A Remote Substrate Docking Mechanism for the Tec Family Tyrosine Kinases†

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ABSTRACT: During T cell signaling, Itk selectively phosphorylates a tyrosine within its own SH3 domain and a tyrosine within PLCγ1. We find that the remote SH2 domain in each of these substrates is required to achieve efficient tyrosine phosphorylation by Itk and extend this observation to two other Tec family kinases, Btk and Tec. Additionally, we detect a stable interaction between the substrate SH2 domains and the kinase domain of Itk and find that addition of specific, exogenous SH2 domains to the in vitro kinase assay competes directly with substrate phosphorylation. On the basis of these results, we show that the kinetic parameters of a generic peptide substrate of Itk are significantly improved via fusion of the peptide substrate to the SH2 domain of PLCγ1. This work is the first characterization of a substrate docking mechanism for the Tec kinases and provides evidence of a novel, phosphotyrosine-independent regulatory role for the ubiquitous SH2 domain.

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

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

This study focuses on Itk (interleukin-2 tyrosine kinase), the Tec kinase that participates in signaling processes following T cell receptor engagement by phosphorylating Tyr 783 of phospholipase Cγ1 (PLCγ1) (*14*−*19*). Phosphorylation of PLCγ1 on specific tyrosine residues, including Tyr 783, leads to activation of lipase activity (*20*). In addition to the PLCγ1 substrate, Itk also undergoes autophosphorylation on Tyr 180 within its SH3 domain (*18*, *21*). The local amino acid sequences surrounding these two phosphorylation sites are shown in Figure 1a and reveal little sequence similarity. Moreover, the structural context of these target tyrosines differs since Tyr 180 of Itk is embedded within the SH3 domain fold (Figure 1b), while Tyr 783 of PLCγ1 resides in a linker region between the carboxy-terminal SH2 domain (SH2C) and SH3 domain of PLCγ1 (Figure 1c). Thus, sequence and structural differences between two known substrates of the Itk kinase raise questions related to the mechanisms by which Itk recognizes its targets in a sufficiently specific manner to maintain the fidelity of signal transduction.

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

RESULTS

Efficient Phosphorylation by the Tec Kinases Requires the SH2 Domain of the Substrate. In vitro kinase assays using a

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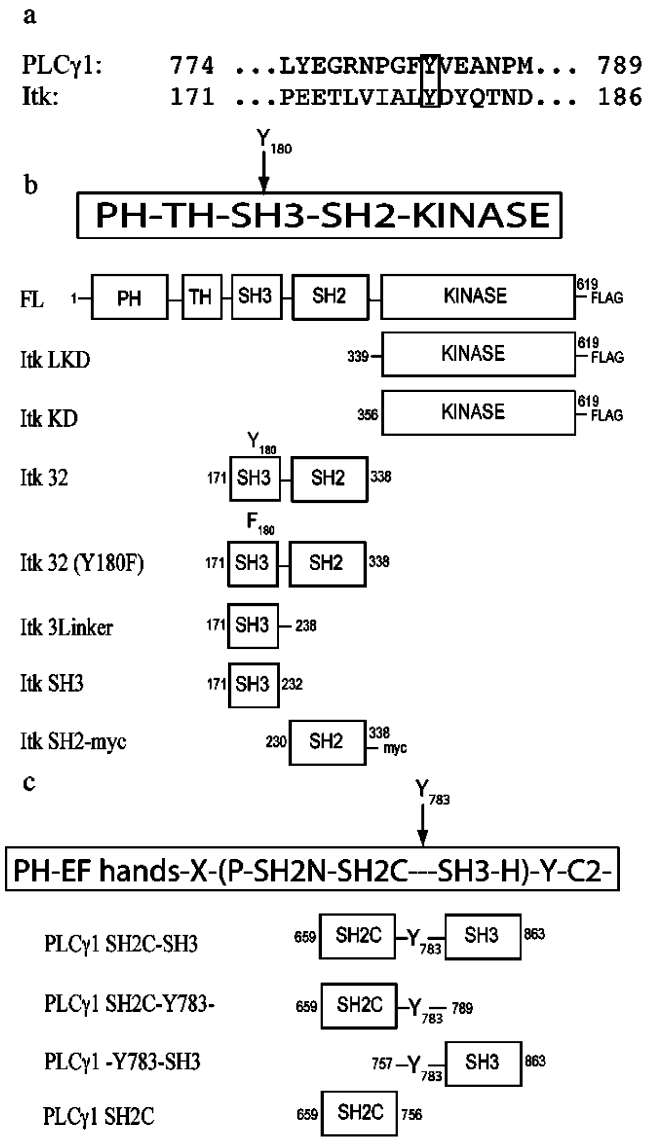


FIGURE 1: Domain structures of Tec kinases and PLCγ1. (a) Sequences surrounding Y783 in PLCγ1 and Y180 in Itk SH3. Tyrosine residues that are phosphorylated by Itk are boxed. (b) Domain structure of Itk and specific Itk constructs used in this study. The autophosphorylation site of Itk, Y180 within the SH3 domain, is indicated. The domain structures of Tec and Btk are the same as Itk, and the Tec and Btk domain fragments used in this study follow the same nomenclature [Btk SH3, Btk 32 (each contains Y223) Tec SH3, and Tec 32 (each contains Y187)]. (c) The domain structure of PLCγ1 contains a PH domain, an EF-hand motif, the catalytic domain (comprising the X and Y domains), two SH2 domains (SH2N and SH2C for the amino and carboxy termini, respectively), the SH3 domain, a split PH domain indicated by (P and H), and the C2 domain. The PLCγ1 fragments used in this study are shown, and substrate fragments each include Y783, the target of Itk kinase activity.

