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Review

Structure and function of Tec family kinase Itk

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Abstract

Itk is a member of the Tec family of kinases that is expressed predominantly in T cells. Itk regulates the T cell receptor signaling pathway to modulate T cell development and T helper cell differentiation, particularly Th2 differentiation. Itk is also important for the development and function of *i*NKT cells. In this review we discuss current progress on our understanding of the structure, activation and signaling pathway of Itk, in addition to inhibitors that have been developed, which target this kinase. We also place in context the function of Itk, available inhibitors and potential use in treating disease.

Keywords: kinase; T cells; Tec.

List of abbreviations

AP-1, activator protein-1; APC, antigen presenting cell; BiFC, bimolecular fluorescence complementation; Btk, Bruton's tyrosine kinase; CD3, cluster of differentiation 3; CRAC, calcium release activated current; Csk, C-terminal Src kinase; CypA, cyclophilin A; DAG, diacyl glycerol; FRET, fluorescence resonance energy transfer; GADS, Grb-2, growth factor receptor binding protein 2; Grb2-related adapter downstream of Shc; HS-1, hematopoietic lineage cell specific protein-1; *i*NKT, invariant natural killer T cells; IP_3, inositol-1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; ITAM, immunoreceptor tyrosine based activation motif; Itk, interleukin-2 inducible T cell kinase; ItpkB, IP_3 3-kinase B; KSP, kinase specificity pocket; LAT, linker of activated T cells; MHC, major histocompatibility complex; NK, natural killer; PH, pleckstrin homology domain; PI, phosphatidyl inositol; PIP_3, phosphatidylinositol (3,4,5) trisphosphate; PI3K, phosphatidylinositol 3 kinase; PKA, protein kinase A; PLC, phospholipase C; PRR, proline rich region; PRS, proline rich sequence; pTyr, phosphotyrosine; RasGRP, Ras guanyl nucleotide release protein; Sam68, Src associated in mitosis 68; SHC, SH2 containing protein; SLP-76, SH2 domain containing leukocyte protein of 76 kilodalton; SRF, serum response factor; TCR, T cell receptor ; TH, Tec homology; Th2, T helper 2; ZAP-70, zeta chain associated protein of 70 kilodaltons.

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Introduction

Tec family tyrosine kinases, which include Btk, Itk/Emt, Tec, Txk/Rlk, and Bmx/Etk, are the second largest family of non-receptor tyrosine kinases expressed in lymphocytes (1, 2). They play critical roles in lymphocyte signaling, development and function, and have been implicated in various diseases (2–4). Three Tec family tyrosine kinases are expressed in T cells: Itk, Txk and Tec, with Itk being the predominant one expressed. However, Itk is also expressed in mast cells, NK cells and NKT cells (2). Compared to Itk, Txk is expressed at approximately 10–30% of Itk in resting mature T cells and NKT cells. In contrast, Tec is expressed at a much lower level, approximately 1% of Itk levels in T cells (2, 5).

Itk acts as an amplifier during T cell receptor signaling and its deficiency leads to reduced intensity and duration of downstream signaling pathways (6). Consistent with its important role in T cell signaling, Itk has been shown to be important for the development of various T cell subsets in humans and animals. Itk affects positive and negative selection of αβ T cells, in addition to CD4/CD8 cell commitment (7–10). Itk is also required for the development of conventional or naïve phenotype ($CD44^{low}$) $CD8^+$ and $CD4^+$ T cells, but not for the development of innate or memory phenotype ($CD44^{hi}$) $CD8^+$ and $CD4^+$ T cells (11–15). Besides conventional T cells, Itk has been shown to regulate the development of innate-like T cell populations, such as invariant NKT cells and NKT-like Vγ1.1/Vδ6.2/3 γδ T cells (5, 16–19).

Itk also plays important roles in the function of T cells. Itk null mice are defective in Th2 cell function, leading to defects in developing allergen-induced allergic asthma and responding to Th2-induced parasitic infections (9, 10, 20). Itk has also been demonstrated to regulate IL-17A production in Th17 cells *in vitro* and *in vivo* via regulation of NFATc1 (21). In addition, in the absence of Itk, cytokine production by *i*NKT cells is significantly reduced (5, 16, 17). Two recent studies have also found that the increased IL-4 producing NKT-like Vγ1.1/Vδ6.2/3 γδ T cells in Itk null mice contribute to the elevated serum IgE level in these mice (18, 19). Mutations in Itk leads to similar deficiencies in humans, although these patients have not been as well characterized as the animal models of Itk deficiency. In humans, Itk deficiency is associated with fatal EBV-associated lymphoproliferative disorders (22). SNP analysis also links Itk with seasonal allergic rhinitis (23). Given the critical importance of Itk in T cell development and function, it is important for us to understand the mechanism by which Itk is regulated and activated in addition to its role in the signaling pathways affecting those functions.

