Published in final edited form as:

Int J Biochem Cell Biol. 2013 June ; 45(6): 1074–1082. doi:10.1016/j.biocel.2013.02.011.

The zinc-binding region of IL-2 inducible T cell kinase (Itk) is required for interaction with Gα13 and activation of Serum Response Factor

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Abstract

Tec family kinases play critical roles in the activation of immune cells. In particular, Itk is important for the activation of T cells via the T cell Receptor (TcR), however, molecules that cooperate with Itk to activate downstream targets remain little explored. Here we show that Itk interacts with the heterotrimeric G-protein α 13 subunit Gα13 during TcR triggering. This interaction requires membrane localization of both partners, and is partially dependent on GDP- and GTP-bound states of Gα13. Furthermore, we find that Itk interacts with Gα13 via the zinc binding regions within its Tec homology domain. The interaction between Itk and Gα13 also results in tyrosine phosphorylation of Gα13, however this is not required for the interaction. Itk enhances Gα13 mediated activation of Serum Response Factor (SRF) transcriptional activity dependent on its ability to interact with Gα13, but its kinase activity is not required to enhance SRF activity. These data reveal a new pathway regulated by Itk in cells, and suggest cross talk between Itk and G-protein signaling downstream of the TcR.

Keywords

Tec kinase; G-protein; Btk homology domain; Serum response factor

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Authors' contributions

W.H. and A.A. designed the research, analyzed data, and wrote the manuscript; W.H., J.L.M., V.P.G. and J.L. conducted experiments; Q.Q. provided critical reagents.

Conflict of interest

The authors declare no conflict of interest.

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1. Introduction

Itk is a member of the Tec family of non-receptor tyrosine kinases and plays a critical role in regulating signals emanating from the TcR. Itk has an N-terminal PH domain, allowing it to interact with PI(3,4,5)P₃ lipids at the plasma membrane, the latter generated by PI3 kinase (August et al., 1997). This PH domain is critical for the function of Itk as membrane interaction is a requirement for its activation by upstream receptors via Src kinases such as Lck (August et al., 1997, Ching et al., 1999, Woods et al., 2001). Itk also has SH3 and SH2 domains allowing it to interact with other proteins in signaling complexes. In addition, and unique to Tec kinases, Itk has a Tec-homology (TH) domain, composed of a Zn²⁺-binding Btk homology (BH) motif and a proline-rich region (PRR). The function of this TH domain is currently unclear. Downstream of the TcR, Itk activates PLC γ 1 and regulates increases in intracellular Ca²⁺, and the absence of Itk preferentially affects influx of extracellular calcium from the outside of the cell (Berg et al., 2005, Liu et al., 1998). Itk also regulates the ERK/MAPK pathway downstream of DAG, released as a consequence of activation of PLC! 1. These pathways place Itk as a regulator of transcription factors such as NFAT, NF-! B and AP-1 downstream of the TcR (Dolmetsch et al., 1997, Dolmetsch et al., 1998, Fowell et al., 1999, Schaeffer et al., 1999, Schaeffer et al., 2001, Miller and Berg, 2002).

The presence of SH2 and SH3 domains allow Itk to interact with upstream and downstream signaling nodes and substrates, including adaptor proteins such as LAT and SLP-76 (Smith-Garvin et al., 2009). The latter interaction is critical for the efficient activation of Itk as well as in TcR signaling. This interaction between Itk and SLP-76 is part of multi-protein complexes that include Arp2/3, Cdc42 and WASP, which are able to regulate the actin cytoskeleton (Andreotti et al., 2010). The ability to regulate the actin cytoskeleton may in part, be kinase independent since kinase inactive mutants of Itk retain the ability to induce changes in the actin cytoskeleton (Dombroski et al., 2005, Grasis et al., 2003). Kinase inactive Itk is also able to activate the actin sensitive transcription factor SRF downstream of antigen receptors (Hao et al., 2006). SRF is regulated by changes in actin cytoskeleton, such that the concentration of G-actin regulates the cytoplasmic location of Myocardin-related transcription factors, which regulate the activity of SRF (Olson and Nordheim, 2010). The heterotrimeric G-protein ! subunit G! 13 also modulates the actin cytoskeleton to regulate SRF via Rho GTPases acting on ROCK and actin regulating proteins (Mao et al., 1998a, Mao et al., 1998b). Itk also regulates the activity of Rho GTPases downstream of the CXCR4 chemokine receptor (Labno et al., 2003).

