A kinase independent function for Tec kinase ITK in regulating antigen receptor induced serum response factor activation

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Abstract The Tec family kinases are critical downstream regulators of antigen receptor signals in lymphocytes. As kinases, they act on critical substrates to regulate signals such as calcium increase leading to activation of transcription factors such as NFAT, NFκB and SRF. We now show here that ITK, a member of the Tec family of tyrosine kinases, has a kinase independent function. Mutants of ITK that lack kinase activity or a kinase domain can rescue cells lacking Tec family kinases for antigen receptor induced SRF activation, but not for NFAT, AP-1 or NFκB activation. Furthermore, expression of these mutants in WT cells enhanced SRF activation. This kinase independent function required the SH2 domain since a mutant lacking both the kinase and SH2 domains was much less effective at rescuing SRF activation. This kinase-deleted mutant could partially rescue ERK activation, and interact with multiple tyrosine phosphorylated proteins during antigen receptor signaling, suggesting that ITK uses a scaffolding function that regulates signals leading to specific regulation of SRF activation.

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1. Introduction

The Tec family of non-receptor tyrosine kinases is the second largest family of its kind and is predominantly expressed in cells of the hematopoietic lineage (for review see [1]). This family includes Bruton’s tyrosine kinase (BTK), expressed in B cells and mast cells, and interleukin-2 inducible T cell kinase (ITK) expressed in T cells and mast cells. A large body of work indicates that these kinases are critical for antigen receptor signaling in B and T cells. Humans or mice carrying mutations in BTK develop agammaglobulinemia (humans) or X-linked immunodeficiency (mice) due to defects in B cell maturation, as well as B cell activation [2]. Similarly, mice lacking ITK exhibit defects in T cell maturation, although not as severe as BTK, perhaps due to compensation from the other Tec kinase expressed in T cells, Rlk/Txk, since ITK/Rlk double knockouts exhibit more severe phenotypes [3,4]. More importantly, mice lacking ITK exhibit defects in T cell activation and differentiation, such that naïve T cells from these mice differentiate poorly to either Th1/2 lineages, or both Th11 and Th12 lineages, depending on the background of these mice, accompanied by reduced secretion of IL-2, -5, -13 and to a lesser extent IFN-γ [5–11]. These mice also exhibit reduced responses to infection with lymphocytic choriomeningitis virus, vaccinia virus, vesicular stomatitis virus, and Toxoplasma gondii, and are resistant to developing allergic inflammation in the lung [5,9,12]. Thus signals from ITK are critical for efficient T cell activation and subsequent differentiation.

ITK null T cells have reduced calcium increase following T-cell receptor (TcR) crosslinking, perhaps due to reduced tyrosine phosphorylation of the enzyme PLCγ1 [10]. PLCγ1 activation is required for the generation of inositol(1,4,5)triphosphate (IP<sub>3</sub>) and diacyl glycerol [13]. IP<sub>3</sub> generates calcium increases by binding to IP<sub>3</sub> receptors (IP<sub>R</sub>s) in the endoplasmic reticulum, releasing intracellular stores of calcium. ITK is also required for the subsequent calcium entry via store operated channels that lead to sustained calcium increases in cells stimulated via the TcR [10]. Thus one critical substrate for ITK is PLCγ1, which requires tyrosine phosphorylation for subsequent function. Downstream of PLCγ1, ITK is also required for optimal activation of the transcription factor nuclear factor of activated T cells (NFAT), a consequence of reduced calcium increases in cells lacking ITK [6,8], and for downregulation of T-Bet, another critical transcription factor involved in regulating Th1 cell differentiation [11]. Like other non-receptor tyrosine kinases, ITK has a kinase domain that carries out the phosphorylation of its substrates [1]. In addition, ITK has a Pleckstrin homology (PH) domain that interacts with membrane phosphatidyl-inositol lipids phosphorylated at the D3 position of the inositol ring of these lipids [1,14]. ITK also has a Tec homology (TH) domain that is only found in Tec kinases, which has an N-terminal region of homology with Ras GAP and a proline rich C-terminal region [1]. By regulating membrane localization of ITK, the PH domain of ITK controls its activation, and the proline rich region of the TH domain may also be involved in the regulation of ITK kinase activity [14–16]. SH2 and SH3 domains round
out the structure of ITK. Both of these domains have been shown to interact with other proteins in vitro, including cbl, SLP-76, PLCγ1 and CD28 (for review see [17]), suggesting that ITK may be able to interact with many proteins in vivo, however, the significance of these interactions are unclear.

