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Substrate Recognition of PLC γ 1 via a Specific Docking Surface on Itk

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Abstract

Itk (interleukin-2 inducible *T* cell kinase) is a non-receptor protein tyrosine kinase expressed primarily in T cells. Itk catalyzes phosphorylation on tyrosine residues within a number of its natural substrates, including the well-characterized Y783 of PLC γ 1. However, the molecular mechanisms Itk exploits to recognize its substrates are not completely understood. We have previously identified a specific docking interaction between the kinase domain of Itk and the C-terminal Src homology 2 (SH2C) domain of PLC γ 1 that promotes substrate specificity for this enzyme/substrate pair. In the current study, we identify and map the interaction surface on the Itk kinase domain as an acidic patch centered on the G helix. Mutation of the residues on and adjacent to the G helix within the Itk kinase domain impairs the catalytic efficacy of PLC γ 1 substrate phosphorylation by specifically altering the protein–protein interaction interface and not the inherent catalytic activity of Itk. NMR titration experiments using a Btk (*Bruton's tyrosine kinase*) kinase domain as a surrogate for the Itk kinase domain provide further support for an Itk/PLC γ 1 SH2C interaction surrounding the G helix of the kinase domain. The work presented here provides structural insight into how the Itk kinase uses the G helix to single out Y783 of PLC γ 1 for specific phosphorylation. Comparing these results to other well-characterized kinase/substrate systems suggests that the G helix is a general structural feature used by kinases for substrate recognition during signaling.

Receptor stimulation triggers intracellular phosphorylation cascades that are controlled by the action of protein kinases. The exquisite fidelity of protein kinases in targeting a specific substrate arises from protein-protein interactions remote from the kinase active site. A focused study of the Itk (interleukin-2 inducible T cell kinase)/PLC γ 1 kinase/substrate pair in the T cell signaling pathway reveals that the G helix in the large lobe of the Itk kinase domain serves as a substrate handle, directly mediating interaction with PLC γ 1 C-terminal Src homology 2 domain to ensure proper substrate selection and site selective phosphorylation during signaling.

Keywords

Tec family kinase Itk; substrate recognition; PLC γ 1 phosphorylation; kinase domain; G helix

Introduction

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

determinants for substrate specificity are present in most kinases, as substrate preferences are often observed *in vitro*.⁶

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

Itk (interleukin-2 inducible T cell kinase) is a non-receptor protein tyrosine kinase that belongs to the Tec kinase family. Itk is primarily expressed in T cells and plays an important regulatory role in T cell signaling and development.^{10–12} Itk contains, from amino-terminus to carboxy-terminus, a Pleckstrin homology domain, a Tec homology domain, a Src homology 3 domain, a Src homology 2 (SH2) domain and a kinase domain responsible for the phosphotransferase activity.¹² A number of specific tyrosines targeted by Itk have been identified, including Y783 of PLC γ 1, Y180 of Itk, Y173 of SLP-76 and, recently, Y210 as well as Y222 of DEF6.^{13–17} Among these Itk-mediated phosphorylation on Y783 of PLC γ 1 is the best studied and most important in the context of T cell signaling. Phosphorylation on PLC γ 1 Y783 activates the lipase activity of PLC γ 1, which generates two essential secondary messengers, diacylglycerol and inositol trisphosphate, by hydrolyzing phosphatidylinositol 4,5-bisphosphate.^{18,19} Diacylglycerol and inositol trisphosphate lead to activation of protein kinase C and release of calcium ions from the endoplasmic reticulum, respectively.

We have previously described a specific docking interaction between Itk and PLC γ 1 that is required for phosphorylation on Y783 and subsequent downstream signaling in T cells.¹⁴ The specific site on PLC γ 1 that is required for efficient phosphorylation by Itk has been delineated and consists of a largely basic cluster of surface-exposed residues on the C-terminal SH2 domain (SH2C) of PLC γ 1.²⁰ This recognition site is remote from the site of tyrosine phosphorylation, Y783, which resides 29 amino acids beyond the carboxy-terminus of the PLC γ 1 SH2C domain in what appears, based on NMR data, to be a flexible linker region (data not shown). The PLC γ 1 SH2C domain interacts directly with the kinase domain of Itk in a manner that is completely independent of the normal SH2-mediated phosphotyrosine interaction.¹⁴ Mutation of this recognition site within full-length PLC γ 1 leads to loss of phosphorylation on Y783 and significantly reduced calcium flux in T cells.²⁰