The ability of Itk to regulate SRF as well as Rho GTPases prompted us to examine the relationship between Itk and G! 13. We show here that Itk interacts directly with G! 13, which is partially dependent on its GTP- or GDP-bound state, and is dependent on membrane localization of both Itk and G! 13. We also show that the Zn²⁺-binding BH motif in the TH domain of Itk is required for the interaction between Itk and G! 13. In addition, we identify mutants of G! 13 that are unable to interact with Itk, and show that these mutants are also unable to regulate SRF activity. We conclude that the ability of Itk to interact with G! 13 is critical in regulating its ability to activate SRF activity.

2. Materials and methods

2.1. Reagents

The pcDNA3.1+/Gα13 construct was purchased from cDNA Resource Center (Missouri University of Science and Technology, Rolla, MO). Primary antibodies include: mouse anti-human CD3ε antibody (OKT3, eBioscience, San Diego, CA), mouse anti-human CD28 (eBioscience), mouse anti-Itk monoclonal (2F12, Cell Signaling Technology, Inc., Danvers, MA), rabbit anti-Itk polyclonal antibodies (a gift from Dr. Gordon Mills, M. D. Anderson Cancer Center (August et al., 1994)), mouse anti-GFP monoclonal (Roche Applied Science, Indianapolis, IN), rabbit anti-GFP polyclonal antibodies (a gift from Dr. Maurine E. Linder, (Seedorf et al., 1999, He and Linder, 2009)), mouse anti-β-Actin monoclonal antibody (AC-74, Sigma-Aldrich, St. Louis, MO), and rabbit anti-human Gα13 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase (HRP) conjugated anti mouse/rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were detected by ECL system (GE Healthcare Biosciences, Pittsburgh, PA). Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were purchased from Sigma.

2.2. Site directed mutagenesis

The pcDNA3.1-YFP1-Itk (Y1-Itk) and pcDNA3.1-Itk-YFP2 (Itk-Y2) were generated as described (Hao et al., 2006). The Gα13-YFP2 construct was made by replacing the Itk sequence in pcDNA3.1-Itk-Y2, with the cDNA sequence of Gα13. Both Itk and Gα13 sequences were of human origin. Mutants were generated by site-directed mutagenesis (Stratagene, Santa Clara, CA).

2.3. Transfection and RNA interference

HEK293T cells were cultured in complete DMEM, supplemented with 10% fetal bovine serum (FBS). Human Jurkat T cells were cultured in complete RPMI 1640, supplemented with 10% FBS. Plasmids were transfected into HEK293T and Jurkat T cells using TransIT-293T and TransIT-Jurkat transfection reagents (Mirus Bio LLC, Madison, WI) respectively. RNA interference to knockdown Itk or Gα13 expression was performed with lentiviral transduction. Briefly, pLKO.1-puro plasmid expressing small hairpin RNA (shRNA) against Itk (TRCN0000010020, Sigma) or Gα13 (TRCN0000036887, Sigma) was co-transfected with pCMV-delta 8.9 and pCMV-VSV-G into HEK293T cells, and lentiviral particles collected from supernatants 48 hours later were used to infect Jurkat T cells. Infected cells were selected by puromycin (Sigma). pLKO.1 plasmid expressing control shRNA (SHC002, Sigma) was used for control. Protein knockdown efficiency was verified by western blotting.

