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Kinase-Independent Functions for Itk in TCR-Induced Regulation of Vav and the Actin Cytoskeleton


The Tec family kinase Itk is an important regulator of Ca\textsuperscript{2+} mobilization and is required for in vivo responses to Th2-inducing agents. Recent data also implicate Itk in TCR-induced regulation of the actin cytoskeleton. We have evaluated the requirements for Itk function in TCR-induced actin polarization. Reduction of Itk expression via small interfering RNA treatment of the Jurkat human T lymphoma cell line or human peripheral blood T cells disrupted TCR-induced actin polarization, a defect that correlated with decreased recruitment of the Vav guanine nucleotide exchange factor to the site of Ag contact. Vav localization and actin polarization could be rescued by re-expression of either wild-type or kinase-inactive murine Itk but not by Itk containing mutations affecting the pleckstrin homology or Src homology 2 domains. Additionally, we find that Itk is constitutively associated with Vav. Loss of Itk expression did not alter gross patterns of Vav tyrosine phosphorylation but appeared to disrupt the interactions of Vav with SLP-76. Expression of membrane-targeted Vav, Vav-CAAX, can rescue the small interfering RNA to Itk-induced phenotype, implicating the alteration in Vav localization as directly contributing to the actin polarization defect. These data suggest a kinase-independent scaffolding function for Itk in the regulation of Vav localization and TCR-induced actin polarization. The Journal of Immunology, 2005, 174: 1385–1392.

Upon stimulation with Ag, T lymphocytes undergo a dramatic cellular reorganization leading to cell polarization, which is accompanied by the accumulation of F-actin at the site of contact with the APC (1). This regulation of the actin cytoskeleton is required for full T cell activation. Accordingly, mice deficient in the guanine nucleotide exchange factor Vav or the Wiskott-Aldrich syndrome protein, two critical regulators of the actin cytoskeleton, exhibit defective T cell responses and development (2–5). However, actin reorganization is not only required for proper T cell signaling, it also is initiated by and requires signals emanating from the TCR. A number of studies have helped delineate the pathways between the TCR and the actin cytoskeleton and have revealed essential roles for proximal tyrosine kinases and adaptor molecules in addition to Vav and Wiskott-Aldrich syndrome protein (6–8). Recent data suggest that the Tec kinase Itk is an important contributor to these pathways.

The Tec family tyrosine kinases are key signaling intermediates that are required for full activation of phospholipase C-γ (PLC-γ)<sup>6</sup> and Ca\textsuperscript{2+} mobilization downstream from Ag receptors (9). Mutations affecting the Tec kinase Btk give rise to the human primary immunodeficiency X-linked agammaglobulinemia, which is associated with impaired responses to BCR engagement (10, 11). Similar defects have been observed in T cells from animals deficient in the Tec kinases Itk and Rlk and are associated with decreased ability to respond to infectious agents (12, 13). Notably, Itk is required for generating appropriate Th2 responses in vivo, a defect that may be linked to impaired activation of the Ca\textsuperscript{2+}-sensitive NFAT transcription factors and/or altered T-bet expression (14–17). More recently, Itk-deficient cells have been shown to have defects in actin polarization and the localized activation of the Rho family GTPase Cdc42 (18, 19). Overexpression of the Itk pleckstrin homology (PH) domain or Itk containing a mutation affecting the Src homology 2 (SH2) protein interaction domain in Jurkat cells also prevents TCR-induced actin polymerization (18, 20), suggesting that the Tec kinases may have important functions in the regulation of the actin cytoskeleton. However, Itk deficiency also causes altered thymic development and selection, and mature cells from Itk<sup>−/−</sup> animals can express decreased TCR levels and altered cell surface markers (21, 22), complicating interpretation of these results. Experiments in cell culture have to date relied on overexpression of mutant versions of Itk, which also may not accurately reflect protein function in vivo (18, 20, 23). Indeed, the first report to address the role of Itk in actin reorganization and cell polarization concluded that Itk was not involved in this process because a kinase-inactive version of Itk failed to disrupt cell contact and polarization to the APC (23).