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

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

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

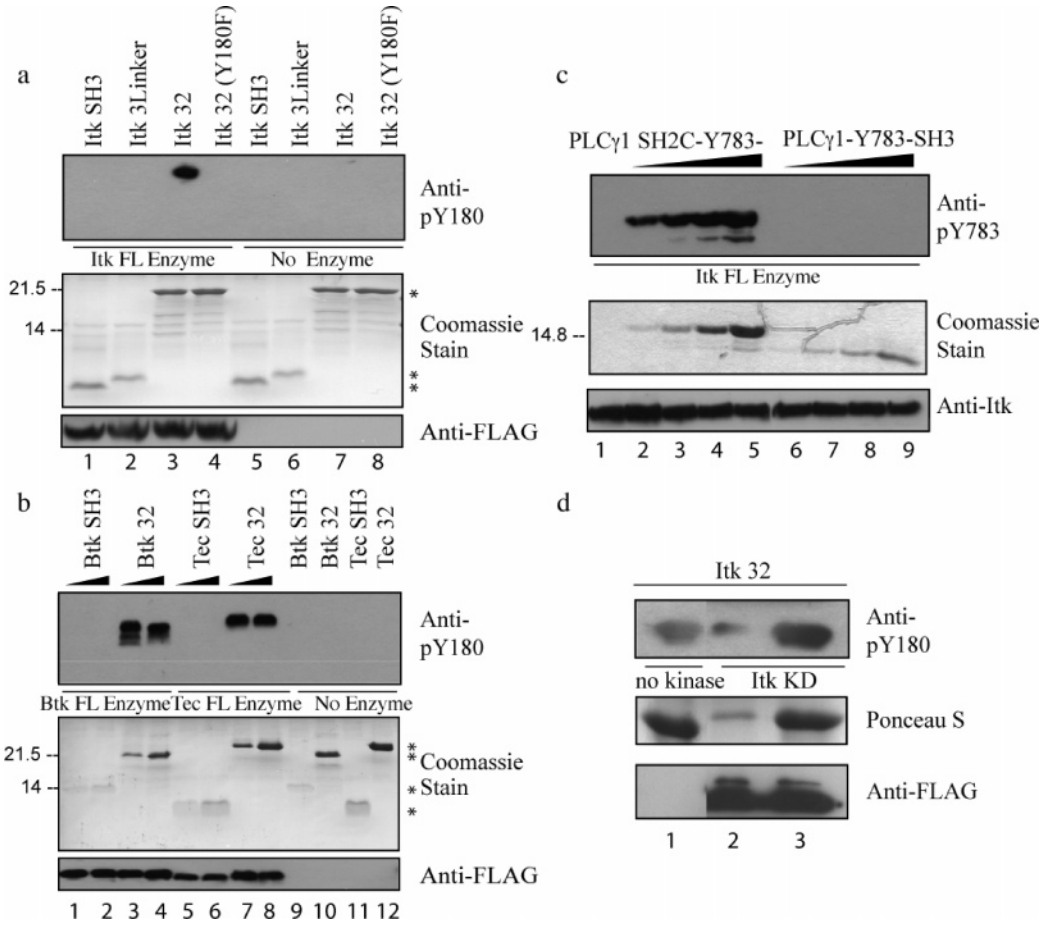


FIGURE 2: Tec-mediated phosphorylation requires the SH2 domain adjacent to the target tyrosine. (a) Only Itk fragments that contain the SH2 domain and Y180 are phosphorylated by Itk. Itk SH3, Itk SH3 linker (Itk 3Linker), Itk SH3−SH2 (Itk 32), or Itk SH3−SH2 with a phenylalanine in place of the Y180 phosphorylation site [Itk 32(Y180F)] (each at 10 μM) was incubated with 250 nM FLAG-tagged Itk full-length (FLItk) enzyme in an in vitro kinase assay. Lanes 5−8 contained no enzyme controls. Phosphorylation at Y180 in the SH3 domain is detected with an anti-pY223 Btk antibody (denoted anti-pY180 throughout). Throughout, the Coomassie or Ponceau S stain of the gel, anti-FLAG blots, and the anti-Itk blot (bottom panels) show protein levels. For panels a−c, the Coomassie staining of the SH3 domain fragments is consistently less efficient than that of the larger constructs, but protein amounts across these experiments are uniform on the basis of measured absorbances. Asterisks next to the Coomassie-stained gel indicate the positions of Itk 32, Itk 3Linker, and Itk SH3. (b) Phosphorylation of the Tec and Btk substrates was carried out in a manner similar to that shown in panel a for Itk. Btk SH3 (5 and 10 μM in lanes 1 and 2, respectively) or Btk SH3−SH2 (Btk 32) (5 and 10 μM in lanes 3 and 4, respectively) was incubated with 100 nM FLAG-tagged Btk full-length enzyme in an in vitro kinase assay. Tec SH3 (5 and 10 μM in lanes 5 and 6, respectively) or Tec SH3−SH2 (Tec 32) (5 and 10 μM in lanes 7 and 8, respectively) was incubated with 100 nM FLAG-tagged Tec full-length enzyme in an in vitro kinase assay. Lanes 9−12 contained no enzyme controls at substrate concentrations of 10 μM. Phosphorylation at Y223 in the Btk SH3 domain and Y187 in the Tec SH3 domain is detected with an anti-pY223 Btk antibody. Asterisks next to the Coomassie-stained gel indicate positions of Tec 32, Btk 32, Btk SH3, and Tec SH3. (c) Efficient phosphorylation of PLCγ1 Y783 by Itk occurs only when the adjacent PLCγ1 SH2 domain is present. Lane 1 contained the enzyme alone control. Varying concentrations (1, 3, 5, and 10 μM in lanes 2−5, respectively) of the fragment of PLCγ1 that contains both the SH2C domain and the following 33 amino acids (PLCγ1 SH2C-Y783-) or the PLCγ1 fragment that contains the 33-residue linker followed by the SH3 domain (PLCγ1 -Y783-SH3, 1, 3, 5, and 10 μM in lanes 6−9, respectively) were subjected to in vitro phosphorylation by 1.2 μM FLAG-tagged Itk full-length enzyme. Phosphorylation at Y783 in the PLCγ1 constructs is detected using an anti-pY783 PLCγ1 antibody. (d) Purified FLAG-tagged Itk kinase domain (2 μM) alone (Itk KD) was incubated with 1 (lane 2) or 10 μM (lane 3) Itk 32 domain in an in vitro kinase reaction mixture as described previously. Lane 1 contained 10 μM Itk 32 domain alone with no enzyme. All data are representative of at least three independent experiments.

domains of Itk and PLCγ1 that mediates recognition and phosphorylation of these physiological substrates.

The Free SH2 Domain Competes with the SH2 Domain-Containing Substrate and Reduces the Extent of Phosphorylation of the Substrate. If the Itk SH2 domain and PLCγ1 SH2C domain are indeed docking sites required for the phosphorylation of Itk substrates, we expect that exogenous SH2 domain (either Itk SH2 or PLCγ1 SH2C) should effectively compete for the binding site on the Itk kinase domain and inhibit phosphorylation of the substrates. To address this, phosphorylation of Itk 32 and PLCγ1 SH2C-Y783- was monitored in the presence of increasing concentrations of either the free Itk SH2 domain or the free PLCγ1 SH2C domain (Figure 4a,b). For both substrates, the corresponding free SH2 domain inhibits substrate phosphorylation (Figure 4a,b), while two different control SH2 domains (derived from PI3K and Grb2) do not inhibit the phosphorylation of the substrates even at a large molar excess (Figure 4a,b). Inhibition by the exogenous SH2 domain extends to phosphorylation of the full-length protein. In this case, Tyr 180 within full-length Itk is the substrate, and the extent of autophosphorylation at this site is greatly diminished upon addition of the free Itk SH2 domain (Figure 4c).

Finally, we tested whether addition of the free SH2 domain to the in vitro kinase assay affects the phosphorylation of peptide B, a small model peptide substrate used previously

