Disrupting the Intermolecular Self-Association of Itk Enhances T Cell Signaling

Lie Min, Wenfang Wu, Raji E. Joseph, D. Bruce Fulton, Leslie Berg and Amy H. Andreotti

*J Immunol* 2010; 184:4228-4235; Prepublished online 17 March 2010;
doi: 10.4049/jimmunol.0901908
http://www.jimmunol.org/content/184/8/4228

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/03/15/jimmunol.090190.8.DC1

**References**
This article cites 54 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/184/8/4228.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Disrupting the Intermolecular Self-Association of Itk Enhances T Cell Signaling

Lie Min,*† Wenfang Wu, † Raji E. Joseph,* D. Bruce Fulton,* Leslie Berg, † and Amy H. Andreotti*

The Tec family tyrosine kinase (Itk), is a key component of the TCR signaling pathway. Biochemical studies have shown that Itk activation requires recruitment of Itk to the membrane via its pleckstrin homology domain, phosphorylation of Itk by the Src kinase, Lck, and binding of Itk to the SLP-76/LAT adapter complex. However, the regulation of Itk enzymatic activity by Itk domain interactions is not yet well understood. In this study, we show that full-length Itk self-associates in an intermolecular fashion. Using this information, we have designed an Itk variant that exhibits reduced self-association but maintains normal binding to exogenous ligands via each of its regulatory domains. When expressed in insect cells, the Itk substrate phospholipase Cγ1 is phosphorylated more efficiently by the Itk variant than by wild-type Itk. Furthermore, expression of the Itk variant in primary murine T cells induced higher ERK activation and increased calcium flux following TCR stimulation compared with that of wild-type Itk. Our results indicate that the Tec kinase Itk is negatively regulated by intermolecular clustering and that disruption of this clustering leads to increased Itk kinase activity following TCR stimulation. The Journal of Immunology, 2010, 184: 4228–4235.

 interleukin-2 tyrosine kinase (Itk) is a nonreceptor protein tyrosine kinase of the Tec family that is expressed in T cells, mast cells, and NK cells (1–5). Itk participates in signaling processes following TCR engagement by phosphorylating and activating phospholipase Cγ1 (PLCγ1), leading to production of two second messengers, 1,2-diacylglycerol and inositol 1,4,5-triphosphate (6–9). In addition to Itk, the Tec family includes Btk, Tec, Rlk, and Bmx, each of which shares a similar domain structure with the Src kinase family members (10). Both families contain a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, and the catalytic domain. With the exception of Rlk, the Tec kinases also contain a pleckstrin homology (PH) domain and a Tec homology domain at the N terminus (Fig. 1A).

The mechanisms promoting Itk activation following TCR stimulation have been well described. To date, three upstream signals are required for Itk activation, including Itk recruitment to phosphatidylinositol 3,4,5-trisphosphate in the membrane via its PH domain, Itk binding to the SLP-76/LAT adapter complex via its SH2 and SH3 domains, and finally Itk phosphorylation by Lck at the activation loop tyrosine in its kinase domain (11). However, the structural changes in Itk that accompany this activation process, as well as the mechanism(s) by which Itk activity is turned off when TCR signaling is terminated, have not been established. One reason for this lack of information is that, despite similarities to the Src kinases in primary structure, there are also significant differences between Itk and Src (Fig. 1A). Most notably, the Tec kinases all lack the C-terminal autoinhibitory sequence that serves to negatively regulate the Src kinases (12–15). Thus, the well-characterized inhibitory interaction in Src between the Src SH2 domain and the phosphorylated Tyr527 in the C-terminal tail cannot occur for the Tec kinase family. The absence of the Src regulatory tail sequence in Itk and the related Tec family members raises questions about the domain interactions and conformational changes that regulate Itk activity during the course of T cell signaling.