We now define the complementary interaction surface on the Itk kinase domain that serves as the specific substrate-docking surface for PLC γ 1. Coupling mutagenesis and functional screening, we narrowed the potential interaction area to an acidic patch surrounding the G helix in the C-terminal lobe of the Itk kinase domain. Kinetic parameters support the importance of the mapped residues in the phosphorylation efficiency toward PLC γ 1, and mutation of the docking surface on Itk leads to diminished binding of the kinase domain to PLC γ 1 SH2C. Analysis of the enzyme/substrate interaction using NMR spectroscopy provides further support for the location of the docking site centered on the G helix. These data contribute to our understanding of the specificity-determining elements that control Itk function.

Results

Mapping the PLC γ 1 docking surface on the Itk kinase domain

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

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

Next, we extended the linker length of the PLC γ 1 L4 construct by insertion of a single glycine or a glycine-serine-glycine tripeptide spacer to produce a substrate with a linker length of five amino acids (PLC γ 1 L5) or seven amino acids (PLC γ 1 L7) (Fig. 2b). Phosphorylation of PLC γ 1 L5 remains poor, but the slightly longer linker in PLC γ 1 L7 restores Y783 phosphorylation to wild-type levels. We conclude that the minimal linker length between the SH2C domain and Y783 that is necessary to satisfy both the SH2C/kinase docking interaction and phosphorylation of Y783 within the Itk kinase domain active site is seven amino acids.

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

Alanine scan of the acidic Itk kinase residues identifies the G helix as docking site

Following previous work that successfully mapped the substrate-docking site on the surface of the Csk kinase,²¹ we mutated each of the 12 Itk kinase domain residues identified by the analysis described above to alanine. The activated forms of wild-type Itk and the Itk alanine

mutants were produced from insect cells by co-expression of Lck (lymphocyte-specific protein tyrosine kinase) as described previously.²² Each Itk enzyme (wild type or alanine mutant) was purified and assessed using equalized amount of enzyme in *in vitro* kinase assays for phosphorylation of two separate substrates (peptide B alone and peptide B fused to the PLC γ 1 SH2C domain) (Fig. 3a). Peptide B has been previously characterized as a docking-independent Itk substrate ($K_m \approx 80 \mu\text{M}$) while Itk phosphorylation of peptide B fused to PLC γ 1 SH2C is enhanced ($K_m \approx 5 \mu\text{M}$) by virtue of the PLC γ 1 SH2C/Itk kinase domain docking interaction.¹⁴ Comparison of the two substrates (peptide B and PLC γ 1 SH2C-peptide B) allows us to evaluate the extent to which alanine mutations in Itk alter the intrinsic kinase activity (peptide B phosphorylation will decrease) *versus* the extent to which alanine mutation disrupts the substrate-docking site (PLC γ 1 SH2C-peptide B phosphorylation will decrease).

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

Initial velocity measurements for 10 glutamate/aspartate-to-alanine Itk mutants revealed that three mutants (E565A, E568A/D569A and E596A) result in decreased activity toward the docking substrate (PLC γ 1 SH2C-peptide B) compared to the generic peptide B substrate (Fig. 3b). Examining the location of these acidic residues on the structure of the Itk kinase domain shows that E565, E568 and D569 cluster on the G helix, while E596 is located on the adjacent αH - αI loop (Fig. 3c). A second round of mutagenesis was then carried out to assess the contribution of 16 additional residues surrounding the G helix and the αH - αI loop (Fig. 3d). The results indicate that F529, S530, S571, T572, Y577, K595 and K597 are also part of the substrate-docking site (Fig. 3d and e) while the other nine residues examined by mutation to alanine do not contribute to PLC γ 1 docking on the Itk kinase domain (Fig. 3d). S571 and T572 reside on the G helix further supporting a role for this region of the Itk kinase domain in substrate docking, F529 and S530 are located on the short helix in between the P+ 1 loop and F helix and, like E596 identified in the initial screen, K595 and K597 are located on the αH - αI loop (Fig. 3e). All of these residues cluster to a contiguous region on the C-terminal lobe of the Itk kinase domain (Fig. 3f). Y577 on the αG - αH loop is the exception as it is somewhat removed from the other residues identified to be important for docking (Fig. 3f). We speculate that mutation of Y577 to alanine might indirectly perturb the docking interaction surface on the Itk kinase domain by altering the structure or position of the G helix.

