 4B,C, Table 1). Introduction of a negative charge into this pocket by mutating Y421 to Asp leads to a complete loss of activity (Supporting Information Figure 1a). The Itk L350A mutant retains three-quarters of the activity of the wild-type enzyme (Figure 4B,C, Table 1). The identical position in both Src and Csk has been extensively mutated. For Src, mutation at this site (L255A) increases kinase activity several fold, consistent with the structure of Src showing a critical role for L255 in stabilizing the inactive conformation (35). Mutation of the corresponding position in Csk (F183) leads to a decrease in kinase activity (36, 37). It should be noted that, unlike the other Itk point mutants in this study, the Y511 phosphorylation level of the L350A mutant differs significantly from that of wild-type full-length Itk. Specifically, the data in Figure 4A lanes 1 and 2 indicate that a larger fraction of the total Itk L350A enzyme is phosphorylated on Y511 when compared to wild-type enzyme, a result that suggests that the measured catalytic activity of the L350A mutant (Table 1) is probably artificially high. Thus, the actual decrease in catalytic efficiency for the Itk L350A mutant could be even more pronounced than

indicated by our measured kinetic parameters and further suggests that the active conformation of Itk in this region more closely resembles that of Csk. Together with the loss of activity upon mutation of Y421 and M410, these findings highlight the importance of the putative hydrophobic pocket surrounding W355 in Itk.

**E394 in the N-Terminal Kinase Lobe Regulates Activity.** Results of our deletion study provide evidence that the SH2 domain of Itk contributes to the intrinsic kinase activity of Itk (Figure 1). This is interesting in light of previous work pointing to the importance of the SH2 domain in T cell activation (22). Moreover, structural work suggests that phospholigand binding to the Itk SH2 domain induces structural changes on the distal surface of the SH2 domain, including a part of the B-helix, the  $\beta$ E and  $\beta$ F strands (38). In the full-length Csk structure it is these precise secondary structural elements of the SH2 domain that are in close proximity to the small lobe of the Csk kinase domain (7). The crystal structure of Csk shows that N226 in the  $\beta$ 3 C-helix loop of the N-terminal lobe of the kinase domain is in close proximity to the SH2 domain of Csk (7) and mutation of this residue to Ala results in a 50% decrease in Csk activity (36). Structural alignment of the isolated Itk kinase domain with full-length Csk brings Itk E394 within the  $\beta$ 3 C-helix loop in close proximity to the Csk SH2 domain (Figure 3A). Moreover, SAXS analysis of full-length Btk shows that the SH2 domain is adjacent to the N-terminal lobe of the kinase domain (39). To determine if this residue is in fact involved in regulating Itk activity, we measured the kinetic parameters for the Itk E394A mutant. Mutation of E394 to Ala leads to a decrease in Itk activity to less than half that of the full-length wild-type enzyme (Figure 4B,C, Table 1). Again, the  $K_m$  values for both peptide B and ATP remain unchanged, showing that substrate recognition is not affected by this mutation (Table 1). These data suggest that E394 is important for positively modulating Itk activity, and may be in direct contact with the SH2 domain in the active conformation of this enzyme.

**In Vivo Activity of Itk Full-Length Mutants.** The *in vitro* kinetic analyses of the Itk mutants described above revealed varying effects on activity and allowed precise measurement of  $K_m$  and  $k_{cat}$ . To examine the extent to which these kinetic parameters reflect the behavior of each mutant *in vivo*, we infected *Sf9* cells with the panel of Itk baculoviruses, and tested overall phosphotyrosine levels within the cell by Western blotting with a phosphotyrosine specific antibody (Figure 4D). ItkFL, L350A, and Y421F mutants all showed intense tyrosine phosphorylation, consistent with the *in vitro* kinase data indicating that these mutations do not adversely affect kinase activity. The other Itk mutants, W355A, E394A, M410A, Y421A as well as the kinase inactive Itk mutant (K390R) all showed low overall phosphorylation levels, consistent with their poor activity *in vitro*.