We and others have previously reported detailed structural studies for regulatory domain fragments of Itk, Btk, Tec, and Rlk (16–25). An emerging theme for each of these kinases is that the noncatalytic domains form dimeric and higher-order oligomeric structures in solution. For Itk, self-association of the regulatory domains occurs via intermolecular interactions between the SH3 domain and the SH2 domain (18). The structure of the intermolecular Itk SH3/SH2 complex has been solved (16), providing a molecular basis for probing the functional significance of intermolecular association. An intermolecular interaction has also been described for the Itk PH domain (26). The isolated PH domain interacts both with itself and with the PH domain within full-length Itk in coimmunoprecipitation experiments, suggesting that multiple contacts across the regulatory domains stabilize a self-associated form of Itk. Finally, a split yellow fluorescent protein system has previously allowed visualization of intermolecular interactions between full-length Itk molecules in cells (27). Thus, abundant data point to intermolecular clustering of Itk, most likely mediated by multiple Itk regulatory domains, yet to date the functional significance of this self-association has not been explained.

In this paper, we extend the earlier studies of Itk domain fragments and demonstrate that full-length Itk self-associates in an intermolecular fashion in vitro. To evaluate the functional significance of the observed self-association, we then designed a mutant Itk molecule that retains all of the structural features of the wild-type...
enzyme yet exhibits diminished self-association. We next examined substrate phosphorylation levels by the wild-type and mutant Itk molecules as well as the signaling properties of wild-type and mutant Itk following expression in primary CD4+ T cells. The results of these experiments indicate that the Itk mutant exhibiting diminished self-association has increased activity and signaling capacity both in vitro and upon TCR engagement in primary T cells. On the basis of these findings, we discuss a mechanistic explanation for this observation and propose a model for the control of Itk activity during TCR signaling.

Materials and Methods

Con structs and baculovirus production

V5- or myc-tagged proteins were cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). Itk(BtkSH3) was generated by replacing the Itk SH3 domain sequence from Pro215 to Asn232 with the human Btk sequence spanning Ser215 to Ser235 by PCR. Flag-tagged full-length, wild-type (mouse Itk) or Itk(BtkSH3) were cloned into the pENTR/D-TOPO vector (Invitrogen) by TOPO cloning. Point mutations (Y180F) were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pENTR vectors with various inserts were recombined in vitro with BaculoDirect C-Term Linear DNA (Invitrogen) according to the manufacturer’s instructions (Invitrogen) for virus production. The PLCγ1 baculovirus has been described previously (9).

Immunoprecipitation and Western blot

NIH 3T3 cells were transfected with V5- or myc-tagged DNA using Effectene Transfection Reagent from Qiagen (Valencia, CA). Twenty-four hours post-transfection, cells were lysed in buffer containing 0.5% NP-40, 50 mM Tris (pH7.4), 150 mM NaCl, 10 mM MgCl2, and protease inhibitors (Roche Applied Science, Indianapolis, IN). Immunoprecipitation, Western transfer, and Western blotting were performed using standard techniques. Antibodies used throughout are as follows: anti-Erk (Cell Signaling Technology, Beverly, MA), anti-phospho-Erk (Cell Signaling Technology), anti-IkB (Upstate Biotechnology, Lake Placid, NY), anti-PLCγ1 (Upstate Biotechnology), anti-pY783 (Upstate Biotechnology), anti-myc (Invitrogen), and anti-V5 (Invitrogen). Anti-Btk pY223 (used to probe the analogous site in Itk, pY180) and anti-Btk pY551 (used to probe the analogous site in Itk, pY511) are from Dr. Owen Witte.

Protein expression and purification

The purification method for bacterially expressed protein has been described previously (18). For production of Itk enzyme for in vitro kinase assays, baculovirus encoding either full-length, wild-type Itk or Itk(BtkSH3) along with Lck virus (if indicated) were used to infect Sf9 cells in a 1:1 ratio. Proteins were purified using methods reported previously (28–30). A kinase inactive mutant of Itk is used to ensure that activity measurements reflect Itk activity alone and not copurifying Lck. Coexpression of Itk [or Itk(BtkSH3)] with PLCγ1 in Sf9 cells did not include Lck expression to avoid spurious phosphorylation of the substrate by Lck. It has been demonstrated previously that Itk produced in this manner is active (31).