Antigen receptor stimuli results in the assembly of critical signal complexes that control phosphorylation and the duration of signals, leading to full activation. We show here that ITK has a kinase independent function in regulating antigen receptor activation. Expression of a kinase-deleted form of ITK enhances antigen receptor signals for serum response factor (SRF) activation. Furthermore, this kinase-deleted form of ITK can rescue antigen receptor activation of SRF, but not NFAT, AP-1 or NFκB in cells lacking Tec kinases. These data suggest that the kinase domain of ITK is dispensable for some functions, and that the non-kinase regions of ITK may function as an adaptor to enhance antigen receptor signals under certain conditions.

2. Materials and methods

2.1. Cells, reporter plasmids and reagents

DT40 cells (WT and BTK null and phospholipase Cγ (PLCγ) null, [18]) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% chicken serum. The SRF-, SRE-, NFκB- and AP-1-luciferase reporters were from Stratagene (Stratagene, La Jolla, CA), and contained a luciferase gene driven by tandem copies of the SRF binding element CArg box (GTCCATATTGACG), the c-fos SRE binding element (AGGATGTCCATATTAGGACATCT), NFκB binding element (TGGGGACTTTCGC), or AP-1 binding element (TGACCTGACG). The NFAT-luciferase construct was a gift of Dr. Gary Koretzky (University of Pennsylvania), and the pEBO-ERK, plasmid from Dr. Bruce Mayer (University of Connecticut Health Center). Antibodies to Gst were a kind gift of Drs. K. Sandeep Prabhu and C. Channa Reddy (Penn State University), against phospho-ERK and ERK from Cell Signaling Technologies (Beverly, MA), and against hemagglutinin (HA) from Sigma (St. Louis MO). ITK mutants have been previously described [14,15], except the ITKΔKin/GFP (kinase domain deleted and replaced with EGFP), and the ITKΔSH3ΔKin/GFP (SH3 and kinase domain deleted and replaced with EGFP) which were constructed by PCR. Kinase inactive BTK was a kind gift of Dr. Owen Witte (Howard Hughes Medical institute, University of California, Los Angeles). The MEK inhibitor PD98059 was from Research Biochemicals International (Natick, MA).

2.2. Western blot

Immunoprecipitation, GST pull-downs and Western blots were performed as previously described [19]. Blots were probed with anti-HA, phospho-ERK, -ERK, or -Gst primary antibodies and appropriate secondary antibody conjugated with HRP and detected using the ECL system (Amersham).

2.3. Cell transfection and luciferase activity assay

Cells were transfected with SRF-, NFAT-, AP-1, NFκB-luciferase or pEBO-ERK plasmids using a BTX electro-square-porator T820 (BTX, San Diego, CA). After recovering in growth medium for 8 h, cells were