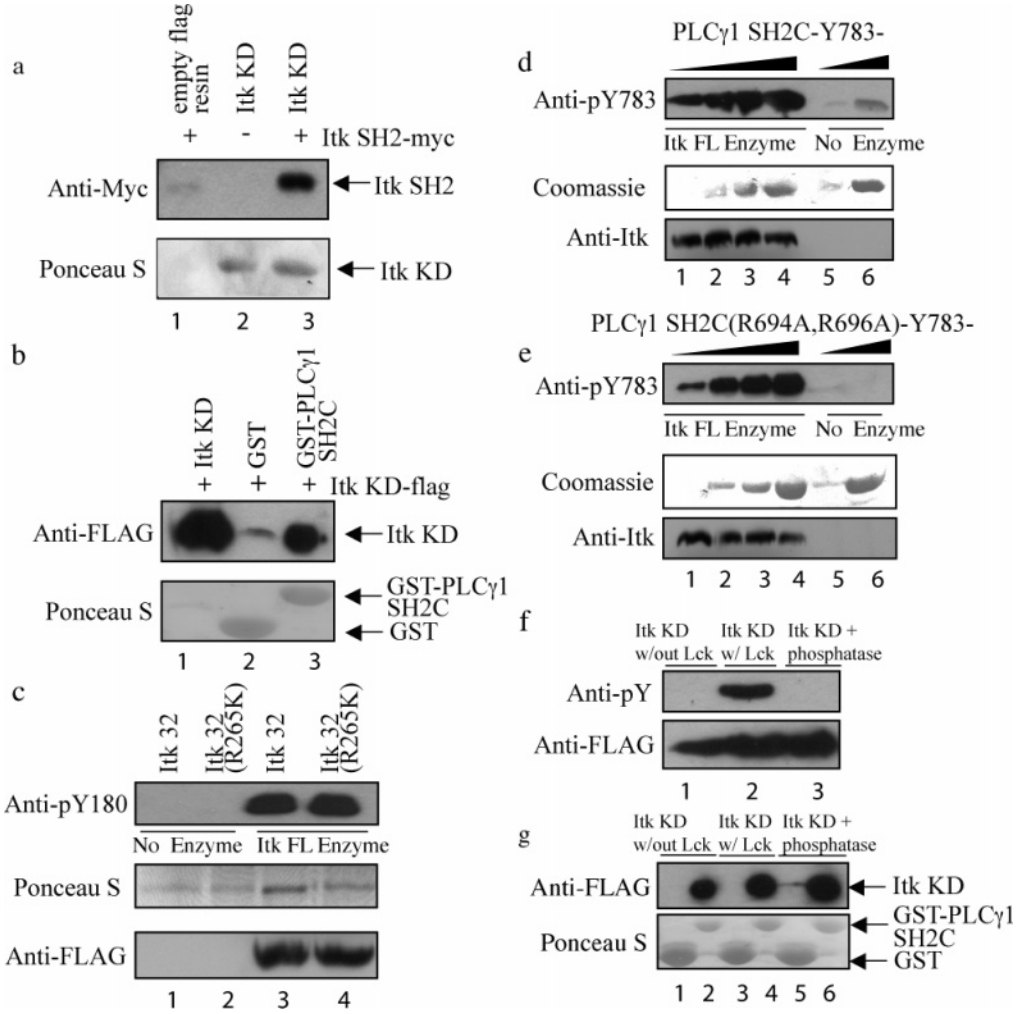


FIGURE 3: Itk kinase domain interacts directly with PLCγ1 and Itk SH2 domains in a phosphotyrosine-independent manner. (a) Empty anti-FLAG resin (lane 1) or purified FLAG-tagged Itk kinase domain (Itk KD) immobilized on an anti-FLAG resin (lanes 2 and 3) was incubated with purified myc-tagged control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

to measure Itk activity in vitro (*25*). The levels of peptide B phosphorylation as indicated by initial velocity measurements do not change significantly with increasing concentrations of the Itk SH2 domain (Figure 4d), suggesting that binding of SH2 to the kinase domain does not occur in a manner that directly interferes with binding of peptide B to the active site. Furthermore, this result points to the absence of toxicity effects on Itk catalytic activity upon addition of the free SH2 domain, providing further support for the direct competition with substrate shown in Figure 4a−c. Thus, a specific interaction between the SH2 domain of the Itk protein substrates and the Itk kinase domain is required for efficient substrate phosphorylation and appears to be localized outside of the catalytic cleft.

The SH2 Domain Enhances the Substrate Binding Affinity of a Generic Substrate. The data described above predict that the SH2 domain within the substrates of the Tec kinases serves a docking role and likely increases the affinity of substrates for the catalytic domain. To directly test this hypothesis, the PLCγ1 SH2C domain was covalently linked