## DISCUSSION

Orchestrated changes in phosphorylation status and/or domain interactions regulate enzyme activity during cell signaling. Using Itk as a model system for the Tec kinases, we have uncovered the positive role played by the SH2–kinase linker in maintaining the catalytic activity of Itk. Moreover, extension of the construct to include the SH2



domain leads to further enhancement of Itk activity. While this could be due in part to higher levels of Y511 phosphorylation for this Itk fragment, it is also likely that the presence of the SH2 domain serves to stabilize the proper orientation of the 17 amino acid linker region. This is consistent with the results of mutating E394, putatively a site of contact between the kinase lobe and the SH2 domain, and previous *in vivo* data showing that deletion of the SH2 domain from full-length Itk leads to decreased levels of Itk phosphorylation (22). Thus, our *in vitro* kinase assay shows that, in addition to playing a role in mediating protein–protein interactions *in vivo*, the Itk SH2 domain may also play a key role in maintaining the active conformation of Itk.

Based on the mutational analysis of Itk presented here, it is tempting to predict an arrangement for the active state of the Tec family kinases that is similar to that of Csk. We have reported significant similarities in the behavior of Itk and Csk, yet some details of the Itk and Csk regulatory apparatus seem distinct. For example, both the SH3 and SH2 domains positively modulate Csk activity (8), while our results indicate that Itk requires the 17 amino acid linker preceding the kinase domain to regain much of the catalytic activity that is lost on deletion of the entire regulatory region and only the SH2 domain and not the entire SH3–SH2 unit appears to further stabilize the active conformation of the kinase. The activity of the isolated kinase domain of Csk can be enhanced by the addition of the SH3 or SH3–SH2 domains *in trans* (40). In contrast, we found that addition of neither the Itk SH2 domain, the SH2-linker portion, nor the SH3–SH2 fragment *in trans* to the isolated kinase domain had any measurable effect on the *in vitro* activity of the Itk kinase domain (data not shown). Previous *in vivo* work showed that an Itk SH2 domain (R265K) mutant that lacks the ability to bind phospholigand exhibits poor activity (22). However, our efforts to directly test the effect of phosphopeptide binding to the SH2 domain on the catalytic activity of Itk were thwarted due to the low affinity and toxicity effects of the phosphopeptide on *in vitro* kinase activity (data not shown). Finally, it is also notable that SAXS analysis of full-length Btk predicts a linear arrangement for the regulatory domains that, if representative of the active conformation, would be inconsistent with a Csk-like arrangement (39). Thus, the non-receptor tyrosine kinases that share the SH3–SH2–kinase domain cassette (i.e., Src, Csk, Abl, and Itk) are distinguished by variations in their regulatory mechanisms. Ultimately, the regulatory differences that are becoming evident among these apparently closely related tyrosine kinases could be exploited to develop reagents that selectively alter the activity of one enzyme over others. The appropriate targets of such reagents may in fact be the noncatalytic regulatory domains instead of the active sites of each of these kinases.

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#### SUPPORTING INFORMATION AVAILABLE

The kinase activities of Itk full-length Y421D mutant and various catalytically inactive (K390R) deletion fragments were compared with that of the wild-type (WT) enzyme. The stability of Itk full-length W355A and M410A mutants was compared to that of the WT enzyme using urea denaturation curves. This material is available free of charge via the Internet at <http://pubs.acs.org>.