Structure-activity relationship of Itk

Structure of Itk

Tec kinases are characterized by a common domain architecture, portions of which they share with other protein tyrosine kinase families like Src, Csk and Abl. These protein tyrosine kinase families have a consensus SH3-SH2-kinase domain structure. Itk and other Tec kinases also have a TH domain and the amino-terminal PH domain. The ability to be inducibly recruited to the plasma membrane through the PH domain is a distinguishable feature of Tec kinases, with the exception of Txk/Rlk, which has a cysteine string motif instead of TH and PH domains, constitutively anchoring it at the membrane (6, 24, 25). The crystal structure of the kinase domain of Itk has been solved and it closely resembles the kinase domain of Src family of kinases, with a small N-terminal lobe and a large C-terminal lobe connected by a flexible hinge. The flexible hinge region connecting the two lobes forms part of the ATP binding site, with the activation loop found between the two lobes of the kinase domain. Based on the structures of Src and Btk kinase domains, the activation loop covers the active site of the kinase domain and sterically blocks substrate binding prior to activation. Activation of the kinase results in phosphorylation of the activation loop, which induces extensive conformational changes and the activation loop moves out of the active site exposing a substrate binding site. The C-helix moves towards and forms extensive contacts with the N-terminal lobe of the kinase domain, thus stabilizing the active conformation of the kinase (2, 26, 27).

Although the signaling events upstream and downstream of Itk, and the functional significance of Itk signaling has been elucidated in reasonable detail (6), there are still gaps in our understanding of the structural changes accompanying Itk's activation *in vivo*. In addition, the mechanism of down regulation of its kinase activity following the termination of TCR stimulus is not well defined. This is mainly because the structure of full length Itk protein has not been solved to date (24, 25). Furthermore, although Itk shares catalytic and non-catalytic domains with other protein tyrosine kinase families, available evidence points towards multiple levels of regulation that may be unique to Itk.

A number of tyrosine kinases form intramolecular and intermolecular interactions that maintain them in an inactive state until they receive their activation stimulus. For instance, Src family kinases are maintained in a closed/inactive conformation by an intramolecular interaction between a phosphorylated Tyr^{527} residue in their C terminus and their SH2 domain prior to stimulation. Activation of these kinases can be induced by ligands for these SH2 and SH3 domains and/or dephosphorylation of the regulatory tyrosine residue followed by the phosphorylation of the activation loop of the kinase (26, 28). Soon after the identification and characterization of Itk, it became apparent that it lacks this regulatory tail, but since then a number of studies have shown that Itk's activity may be regulated by canonical and non-canonical intramolecular and intermolecular interactions (6, 25, 29, 30). The presence of a PRR adjacent to the SH3 domain is unique to Tec kinases and an SH3 ligand has been identified in the PRR of Itk (31). Using isolated domains of Itk and NMR approaches, this PRR has been shown to interact in an intramolecular fashion with SH3 domain (32). The formation of this intramolecular interaction was also shown to prevent the domains from binding their respective protein ligands. It is interesting to note that the ligand binding pocket of the SH2 domain is not blocked during this interaction, suggesting a putative mechanism by which Itk can switch to an 'open' state when they interact with binding partners containing pTyr ligands for the SH2 domain (32). These results were further substantiated when similar observations were made with related Tec kinases Tec and Btk, which both have two PRS (PRS-1 and PRS-2) as part of their PRR. PRS-1 sequences tend to bind to their analogous SH3 domains with low affinity, favoring the formation of monomers via intramolecular interactions, whereas PRS-2 sequences tend to have higher affinities and favor the formation of intermolecular interactions with SH3 domain of an adjacent molecule to form dimers. In line with this, Andreotti and colleagues have identified two factors that determine the formation of intramolecular versus intermolecular interaction between these regulatory domains. While a longer linker region and a lower affinity between the PRR and SH3 domain favors intramolecular interactions, the opposite is true for intermolecular interactions. It is noteworthy that Itk has a longer linker region and only has PRS-1, which binds SH3 domain with a lower affinity than PRS-2 (K_d=0.77±0.16 mM) (33, 34).

Non-canonical interactions between Itk domains

Non-canonical intermolecular interactions can occur between SH3 and SH2 fragments of Itk. Such interactions can occur between individual SH3 and SH2 domains, as well as between two SH3-SH2 fragments, the latter with a K_d of approximately 25 μM (34, 35). This interaction uses the classical ligand-binding pocket of the SH3 domain to interact with the SH2 domain, as the interaction is abrogated in a mutant Itk where a critical residue in the SH3 ligand-binding pocket, Trp^{208}, is replaced by a lysine. The interface for this interaction partially overlaps with the pTyr-binding pocket of the SH2 domain (35). Interactions between the SH3 domain of Itk and PRR has been estimated to be 0.77±0.16 mM, those between the SH2 domain and a pTyr peptide from SLP-76 have been estimated to be 0.41±0.12 mM and between the SH3-SH2 and pTyr peptide as 1.78±1.09 mM. Although the proximity between the SH3 ligand-binding pocket and proline rich ligand made the estimation of Kd unreliable, a proline rich peptide from SLP-76 can potentially displace the SH2 domain from the SH3 binding pocket. In contrast, the pTyr peptide from SLP-76 was not able to displace the SH3 domain from the SH2 binding pocket. This implies that the SH3-SH2 intermolecular interactions can influence exogenous ligand binding and the PRR of other proteins can compete with this interaction for the SH3 binding pocket. Consistent with this, the SH3-SH2 intermolecular interaction has been suggested to negatively regulate the activity of Itk (34).