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded at 298 K on a DRX500 spectrometer (Bruker, Billerica, MA) operating at a 1H frequency of 499.867 MHz. Protein concentrations were adjusted to 1 mM. Global measurements at varying enzyme concentrations, the peptide B concentrations were adjusted to 1 mM. Global least-squares parameter fitting of the titration data were performed using the NMR Spectroscopy Software (NMI) version 5.3.1 (The Mathworks, Natick, MA).

Statistical analysis

The Student’s t test was used to compare differences.

Results

The Itk SH3 domain interacts specifically with the Itk SH2 domain in a noncanonical manner

SH2 and SH3 domains are well-studied signal transduction modules that mediate protein–protein interactions to alter protein localization and to promote proximity for enzyme–substrate interactions. SH2 and SH3 binding domains typically mediate interactions with target proteins that contain phosphotyrosine motifs or proline-rich sequences, respectively. For instance, the Itk SH2 domain binds to phosphorylated tyrosine residues on SLPI-76, and the Itk SH3 domain binds to proline-rich regions in SLPI-76 and Vav1 (11). However, the regulatory SH2 and SH3 domains of Itk interact with each other via an intermolecular association that does not fit these established paradigms for SH2 and SH3 recognition (18). The Itk SH3/SH2 interaction is neither phosphotyrosine-dependent nor mediated by a polyproline motif (16). Thus, the Itk amino acid residues mediating this self-association are distinct from those involved in binding to other signaling proteins, such as SLPI-76. Given this distinction, Itk provides us with an ideal opportunity to specifically disrupt self-association by altering the SH3 and/or SH2 domains in a manner that does not affect binding to canonical proline-rich or phosphotyrosine ligands. These alterations then would permit functional assays, allowing us to compare full-length, wild-type Itk with a full-length Itk variant in which self-association is disfavored.

Molecular determinants of the intermolecular SH3/SH2 interaction

The three-dimensional structure of the binary Itk SH3/SH2 complex is shown in Fig. 1B. Using this structure, we aimed to identify sequence changes that would abolish the interaction between the Itk

Spleen and lymph node cells were isolated from wild-type or Itk−/− (6) C57BL/10 (The Jackson Laboratory, Bar Harbor, ME) mice, and CD4+ T cells were purified by positive selection using anti-CD4 Ab-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA).

Retrovirus production

Itk and Itk(BTKSH3) were cloned into the retroviral mouse stem cell virus vector MSCV2.2-internal ribosomal entry site (IRES)-GFP (32). Phoenix-E retroviral packaging cells (Orbigen, San Diego, CA) were transfected with each retrovirus construct plus the pCL-Eco retrovirus packaging vector (Imgenex, San Diego, CA) as described previously (9), and virus particles were harvested and stored at −80°C. For each infection, 2 × 106 primary CD4+ T cells were stimulated with 5 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 375 ng/ml ionomycin (Calbiochem, La Jolla, CA) for 24 h and then incubated with 2 ml viral supernatant plus IL-2 (30 ng/ml) and Lipofectamine (Invitrogen). After 3–4 d, CD4+ GFP+ infected cells were sorted by flow cytometry and cultured a further 2 wk in IL-2. For analysis, 3 × 105 cells were resuspended in incubation buffer with biotinylated anti-CD3 Ab (25 μg/ml; eBioscience, San Diego, CA) for 10 min, followed by streptavidin (50 μg/ml; PerkinElmer Life and Analytical, Rockford, IL) crosslinking for 5 min. Cell lysates were analyzed for phospho-ERK by immunoblot; alternatively, cells were permeabilized and stained with anti-phospho-ERK Ab followed by flow cytometry.