It is worth noting that certain mutations in the Itk kinase domain appear to enhance the initial velocity for the PLC γ 1 SH2C-peptide B substrate compared to the peptide B substrate alone. The most notable example of this is the R561A/S562A mutant (Fig. 3d). R561 and S562 are located at the amino-terminus of the G helix and are therefore quite close to the SH2C binding site. The observed increase in initial velocity for this mutant may reflect an overall reduction of positive charge that stabilizes binding to the net positively charged SH2C binding partner. That said, the K595A/K597A mutant, located on the opposite side of the G helix, also reduces the net positive charge of the docking site but has a deleterious

effect on substrate docking. It is possible that the side chain of K595 and/or K597 forms favorable contacts to the acidic side chain of PLC γ 1 E709, previously identified as a component of the PLC γ 1 SH2C docking surface.

Mutation of the substrate-docking site on Itk increases K_m for the PLC γ 1 SH2C-peptide B substrate but not for peptide B alone

To more quantitatively assess how mutation of the substrate-docking site on the Itk kinase domain affects phosphorylation of PLC γ 1, we next determined the K_m and k_{cat} values for the peptide B and PLC γ 1 SH2C-peptide B substrates for both wild-type Itk kinase domain and the Itk E565A mutant (Fig. 4). For the peptide B substrate, both wild-type Itk (Fig. 4a) and Itk E565A (Fig. 4c) have similar K_m and k_{cat} values (Fig. 4e). In contrast, for the docking-competent PLC γ 1 SH2C-peptide B substrate, the Itk E565A mutant exhibits an increased K_m value (Fig. 4d) compared to wild-type Itk for the same substrate (Fig. 4b). As a result, enzyme efficiency (k_{cat}/K_m) for phosphorylation of PLC γ 1 SH2C-peptide B is lower for the Itk E565A mutant than for the wild-type Itk (Fig. 4e) whereas efficiency of phosphorylation of peptide B alone is not affected by the mutation of E565 on the G helix further supporting a role for this residue in substrate docking (Fig. 4e). Attempts to combine kinase domain mutations in this assay were not successful as triple and quadruple Itk mutations had deleterious effects on k_{cat} .

Itk mutations diminish protein–protein interaction between Itk kinase domain and PLC γ 1 SH2C

To complement the functional assays used to elucidate and characterize the PLC γ 1 docking site on the Itk kinase domain, we next assessed the extent to which mutations in the Itk kinase domain affect the direct interaction between PLC γ 1 SH2C and Itk. Purified GST-PLC γ 1 SH2C fusion protein [or glutathione *S*-transferase (GST) alone] was immobilized on glutathione beads and incubated with purified Itk kinase domain (wild type or mutants). Consistent with previously published data,¹⁴ wild-type Itk kinase domain binds to the GST fusion of PLC γ 1 SH2C domain and not GST alone (Fig. 5a, lane 3). Mutation of the Itk kinase domain in the newly described PLC γ 1 docking site, Itk E568A/D569A and Itk F529A/S530A, results in diminished binding to the immobilized GST-PLC γ 1 SH2C domain (Fig. 5a, lanes 6 and 9). This observation provides additional evidence that the Itk kinase domain, in the region including and adjacent to the G helix, mediates a direct interaction with the PLC γ 1 SH2C domain.

NMR analysis of the kinase/SH2C domain interaction

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

suggests that the molecular details of a docking interaction between Btk and the PLC γ 1 SH2C domain are similar to the Itk/PLC γ 1 interaction.