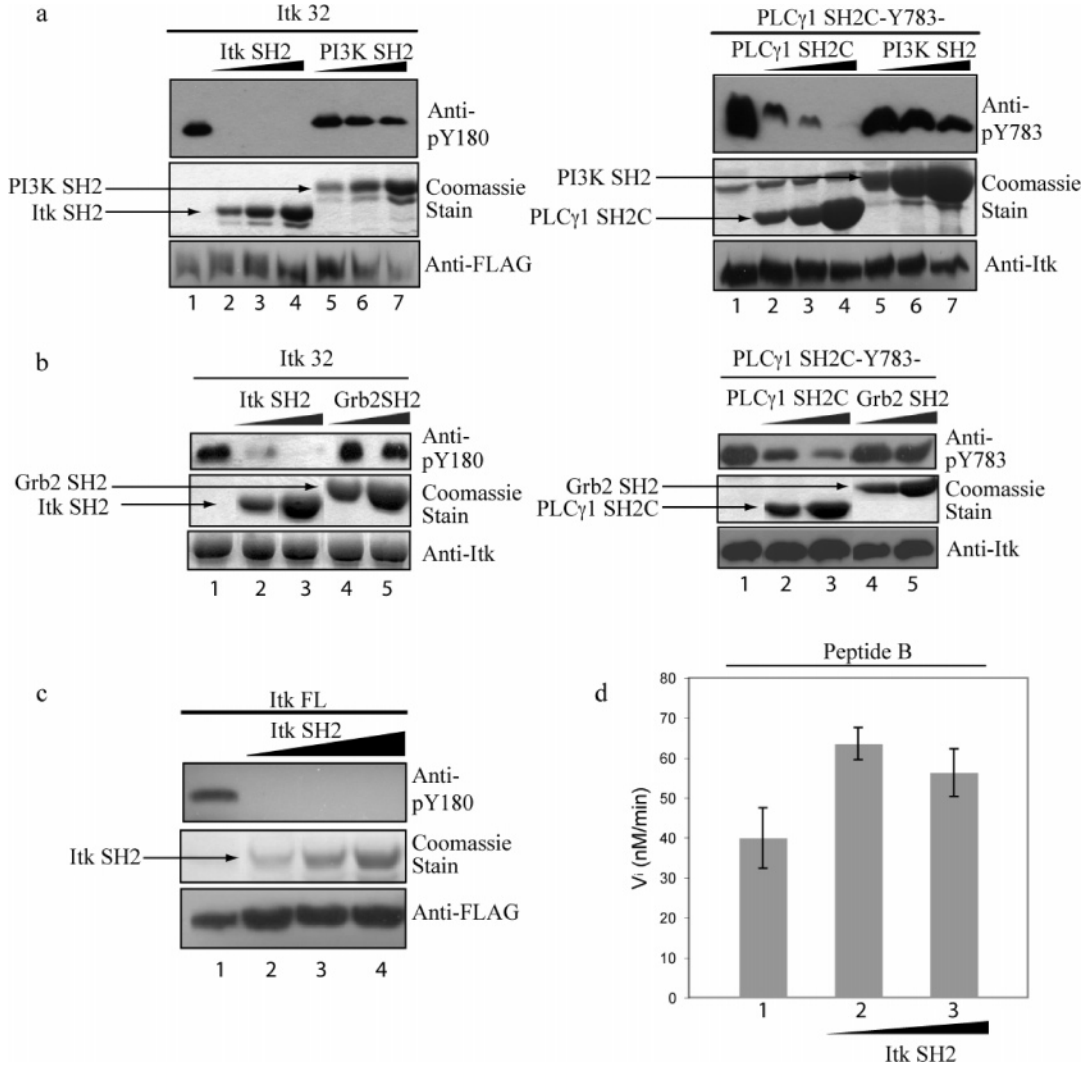
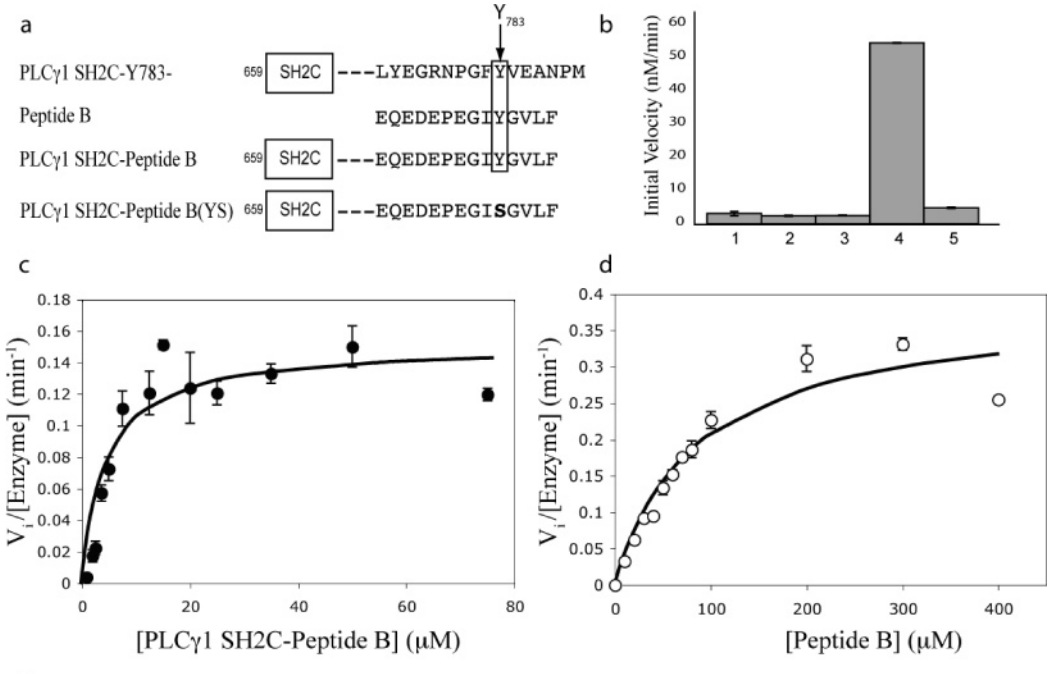