Ca2+ flux assay

Wild-type Itk and Itk(BTKSH3) were cloned into the retroviral vector pMX-IRES-hCD8e (33). Four days postinfection, cells were sensitized with 10 μg/ml biotin–aCD3 for 10 min on ice in OPTI-MEM, washed, and incubated with 16 μg/ml Fura-Red (Invitrogen) and 16 μg/ml Fluo-3 (Invitrogen) in RPMI 1640/5% FBS at 37°C for 45 min. Cells were washed twice with RPMI 1640/5% FBS, incubated at room temperature in Tyrode’s buffer/BSA for 30 min. Cells were analyzed by flow cytometry with the addition of streptavidin at 30 s. Tyrode’s buffer/BSA for Ca2+ influx assay: contained 137 mM NaCl, 1.2 mM HEPES, 2.7 mM KCl, 0.04 mM NaH2PO4·H2O, 5.6 mM glucose, 0.5 mM CaCl2·H2O, 1 mM MgCl2·6H2O, and 2% FBS.

Statistical analysis

Statistical analysis was performed using the Student t test.

Purification of primary CD4+ T cells

The in vitro kinase assay (described in Ref. 28) is adapted from previous in vitro kinase assays developed for Itk (29, 30). Biotin–labeled peptide B (AnaSpec, Fremont, CA) was used as a substrate. For initial velocity measurements at varying enzyme concentrations, the peptide B concentrations were adjusted to 1 mM. Global least-squares parameter fitting of the titration data were performed using the NMR Spectroscopy Software (NMI) version 5.3.1 (The Mathworks, Natick, MA). For production of Itk enzyme for in vitro kinase assays, baculovirus encoding either full-length, wild-type Itk or Itk(BTKSH3) along with Lck virus (if indicated) were used to infect Sf9 cells in a 1:1 ratio. Proteins were purified using methods reported previously (28–30). A kinase inactive mutant of Itk is used to ensure that activity measurements reflect Itk activity alone and not copurifying Lck. Coexpression of Itk [or Itk(BTKSH3)] with PLCγ1 in Sf9 cells did not include Lck expression to avoid spurious phosphorylation of the substrate by Lck. It has been demonstrated previously that Itk produced in this manner is active (31).

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded at 298 K on a DRX500 spectrometer (Bruker, Billerica, MA) operating at a 1H frequency of 499.867 MHz. Protein concentrations were adjusted to 1 mM. Global least-squares parameter fitting of the titration data were performed using the Matlab (version 5.3.1; The Mathworks, Natick, MA) suite of programs.

Phosphorylation status

Purified full-length, wild-type Itk, Itk(BTKSH3), and Y180F full-length Itk were subjected to autophosphorylation assays by incubation at room temperature in a buffer containing 50 mM HEPES (pH 7.0), 10 mM MgCl2, 1 mM DTT, 1 mg/ml BSA, and 0.2 mM ATP at room temperature for 30 min at 0.8 μM final enzyme concentration. The reaction was stopped by the addition of SDS loading dye, run on an SDS-PAGE gel, and Western blotted.

Initial velocity measurements

The in vitro kinase assay (described in Ref. 28) is adapted from previous in vitro kinase assays developed for Itk (29, 30). Biotin–labeled peptide B (AnaSpec, Fremont, CA) was used as a substrate. For initial velocity measurements at varying enzyme concentrations, the peptide B concentration was maintained at 400 μM.
To ensure that the Btk SH3 sequence binds the canonical proline-rich ligand in a manner similar to the Itk SH3 domain, we next compared the affinities of the Itk and Btk SH3 domains for a proline-rich peptide derived from SLP-76 (Q184QPPVPPQRMA195), previously shown to bind the SH3 domain of Itk (36). Separate titrations of the QQQPPVPPQRMA peptide into 1\(^{15}\)N-labeled Itk or Btk SH3 domains induced chemical shift perturbations that were analyzed to determine their respective dissociation constants. The binding curves generated upon addition of increasing concentrations of the SLP-76 peptide into the Btk and Itk SH3 domains are indistinguishable (Fig. 2C). Thus, the Btk SH3 domain does not interact with the Itk SH2 domain yet maintains normal proline binding characteristics. These data provide a strategy for altering the full-length Itk sequence to disfavor the observed self-association of wild-type Itk while maintaining classical proline ligand binding characteristics. A full-length Itk protein [designated Itk\(_{\text{BtkSH3}}\)] that contains the Btk sequence within the region that normally spans the Itk SH3 domain (Fig. 2D) was constructed. All of the other regions of Itk\(_{\text{BtkSH3}}\) are identical to those of full-length, wild-type Itk.