Fig. 1. Differential requirement for domains of ITK in regulating antigen receptor mediated SRF and NFAT activation. (A) BTK-/- DT40 B cells were transiently transfected with SRF-luciferase reporter plasmid along with EGFP, or with the same vector carrying the indicated mutants of ITK. Cells were then left unstimulated, or stimulated with 4 µg/ml anti-IgM, lysed for luciferase assay, and results expressed as fold increase over non-stimulated cells, which was set at 1. (B) BTK-/- DT40 B cells were transiently transfected with NFAT-luciferase reporter plasmid along with EGFP, or with the same vector carrying the indicated mutants of ITK. Cells were then left unstimulated, or stimulated with 4 µg/ml anti-IgM, lysed for luciferase assay, and results expressed as fold increase over non-stimulated cells, which was set at 1. (C) BTK-/- DT40 cells transfected with the indicated ITK mutants were lysed, proteins separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-HA antibody to detect their expression. (D) WT DT40 B cells were transiently transfected with SRF-luciferase reporter plasmid along with EGFP, or with the same vector carrying the indicated mutants of ITK. Cells were then stimulated as in Fig. 1. *P < 0.05 vs. EGFP vector control.
starved in serum free RPMI 1640 supplemented with 10 mM HEPES pH 7.2 for 12 h and then equally split into different groups which were either stimulated by adding 4 μg/ml anti-chicken IgM, or no stimulation as control for 8 h unless stated otherwise. Cells treated with inhibitors were pretreated for 30 min to an hour prior to stimulation. Cells were then harvested, counted and lysed for luciferase assay using Promega luciferase assay kit, and for checking the expression of ITK mutants or ERK activation by Gst pulldown by Western blot. The results are normalized as fold over non-stimulated control and are the mean ± SD of three to six independent experiments done in triplicate unless otherwise indicated. Statistical significance was determined using Student’s t-test.

3. Results

3.1. Differential requirement for domains of ITK in regulating antigen receptor mediated SRF activation

We have previously shown that B cell antigen receptor signals lead to activation of the transcription factor SRF, which is dependent on Tec family kinase expression [19]. Using the DT40 B cell line lacking Tec family kinases, we have therefore investigated the requirements for the various domains of the Tec family kinase, ITK in regulating SRF activation by the BcR. Using a transient transfection system, we show that WT ITK can rescue BcR induced SRF activation (Fig. 1A). Transfection of various domain deletion mutants of ITK demonstrated a requirement for the PH, SH2 and SH3 domains for rescue of BcR induced SRF activation (Fig. 1A). Similar results were observed for activation of the transcription factor NFAT, which also has a dependence on the expression of Tec family kinases for activation ([Fig. 1B]; [6,19,20]). The expression levels of all the mutants were roughly equivalent (Fig. 1C, data not shown). In all cases, bypassing early BcR signals with PMA and ionomycin could rescue SRF and NFAT activation ([19] and data not shown).

In order to determine whether expression of these domain deletion mutants of ITK affected signaling in the presence of endogenous Tec kinases, we introduced them into WT DT40 cells which only express BTK [18] (Fig. 1D). We found that while the mutant lacking the PH domain or the proline rich region did not significantly affect BcR induced SRF activation, the mutants lacking the SH3 or SH2 domains acted as dominant negatives, significantly inhibiting SRF activation (Fig. 1D). Together, these data indicate that the PH, SH3 and SH2 domains are required for BcR induced activation of SRF and NFAT.

3.2. SRF but not NFAT, AP-1 or NFκB activation by the antigen receptor is independent of the kinase domain of ITK

We then focused our attention on the kinase domain of ITK. In order to ensure that no kinase activity was present, we con-

![Fig. 2. Activation of SRF but not NFAT, is independent of the kinase domain of ITK. (A) BTK−/− DT40 B cells were transiently transfected with SRF-, NFAT-, SRE-, NFκB- or AP-1-luciferase reporter plasmids along with either EGFP or with the same vector carrying the ITKAkin/GFP. Cells were then stimulated as in Fig. 1. (B) WT DT40 B cells were transiently transfected with SRF-, NFAT- or AP-1-luciferase reporter plasmids along with EGFP or with the same vector carrying the ITKAkin/GFP mutant of ITK. Cells were then stimulated as in Fig. 1. (C) BTK−/− DT40 B cells stably expressing the ITKAkin/GFP mutant of ITK were transiently transfected with SRF-, NFAT, AP-1 or NFκB-luciferase reporter plasmids and then stimulated as in Fig. 1. *P < 0.05 vs. EGFP vector control.](#)
structured a mutant of ITK lacking its kinase domain, replaced with GFP. Surprisingly, transfection of this ITK mutant rescued BcR induced SRF activation in Tec kinase null cells (Fig. 2A). This mutant could also rescue BcR induced activation of the SRE, which is dependent on both SRF and TCF transcription factors (Fig. 2A).