Initial experiments revealed that the NMR sample containing the Btk kinase domain and the larger PLC γ 1 fragment consisting of both SH2 domains (SH2N-SH2C) is more stable than samples containing the kinase domain plus PLC γ 1 SH2C alone, and thus, we proceeded with the larger PLC γ 1 fragment. Addition of unlabeled PLC γ 1 SH2N-SH2C into the sample of ^{15}N -labeled Btk kinase domain mutant leads to selective line broadening for a subset of resonances in the [^{15}N , ^1H]TROSY (transverse relaxation optimized spectroscopy) spectrum of the Btk kinase domain mutant due to exchange between PLC γ 1-bound and unbound kinase domains (Fig. 5c). Given a protein of this size, complete resonance assignments are a major undertaking, and so as a first step, we have used amino-acid-specific isotopic incorporation to further characterize the kinase/SH2C interaction.

Taking advantage of the excellent expression characteristics of the Btk kinase domain mutant, we produced protein that is selectively ^{15}N -labeled on tyrosine. There are many amino acid types that are good candidates for specific labeling strategies, and our choice of tyrosine reflects a combination of ease of synthesis, limited scrambling of the isotope label and the observation that two tyrosine residues (Y571 and Y598) flank the G helix in the Btk structure (Fig. 5d). Focusing on Btk Y571 and Y598, we were able to assign each specific resonance (Supplementary Fig. 2) and then examine NMR spectral changes as a result of addition of PLC γ 1 (Fig. 5e). We observe significant exchange broadening of the resonance corresponding to Y598 that is consistent with binding of PLC γ 1 to the nearby G helix of the kinase domain (Fig. 5d and e). The resonance corresponding to Y571 broadens only slightly over the course of the titration and does not change to the same extent as Y598 perhaps due to its distance from the G helix. The more modest spectral changes observed for Y571 could indicate that this residue is located at the periphery of the PLC γ 1 binding site or could arise simply due to the longer tumbling time of the higher-molecular-weight PLC γ 1/kinase complex, that is, non-residue-specific line broadening. These data (in particular, the dramatic linewidth changes observed for Y598) provide additional evidence for the substrate-docking site centered on the G helix.

The substrate-docking surface on the C-terminal lobe of the Itk kinase domain also mediates phosphorylation of PLC γ 1 in full-length Itk

In the experiments carried out to this point, we have focused on the Itk kinase domain rather than the multidomain full-length protein. For the purposes of mapping the Itk/PLC γ 1 interaction, use of the active Itk catalytic domain by itself is justified but does not answer the question of how the substrate-docking surface behaves within full-length Itk. To address this question, we examined PLC γ 1 phosphorylation by wild-type, full-length Itk and the full-length Itk mutants E565A and F529A/S530A (Fig. 6). All three full-length Itk enzymes were expressed, purified and subjected to the same activity assay as described above for the Itk kinase domain (Fig. 3). For both Itk E565A and Itk F529A/S530A mutants, phosphorylation of the PLC γ 1 SH2C-peptide B substrate is selectively compromised compared to phosphorylation of peptide B (Fig. 6a). This is consistent with the results obtained using the truncated Itk kinase domain and suggests that the substrate-docking surface on the C-terminal lobe of the Itk kinase domain is an important specificity determinant in phosphorylation of PLC γ 1 by full-length Itk.

Discussion

G helix is a recurring substrate-docking site on protein kinases

Direct interaction of PLC γ 1 SH2C with the Itk kinase domain leads to specific and efficient phosphorylation of the PLC γ 1 Y783 target site. The data presented here point to a largely acidic surface centered at the G helix of the Itk kinase domain (Fig. 6b) that serves as a recognition site for the previously identified basic region of the PLC γ 1 SH2C domain.²⁰ Comparison of the model that emerges for Itk-mediated phosphorylation of PLC γ 1 (Fig. 7a) with substrate-docking surfaces that have been characterized for other kinase domains reveals interesting similarities (Fig. 7). Compiling the kinase structures for which docking sites have been mapped and those for which enzyme/substrate co-crystal structures are available shows that the large C-terminal kinase lobe is the primary site for substrate recognition. Moreover, the G helix, in particular, plays a central role in mediating remote substrate recognition. The structures of PKR in complex with eIF2 α and the Rho-associated protein kinase I (ROCKI) in complex with RhoE are strikingly similar to the Itk/PLC γ 1 interaction (Fig. 7b and c).^{8,23} The substrate proteins RhoE, eIF2 α and PLC γ 1 all interact with their cognate kinase domain at and around the G helix. This structural similarity occurs despite differences in the chemical nature of the complementary interaction surfaces. In yet another example of the role of the G helix in mediating phosphorylation, the serine/threonine kinase, p21-activated kinase 1/2, appears to dimerize and autophosphorylate via an interface that includes the G helix.²⁴ The similarities in these different systems suggest that the exposure and flexibility of the G helix, as well as its location with respect to the active site, make this structural sub-element an ideal platform for substrate-docking interactions.