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Abbreviations used in this paper: PLC-γ, phospholipase C-γ; PH, pleckstrin homology; PBT, peripheral blood T lymphocyte; si, small interfering; WT, wild type; ITAg, Jurkat-TAg; CAT, chloramphenicol acetyltransferase; EGFP, enhanced GFP; RNAi, RNA interference; LAT, linker for activation of T cells; SH2, Src homology 2; Itk-KD, Itk-kinase defective.
Ideally, an Itk-deficient cell line would help address these concerns and aid the study of Itk function in T cell signaling. Indeed, analyses of mutant Jurkat cell lines that are deficient in signaling molecules have greatly advanced our understanding of TCR signal transduction (24). However, the majority of mutant Jurkat lines have been isolated by virtue of their inability to mobilize Ca$^{2+}$ or induce expression of reporter genes upon TCR engagement, and mutants that permit partial T cell function, such as seen in Itk-deficient cells (13, 25), may not be isolated by such approaches. Although additional mutant T cell lines have been generated via somatic cell gene targeting, this process is highly labor-intensive, and few genes have been disrupted successfully (26, 27).

To evaluate functions of Itk, independent of altered T cell development, we have inhibited Itk expression in Jurkat cells and human peripheral blood T lymphocytes (PBT) using small interfering (si)RNA (28, 29). We demonstrate that reduction in endogenous Itk reproduces the functional defect in actin organization seen in Itk$^{-/-}$ mice, allowing us to re-express wild-type (WT) and mutant versions of murine Itk and identify domains of Itk required for TCR-stimulated actin cytoskeleton rearrangement.

We confirm that Itk plays a critical role in TCR-induced actin cytoskeletal reorganization and demonstrates that the actin defects in Itk-deficient cells correlate with the ability of the Vav guanine nucleotide exchange factor to localize to the site of TCR stimulation both in Jurkat cells and in T cells derived from normal donors. Additionally, we show that Itk can directly interact with Vav and regulates Vav localization in a kinase-independent manner that requires function of the Itk SH2 domain. Finally, expression of a membrane-targeted mutant of Vav can rescue the defect in actin polarization in Itk-deficient cells. Our data extend recent observations from the Tsoukas laboratory (18) by demonstrating a kinase-independent role for Itk in regulating Vav localization, and suggest a mechanism for the actin defect in Itk-deficient cells.

Materials and Methods

Cells and reagents

Jurkat-TAg (JTAg) cells were grown in RPMI 1640 containing 10% FBS (HyClone), 10 mM HEPES, 2 mM l-glutamine, 50 μM 2-ME, 100 U of penicillin/100 μg/ml streptomycin, and 1 μg/ml ciprofloxacin. In some experiments, antibiotics were removed to improve electroporation. Human PBT cells were obtained from donors at the National Institutes of Health Clinical Center and were purified by Ficoll separation. Cells were washed several times and cultured for 2 days in medium (RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 10 μg/ml ciprofloxacin, and 20 U/ml human IL-2 (Roche)) containing 2 μg/ml Con A. Con A was removed by 30 min of incubation with 10 ng/ml α-methyl-pyranoside at 37°C, followed by three washes in PBS; and the cells were rested for 5–9 days in IL-2 containing media before treatment. Reagents used included pEGFP-N1 (Clontech Laboratories), anti-Lck (BD Pharmingen), anti-Vav and anti-Itk 2F12 (Upstate Biotechnology), anti-tubulin (ICN Pharmaceuticals), anti-FLAG (M2; Sigma-Aldrich), and anti-SLP-76 (Antibody Solutions); anti-clonotypic TCR (CD3/OKT3 and anti-CD3 OKT3) were concentrated from hybridoma supernatants. Myc-tagged Itk constructs were kindly gifted from Drs. L. Berg (University of Massachusetts, Worcester, MA) and S. Bunnell (National Cancer Institute, Bethesda, MD).