To determine if this behavior was specific to SRF, we also tested whether this kinase deletion mutant of ITK could rescue or enhance BcR activation of NFAT, AP-1 or NFkB. We found that the kinase deletion mutant could not rescue BcR induced NFAT, NFkB or AP-1 activation, all of which could be rescued by WT ITK (Fig. 2A). Confirming the effect of this mutant on SRF activation, expression of the kinase deleted ITK mutant in WT DT40 cells lead to a significant enhancement of BcR induced SRF, but not NFAT or AP-1 activation (Fig. 2B). A point mutation in the kinase domain which significantly reduces kinase activity [14], was also able to rescue BcR induced SRF activation in Tec kinase null DT40 cells, and was less efficient, although still able to enhance BcR induced SRF activation in WT DT40 cells (data not shown). Similarly, a mutant of BTK with a point mutation in the kinase domain could rescue SRF activation to a similar level as the kinase deleted ITK mutant (EGFP (control) = 1.2 ± 0.1 fold, kinase deleted ITK = 2.8 ± 0.5 fold and kinase inactive BTK = 2.5 ± 0.2 fold, data not shown). The ability of the ITK kinase domain deleted mutant to rescue antigen receptor induced SRF activation was not due to transient expression. We generated a stable cell line of BTK null DT40 cells carrying the kinase deleted ITK mutant, and analysis of SRF, AP-1, NFkB and NFAT activation in this cell line revealed that the kinase deleted ITK could selectively rescue antigen receptor induced SRF activation (Fig. 2C). Similar results were observed in another independent line (data not shown).

This kinase independent activation of SRF by ITK was dependent on the SH2 domain since removal of this domain from the kinase-deleted mutant dramatically reduced the ability of ITK to rescue SRF activation (Fig. 3A). Similarly, expression of the PH domain of ITK alone could not rescue SRF activation (Fig. 3A). Since PLCγ is a major substrate of Tec kinases and is required for SRF activation, we determined if the ability of the kinase deleted mutant of ITK to activate SRF was dependent on PLCγ. Expression of this mutant in DT40 cells lacking PLCγ demonstrated that this lipase was required for the ability of this mutant to activate SRF in these cells (Fig. 3B). Thus there is a Tec kinase independent pathway that leads to SRF and SRE activation, but not NFAT, AP-1 or NFkB. Furthermore in WT cells, this pathway can enhance the activities of SRF, but not NFAT or AP-1. Since this is kinase domain independent, it suggests that there is perhaps an adaptive function for ITK in regulating pathways that lead to this transcription factor.

### 3.3. Antigen receptor mediated early activation of ERK is independent of the kinase domain of ITK

We have previously shown that activation of SRF by the BcR is critically dependent on activation of the ERK [19]. Activation of ERK is partially dependent on Tec kinases in DT40 cells [21]. To determine if the kinase deleted mutant of ITK could rescue ERK activation, we transfected Tec kinase null cells with a tagged version of ERK along with control GFP or the ITK mutant and examined BcR induced ERK activation. Our analysis indicated that the kinase-deleted form of ITK could partially rescue ERK activation in Tec kinase null DT40 cells, and enhance the activation of ERK in WT DT40 cells (Fig. 4A), with similar results observed in BTK null DT40 cells stably expressing the kinase deleted ITK mutant (Fig. 4B). Indeed, an inhibitor of the MEK-ERK pathway, PD98059 could inhibit the ability of the kinase deleted ITK to rescue antigen receptor induced SRF activity (Fig. 4C). This suggests that the ERK/MAPK pathway is one potential pathway downstream of the B cell antigen receptor that may be regulated in a kinase independent way.