This intermolecular SH3-SH2 interaction is regulated by the activity of a peptidyl-prolyl *cis*/*trans* isomerase CypA on the Asn^{286}-Pro^{287} prolyl imide bond in the SH2 domain of Itk. Proline isomerization around Pro^{287} of Itk can result in two distinct conformers, *cis* and *trans*, in solution. Because Pro^{287} in Itk is on a flexible loop, it is probable that both the *cis*- and *trans*-configuration occurs in solution. The effects of this proline isomerization on the SH2 domain function is profound, with the process affecting the conformation of more than one-third of the residues in the domain and thus having the potential to directly regulate the ligand specificities of the SH2 domain (35). Proline isomerization is known to be a rate-limiting step in protein folding, as the activation energy required for the conversion is high and reaction proceeds at a slow rate (36). This could functionally segregate the two conformers. According to the model CypA binds to a conserved Pro^{287} in the SH2 domain of Itk and maintains it in the *cis*-conformation. This conformation has high affinity for the SH3 domain of Itk and hence would favor the formation of homodimers over binding to phosphotyrosine containing ligands. The structure of the Itk *trans*-SH2/pTyr complex suggests that the *trans*-configuration of the proline containing loop allows the SH2 domain to better bind phosphotyrosine residues than when in the *cis*-configuration (37). Although the kinase activity of Itk appears to be blocked in the *cis*-configuration, the pTyr binding pocket of the SH2 domain is not allosterically blocked implying that pTyr ligands can compete with CypA to change the configuration of the prolyl imide bond between Asp^{286} and Pro^{287} into the *trans*/open conformation. Indeed the binding of pTyr ligands to the SH2 domains of kinases can stabilize open configurations and in some cases, activate these kinases (38, 39).

Thus this proline driven conformational switch between *cis*/*trans*-conformers has the potential to regulate the activity of Itk by determining the ligand interactions of its SH2 domain to favor either dimerization (*cis*-conformer, via binding to the SH3 domain of another Itk molecule) or to binding pTyr ligand (*trans*-conformer) (35, 40). This interaction between CypA and Itk has been suggested to be physiologically relevant because CypA null T cells exhibit altered TCR signal transduction and T helper cytokine production (41). In addition, swapping the SH3 domain of Itk with one from Btk reduces intramolecular interactions, while retaining the ability to bind exogenous protein ligands. This altered Itk was shown to enhance Itk signaling both in cell lines and primary murine $CD4^+$ T cells (42).

PH domain interactions

In addition to the interactions between the SH3 and SH2 domains described above, the PH domain of Itk can interact intramolecularly, although the determinants and consequence of this interaction is not clear and is likely to be stabilized by other interactions (24, 43). A common feature of Itk activation in response to the cell surface receptor engagement is the activation of PI3-Kinase, which generates $PI(3–5)P_3$ that can interact with the PH domain of Itk. This suggests that specific location of Itk at the plasma membrane is critical for its activation. Consistent with this, a mutant of Itk that lacks the PH domain and a portion of the TH domain shows abpproximately a 4-fold reduction in the catalytic efficiency (k_{cat}/K_m) towards SAM68, a substrate of LAT and ZAP-70. The K_m values for SAM68 and ATP were very similar suggesting that PH domain is unlikely to affect the ability of Itk to interact with its substrates (44, 45). While localization of Itk to the plasma membrane via $PI(3–5)P_3$ is clearly critical for its activation, Itk can also form dimers that are $PI(3–5)P_3$ dependent. Indeed, we have found that Itk interacts with other Itk molecules, forming head-to-head dimers or higher order clusters specifically at the plasma membrane, but not the cytoplasm (46). In addition, this dimerization or clustering of Itk at the plasma membrane is not random, but occurs specifically in the vicinity of receptors that can recruit PI3-Kinase (46). The PH domain, but not other domains of Itk, is required for this process (46). Studies from Huang et al. also show that the production of IP4 by ItpkB induces the binding of Itk to $PI(3–5)P_3$ through its PH domain, and is required for the recruitment and activation of Itk (43). They have also shown that Itk aggregates via its PH domain, further confirming that Itk forms dimers or clusters at the plasma membrane (43). All the data suggest that the high density of Itk molecules at the plasma membrane could be a result of dimerization or clustering of Itk in close vicinity with other signaling proteins at the plasma membrane, which may help the formation and stability of proximal TCR signaling complex.