siRNA

Synthetic siRNA oligonucleotides were purchased from Xeragon (Qiagen) and treated per the manufacturer’s recommendation. siRNA against human Lck or Itk included the following: Lck-1 sense, 5’-CAUCGAUUUUGUGUAGACAUCG-3’; Lck-1 antisense, 5’-AGUUCUCACACACACCAUGU-3’; Itk-1 sense, 5’-UGUUCUCAGGGUCAGAAGC-3’; Itk-1 antisense, 5’-GCUUCCUCACGAGAGAAGC-3’; Itk-2 sense, 5’-GGAGCCUUCAUGGAAGGCAUU-3’; and Itk-2 antisense, 5’-UCCCUUACCAUGAGGCGCUU-3’. Both sets of Itk oligos worked with similar efficiencies. siRNA against GFP and chloramphenicol acetyltransferase (CAT) have been described previously (30).

Electroporation

JTAg cells (10$^7$ cells/0.5 ml in RPMI 1640 plus 20% FBS and 10 mM HEPES) were electroporated with siRNA (1–2 μg) and/or plasmids (3–5 μg) in 0.4-mm cuvettes (300 V, 20 ms, BTX-850). To examine Vav localization, multiple cuvettes were electroporated with siRNA, combined, and 24 h later, live cells were purified over Ficoll and transfected with 10 μg of Flag-tagged Vav and/or 3–10 μg of Myc-tagged murine Itk constructs using the Amaxa nucleofector (program C16 in Solution T; Amaxa). Rested human PBT were transfected with siRNA (0.5 μg/5 × 10$^6$ cells) and/or murine Myc-tagged Itk (3–10 μg) using the Amaxa nucleofector (program T23 in solution human T cells). The cells were placed into 12-well plates containing 2 ml of fresh media supplemented with 20% FBS and 20 U/ml IL-2.

T cell/bead conjugations

T cell/bead conjugates were prepared and evaluated in a manner similar to Lowin-Kropf et al. (8) and Labino et al. (19). In these studies, 5-μm latex beads (10$^3$ beads/ml (Interfacial Dynamics)) were coated with IgM, anti-TCR C305 (1/130 dilution in PBS), or anti-CD3 OKT3 (for human PBT) as described previously (13). For T cell/bead conjugations, 1.25 × 10$^6$ cells in serum-free media were mixed with 2 × 10$^6$ Ab-coated beads in 70 μl, spun for 1 min at 100 × g, flicked to mix, and incubated at 37°C for 10 min. Conjugates were fixed with 4% paraformaldehyde at room temperature for 10 min, 0.7 ml of serum-free RPMI 1640 was added, and cells were stored rotating at 4°C until stained.

Immunofluorescence staining and microscopy

T cell/bead conjugates were stained with Alexa594-phalloidin (Molecular Bioprobes) as described previously (19). For Vav localization, cells were stained with anti-FLAG (1:500 in 1.25% BSA/0.1% saponin in PBS at room temperature for 40 min), followed by two washes and incubation with a solution of FITC-anti-mouse IgG (1:1000) and Alexa594-phalloidin (1:67) (19). Conjugates were examined on an inverted Zeiss Axiopt microscope with ×40 and ×100 oil immersion objectives. Cells were scored as having polarized actin if they bound a single bead and showed increased phalloidin staining at the T cell/bead interface. Thirty to 50 conjugates were scored per condition. Data are presented as the percent cells with polarized actin or Vav per conjugates scored ±SE for a minimum of three experiments. For human PBT cells, data were obtained using cells from two or more donors.

Immunofluorescence analysis of Jurkat-Nalm6 conjugates

Conjugations and microscopy were performed as previously described (31), except that CMAC stained Nalm 6 cells were allowed to interact with JTAg cells for only 5 min at room temperature, then plated onto poly-l-lysine-coated (Sigma-Aldrich) slides for 1–5 min, fixed, and stained.