The G helix is not the only site for substrate docking on the C-terminal kinase lobe. ERK1/2 is considered a paradigm for modular docking interactions; this well-studied MAPK (*mitogen-activated protein kinase*) contains two distinct sites on the kinase domain functioning independently to recruit different substrates.²⁵ One of the docking sites, which binds to the DEF (*docking site for ERK, FXFP*) motif or F-site, maps to part of the activation segment, the N-terminal tip of the F helix, the MAPK insert and the G helix.²⁶ It is clear that this docking site resembles the Itk substrate-docking site (Fig. 7d and e) despite the structural differences between these two kinases in this region due to the MAPK insert. The other ERK2 docking site, which binds to the DEJL (*docking site for ERK and JNK, LXL*) motif or D-site, is located at the β 7– β 8 and α D– α E loops on the C-terminal lobe (Fig. 7e).²⁷ This surface is reminiscent of the surface the Csk exploits to engage in the docking interaction with its Src family kinase substrate (Fig. 7f).⁹ Thus, while the G helix is certainly a recurring site for substrate-docking interactions, kinase domains also use other motifs to mediate substrate recognition.

For Itk, we have previously characterized an intramolecular docking interaction with its own SH2 domain that controls autophosphorylation of Y180 on the Itk Src homology 3 domain.¹⁴ Our data for that system suggest a docking interaction surface on the back side of the Itk kinase domain, including both N-lobe and C-lobe residues.²⁸ Thus, like ERK2, Itk appears to exploit at least two distinct surfaces on the kinase domain to recognize different substrates. Consistent with nonoverlapping substrate-docking surfaces, we find that addition of the isolated PLC γ 1 SH2C effectively competes with Itk-mediated phosphorylation of PLC γ 1 Y783,¹⁴ while addition of the other substrate recognition element, Itk SH2, to the same Itk kinase assay has no effect on phosphorylation of PLC γ 1 Y783 (R. Xu, data not shown). The presence of multiple docking interaction surfaces explains, at least in part, how Itk and other kinases distinguish between distinct substrates.

Does posttranslational modification enhance substrate docking?

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

Does the docking interaction with PLC γ 1 SH2C domain regulate Itk activity?

Docking interactions in general, apart from acting as a passive substrate specificity determinant, are also known to be capable of directly regulating the kinase activity for enzymes such as ERK2 and PDK1.^{27,31} To assess whether the docking interaction between the PLC γ 1 SH2C domain and the Itk kinase domain modulates Itk activity, we titrated isolated PLC γ 1 SH2C domain into the *in vitro* Itk kinase assay and determined the initial velocity for peptide B phosphorylation by Itk. Since we have not yet directly measured the affinity between PLC γ 1 SH2C domain and Itk kinase domain, K_m of PLC γ 1 SH2C-peptide B substrate ($\sim 5 \mu\text{M}$) was used as an estimate. PLC γ 1 SH2C domain was added from 0- to 10-fold K_m of PLC γ 1 SH2C-peptide B substrate ($50 \mu\text{M}$) to ensure saturation of binding. Within this range, the initial velocity of peptide B phosphorylation remained largely unchanged (data not shown), indicating that the docking interaction between PLC γ 1 SH2C domain and Itk kinase domain by itself has no direct effect on the intrinsic activity of Itk. This is consistent with the k_{cat} value measured for PLC γ 1 SH2C-peptide B substrate being similar to that measured for peptide B substrate.¹⁴ Our *in vitro* assay is a minimal system, however, and it is likely that, in the signaling complex that contains full-length Itk and PLC γ 1 peripheral to the T cell membrane, the interactions between Itk, PLC γ 1 and other signaling proteins as well as transient posttranslational modifications might indeed serve to enhance Itk catalytic efficiency.