Conformation of activated Itk

A number of studies have suggested that protein tyrosine kinases are usually maintained in an inactive conformation due to self-interactions between domains, which block the ATP binding pocket of the kinase domain (47). When protein tyrosine kinases are activated by upstream signals, these intramolecular interactions are disrupted and the kinase domain undergoes conformational changes, allowing the binding of ATP, thus increasing their kinase activity, as well as allowing interaction with substrates. As mentioned earlier, the crystal structure of Src kinases shows that in the resting state Src kinases are folded as monomers, with the structure held together via intramolecular interactions between the carboxyl-terminal negative regulatory phosphotyrosine and the SH2 domain, as well as the SH3 domain and the SH2 linker peptide, keeping them in the inactive state (47).

Intramolecular interactions between the PRR and SH3 regions of Itk block the ligand binding pocket of the domains and the activity of Itk increases when the SH3-SH2 intermolecular interaction is disrupted (42). This would imply that like the Src kinases, the non-catalytic domains of Itk negatively regulate the enzymatic activity of Itk. However, unlike Src and Abl kinases, the kinase activity of Itk is completely abrogated in the absence of these domains. Thus the non-catalytic domains of Itk might assist in maintaining its inactive conformation, possibly in the resting state but could also stabilize the active conformation.

In order to completely appreciate this regulatory feature we next consider the ‘regulatory spine’ of Itk. The comparison of active and inactive conformations of a number of

serine/threonine and tyrosine kinases led to the identification of residues whose spatial regulation is critical to the activation of these kinases (48). Using PKA as a model, Eyck and colleagues defined this to be five single hydrophobic residues scattered throughout the three-dimensional structure of the kinase domain that are disrupted in the resting state but are dynamically assembled following activation (45). Based on this definition, the regulatory spine of Itk would include Met^{409}, Leu^{420}, His^{479}, Phe^{500} and Asp^{539} residues of the kinase domain. Studies with mutants of kinase domain fragments and full-length Itk protein have confirmed that these residues form the core of Itk's regulatory spine. With the lack of a crystal structure for full length Itk, groups have used the structure of the Src kinase, Csk to predict the structure of full length Itk. There are a number of advantages of using Csk for comparative structure building studies. Like Itk, Csk lacks the C-terminal regulatory tyrosine residue. In addition, the isolated kinase domain of Csk is catalytically inert. The crystal structure of Csk in the active conformation reveals that the linker regions of the SH2-kinase and the SH2-SH3 domains are in close contact with the N-terminal kinase domain, which stabilizes the C-helix region of the active kinase (49). In kinetic assays, the SH2-kinase linker residues were found to be critical for the kinase activity of Itk (50, 51).

The similarities in the structure of solved domains of Itk with the structure of full length Csk and the shared regulatory features lead to the design of mutants of Itk that test the similarity of the proposed structures. As with Csk, mutation of conserved Trp^{355} residue in the SH2-kinase linker region of Itk reduced its kinase activity. Also, Itk has a well conserved Met^{410} residue found only among Tec kinases (42, 50). When superimposed with the structure of Csk, this residue would be on the C-helix and could make contacts with the Trp^{355} equivalent residue to stabilize the active conformation (24). With the inclusion of residues Met^{409}, Leu^{420}, His^{479}, Phe^{500}, Asp^{539}, Met^{410} and Trp^{355} would form the extended regulatory spine of Itk, which controls its activity. Thus interactions between residues in the SH2-kinase linker and the kinase domain may stabilize the C-helix of Itk to maintain its active conformation. In the inactive conformation where the regulatory spine is not assembled the C-helix can move outward, disrupting the contacts between the linker region and kinase domain, which is critical for the kinase activity. These studies might help to explain why the isolated kinase domain of Itk is catalytically inert. This idea is supported by the fact that the reported crystal structures of phosphorylated and unphosphorylated forms of the kinase domain of Itk are structurally similar (52). However, this could be secondary to the fact that the constructs used to generate the structure of the kinase domains did not contain the SH2-kinase linker region, preventing the assembly of the regulatory spine known to induce conformational changes (42, 53, 54).

Another regulatory feature shared by a number of tyrosine kinase families is the presence of a gatekeeper residue at the end of the regulatory spine. Gatekeeper residues are found within the ATP binding site of a kinase and control the accessibility to the hydrophobic pocket. In Src and Abl kinases the substitution of the gatekeeper residue with a bulkier hydrophobic residue stabilizes the regulatory spine to give a constitutively active kinase (55). A gatekeeper residue Phe^{434} has been identified in Itk. The substitution of this residue with less hydrophobic alanine or glycine can inactivate Itk, but this can be rescued by a second mutation Leu^{432} Ile, possibly because isoleucine compensates for the loss hydrophobicity of the regulatory spine due Phe^{434}Ala/Gly mutation. Interestingly, even isolated kinase domains of Itk show kinase activity when Phe^{434} is replaced by a methionine, a bulkier residue shown to stabilize the regulatory spine of other tyrosine kinases (53, 54).