Immunoblotting

For verification of Itk levels, cells (4 × 10$^7$ cells/ml) were lysed in 1% Triton X-100 in PBS (pH 7.4) with protease inhibitors (Roche) for 20 min on ice and clarified (14,000 rpm, 10 min at 4°C). For biochemical assays, cells (10$^7$/100 μl) were stimulated with either soluble C305 or OKT3 for 2 min unless otherwise indicated, lysed in 1% Triton X-100, 150 mM NaCl, 20 mM HEPES, 50 mM β-glycerophosphate, 2 mM EGTA, 10% glycerol, 10 mM NaF, 1 mM Na3VO4, and protease inhibitors, and clarified as above. Proteins were immunoprecipitated with anti-FLAG, separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with primary and HRP-conjugated secondary Ab as per the manufacturer, and visualized by ECL (Amersham Biosciences). In some experiments, FLAG peptide (Sigma-Aldrich) was included in the immunoprecipitation as a specificity control.

Results

Establishment of siRNA to reduce Itk expression in Jurkat cells

To confirm the feasibility of using RNA interference (RNAi), JTAg cells were electroporated with a plasmid encoding enhanced GFP (EGFP), and siRNA against EGFP or a control siRNA (si-CAT) (30) and GFP expression was monitored by flow cytometry at various times postelectroporation. Cotransfection of the siGFP oligonucleotide, but not the control siCAT nor the sense strand ribonucleotide, markedly reduced expression of GFP (5- to 12-fold; Fig. 1A). Reductions in GFP expression were observed both by percentage of cells positive for GFP and by mean fluorescence intensity of the GFP signal (Fig. 1B). Although optimal gene silencing required dsRNA, in some experiments, antisense RNA (30)

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showed a modest reduction in expression of GFP. GFP silencing was dose dependent, reaching a plateau at 1–2 μg of a 22-bp siRNA electroporated per 10^7 cells (Fig. 1B). At doses of ≥5 μg of siRNA, nonspecific effects were observed with decreased expression of multiple proteins, whereas at doses <0.6 μg, less efficient silencing was seen (Fig. 1B and data not shown). On the basis of these results, we routinely used 1–2 μg of siRNA per 10^7 cells.

To test whether RNAi could reduce expression of endogenous Itk, we used siRNA against the genes encoding Itk or the Src family kinase Lck (siItk or siLck). Transfection of siItk or siLck led to specific reductions in expression of Itk or Lck, respectively (Fig. 1C). Similar results were found using siltk in human PBT (Fig. 1D). TCR and Tubulin levels were also unaffected by siRNA treatment (Fig. 1, C and D, and data not shown), confirming the specificity of the effects. Time course studies revealed that maximal suppression of Itk occurred in the first 2 days posttransfection with normal protein levels restored by day 4 (Fig. 1E). Thus, for functional studies of Itk, we used cells to which siItk had been introduced 24–36 h earlier.

**Itk and actin polarization**

Recent studies using both Itk-deficient mouse primary T cells and Jurkat cells expressing mutant versions of Itk suggest that Itk plays an important role in TCR-induced regulation of the actin cytoskeleton (18–20). To dissect this phenotype, JTAg cells were transiently transfected with siltk, and 24 h later, the cells were stimulated with either mouse IgM-coated beads as a negative control or anti-TCR-coated beads, stained with rhodamine-phalloidin, and examined by fluorescence microscopy. Conjugates between a single cell and bead were counted and scored positive if the conjugate showed increased actin accumulation at the site of bead contact (Fig. 2, A and B). Conjugates of T cells to IgM-coated beads exhibited a basal level of polarized actin of 25–30%, which was unaffected by treatment with siltk (Fig. 2B). However, similar to Itk-deficient cells, JTAg cells treated with silkt showed reduced TCR-induced actin polarization (Fig. 2B). This effect appeared to be specific for siltk because actin polarization remained normal in