FIGURE 4: Presence of isolated SH2 domain that diminishes the level of phosphorylation of Itk Y180 or PLCγ1 Y783. Substrate used in each experiment is indicated above each set of blots. (a) At the left, the Itk 32 substrate was incubated with the Itk full-length enzyme as before (lane 1) or with the Itk 32 domain and free Itk SH2 domain in ratios of 1:10, 1:25, and 1:50 (lanes 2−4, respectively) or equivalent ratios of PI3K N-terminal SH2 domain (lanes 5−7) and subjected to an in vitro kinase assay. An anti-pY180 antibody shows that the free Itk SH2 domain competes with the Itk 32 substrate, while the SH2 domain from PI3K does not. Coomassie staining and an anti-FLAG blot (bottom panels) show protein levels. At the right, the PLCγ1 SH2 domain competes with the PLCγ1 substrate in a similar manner. Lane 1 contained only Itk enzyme and PLCγ1 SH2C-Y783- substrate without additional free SH2 domain. Ratios of free SH2 domain to substrate are 1:5, 1:10, and 1:50 in lanes 2−4, respectively, and equivalent ratios of PI3K SH2 domain are used in lanes 5−7. Coomassie staining of the gel and an anti-Itk blot (bottom panels) show protein levels. (b) Same experiment as in panel a using the Grb2 SH2 domain as a control. At the left, the Itk 32 substrate was incubated with Itk full-length enzyme (lane 1) or with Itk 32 domain and free Itk SH2 domain in ratios of 1:10 and 1:25 (lanes 2 and 3, respectively) or equivalent ratios of Grb2 SH2 domain (lanes 4 and 5) and subjected to an in vitro kinase assay. At the right is an identical experiment following PLCγ1 substrate phosphorylation in the presence of the PLCγ1 and Grb2 SH2 domains. Lane 1 contained PLCγ1 substrate alone; lanes 2 and 3 contained the PLCγ1 substrate and PLCγ1 SH2 domain in ratios of 1:10 and 1:25, respectively, and lanes 4 and 5 contained identical ratios of the PLCγ1 substrate and Grb2 SH2 domain. (c) The Itk SH2 domain competes with autophosphorylation of Y180 within full-length Itk. For lanes 2−4, the amount of free SH2 domain is the same as that used in lanes 2−4 of panel a, respectively. No exogenous SH2 domain has been added in lane 1, and full-length Itk is both the enzyme and the substrate. Coomassie staining and an anti-FLAG blot (bottom panels) show protein levels. (d) Free Itk SH2 domain does not compete with phosphorylation of a peptide substrate. Initial velocities are shown for phosphorylation of peptide B (sequence shown in Figure 5a) by full-length Itk in the absence (column 1) and presence (columns 2 and 3) of the Itk SH2 domain. The amount of SH2 domain used here is the same as that used in lanes 3 and 4 in panel c.

to the amino terminus of peptide B (Figure 5a). To ensure that the desired tyrosine residue is the only site within this construct that undergoes phosphorylation, we constructed a PLCγ1 SH2C−peptide B mutant that replaced the putative site of tyrosine phosphorylation with serine (Figure 5a). This mutant did not incorporate phosphate, indicating that the tyrosine within the peptide B sequence is the only site in the PLCγ1 SH2C−peptide B fusion protein that is phosphorylated by Itk (Figure 5b).

Quantitative kinetic constants were compared for peptide B alone and the PLCγ1 SH2−peptide B fusion (Figure 5c−e). Covalent attachment of the PLCγ1 SH2C domain to peptide B increases substrate affinity as indicated by a reduced K_m value: 5.64 μM for the SH2−peptide B fusion protein compared to 87 μM for peptide B alone. The k_{cat} value changed to only a small extent, suggesting that the SH2 domain primarily serves as a docking site to facilitate substrate recognition and binding by the Itk kinase domain.



e

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}μM^{-1})
Peptide B	87.1 ± 18.5	0.39 ± 0.03	0.004
PLCγ1 SH2C-Peptide B	5.64 ± 1.78	0.16 ± 0.02	0.028

FIGURE 5: PLCγ1 C-terminal SH2 domain that can mediate docking of peptide B into the kinase active site of Itk. (a) Construction of a PLCγ1 SH2C−peptide B fusion protein was based on alignment of Y783 within the PLCγ1 linker and the Tyr of peptide B. The 14 amino acids of peptide B were used to replace the last 16 amino acids of PLCγ1 SH2C-Y783-. The Tyr that is phosphorylated in each substrate is boxed. A mutant construct is also shown that contains serine in place of the tyrosine of peptide B. This site was mutated to serine instead of phenylalanine due to solubility problems associated with the Tyr to Phe mutation. (b) The FLAG-tagged Itk LKD enzyme (1 μM) was incubated with 20 μM PLCγ1 SH2C−peptide B fusion protein (lane 4) or 20 μM PLCγ1 SH2C−peptide B(YS) mutant protein (lane 5) in an in vitro kinase assay. Lanes 1−3 contained Itk enzyme alone, PLCγ1 SH2C−peptide B substrate alone, and PLCγ1 SH2C−peptide B(YS) substrate alone, respectively, that have each been subjected to the same in vitro kinase assay conditions. (c and d) The FLAG-tagged Itk LKD enzyme was incubated with increasing amounts of biotinylated PLCγ1 SH2C−peptide B fusion protein (c) or biotinylated peptide B alone (d) in an in vitro kinase assay. (e) Kinetic parameters describing phosphorylation of peptide B and the PLCγ1 SH2C−peptide B fusion protein by Itk. Data are the averages of two independent experiments.

Thus, when linked to the PLCγ1 SH2C domain, peptide B is a better substrate for Itk (exhibiting a 15-fold increase in substrate affinity), providing further support for the finding that the Itk catalytic efficiency selectively depends upon docking interactions with the substrate SH2 domain.