#### REFERENCES

- Hantschel, O., and Superti-Furga, G. (2004) Regulation of the c-Abl and Bcr-Abl tyrosine kinases, *Nat. Rev. Mol. Cell Biol.* 5, 33–44.
- Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism, *Mol. Cell* 3, 629–638.
- Roskoski, R., Jr. (2004) Src protein-tyrosine kinase structure and regulation, *Biochem. Biophys. Res. Commun.* 324, 1155–1164.
- Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck, *Nature* 385, 602–609.
- Xu, W., Harrison, S. C., and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src, *Nature* 385, 595–602.
- Williams, J. C., Weijland, A., Gonfloni, S., Thompson, A., Courtneidge, S. A., Superti-Furga, G., and Wierenga, R. K. (1997) The 235 Å crystal structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions, *J. Mol. Biol.* 274, 757–775.
- Ogawa, A., Takayama, Y., Sakai, H., Chong, K. T., Takeuchi, S., Nakagawa, A., Nada, S., Okada, M., and Tsukihara, T. (2002) Structure of the carboxyl-terminal Src kinase, Csk, *J. Biol. Chem.* 277, 14351–14354.
- Sun, G., and Budde, R. J. (1999) Mutations in the N-terminal regulatory region reduce the catalytic activity of Csk, but do not affect its recognition of Src, *Arch. Biochem. Biophys.* 367, 167–172.
- Veillette, A., Caron, L., Fournel, M., and Pawson, T. (1992) Regulation of the enzymatic function of the lymphocyte-specific tyrosine protein kinase p56lck by the non-catalytic SH2 and SH3 domains, *Oncogene* 7, 971–980.
- Smith, C. I., Islam, T. C., Mattsson, P. T., Mohamed, A. J., Nore, B. F., and Vihinen, M. (2001) The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species, *Bioessays* 23, 436–446.
- Berg, L. J., Finkelstein, L. D., Lucas, J. A., and Schwartzberg, P. L. (2005) Tec family kinases in T lymphocyte development and function, *Annu. Rev. Immunol.* 23, 549–600.
- Schwartzberg, P. L., Finkelstein, L. D., and Readinger, J. A. (2005) TEC-family kinases: regulators of T-helper-cell differentiation, *Nat. Rev. Immunol.* 5, 284–295.
- Heyeck, S. D., Wilcox, H. M., Bunnell, S. C., and Berg, L. J. (1997) Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity, *J. Biol. Chem.* 272, 25401–25408.
- Wilcox, H. M., and Berg, L. J. (2003) Itk phosphorylation sites are required for functional activity in primary T cells, *J. Biol. Chem.* 278, 37112–37121.
- Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997) Regulatory intramolecular association in a tyrosine kinase of the Tec family, *Nature* 385, 93–97.
- Baraldi, E., Carugo, K. D., Hyvonen, M., Surdo, P. L., Riley, A. M., Potter, B. V., O'Brien, R., Ladbury, J. E., and Saraste, M. (1999) Structure of the PH domain from Bruton's tyrosine kinase in complex with inositol 1,3,4,5-tetrakisphosphate, *Structure* 7, 449–460.
- Brown, K., Long, J. M., Vial, S. C., Dedi, N., Dunster, N. J., Renwick, S. B., Tanner, A. J., Frantz, J. D., Fleming, M. A., and Cheetham, G. M. (2004) Crystal structures of interleukin-2 tyrosine kinase and their implications for the design of selective inhibitors, *J. Biol. Chem.* 279, 18727–18732.
- Mallis, R. J., Brazin, K. N., Fulton, D. B., and Andreotti, A. H. (2002) Structural characterization of a proline-driven conformational switch within the Itk SH2 domain, *Nat. Struct. Biol.* 9, 900–905.
- Mao, C., Zhou, M., and Uckun, F. M. (2001) Crystal structure of Bruton's tyrosine kinase domain suggests a novel pathway for