Using the newly constructed Itk\(_{\text{BtkSH3}}\) Protein, we examined the phosphorylation status of both Tyr\(_{511}\) in the Itk activation loop and Tyr\(_{180}\) within the Itk SH3 domain. Tyr\(_{180}\) is strictly conserved among SH3 domains, and the corresponding residue in the Btk SH3 domain is Tyr\(_{225}\). Phosphorylation at both tyrosine sites (Tyr\(_{511}\) and Tyr\(_{180}\)) accompanies activation of Itk in T cells and if disrupted would interfere with Itk function in T cells (9). Full-length, wild-type Itk and Itk\(_{\text{BtkSH3}}\) were separately expressed as flag-tagged fusion proteins and were coexpressed with Lck in insect cells. The proteins were purified, separated by SDS-PAGE, and then probed by Western blotting with a Btk pY223-specific Ab [previously shown to recognize specific phosphorylation on both Itk Tyr\(_{180}\) and Btk Tyr\(_{225}\) (9)] or an itk pY511-specific Ab. Both wild-type Itk and Itk\(_{\text{BtkSH3}}\) were phosphorylated to the same degree at the expected sites: Tyr\(_{180}\) and Tyr\(_{511}\) (Fig. 2E). Thus, sequence changes in the full-length Itk\(_{\text{BtkSH3}}\) diminish intermolecular self-association but do not alter the normal phosphorylation patterns associated with wild-type Itk activation.

To examine the extent that the intermolecular Itk SH3/SH2 interaction mediates self-association of the full-length Itk kinase, we generated differentially tagged versions of full-length, wild-type Itk and Itk\(_{\text{BtkSH3}}\) for coimmunoprecipitation experiments (Fig. 3A). First, the V5- and myc-tagged wild-type Itk were transiently co-transfected into NIH 3T3 cells and subjected to immunoprecipitation using anti-myc Ab. The immunoprecipitated fraction was then resolved by SDS-PAGE and probed with an Ab to the V5 tag. As a control, NIH 3T3 cells were also transiently transfected with V5-tagged Itk and/or the empty vector alone. The V5-tagged full-length Itk is readily detected in anti-myc immunoprecipitates from cotransfected NIH 3T3 cells, indicating that full-length, wild-type Itk self-associates in an intermolecular fashion (Fig. 3A). These data are consistent with previous in vivo data that also indicate intermolecular association of full-length Itk molecules (27).

**Btk SH3 sequence limits Itk self-association**

In a manner identical to the wild-type Itk sequence, V5- and myc-tagged Itk\(_{\text{BtkSH3}}\) were transiently cotransfected into NIH 3T3 cells and subjected to coimmunoprecipitation. Consistent with the absence of an intermolecular interaction between the isolated Itk SH2 domain and the Btk SH3 domains, coimmunoprecipitation data indicate that full-length Itk\(_{\text{BtkSH3}}\) does not self-associate to a level that is detected in the coimmunoprecipitation experiments for wild-type Itk (Fig. 3B). The observed differences in the coimmunoprecipitation data for Itk and Itk\(_{\text{BtkSH3}}\) suggest that...
disruption of the noncanonical Itk SH3/SH2 interaction reduces intermolecular self-association of full-length Itk compared with that of wild-type protein. The functions of these Itk proteins were next examined both in vitro and in primary T cells.