Although the above-mentioned studies have provided intriguing insights into the possible structure-function relationship of Itk, it should be noted that these interpretations, with the exception of mutagenesis based studies, are based on isolated domain fragments of Itk and the exact conformation of full length Itk protein *in vivo* could be very different. Indeed, while the deletion of PRR or PH domain even in the membrane targeted mutant of Itk significantly reduced its basal activity, deletion of the SH3 domain, which would disrupt the SH3-SH2 intermolecular interaction, results in a two-fold increase in activity (56).

In the absence of a crystal structure for full length Itk, we have examined the conformation of Itk and its role in the activity of Itk *in vivo* using both BiFC and FRET assays (57). These two methods are widely used in the study of protein-protein interactions *in vivo*, and can directly visualize protein interactions in living cells (58–61). Based on these assays, we have found that Itk forms head-to-tail dimers only in the plasma membrane, but not in the cytoplasm, suggesting that Itk does not form dimers in the inactive state (57). The dimerization at the plasma membrane might explain previously reported co-immunoprecipitation results. However, we cannot exclude the possibility that small amounts of Itk head-to-tail dimers might exist in the inactive state. We have also shown that in its inactive state *in vivo*, Itk exists as monomers with their N- and C-terminal regions in close proximity (within 80 Å). This conformation is dependent on the Zn^{2+} binding region in the TH domain of Itk and is stabilized by intramolecular interactions, even in the absence of the Itk SH3 ligand-binding pocket and its PRR (57). Instead, our results suggest that Itk exists predominantly as intramolecularly folded monomers in the cytoplasm in the inactive state (57). We also identified that the zinc-binding region in BH motif is important for maintaining this conformation of Itk (57). More importantly, our study suggested that disrupting the zinc-binding region of Itk by point mutation dramatically increases the phosphorylation level of Itk in the presence of Lck stimulation, suggesting that opening the intramolecularly folded conformation of Itk can lead to increases in the activation of Itk (57). Combining the NMR derived structures of Itk PRR, SH3 and SH2 domain, the crystal structure of Itk kinase domain and the crystal structure of Btk PH and BH motif (which are predicted to be very similar to Itk), we can suggest a model of the conformation of an Itk monomer in the inactive state (Figure 1). We next consider the signaling events that activate Itk and the downstream signaling cascade.

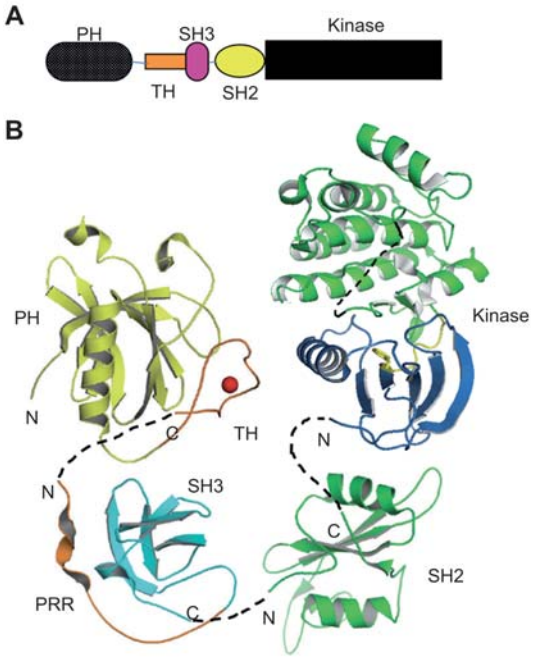

Figure 1 Proposed structure inactive of Itk.
(A) Linear organization of Itk domains. (B) Structure of inactive Itk includes proposed organization of the N-terminal Pleckstrin homology (PH) domain, Tec homology (TH) domain, Src-homology 3 (SH3) domain, Src-homology 2 (SH2) domain and a C-terminal Kinase domain of Itk. The C-lobe is depicted in light green, the hinge region and gatekeeper residues are in yellow. The activation loop is very flexible and could not be seen in the crystal structure, so is drawn using a black dashed line. The linkers between the known domain structures are also unknown and thus drawn as black dashed lines.

Activation of Itk downstream of the T cell receptor

T cells are activated when they recognize their cognate peptide MHC and costimulatory molecules on the surface of APCs. The first intracellular signaling event following TCR ligation is the activation of the CD4/CD8 co-receptor associated Src family of protein tyrosine kinases Lck and Fyn. Lck then phosphorylates the ITAM residues in the ζ-chain of CD3 creating a docking site for the recruitment and activation of ζ-chain associated protein of 70 kDa (ZAP-70). ZAP-70 phosphorylates the transmembrane adapter protein, LAT. This initiates the assembly of the 'proximal signaling complex' that is responsible for the translation of the TCR signal into a number of signaling outcomes. LAT binds the adapter proteins, GRB2 and GADS, which then binds the cytosolic adapter protein SLP-76 (62). SLP-76 is phosphorylated by ZAP-70 and it interacts with the guanine nucleotide exchange factor Vav1 as well as Itk (6). Itk is recruited to the signaling complex via its PH domain that interacts with PIP_3 that is produced at the plasma membrane in close proximity to the signaling complex by PI3K (Figure 2).