**FIGURE 1.** RNAi is functional in Jurkat cells. A, JTAg cells were electroporated with pEGFP-N1 with or without siRNA against EGFP or CAT. Percentages of GFP^+^ cells 24 h posttransfection are shown. Data represent one of three independent experiments. B, Cells were transfected with pEGFP-N1 plus varying amounts of siGFP. Data are presented as the mean fluorescence intensity (MFI). C, Reduction of endogenous gene expression using siRNA. JTAg cells were electroporated with siRNA against GFP, Lck, or Itk, and 24 h later, cell lysates were immunoblotted for Itk, Lck, and Tubulin. D, PBT cells from a normal donor were treated with siRNA against Itk, and 24 h later, cell lysates were immunoblotted for Itk and Tubulin. E, JTAg cells were electroporated with siltk, lysed at the indicated times after electroporation, and immunoblotted for Itk and Tubulin.

**FIGURE 2.** Reduction of Itk expression impairs actin polarization. A, JTAg cells were electroporated with siltk2 or siGFP. Twenty-four hours later, cells were stimulated with anti-TCR-coated beads, fixed, and stained with rhodamine-phalloidin. Representative polarized (positive) and nonpolarized (negative) cells are shown. B, JTAg cells were scored for actin polarization in response to IgM- or C305-coated beads. C, WT murine Itk rescues the actin polarization defect. JTAg cells were transfected with siltk plus plasmid encoding murine WT Itk and scored for actin polarization to C305-coated beads. Corresponding levels of endogenous Itk, murine myc-tagged Itk, and tubulin are shown below each lane.
cells treated with siGFP. Importantly, re-expression of WT murine Itk, which is not affected by the siRNA directed against human Itk, rescued actin polarization in siItk-treated cells, demonstrating the specificity of the defect for the loss of Itk expression (Figs. 2C and 6A).

Requirements for Itk function in actin regulation
The ability of murine Itk to rescue the siItk effects on actin organization allowed us to address the requirements for Itk function.

Although kinase-inactive mutants are often used to block functions of tyrosine kinases, data suggest that overexpression of kinase-inactive Itk may not affect actin cytoskeletal reorganization (18, 23). However, such experiments only address the ability of mutants to act as dominant-negative proteins but do not necessarily address requirements for protein function, which are better addressed by genetic complementation. To definitively address whether actin cytoskeleton regulation required Itk kinase activity, we re-expressed a point mutant of murine Itk that impaired its kinase activity (Itk- kinase defective (Itk-KD)) in cells transfected with siItk. Although this mutant has previously been shown to fail to rescue IL-2 transcription in Itk-deficient mouse cells, Itk-KD was able to rescue actin polarization to a similar extent as WT Itk in JTAg cells treated with silks (Fig. 3C). In contrast, Itk-KD that also carried a mutation of the PH domain failed to rescue actin polarization (data not shown). Thus, the PH domain, but not kinase activity of Itk, is required to rescue the TCR-induced actin polarization defect in silktreated cells. Finally, similar to findings from Tsoukas et al. (18), we observed that expression of a SH2 mutant of Itk dominantly inhibited actin polarization in JTAg cells (Fig. 3D). This effect occurred to a similar extent as that caused by transfection of silks. Consistent with these observations, the SH2 mutant of Itk also failed to rescue actin polarization in silktreated cells.

Kinase-independent functions of Itk in primary human T cells
Jurkat T cells have been shown to be deficient in the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an inositol phosphatase that acts on the products of PI3K (33, 34). Deficiency of this phosphatase has been found to alter Itk localization (33). To confirm that our findings are not an artifact of the use of a Jurkat subline, we examined actin polarization in human PBT cells from normal donors that were treated with siSilk. Because our studies with JTAg cells used the C305 anti-TCR Ab that is specific for the clonotypic TCR on Jurkat cells, we first confirmed that we could visualize actin polarization using PBT lymphocytes or JTAg cells that were stimulated with beads coated with OKT3, an anti-CD3 Ab (Fig. 4, A and B, and data not shown). We then demonstrated that silk could also reduce actin polarization in PBT cells and that both WT and the kinase-defective mutant of Itk could rescue the actin defect in PBT treated with silk (Fig. 4, A and C). Finally, we found that expression of the SH2 mutant of Itk also blocked anti-CD3-induced actin polarization in human PBT cells (Fig. 4D). Thus, a kinase-independent function for Itk in actin polarization is also seen in primary human T cells.