DISCUSSION

We have demonstrated a previously unrecognized docking role for the SH2 domain within the substrates of the Tec kinases that facilitates phosphorylation at a remote tyrosine. Many kinase sequences contain SH2 domains, and these binding modules are known to affect the association of the parent kinase molecule within a signaling complex (*26*). Additionally, processive phosphorylation mediated by SH2−phosphotyrosine interactions has been described previously (*27*−*29*). In those examples, a tyrosine kinase creates a binding site on the substrate for its own internal SH2 domain; the ensuing SH2−substrate interaction leads to efficient phosphorylation at additional sites on the substrate. In contrast to the role of the SH2 domain in the processive phosphorylation mechanism, the results presented here suggest that SH2 domains within the substrates of the Tec kinases harbor a recognition motif that is required for efficient and selective phosphorylation of the target tyrosine. For example, the direct substrate of Itk in T cells, PLCγ1, harbors an SH2 domain (SH2C) that binds directly to the Itk kinase domain, allowing the downstream PLCγ1 tyrosine (Tyr 783) to be efficiently phosphorylated by Itk. Also, autophosphorylation of Try 180 in the Itk SH3 domain depends on Itk SH2-mediated interactions with the Itk kinase domain. This mode of substrate recognition is evident within the full-length enzyme as addition of the competing exogenous Itk SH2 domain to full-length Itk completely eliminates Itk autophosphorylation (see Figure 4c). To our knowledge, this direct kinase domain−SH2 interaction is a novel mode of substrate recognition that provides significant insight into how the Tec kinases achieve fidelity in their interactions with appropriate substrates and avoid deleterious "cross talk" with other substrates.

Our findings are consistent with a previous study of autophosphorylation within the Tec family kinases (*21*) that provided a hint about the docking role of the SH2 domain. Smith and co-workers qualitatively showed that the SH3−SH2 fragments of Btk and Itk are both phosphorylated preferentially to the isolated Btk SH3 domain by the Btk kinase (*21*). The authors speculated that the SH3−SH2

substrate may have additional interactions with the kinase or that the site of phosphorylation in the SH3 domain becomes more accessible in the larger SH3−SH2 substrate. They also left open the possibility that there are new phosphorylation sites on the larger SH3−SH2 substrate but report that the SH2 domain of Btk is not a substrate for Btk. The data we present strongly support a model in which the SH2 domain serves a direct docking role that is a significant determinant of substrate specificity for the Tec kinases. It is especially interesting to note that Smith and co-workers report phosphorylation levels for the SH3−SH2 fragment that are 5−8-fold higher than that of the isolated SH3 domain (*21*). This is in excellent agreement with kinetic data shown in Figure 5 that indicate that peptide B is a better substrate by 7-fold (k_{cat}/K_m) when tethered to the SH2 domain of PLCγ1. Finally, our use of phosphotyrosine specific antibodies and mutation of the target tyrosine support the notion that additional phosphorylation sites in the SH2 domain containing substrates are not leading to the observed increase in the level of substrate phosphorylation (*21*).

The phosphotyrosine-independent nature of the binding event between the Itk kinase domain and SH2 domains highlights alternative means by which SH2 domains can engage their targets. To date, targeting the conserved arginines in the phosphotyrosine binding pocket of the SH2 domain has become the traditional "loss-of-function" mutation for this binding module. A full appreciation of phosphotyrosine-independent SH2 binding will lead to revised models of signaling complexes. For PLCγ1 signaling in particular, traditional mutagenic approaches targeting the phosphotyrosine binding site of the SH2C domain have previously led to conclusions that this portion of PLCγ1 is dispensable for PLCγ1 recruitment and phosphorylation (*30*, *31*). More recently, the extended experimental approach of Samelson and co-workers showed that all three SH domains, including SH2C, are required for phosphorylation of PLCγ1 in T cells (*32*), yet these studies still relied on the mutation of arginine in the phosphotyrosine binding pocket of the SH2C domain. Certainly, the phosphotyrosine-independent docking role for the PLCγ1 SH2C domain would have been missed by standard loss-of-function mutations in the SH2 domain.

To fully characterize this novel, SH2-dependent substrate recognition mechanism, the interaction sites on both the Itk kinase domain and the substrate SH2 domains must be mapped at the molecular level. In addition to providing insight into the PLCγ1 activation mechanism, the precise arrangement of the Itk SH2 domain within the full-length Itk enzyme is of particular interest. In other tyrosine kinases, the noncatalytic SH2 domain plays a defined role in regulating kinase activity by forming direct intramolecular contacts to the small lobe of the kinase domain (*33*, *34*). For Itk, we have also found that the SH2 domain positively contributes to the regulation of catalytic activity (R. E. Joseph and A. H. Andreotti, *Biochemistry*, in press). Thus, the emerging bifunctional nature of the Itk SH2 domain (as substrate recognition module and regulatory domain) raises interesting questions related to how the Itk SH2 domain orchestrates its various roles during enzymatic catalysis. Does this SH2 domain interact with its neighboring kinase domain in a single mode that achieves both regulation of kinase activity and substrate recognition of the autophosphorylation site, or does the SH2 domain shift between multiple interaction sites on the kinase domain to achieve these functions? With molecular level details still forthcoming, it is nevertheless clear that the data we present here point to a specific SH2 domain-mediated docking mechanism by which the Tec kinases recognize and phosphorylate their substrates. One extension of this result is that screening for interactions between the Tec kinase domains and unrelated SH2 domains may provide leads for identifying additional substrates for this important family of tyrosine kinases. In addition, we have shown that Itk substrates can be displaced from the active site by addition of the exogenous SH2 domain. This result promises an exciting strategy for attenuating Itk-mediated signaling that may have significant selectivity advantages since it would not require the development of molecules that discriminate between very similar kinase active sites.