The docking surface on Itk expands target sites for small molecules

Interest in small-molecule inhibitors for Itk has increased in recent years as drug leads are sought for treatment of inflammatory disorders such as asthma and rheumatoid arthritis. Currently, the reported small-molecule Itk inhibitors target the ATP binding pocket,^{32–34} a binding site that promotes high-affinity binding but generally lacks the unique features required to achieve selectivity over other kinases. Our current findings suggest an alternative strategy to target Itk kinase activity in a pathway-specific manner. Small molecules that block the PLC γ 1 substrate-docking surface on Itk, or bidentate inhibitors that bind to both the Itk active site and at least a portion of the substrate-docking surface, are likely to exhibit greater specificity than active-site inhibitors alone. As the Itk/PLC γ 1 and other kinase/substrate recognition motifs are further characterized, it will be interesting to determine the extent to which substrate-docking mechanisms can be exploited in inhibitor design.

Materials and Methods

Constructs

The baculoviral expression constructs for full-length Itk and Itk kinase domain (342–619) (previously referred to as linker-kinase²²) and the bacterial expression constructs for PLC γ 1-

derived fragments have been described previously.^{14,20,22} To improve protein solubility, sample stability and protein dynamics, the Btk kinase domain (residues 396–659) used for the NMR experiments contains the following mutations: Y617P, L542M, S543T, V555T, R562K, S564A and P565S. All mutations were introduced by using the site-directed mutagenesis kit (Stratagene). All constructs were verified by sequencing at Iowa State University DNA Synthesis and Sequencing Facility. Mouse numbering is used for Itk and Btk sequences and bovine numbering is used for PLC γ 1 sequences throughout the text.

Protein expression and purification

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

Uniformly ^{15}N -labeled Btk kinase domain was expressed in BL21(DE3) cells in minimal medium containing ^{15}N - NH_4Cl (Cambridge Isotope Laboratories) as the sole source of nitrogen. When the optical density at 600 nm reached 0.6–0.8, 1 mM IPTG was added to induce protein expression at 17°C for 24 h. ^{15}N -Tyr selectively labeled Btk kinase domain was expressed in minimal medium containing ^{14}N - NH_4Cl . When the optical density at 600 nm reached 0.6–0.7, 0.1 g of ^{15}N -Tyr (Cambridge Isotope Laboratories) and 0.1 g of the other 19 unlabeled amino acids were supplemented to each 500-ml culture and grown at 37°C for 30 min. Standard induction at 17°C for 24 h was then commenced with the addition of 1 mM IPTG. The harvested cell pellets were resuspended in lysis buffer [0.5 mg/ml lysozyme, 50 mM Tris (pH 7.8), 75 mM NaCl and 20 mM imidazole] and stored overnight at -80°C . Cells were lysed at room temperature with addition of 3000 U DNase I (Sigma) and 1 mM PMSF. The lysate was clarified by centrifugation at 16 K for 45 min at 4°C , and the supernatant was incubated with Ni-NTA resin (Qiagen) pre-equilibrated with lysis buffer. The resin was washed with wash buffer [50 mM Tris (pH 7.8), 75 mM NaCl and 40 mM imidazole] and then eluted with elution buffer [50 mM Tris (pH 7.8), 75 mM NaCl, 250 mM imidazole and 10% glycerol]. The eluent was concentrated and dialyzed overnight against NMR buffer [50 mM *N,N*-bis(2-hydroxyethyl)glycine (pH 8.0), 75 mM NaCl, 2 mM DTT, 0.02% NaN_3 and 5% glycerol].