Regulation of Vav localization
One of the key regulators of the actin cytoskeleton in T cells is the guanine nucleotide exchange factor Vav, which is activated by tyrosine phosphorylation upon TCR stimulation and helps regulate the Rho family GT Pases, Rac, Rho, and Cdc42 (35). We have recently found that the altered actin polarization in Itk−/− T cells correlates with defective Cdc42 activation and a failure to localize Vav to the site of TCR stimulation, despite relatively normal TCR-induced Vav phosphorylation (19, 21). To determine whether Vav
localization correlated with the ability of Itk mutants to rescue actin polarization, we expressed a FLAG-tagged version of Vav, which facilitated subcellular localization studies. Although we introduced siItk for 24–36 h to allow maximal gene silencing, expression of Vav-FLAG was performed for short periods of time (4–8 h), which did not alter cellular viability, adhesion of cells to anti-TCR-coated beads, nor polarization of actin (Fig. 5A and data not shown). Similar to primary cells, siItk-treated Jurkat cells showed a reduction in Vav localization that correlated with the extent of actin polarization (Figs. 5A and 6A). This defect in Vav localization was also observed in human PBT cells from normal donors treated with siRNA to Itk (data not shown). Moreover, defective actin polarization and Vav localization were observed both in siItk-treated cells stimulated with Ab-coated beads, as well as those stimulated with Staph enterotoxin E-treated Nalm6 cells, a system which more closely resembles Ag stimulation (Fig. 5B; Ref. 8).

We then examined how re-expression of WT or mutant Itk affected Vav localization in cells transfected with siItk. Expression of either WT or KD murine Itk rescued both actin polarization and Vav localization (Fig. 6, A and B). In contrast, cells expressing the SH2 mutant of Itk showed abnormal Vav localization, which paralleled their defect in actin polarization, and the SH2 mutant failed to rescue Vav localization in siItk-treated cells (Fig. 6C). Thus, the ability to polarize actin correlated with proper Vav localization, and both regulation of Vav localization and actin polarization appeared to be independent of Itk kinase activity but dependent on SH2 domain interactions.

**Membrane-targeted Vav rescues actin polarization**

Although the defect in Vav localization could lead to an actin defect in siItk-treated cells, it is also possible that the defective Vav localization was actually secondary to the actin defect. To determine whether the altered Vav localization directly contributes to the actin defect in siItk-treated cells, we expressed a membrane-targeted construct of Vav, Vav-CAAX (36). Expression of Vav-CAAX restored actin polarization to the site of TCR stimulation in siItk-treated cells (Fig. 7), suggesting that the actin defect in

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**FIGURE 4.** Reduction of Itk expression impairs actin polarization in human PBT cells. A, Human PBT cells were treated with siItk2 or no siRNA and, 24 h later, stimulated with OKT3-coated beads and fixed and stained with rhodamine-phalloidin. Representative polarized and nonpolarized cells are shown. B, Human PBT cells were stimulated with either control (IgM) or OKT3-coated beads, and actin polarization was scored. C, Human PBT cells were transfected with siItk in the presence or absence of plasmids encoding WT and mutant murine Itk, stimulated with OKT3-coated beads and scored for actin polarization. Si0 cells received no siRNA. Data represent the average ± SD of results from two to three donors. D, Itk-SH2 mutant blocks actin polarization in human PBT cells. Data represent the average ± SD of results from two donors.

**FIGURE 5.** Reduction of Itk expression impairs Vav polarization. Cells were electroporated with siItk and, 24 h later, transfected with FLAG-Vav and murine Itk constructs. After 4–6 h, cells were stimulated with anti-TCR-coated beads (A; ∗ marks beads) or Staph enterotoxin E-pulsed Nalm6 cells (B; labeled with 4′,6′-diamidino-2-phenylindole and an arrowhead) and stained with rhodamine-phalloidin and anti-FLAG. T cells in B are marked with a star.