MATERIALS AND METHODS

Baculoviral and Bacterial Constructs. Full-length Itk, Btk, and Tec and the Itk kinase domain fragments were PCR-amplified using a reverse primer that encoded a FLAG epitope tag. The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. All of the PLCγ1, Itk, Btk, and Tec fragments that do not contain the kinase domain were subcloned into the pGEX-2T expression vector (GE Healthcare) for production and purification from bacteria as described previously (*35*). The N-terminal phosphatidylinositol 3-kinase (p85α) SH2 domain (PI3K SH2) and the Grb2 SH2 domain were cloned into pGEX-5X-1 by PCR. The pGEX-2T PLCγ1 C-terminal SH2 domain−peptide B fusion protein was created by PCR and included an N-terminal biotinylation sequence. For this construct and the pGEX-2T PLCγ1 SH2C−peptide B (YS) mutant, Tyr 771 was mutated to Phe to prevent phosphorylation at this site during analysis of the kinetics, and Tyr 775 has been deleted by replacement with the peptide B sequence (*36*). Biotinylated protein was produced in BL21 cells by coexpressing biotin ligase. The biotinylated protein was purified by affinity purification using an avidin resin (Pierce). For the Itk substrate constructs that include both the SH3 and SH2 domains [Itk32 and Itk32(Y180F)], we also introduced two point mutants (W208K and I282A) to eliminate complications that may arise from dimerization of the substrate as reported previously (*35*). The wild-type Itk SH3−SH2 construct is phosphorylated by Itk to the same extent as the double mutant (data not shown). All mutations were made using the site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing at the Iowa State University DNA Synthesis and Sequencing Facility.

Baculovirus Production. The pENTR vectors with various inserts were recombined in vitro with BaculoDirect C-Term Linear DNA (Invitrogen) using the LR Clonase II enzyme (Invitrogen). The DNA was then transfected into *Sf*9 cells using Effectene (Qiagen). Three rounds of viral selection and amplifications were carried out. For protein production, the cells were infected with a 1:1 Itk (or Btk or Tec):Lck baculovirus ratio unless otherwise indicated. The cells were harvested 72 h postinfection, rinsed once with phosphate-buffered saline (PBS), and stored at −80 °C. Following purification, each Itk enzyme construct was assessed for Tyr 511 phosphorylation using a Btk pY551 specific antibody.

This step ensured that coexpression with Lck produced appropriately activated Itk (*18*).

Pull-Down Assays. The purified 0.5 μM FLAG-tagged Itk kinase domain (Itk KD) immobilized on an anti-FLAG resin was incubated with 1 μM purified Myc-tagged Itk SH2 (Itk SH2-myc) domain in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1% NP40, 1 mM EDTA, and 1 mM NaF] at 4 °C overnight. The samples were washed, boiled, resolved on a SDS−PAGE gel, transferred to a PVDF membrane, and Western-blotted with an anti-Myc antibody (Invitrogen). For PLCγ1 pull-down assays, 0.22 μM FLAG-tagged Itk kinase domain (Itk KD) was incubated with 3.8 μM purified GST or 3.8 μM GST−PLCγ1 SH2C protein immobilized on glutathione beads. The samples were treated as described above and Western-blotted using an anti-FLAG antibody. The unphosphorylated FLAG-tagged Itk kinase domain (Itk KD) was prepared by treating 13.6 μM Itk KD with 1 unit/μL alkaline phosphatase (New England Biolab) for 1 h at 37 °C.

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

Kinase Assays and Western Blotting. Full-length Itk (FL), the linker kinase domain (Itk LKD), the kinase domain (Itk KD), full-length Btk, or full-length Tec was incubated with the indicated substrates in an in vitro kinase assay buffer [50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, 1 mM Pefabloc, and 200 μM ATP] for 1 h at room temperature. The samples were boiled, separated by SDS−PAGE, and transferred onto a PVDF membrane. The membranes were then blotted with either phosphotyrosine specific antibodies [anti-pY783 for PLCγ1 or pY223 Btk antibody used to detect pY180 in Itk, pY187 in Tec, and pY223 in Btk (*18*)], an anti-phosphotyrosine antibody (4G10 from Upstate), or an anti-FLAG antibody (Sigma) and developed by standard chemiluminescence (Pierce) methods. In quantitative kinase assays (*25*), K_m determinations for peptide B [aminohexanoylbiotin-EQEDEPEGIYGVLF-NH_2 (Anaspec Inc.)] and the biotinylated PLCγ1 SH2C−peptide B fusion protein were carried out by incubating the purified Itk LKD enzyme in reaction buffer {50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, 1 mM Pefabloc SC [4-(2-aminoethyl)benzenesulfonyl fluoride], and 200 μM ATP} and 5 μCi of [^{32}P]ATP (GE Healthcare) at room temperature. The peptide B concentration was varied between 0 and 400 μM, and PLCγ1 SH2C−peptide B fusion protein concentration was varied between 0 and 80 μM. The enzyme concentration used for kinetic analysis was 0.9 μM. Each assay was carried out in duplicate. The data that were obtained were fitted to the Michealis−Menten equation using GraphFit 5, and the kinetic parameters were obtained.

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