Upon recruitment, Itk interacts with the SLP-76/LAT complex through its SH3 and SH2 domains and is phosphorylated at the Tyr^{511} residue on its activation loop by Lck. Itk can also autophosphorylate Tyr^{180} residue in its SH3 domain, to alter its SH3 ligand binding pocket. Although it is not clear if transphosphorylation at Tyr^{511} precedes Tyr^{180} autophosphorylation, the fact that an Itk variant with a mutation in Tyr^{180} can still be phosphorylated at Tyr^{511} would support this notion (63). In this complex, the SH3 and SH2 domains of Itk interacts with the PRR and phosphorylated Tyr^{145} of SLP-76, respectively (25, 38, 64). Substitution of this tyrosine with a nonphosphorylatable phenylalanine residue did not affect Itk recruitment to SLP-76 but the mutation affected downstream Itk signaling, resulting in a phenotype that mimics Itk deficiency (65). This is consistent with the fact that this interaction has been shown to be critical for Itk signaling, with almost all of the catalytically active Itk molecules interacting with SLP-76 (66).

PLCγ is brought to the complex through its interaction with LAT but also binds SLP-76, Vav1 and is activated by Itk (6, 62). Itk phosphorylates Tyr^{783} and Tyr^{775} residues of PLCγ, and there is considerable interplay between all these components, which is critical for the stability and functionality of the complex (66–70). Most tyrosine kinases strictly depend on characteristic recognition residues around the substrate Tyr (pTyr-1, pTyr+1, pTyr+3), to form a stable complex to ensure substrate specificity and efficient phosphorylation (71). Itk phosphorylates Tyr^{783} of PLCγ and it phosphorylates its own SH2 domain. These two substrates do not have enough similarity to suggest a sequence dependence of Itk for its substrate targets. Also, while Itk efficiently phosphorylates the isolated SH3-SH2 domains of PLCγ, Itk is not able to phosphorylate the isolated SH3 domain of PLCγ, despite the presence of Tyr^{783} (50, 72). This suggests that Itk might use the substrate docking mechanism to achieve substrate specificity by binding to the substrate SH2 domain in a phosphotyrosine independent manner (50, 72). This feature of substrate recognition in Tec kinases has been reviewed in detail recently (24).

Upon activation by Itk, PLCγ catalyzes the breakdown of IP_3 and DAG. DAG can bind and activate PKCθ and RasGRP. PKCθ activates and allows the nuclear translocation of NFκB, whereas RASGRP initiates the MAPK signaling cascade leading to the activation of Erk and downstream transcription factors, such as AP-1. The activation of the MAPK signaling cascade is implicated in the production of cytokines and expression of activation markers by T cells (6). IP_3, the other second messenger produced, binds to the IP_3 receptor on the membrane of ER, resulting in the release of Ca^{2+} from the ER. This depletion of intracellular calcium stores results in a steady influx of extracellular calcium through the Ca^{2+} release activated current (CRAC) channels in the plasma membrane. The Ca^{2+} influx leads to the activation and nuclear translocation of NFAT through calcineurin and calmodulin (2). Consistent with this, in the absence of Itk, there are severe defects in activation of key signaling components, such as PLC-γ that leads to a defective regulation of transcription factors NFAT, AP-1, reduced influx of Ca^{2+} and activation of MAPKs (6, 9, 73). Tec kinases can also influence actin polarization, activation