Recombinant flag-tagged, wild-type Itk and Itk(BtkSH3) were separately purified from baculovirus-infected insect cells and used for in vitro kinase assays. With the in vitro kinase assay that we have described previously for wild-type Itk (28), a biotinylated peptide substrate (30) was used to measure the initial velocity of wild-type Itk and Itk(BtkSH3) at different concentrations of enzyme (Fig. 4A).

The concentration of peptide substrate in these assays is 400 μM (approximately five times $K_m$) to ensure that substrate is not limiting.
Increased activity of Itk(BtkSH3) versus wild-type Itk in vitro.
A. Initial velocity (V, in μM/min) is measured for the wild-type Itk enzyme and the Itk(BtkSH3) enzyme at three different enzyme concentrations, 0.12, 1.2, and 1.6 μM, using biotin-labeled peptide B as a substrate. At each concentration, the wild-type Itk data are shown in black, and the Itk(BtkSH3) data are shown in gray. Experiment was carried out in duplicate, and the data shown are representative of three independent experiments. B. Full-length, wild-type Itk (lane 1) or Itk(BtkSH3) (lane 2) were coexpressed with PLCγ1 in Sf9 insect cells. After 3 d, cell lysates were prepared, and immunoblotted for phospho-PLCγ1 (top panel), total PLCγ1 (middle panel), or Itk (bottom panel). The bands on the Western blot were quantified using Quantity One software and setting wild-type Itk to one.

Increased activity of Itk(BtkSH3) versus wild-type Itk in vitro.

A. Initial velocity (V, in μM/min) is measured for the wild-type Itk enzyme and the Itk(BtkSH3) enzyme at three different enzyme concentrations, 0.12, 1.2, and 1.6 μM, using biotin-labeled peptide B as a substrate. At each concentration, the wild-type Itk data are shown in black, and the Itk(BtkSH3) data are shown in gray. Experiment was carried out in duplicate, and the data shown are representative of three independent experiments. B. Full-length, wild-type Itk (lane 1) or Itk(BtkSH3) (lane 2) were coexpressed with PLCγ1 in Sf9 insect cells. After 3 d, cell lysates were prepared, and immunoblotted for phospho-PLCγ1 (top panel), total PLCγ1 (middle panel), or Itk (bottom panel). The bands on the Western blot were quantified using Quantity One software and setting wild-type Itk to one.

Increased activity of Itk(BtkSH3) versus wild-type Itk in vitro.

A. Initial velocity (V, in μM/min) is measured for the wild-type Itk enzyme and the Itk(BtkSH3) enzyme at three different enzyme concentrations, 0.12, 1.2, and 1.6 μM, using biotin-labeled peptide B as a substrate. At each concentration, the wild-type Itk data are shown in black, and the Itk(BtkSH3) data are shown in gray. Experiment was carried out in duplicate, and the data shown are representative of three independent experiments. B. Full-length, wild-type Itk (lane 1) or Itk(BtkSH3) (lane 2) were coexpressed with PLCγ1 in Sf9 insect cells. After 3 d, cell lysates were prepared, and immunoblotted for phospho-PLCγ1 (top panel), total PLCγ1 (middle panel), or Itk (bottom panel). The bands on the Western blot were quantified using Quantity One software and setting wild-type Itk to one.
expressing wild-type Itk, consistent with the earlier observations of increased activity for the nonclustering Itk mutant. Efforts to assess IL-2 responses by the retrovirus-transduced Itk$^{+/+}$ T cells indicated a trend toward higher activity in cells expressing Itk (BtkSH3) versus that in cells expressing wild-type Itk, but the differences seen were not statistically significant. Nonetheless, these data overall provide further support for a model in which disruption of intermolecular Itk clustering leads to an increase in Itk-mediated signaling following TCR stimulation.