Kinase assays and Western blotting

For the *in vitro* kinase assay, Itk kinase domain was incubated with the indicated substrates in reaction buffer [50 mM Hepes (pH 7.0), 10 mM MgCl_2 , 1 mM DTT, 1 mg/ml bovine serum albumin, 1 mM Pefabloc and 200 μM ATP] at room temperature for 1 h. The samples were boiled, separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Y783 phosphorylation was monitored using anti-pY783 antibody (Biosource) as described previously.¹⁴

For determination of kinase activity, a previously described procedure was followed with minor modifications. Briefly, 100 nM full-length Itk or 500 nM Itk kinase domain was incubated with either biotinylated peptide B [aminohexanoyl biotin-EQEDEPEGIYGVLF-NH₂ (Ana-spec)] or biotinylated PLC γ 1 SH2C-peptide B at a concentration four times the

previously determined K_m ,¹⁴ in reaction buffer {50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mg/ml bovine serum albumin, 1 mM Pefabloc, 200 μ M ATP and 5 μ Ci of [^{32}P]ATP (PerkinElmer)}. To determine the initial velocity of phosphorylation, we removed 10 μ l of the reaction mixture and mixed it with 5 μ l of 8 M guanidine hydrochloride to terminate the reaction, after a 7.5-min and a 15-min reaction time, respectively. We spotted 10 μ l of this mixture onto the biotin capture membrane (Promega), which was washed two times with 1 M NaCl, two times with 1 M NaCl with 0.1% phosphoric acid, one time with H_2O and one time with 95% ethanol. The radioactivity incorporated on biotinylated peptide B or biotinylated PLC γ 1 SH2C-peptide B was quantified by scintillation counting. Each assay was performed in duplicate, and the experiment was repeated at least twice to ensure reproducibility.

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

NMR spectroscopy

All NMR spectra were acquired at 30 °C on a Bruker AVII700 spectrometer with a 5-mm HCN z-gradient cryoprobe operating at 1H frequency of 700.13 MHz using standard experimental protocols (Bruker pulse program troyf3gppsi19). For the NMR titration experiment, increasing amounts of PLC γ 1 SH2N-SH2C were added stepwise to 160 μ M ^{15}N -labeled Btk kinase domain [a kinase-inactive version of Btk (K430R) was used to ensure sample homogeneity]. The molar ratio of labeled kinase domain to unlabeled SH2N-SH2C in each of the four titration points was 1:0, 1:0.75, 1:2 and 1:4. [^{15}N , 1H] TROSY HSQC (heteronuclear single quantum coherence) spectra were collected at each titration point. To assign the tyrosine peaks of interest on the kinase domain spectrum, we mutated the tyrosine to either alanine or phenylalanine. [^{15}N , 1H]TROSY HSQC spectra were acquired for the ^{15}N -Tyr-labeled Btk kinase domain (400 μ M) as well as the ^{15}N -Tyr-labeled Btk kinase domain carrying either the Y571A or Y598F mutations. Comparison of two spectra permitted unequivocal assignment of the tyrosine in question (either Y571 or Y598).

Binding assay

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

SH2	Src homology 2
GST	glutathione <i>S</i> -transferase

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

Fig. 2.

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

Fig. 3.

(a) Comparison of catalytic efficiency and specificity of Itk kinase domain mutants with respect to the two substrates used in this study. (b) The initial velocity of the active form of the wild-type Itk kinase domain (WT-Itk) toward each of the two substrates shown in (a) was measured (see Materials and Methods) and normalized to 1. The initial velocity value for each mutant Itk enzyme (for both the peptide B and the PLC γ 1 SH2C-peptide B substrate) is then divided by the initial velocity of wild-type Itk and expressed as the ratio: $V_i/V_i(\text{WT-Itk})$. Normalized initial velocity values for peptide B substrate are the dark bars and for PLC γ 1 SH2C-peptide B substrate are the light bars for wild type and each mutant Itk. (c) Surface-exposed acidic residues on the Itk kinase domain are shown in ball and stick; those side chains that, upon mutation to alanine, lead to disruption in docking based on initial velocity measurements are shown in red and labeled. (d) Secondary alanine scan results acquired and presented as described for (b). (e) Results of the alanine scan shown on the ribbon structure of the Itk kinase domain. Red side chains are the same as in (c) and the labeled; orange side chains are those derived from the secondary screen shown in (d). (f) Surface representation of the Itk kinase domain showing the amino acids identified to be involved in the docking interaction with PLC γ 1 SH2C [red and orange as in (c) and (e)]. This view of the kinase domain is rotated to show the “bottom” of the C-terminal lobe.

Fig. 4.