### 3.4. Activation induced association of tyrosine phosphorylated proteins with kinase deleted ITK

In an attempt to understand how an ITK mutant lacking its kinase domain could rescue SRF activation, we determined if this mutant could act as an adaptor. BTK null DT40 cells carrying the kinase deleted ITK mutant were stimulated using pervanadate and the ITK mutant immunoprecipitated and analyzed for the association of tyrosine phosphorylated proteins. We found (Fig. 5) that a number of tyrosine phosphorylated proteins either associated with ITK following antigen receptor stimulation, or alternatively, these proteins are constitutively associated with ITK and become phosphorylated upon...
stimulation. WT ITK similarly tagged with GFP also had the capacity to co-immunoprecipitate with tyrosine phosphorylated proteins (Fig. 5). All together, our data suggests that ITK, and perhaps other Tec kinases, have a kinase independent function leading to the activation of the SRF transcription factor.

4. Discussion

Current understanding of signaling pathways regulated by the Tec family of tyrosine kinases is poor. In addition, whether these pathways have requirements for the different functional domains are unclear. In particular, since the kinase activities usually represent pharmacological targets, whether this domain is required for the activation of specific signaling pathways is of considerably interest. We show here that the Tec family kinase ITK can regulate antigen receptor mediated SRF activation in a kinase independent manner. Thus while ITK mediated activation of the transcription factors NFAT, AP-1 and NFκB are kinase mediated, SRF activation is not. This suggests that ITK, and perhaps other Tec family kinases, may possess adaptor functions that lead to specific signaling pathways.

Precedence for non-receptor kinases using kinase independent activation of signaling pathways exists with the Src family of kinases, where c-Src has a kinase independent function that regulates cell adhesion and migration, which may be related to the interaction of its SH2 and/or SH3 domains with binding partners, as well as function in rescuing specific defects in c-Src null mice [22,23]. More recently, a kinase activity independent function for BTK was reported both in vitro and in vivo. In vitro, a conditional kinase inactive BTK was able to rescue calcium influx in BTK null DT40 cells, which was unlike what had been previously reported for this mutant [18,24]. Our experiments with the kinase deleted ITK also indicates that

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**Fig. 4.** Antigen receptor mediated early activation of ERK/MAPK is independent of the kinase domain of ITK. (A) BTK\(^{-/-}\) DT40 cells were transfected with GST tagged ERK, along with either EGFP (lanes 1–5), the ITK\(^{Kin/GFP}\) mutant of ITK (lanes 6–10) or WT ITK (lanes 11–15). WT DT 40 cells were similarly transfected with GST tagged ERK, along with either the ITK\(^{Kin/GFP}\) mutant of ITK (lanes 16–19) or EGFP (lanes 20–23). Cells were then either not stimulated (lanes 1, 6, 11, 16, 20) or stimulated for the indicated times with anti-IgM (other lanes). Cells were lysed, and tagged ERK immunoprecipitated and activation determined using phospho-specific antibodies and Western blotting (top panels), or anti-Gst to detect ERK (middle and bottom panels. Note that the bottom panel is a shorter exposure of the middle panel). (B) BTK\(^{-/-}\) DT40 cells (lanes 1–5), WT DT40 cells (lanes 6–10), or BTK\(^{-/-}\) DT40 cells stably expressing the ITK\(^{Kin/GFP}\) mutant of ITK (lanes 11–15), were either not stimulated (lanes 1, 6, 11) or stimulated for the indicated times with anti-IgM (other lanes). Cells were lysed, and total cell lysates probed with anti-phospho-ERK specific antibodies and Western blotting (top panels), or anti-ERK to detect ERK (bottom panels). (C) BTK\(^{-/-}\) DT40 B cells stably expressing ITK\(^{Kin/GFP}\) mutant of ITK were transiently transfected with SRF-luciferase reporter plasmid, then stimulated as in Fig. 1 in the presence or absence of ERK pathway inhibitor PD98059. *P < 0.05 vs. anti-IgM without PD98059.
this mutant cannot rescue calcium increase in these cells (data not shown). Other workers have shown that BTK can enhance antigen receptor induced calcium influx in a kinase independent manner [25], while a kinase inactive mutant of BTK can partially rescue B cell development of BTK null B cells in transgenic mice [26]. Similarly, it has been reported that expression of a kinase inactive mutant of ITK does not affect antigen receptor mediated actin rearrangements, and could rescue TCR regulation of Vav and the actin cytoskeleton, supporting a role for a kinase independent function for ITK in regulating actin rearrangements, that may lead to SRF activation [27–29]. Intriguingly, SRF requires actin rearrangements for its activation, both in lymphoid and non-lymphoid cells [19,29], while a role for the actin cytoskeleton in regulating NFAT, AP-1 and NFκB is less understood. Our data also suggest that antigen receptor activation of ERK/MAPK is in part Tec kinase independent, and may explain the preferential activation of SRF over NFAT, AP-1 and NFκB, since we have previously shown that SRF activation is critically dependent on ERK activation [19]. More recently, Tomlinson et al. have shown that Tec can activate NFAT in a partially kinase independent manner, also suggesting a potential adaptor function for Tec [30].