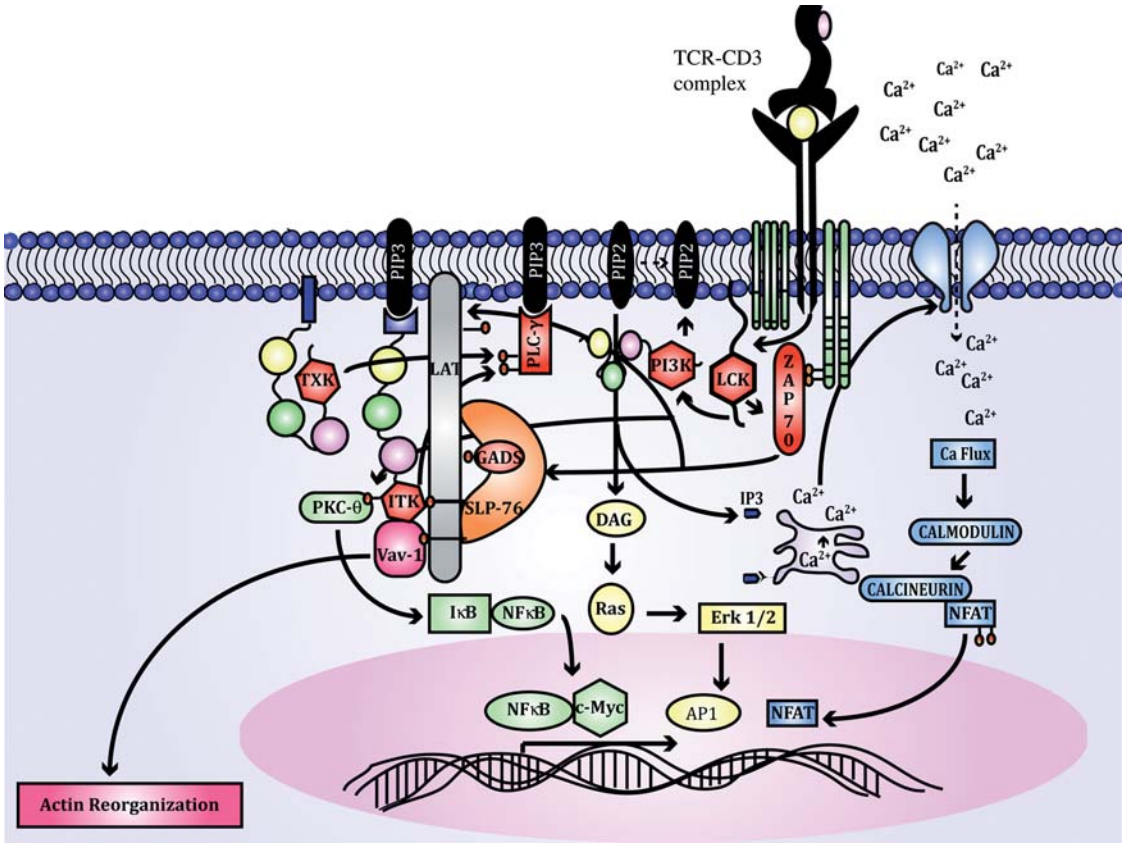

Figure 2 Position of Itk in T cell receptor signaling pathways.

of integrins and cytoskeleton reorganization following stimulation through the TCR and chemokine receptors. This aspect of Tec regulation is secondary to a defect in the recruitment of Vav1, PKCθ and the failure to stabilize the proximal signaling complex (74).

Upon TCR stimulation, both Itk and Vav are phosphorylated and bind SLP-76 in the proximal signaling complex. siRNA based knockdown of Itk in Jurkat and primary human T cells reduces recruitment of Vav and leads to defective TCR induced actin polarization. The expression of wild type Itk or a kinase dead mutant of Itk, but not PH domain or SH2 domain mutants of Itk can rescue both Vav recruitment and actin polarization (66, 75). Itk can interact with Vav even before TCR stimulation and knockdown of Itk reduces Vav-SLP-76 interaction but does not appear to affect the phosphorylation pattern of Vav. Interestingly, T cells deficient in Vav1 show defective activation of PI3K and do not activate Itk and PLC-γ (70). As Vav can interact with Itk, it is possible that this Vav/Itk interaction results in conformational changes in Itk necessary for its activation and stable association with the proximal signaling complex.

Itk can also interact with actin regulatory protein (hematopoietic lineage cell-specific protein 1) HS-1 via its SH2 domain in Jurkat T cells after stimulation. In addition, $HS1^{-/-}$ T cells show similar defects in actin polarization as $Itk^{-/-}$ T cells (76). Therefore, HS-1 may also contribute to the kinase-independent function of Itk in actin reorganization. Tec kinases are required for the activation of transcription factor SRF and antigen-induced activation of SRF is defective in $Btk^{-/-}$ DT40 cells; a B cell line that lacks expression of Tec kinases. We have reported that expression of a kinase-dead Itk mutant rescues antigen stimulated SRF activation in these $Btk^{-/-}$ DT40 cells (77). This rescue is accompanied by partial rescue in ERK/MAPK activation (77). These data suggest that Itk regulates the activation of SRF in a kinase-independent manner, in part, via regulation of the ERK/MAPK pathway. More interestingly, transgenic mice that express a kinase-dead mutant of Itk in T cells on Itk knockout background have a partial rescue of *i*NKT cell maturation (17). Expression of the kinase-dead mutant of Itk restored the expression of CD122 and transcription factors T-bet and eomesodermin in developing *i*NKT cells (17). In contrast, this kinase-dead Itk mutant does not rescue the function and frequency of *i*NKT cells, suggesting that the kinase activity of Itk is required for the function and frequency of *i*NKT cells (17). Together these data suggest that Itk has kinase-independent scaffolding function in multiple signaling pathways and differentially regulates the development of T cell subpopulation.