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**FIGURE 6.** Effect of re-expression of WT or mutant Itk on actin polarization. A, Human PBT cells were transfected with siItk, stimulated with OKT3-coated beads, and cells containing flag-Vav and murine Itk constructs were stained with rhodamine-phalloidin and anti-FLAG. T cells are marked with a star.
Itk-deficient cells results at least in part from improper Vav localization.

Itk expression affects protein interactions with Vav

To additionally investigate the mechanism by which Itk affects Vav, we examined Vav phosphorylation and protein interactions in silenced Itk-treated cells. Unlike Vav localization, which appeared to be dependent on the presence of Itk, patterns of Vav tyrosine phosphorylation were not grossly affected by the loss of Itk (Fig. 8A), consistent with what we have observed in Itk and Rlk/Itk-deficient thymocytes. Thus, tyrosine phosphorylation of Vav appears to occur before or independent of stable recruitment of Vav to the site of TCR contact. To better understand how Itk can affect Vav localization, we performed coimmunoprecipitation experiments to examine whether Itk directly interacts with Vav using cells coexpressing Vav-FLAG and myc-tagged Itk. Immunoprecipitation of FLAG-tagged Vav revealed Itk-myc in the immunoprecipitates (Fig. 8B). The association of Vav and Itk appeared to be constitutive and did not depend on TCR stimulation. The same was also observed for endogenous Itk and Vav (data not shown). Furthermore, coimmunoprecipitation of Itk with Vav was not affected by mutation of the SH2 domain of Itk (Fig. 8B). Thus, the Vav-Itk interaction appears to be independent of Itk SH2 function.

The constitutive association of Itk with Vav suggested that the presence of Itk may influence the ability of Vav to interact with other proteins in TCR-mediated signaling complexes. Therefore, we examined proteins that coimmunoprecipitated with Vav in the presence or absence of Itk. Immunoprecipitation of Vav with an anti-FLAG monoclonal revealed the presence of a 76-kDa tyrosine phosphorylated protein that comigrated with the adaptor molecule SLP-76, as determined by immunoblotting with anti-SLP-76 and Itk Abs (Fig. 8C and data not shown). However, depletion of Itk, via siRNA, reduced the amount of this protein that was observed in the Vav immunoprecipitations. Thus, depletion of Itk appears to impair the ability of Vav to interact with other proteins in the signaling complex.

Discussion

Regulation of the actin cytoskeleton is critical for T cell signal transduction and normal immune cell interactions (1, 37). We have used RNAi to dissect the requirements for Itk in TCR-induced actin polarization. Although certain data suggest that siRNA can give rise to nonspecific effects in mammalian cells, we find that the actin defect in silenced Itk-treated cells is rescued by WT murine Itk, supporting the specificity of this phenotype for the loss of expression of Itk. Our results suggest that Itk has a potential scaffolding function that is independent of its tyrosine kinase activity, which helps regulate Vav localization and cytoskeletal reorganization in response to TCR stimulation.

We have previously reported that Itk-deficient cells show defective TCR-induced actin polarization that is associated with decreased activation of Cdc42, a key regulator of the Wiskott-Aldrich syndrome protein (19). We additionally found that Vav was not properly targeted to the site of TCR contact. The observation that Vav regulates Cdc42 (7) suggested that altered Vav localization contributed to the actin defect in Itk−/− cells. Our demonstration in this study that a membrane-targeted version of Vav can rescue actin polarization in silenced Itk cells directly implicates Vav in the altered actin regulation in these cells. The mechanism(s) by which Itk affects Vav localization remains unclear. Itk has been shown to phosphorylate tyrosine 171 of linker for activation of T cells (LAT), which can bind Vav (38). However, our results argue that proper Vav localization does not require Itk kinase activity. Indeed, the role of Itk kinase activity in...
regulation of the actin cytoskeleton has been somewhat controversial. Work from Donnadieu et al. (23) originally argued that Itk did not participate in actin cytoskeleton regulation because kinase-inactive Itk failed to block T cell polarization. Interpretations of this work have been challenged by two recent studies demonstrating an actin polarization defect in \( \text{Itk}^{-/-} \) cells, despite the inability of \( \text{Itk-KD} \) to inhibit this process (18, 19). Nonetheless, it should be noted that data from Shimizu and colleagues (20) argue that a kinase-inactive Itk mutant can block TCR-induced actin polymerization. Such discrepancies may be the result of differences in assay systems, expression levels, or the use of different mutants of Itk. By demonstrating that a mutant affecting the kinase activity of Itk can indeed rescue the actin defect in \( \text{siItk} \)-treated Jurkat or human PBT cells, our work provides strong evidence for a kinase-independent function of Itk.