(a–d) Substrate [peptide B (a and c) and PLC γ 1 SH2C-peptide B (b and d)] curves for wild-type Itk (a and b) and Itk E565A (c and d) were fit to the Michaelis–Menten equation using GraFit 5 to obtain the kinetic parameters reported in (e). (e) Kinetic parameters for wild-type Itk and Itk E565A.

Fig. 5.

Amino acid residues centered on the G helix are involved in the direct interaction with the PLCγ1 SH2C domain. (a) Purified FLAG-tagged, wild-type Itk kinase domain and Itk kinase domain mutants E568A/D569A and F529A/S530A (lanes 1, 4 and 7) were incubated with either GST (lanes 2, 5 and 8) or the GST-SH2C fusion protein (lanes 3, 6 and 9) immobilized on glutathione beads. Following extensive washing, we assessed the extent to which the FLAG-tagged Itk kinase domains (wild type and mutants) bind to PLCγ1 SH2C by blotting with an anti-FLAG antibody (top panel). The Itk kinase domain mutants, E568A/D569A and F529A/S530A, bind less efficiently to SH2C than wild-type Itk kinase domain. Band intensities were integrated using the Image Lab Software along with the ChemiDoc XRS+ System (BioRad), and normalized values are shown above each band for bound Itk kinase. The bottom panel shows uniform levels of GST and GST-SH2C visualized by Ponceau S staining. (b) ^{15}N , ^1H TROSY HSQC spectra of uniformly ^{15}N -labeled Btk kinase domain. (c) Addition of PLCγ1 SH2N-SH2C to uniformly ^{15}N -labeled Btk kinase domain results in significant broadening of selected peaks (top two panels) in the

[^{15}N , ^1H]TROSY HSQC spectrum. A large subset of kinase domain resonances does not change over the course of the titration (lower panel). (d) Two tyrosines, Y571 and Y598, are adjacent to the G helix in the Btk kinase domain and were targeted for specific resonance assignment. (e) Four [^{15}N , ^1H]TROSY HSQC spectra of ^{15}N -labeled Btk kinase domain with increasing amount of added PLC γ 1 SH2N-SH2C. The peak corresponding to Y598 shows dramatic exchange broadening with the addition of 0.75 molar ratio PLC γ 1 dual SH2 domain and broadens beyond detection with further addition of SH2 domain. The peak corresponding to Y571 broadens only slightly with increasing addition of PLC γ 1 SH2N-SH2C.

Fig. 6.

(a) The initial velocity for full-length wild type and two Itk kinases (E565A and F529A/S530A) is measured and presented for the peptide B and PLC γ 1 SH2C-peptide B substrates in a manner identical with that described in Fig. 3. (b) Structure of the C-terminal lobe of the Itk kinase domain showing the location of E565, F529 and S530 within the substrate-docking surface. Superimposed on the ribbon structure is the electrostatic surface showing the acidic nature of the Itk G helix in red (basic regions are blue). The putative phosphorylation site on the G helix, serine 564 (S564), is circled and labeled on the Itk structure.

Fig. 7. Comparison of Itk/PLCγ1 docking interaction with other enzyme/substrate pairs. (a) Individual structures of the PLCγ1 SH2C domain (2PLD) and the Itk kinase domain (1SNX) are oriented in a manner that brings the two docking surfaces (side chains indicated in red ball and stick) together. The N-terminal kinase lobe (N-lobe) and the C-terminal kinase lobe (C-lobe) are labeled as is the G helix and active site of the Itk kinase domain. (b and c) Co-crystal structures of the ROCK1/RhoE and PKR/eIF2α enzyme/substrate pairs (2V55 and 2A1A, respectively). The ROCK1 and PKR kinase domains are shown in the same orientation as the Itk kinase domain in (a). The G helix and active sites of ROCK1 and PKR are labeled. (d) The Itk kinase domain shown in a different orientation than that shown in (a) with the substrate-docking surface on the C-lobe identified in this study highlighted in red and labeled (PLCγ1 docking site). (e and f) Structures of ERK2 (e) and Csk (f), 2GPH and 3D7T, respectively, are shown with various docking sites highlighted and labeled. Both the DEJL motif docking site on ERK2 (e) and the Src docking site on Csk (f) are orange. The DEF motif docking site on ERK2 (e) is coincident with the PLCγ1 docking site on Itk.