Inactivation of Itk signaling

A critical step in thre regulation of any signaling pathway is the negative regulation of the signal before and after acti-

vation. Any signaling event that can induce a conformational change in Itk to disrupt its regulatory spine and/or induce intra- and intermolecular interactions can regulate the kinase activity of Itk by changing the conformation to the inactive state. For instance, autophosphorylation at Tyr^{180} has been shown to increase the affinity between the SH2 and SH3 domains of Itk (50). Also, the interactions between Itk molecules could displace exogenous protein ligands (50). This is an interesting avenue to explore as it could reveal novel mechanisms by which Itk can regulate its own activity. Clearly a number of other factors can inactivate Itk. Phosphatases, such as PTEN can regulate the recruitment and subsequent activation at the plasma membrane by decreasing the availability of PIP3. Other phosphatases can potentially dephosphorylate ligands of Itk SH2 domain or directly dephosphorylate Itk to destabilize the active conformation.

Expert opinion

Small molecule inhibitors of Itk

The availability of a high resolution crystal structure of phosphorylated and unphosphorylated forms of Itk kinase domain bound to the inhibitor staurosporine has paved the way for the design of a number of small molecule inhibitors of Itk (52). A number of approaches have been used to design inhibitors specific to Itk. One of the most commonly used strategies to date is to target the kinase activity of Itk. This is attractive because inhibitors targeting the unique gatekeeper residue Phe^{435} might be able to specifically inhibit the catalytic activity of Itk without affecting closely related kinases.

Bristol-Myers Squibb designed one of the first small molecule inhibitors selective for Itk, BMS-488516 and later BMS-509744. Both these compounds have an aminothiazole core which binds to the hinge region of Itk's kinase domain. This class of compounds has been shown to selectively inhibit phosphorylation of Itk and subsequent activation of PLCγ, with efficacy demonstrated in the Jurkat T cell line and a murine model of allergic asthma. Although the compound is potent and selective, its poor pharmacokinetics when administered orally may be a major constraint preventing clinical trials (78, 79). Researchers at Boehringer Ingelheim first identified aminobenzimidazoles as potent inhibitors of Itk activity. The structure of the inhibitor bound to Itk has been solved and this showed that the aminobenzimidazole core is bound to the hinge region of Itk, and it also revealed a KSP, which could be used to improve potency and selectivity (80–82). Subsequently, biaryl-thiopene containing aminobenzimidazole compounds were screened to identify lead compounds that bound this KSP in Itk (83). This class of compounds has demonstrated efficacy in an *in vivo* mouse model when administered orally (84). Aza-Indoles and two classes of indoles with thienopyrazole moieties, one by Boehringer Ingelheim and another by Sanofi-Aventis, have been reported to inhibit the activity of Itk by binding to its hinge region. Although these compounds have been shown to be potent, information on selectivity and further characterization has not been published in peer reviewed journals (85). AstraZeneca has reported the potential use of aza-benzimidazoles, benzothiazoles and 1H-pyrazolo[4,3-c]cinnolin-3-ol as Itk inhibitors. These compounds are thought to bind the hinge region of Itk but have not been characterized in detail. Vertex pharmaceuticals has reported three classes of compounds containing pyridone-amide, pyridone-indole and pyridone-amino-benzisothiazole cores as potent Itk inhibitors with broad spectrum therapeutic potential. These compounds have to be further validated for specificity, activity and *in vivo* efficacy. Cellzome reported aminopyridine, diazolodiazine and triazole derivatives as novel inhibitors of Itk with trizoles capable of inhibiting both the activity of Itk and PI3K. Information on the potency and specificity for these compounds in inhibiting Itk has not been published. Bayer Schering has reported the use of pyrimidine compounds with sulfoximine as Itk inhibitors. Along with Itk, this compound can inhibit the activity of a few serine/threonine kinases. These compounds have to be further validated for specificity and efficacy *in vitro* and *in vivo*.

Outlook

As more information becomes available on the function of Itk in T cell activation and cytokine secretion, we recognize that pathways regulated by Itk are complex and myriad. Itk clearly regulates different functions in different cell types, although its mode of regulation and activation will be similar. Thus targeting Itk using specific inhibitors has to be balanced by an understanding of its function in various cell types. Diseases, such as allergic asthma and atopic dermatitis appear to be most amenable to treatments using such inhibitors but it is possible that other conditions, such as transplant rejection or other inflammatory diseases might also respond. However, it will be critical to understand the cell types involved in the various pathologies being targeted so that rationale approaches can be used to use the Itk inhibitors being developed.

Highlights

- Itk is a member of the Tec family of kinases that is expressed predominantly in T cells.
- Itk regulates the T cell receptor signaling pathway to modulate T cell development and T helper cell differentiation, particularly Th2 differentiation. It also regulates *i*NKT cell development and function.
- The structure of each domain has been independently determined including the kinase domain in complex with specific inhibitors. No structure of full-length Itk yet exists.
- A number of inhibitors have been developed that inhibit the activity of Itk; however, Itk also has kinase independent function. Although these Itk inhibitors promise to be useful in a number of inflammatory diseases that are reg-

ulated by T cells, Itk is also expressed in mast cells and has differential function(s) in these cells.
- As we move to understand the function and signaling pathways regulated by Itk, we will get a better understanding of the effect of such kinase inhibitors and their usefulness in treating disease.

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