Our findings suggest that Itk has an adaptor function that requires its PH and SH2 domains, but not kinase activity, for the regulation of Vav recruitment and actin reorganization. Therefore, it is relevant that both Itk and Vav bind via their SH2 domains to the adaptor SLP-76 as part of the LAT-SLP-76 mediated complex that helps orchestrate downstream readouts in TCR signaling (39–42). Moreover, depletion of Itk appears to reduce the amount of SLP-76 found in Vav immunoprecipitates. Because the SH2 domain of Itk is required for actin polarization, it is possible that an interaction between the Itk-SH2 domain and SLP-76 (or LAT) may be required to stabilize the interactions of Vav with other proteins in this signaling complex. Indeed, we observed that Vav and Itk can coimmunoprecipitate and that this does not require that function of the Itk SH2 domain, suggesting that the SH2 domain of Itk is interacting with another protein. Direct binding of Tec kinases and Vav have also been previously reported both in vitro and by coimmunoprecipitation (39, 43). However, it is also possible that a separate function of Itk may also contribute to Vav localization. Of interest are findings that Btk can act in a kinase-independent fashion to increase phosphatidylinositol 3,4,5-phosphate levels (44). Because Vav contains a PH domain, it is possible that Itk contributes to Vav localization through this type of secondary influence.

Nonetheless, the cross-regulation of and interactions between Itk and Vav are complex. Tybulewicz and colleagues (45) have also demonstrated impaired Itk activation in Vav-deficient cells. Although this observation may be secondary to defective PI3K activation in \( \text{Vav}^{-/-} \) cells, rather than a direct effect on Itk, these data underscore the complex regulatory interactions among components of the TCR signaling complex and argue that linear relationships cannot necessarily be used to describe the biochemical pathways regulating T cell activation. Therefore, it is of interest that Tybulewicz and colleagues (45) also noted disruption of the interactions of SLP-76 with PLC-\( \gamma \) in Vav1-deficient cells, suggesting there may be defects in the integrity of the LAT-SLP-76-mediated complex in the absence of either Vav1 or Itk. It should also be noted that our data does not rule out the possibility that Vav1 associates with the LAT-SLP-76 complex in Itk-deficient cells, but does suggest that Vav1 phosphorylation can occur before or independent of stable SLP-76 association.

The Tec kinases have been previously implicated in the regulation of PLC-\( \gamma \) and Ca\(^{2+} \) mobilization (9). Nonetheless, we see...
effects on actin under conditions where we see minimal changes in Ca2+ and NFAT activation (data not shown). It is possible that PLCγ activation may be compensated for by expression of other Tec kinases, as in mice, where T cells lacking two Tec kinases, Rlk and Itk, have more impaired Ca2+ mobilization than Ifk−/− cells (13). Alternatively, it is possible that the defects in prolonged Ca2+ mobilization and immune responses in Itk−/− mice may actually result in part from the altered Vav localization and cytoskeletal organization, which may prevent formation of a stable TCR signaling complex. We have recently found that T cells expressing mutant versions of Tec kinases also show decreased cell polarization and activation of Cdc42 and Rac in response to chemokines (46). It will be of interest to see whether Vav plays a role in these processes and whether Btk-deficient B cells also exhibit similar actin cytoskeleton defects.

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