ITK can interact with a number of partners, at least in vitro, via its non-kinase domains, including the βγ subunits of small G proteins and PKC, and perhaps Gα12 via its PH domain [31–33]. Our data suggests that there is a specific requirement for the PH domain of ITK in rescuing antigen receptor induced SRF; since the PH domain deletion mutant cannot rescue, and a membrane targeted mutant lacking the PH domain does not completely rescue SRF activation (data not shown, although it is possible that localization to the proper membrane compartment is required). Thus binding of one of these effectors, or perhaps other PH domain binding effector(s) may be important for this function. Along these lines, it was recently reported that the PH domain of BTK interacts with the lipid kinase PI5 kinase, and that expression of a kinase deleted form of BTK in B cells results in enhanced calcium influx triggered by the B cell antigen receptor [25]. In addition, expression of a kinase inactive BTK in BTK null mice led to rescue of lambda light chain usage, while other defects remained, leading Middendorp et al. to speculate that BTK may act as an adaptor as well [26]. It is possible that ITK also interacts with PI5 kinase via its PH domain, and that any accompanying increase in calcium could lead to the enhanced SRF activation we observed in WT DT40 cells carrying the kinase deleted mutant of ITK. We have tried to determine if this occurs in the DT40 cells, but the available anti-PI5 kinase antibodies do not react with the chicken PI5 kinase. However, the PH domain and any potential interaction partners are not sufficient since the PH domain alone cannot rescue antigen receptor induced SRF activation (data not shown).

It is also possible that the SH2 and SH3 domains of ITK target critical effectors to enhance or activate the pathway(s), including ERK activation, leading to SRF, since the SH2 domain is required for the ability of the mutant to rescue SRF activation. Indeed, we find that ITK interacts with multiple tyrosine phosphorylated proteins following stimulation. ITK can interact with Src homology 2 domain-containing leukocyte protein of 76 kDa in T cells and Slp-65/BLNK in B cells via its SH2 domain [34]. In addition, the SH3 domain of ITK can interact with c-ebl, and this interaction may be able to enhance pathways leading to SRF activation. Alternatively, these domains of ITK may sequester negative regulator(s) of these pathways leading specifically to SRF activation. Of interest is the finding that a point mutant of ITK in the kinase domain of ITK with significantly reduced kinase activity was less effective at rescuing Tec minus cells for SRF activation, or for enhancing SRF and NFAT activation in WT cells. We speculate that the kinase-deleted form of ITK has its other domains available for interacting with effectors, while the kinase mutant does not. This could then result in more efficient coupling of the kinase-deleted form to downstream effectors leading to SRF activation.

In conclusion, our data suggest that common early signals from the antigen receptor can diverge at the level of Tec kinases, with kinase activation leading to NFAT, AP-1 and NFκB activation, and kinase independent function(s), leading to SRF activation. These data illustrate the importance not only of tyrosine kinase activation, and their potential as pharmacological targets, but also caution in that other protein domains may be critical in differentially regulating specific signaling pathways. However, they also open the door to manipulating specific signaling pathways emanating from ITK that differentially lead to transcription factor activation.

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