- activation and provides insights into the molecular basis of X-linked agammaglobulinemia, *J. Biol. Chem.* 276, 41435–41443.
20. August, A., Sadra, A., Dupont, B., and Hanafusa, H. (1997) Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase, *Proc. Natl. Acad. Sci. U.S.A.* 94, 11227–11232.
21. Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C., and Berg, L. J. (2000) Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade, *J. Biol. Chem.* 275, 2219–2230.
22. Ching, K. A., Grasis, J. A., Taylor, P., Kawakami, Y., Kawakami, T., and Tsoukas, C. D. (2000) TCR/CD3-Induced activation and binding of Emt/Itk to linker of activated T cell complexes: requirement for the Src homology 2 domain, *J. Immunol.* 165, 256–262.
23. Hao, S., and August, A. (2002) The proline rich region of the Tec homology domain of ITK regulates its activity, *FEBS Lett.* 525, 53–58.
24. Lowry, W. E., Huang, J., Lei, M., Rawlings, D., and Huang, X. Y. (2001) Role of the PHTH module in protein substrate recognition by Bruton's agammaglobulinemia tyrosine kinase, *J. Biol. Chem.* 276, 45276–45281.
25. Brazin, K. N., Fulton, D. B., and Andreotti, A. H. (2000) A specific intermolecular association between the regulatory domains of a Tec family kinase, *J. Mol. Biol.* 302, 607–623.
26. Hawkins, J., and Marcy, A. (2001) Characterization of Itk tyrosine kinase: contribution of noncatalytic domains to enzymatic activity, *Protein Expression Purif.* 22, 211–219.
27. Li, T., Rawlings, D. J., Park, H., Kato, R. M., Witte, O. N., and Satterthwaite, A. B. (1997) Constitutive membrane association potentiates activation of Bruton tyrosine kinase, *Oncogene* 15, 1375–1383.
28. Yang, W. C., Ching, K. A., Tsoukas, C. D., and Berg, L. J. (2001) Tec kinase signaling in T cells is regulated by phosphatidylinositol 3-kinase and the Tec pleckstrin homology domain, *J. Immunol.* 166, 387–395.
29. LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J., and Miller, W. T. (1998) Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue, *J. Biol. Chem.* 273, 32129–32134.
30. Lin, X., Ayrappetov, M. K., Lee, S., Parang, K., and Sun, G. (2005) Probing the communication between the regulatory and catalytic domains of a protein tyrosine kinase, Csk, *Biochemistry* 44, 1561–1567.
31. Guo, S., Wahl, M. I., and Witte, O. N. (2006) Mutational analysis of the SH2-kinase linker region of Bruton's tyrosine kinase defines alternative modes of regulation for cytoplasmic tyrosine kinase families, *Int. Immunol.* 18, 79–87.
32. Banavali, N. K., and Roux, B. (2005) The N-terminal end of the catalytic domain of SRC kinase Hck is a conformational switch implicated in long-range allosteric regulation, *Structure (Camb)* 13, 1715–1723.
33. Cowan-Jacob, S. W., Fendrich, G., Manley, P. W., Jahnke, W., Fabbro, D., Liebetanz, J., and Meyer, T. (2005) The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation, *Structure* 13, 861–871.
34. Yamaguchi, H., and Hendrickson, W. A. (1996) Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation, *Nature* 384, 484–489.
35. Gonfloni, S., Frischknecht, F., Way, M., and Superti-Furga, G. (1999) Leucine 255 of Src couples intramolecular interactions to inhibition of catalysis, *Nat Struct Biol* 6, 760–764.
36. Lin, X., Wang, Y., Ahmadibeni, Y., Parang, K., and Sun, G. (2006) Structural basis for domain-domain communication in a protein tyrosine kinase, the C-terminal Src kinase, *J. Mol. Biol.* 357, 1263–1273.
37. Wong, L., Lieser, S., Chie-Leon, B., Miyashita, O., Aubol, B., Shaffer, J., Onuchic, J. N., Jennings, P. A., Woods, V. L., Jr., and Adams, J. A. (2004) Dynamic coupling between the SH2 domain and active site of the COOH terminal Src kinase, Csk, *J. Mol. Biol.* 341, 93–106.
38. Pletneva, E. V., Sundd, M., Fulton, D. B., and Andreotti, A. H. (2006) Molecular details of Itk activation by prolyl isomerization and phospholigand binding: the NMR structure of the Itk SH2 domain bound to a phosphopeptide, *J. Mol. Biol.* 357, 550–561.
39. Marquez, J. A., Smith, C. I., Petoukhov, M. V., Lo Surdo, P., Mattsson, P. T., Knekt, M., Westlund, A., Scheffzek, K., Saraste, M., and Svergun, D. I. (2003) Conformation of full-length Bruton tyrosine kinase (Btk) from synchrotron X-ray solution scattering, *EMBO J.* 22, 4616–4624.
40. Sondhi, D., and Cole, P. A. (1999) Domain interactions in protein tyrosine kinase Csk, *Biochemistry* 38, 11147–11155.
41. DeLano, W. L. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, San Carlos, CA.

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