**Discussion**

Strength and duration of TCR signaling are important in regulating T cell development and lineage commitment. As a key component of the TCR signaling pathway, Itk has been found to modulate several aspects of thymocyte maturation, including positive selection, negative selection, and conventional versus innate CD8$^+$ T cell development (11, 38). These observations suggest that Itk activity is involved in fine-tuning TCR signaling and that this, in turn, regulates T cell fate decisions. Further investigation of this process would be greatly aided by a mouse model in which Itk kinase activity is increased relative to that of wild-type Itk. Previous efforts to increase Itk activity by overexpression have uniformly been unsuccessful (36). The data presented in this report provide a biochemical explanation for these failures. Quite unlike the Src family kinases, there are to date no known mutations that activate Itk signaling. To our knowledge, Itk(BtkSH3) is the first Itk variant to show activity greater than that of the wild-type enzyme in T cells. Thus, in the future, a knock-in mouse expressing Itk(BtkSH3) in place of wild-type Itk could be utilized to study the role of Itk in T cell development and function.
of wild-type Itk could provide a system for examining T cell development and lineage commitment in cells with increased Itk kinase activity.

The quantitative effects of modulating Itk self-association on TCR signaling events are similar to those seen in studies of the Src family kinase, Lck. Src kinases, including Lck, are negatively-regulated by an intramolecular association between the SH2 domain and a phosphorylated tyrosine in the C-terminal tail that is absent in the Tec kinases. A comprehensive examination of Lck autoinhibition was recently reported in which this intramolecular interaction is either disrupted or strengthened by sequence changes close to the phosphotyrosine (39). Lck-deficient Jurkat T cells were reconstituted with wild-type Lck or Lck mutants, and TCR signaling leading to Erk activation was examined. Similar to our findings for wild-type Itk and Itk_{SH3/SH2}-modulation of the Lck autoinhibitory interaction led to detectable but modest changes in Erk activation. Together these studies indicate that shifting the equilibrium between active and inactive kinase conformations alters the strength of TCR-mediated signaling but does so within a limited range of outcomes. This is in contrast to mutations located in active sites that have pronounced, all or nothing, effects on downstream signaling.

In the current study, the sequence changes that we have introduced into full-length Itk have been limited to the SH3/SH2 interface. As already mentioned, the isolated PH domain of Itk also forms intermolecular self-associated complexes (26) and might therefore play a significant role in Itk clustering in T cells. Itk membrane association occurs via PH domain interactions with phosphatidylinositol 3,4,5-trisphosphate in a T cell stimulation-dependent fashion (40–43). Localization of Itk at the membrane could favor PH/PH intermolecular interactions resulting in further stabilization of self-associated Itk complexes. Indeed, the contribution of the PH domain to Itk self-association may be minimal in systems, such as the NIH 3T3 cells used in this study for coimmunoprecipitation experiments in the absence of such activating factors, Itk might remain self-associated and autoinhibited.

It is also possible to envision a role for Itk autoinhibition following activation of Itk by TCR engagement; in this case, self-association would be one mechanism that could terminate Itk signaling (in addition to the activity of phosphatases). In binding studies of the Itk SH3 and SH2 domains, we found that Tyr^{180} phosphorylation in the SH3 domain enhances affinity for the Itk SH2 domain (54). Together with the observations that autophosphorylation at Tyr^{180} in the SH3 domain has no effect on Itk kinase activity and occurs in cis (54), it is reasonable to suggest that autophosphorylation may be a first step toward turning off Itk-mediated signaling by promoting intermolecular self-association leading to a drop in kinase activity. Indeed, a previous report suggests that Itk clustering in T cells occurs following membrane association (27).

Although the precise mechanistic details of when and how intermolecular association of Itk modulates its activity remain a question, this mode of autoinhibition for a nonreceptor tyrosine kinase suggests that reagents (or mutations) that shift the equilibrium toward the self-associated state could dampen T cell activation while disfavoring self-association will increase Itk activity and concomitant T cell activation.

Acknowledgments
We thank Dr. Owen Witte for providing the anti-pY223 and anti-pY551 Btk Abs.

Disclosures
The authors have no financial conflicts of interest.

References