2.4. Imaging and Flow cytometry: Bimolecular Fluorescence Complementation (BiFC)

Transfected HEK293T cells were cultured at 37°C for 24 hours followed by overnight incubation at 30°C to allow fluorochrome maturation of YFP. Live cells on glass bottom 6-well plates or glass cover slips were then directly imaged using a FV1000 (Olympus America, Inc., Center Valley, PA) or a TCS SP5 (Leica Microsystems, Inc., Buffalo Grove, IL) confocal microscope. Stacking images were taken to locate the sub-cellular localization

of the fluorescence, then cells were washed with 2% FBS/PBS (flow buffer), and resuspended in flow buffer followed by flow cytometric analysis on a FC500 (Beckman Coulter, Inc., Brea, CA) or LSRII (BD Biosciences, San Jose, CA) system. YFP fluorescence intensities were determined as previously described (Hao et al., 2006, Qi et al., 2006). Expression of transfected Itk and $\alpha 13$ were confirmed by western blot. Values of YFP intensities were then normalized to that of cells co-transfected with YFP1-WT/Itk and WT/ $\alpha 13$ -YFP2 (positive control), which was set at 1. The change in YFP intensity was used as the primary parameter to determine differences in protein-protein interaction. All combinations of co-transfections were repeated more than 3 times for statistical analysis.

2.5. Co-immunoprecipitation (Co-IP) and Western Blotting

Lysates of transfected HEK293T cells were used to confirm protein expression and phosphotyrosine status. Stimulated Jurkat T cells were used to examine interaction by co-immunoprecipitation. Cells were lysed and used for immunoprecipitation and western blotting as previously described (Iyer et al., 2011).

2.6. Luciferase reporter assay

HEK293T and Jurkat T cells were transfected with plasmids encoding Flag-tagged Tec, HA-tagged Itk and/or pcDNA3.1+/ $\alpha 13$, WT or mutants as indicated, along with SRF-luciferase reporter plasmid (Stratagene, La Jolla, CA), balanced with equivalent quantities of pEGFP plasmid. Following transfection: HEK293T cells were cultured in complete DMEM for 24 hours, starved in serum-free DMEM overnight to reduce background, then stimulated with 20% heat inactivated FBS for 8 hours prior to assay; Jurkat T cells were cultured in complete RPMI for 12 hours, then split and stimulated with PBS, anti-CD3!/anti-CD28 (1! g/ml each), or PMA/Ionomycin (50 ng/ml/0.5 ! M) as a positive control (not shown in data) for 12 hours prior to assay. Luciferase activity assay was performed using luciferase assay kit from Promega (Madison, WI) as previously described (Hao et al., 2006).

2.7. G! 13 Structure rendering

Macromolecular structural data 1ZCB for G! 13 (Hajicek et al., 2011, Kreut et al., 2007), was downloaded from Protein Data Bank, and mutant structures were generated and rendered using MacPyMOL (DeLano, 2002).

2.8. Statistical Analysis

Unpaired two-tailed Student's *t* test was performed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA). Differences with probability $p < 0.05$ were considered statistically significant.

3. Results

3.1. Itk interacts with $\alpha 13$ at the plasma membrane

To determine whether Itk interacts with $\alpha 13$, Jurkat T cells were stimulated with anti-CD3! antibodies and Itk was immunoprecipitated followed by western blotting for $\alpha 13$. We found that Itk transiently interacted with $\alpha 13$ during TcR activation of T cells (Fig. 1A). To more closely examine the interaction between Itk and $\alpha 13$, we used a bi-

fluorescent protein complementation assay as previously described (Qi and August, 2009). We generated YFP1 tagged Itk and YFP2 tagged Gα13 and transfected plasmids encoding these proteins into HEK293T cells. These two proteins were linked to amino acid linkers that would detect interaction within 80Å (Qi and August, 2009) (Fig. 1B). We found that Itk interacted with Gα13 as analyzed by flow cytometry and fluorescence microscopy (Fig. 1D & E, expression of proteins shown in Fig. 1C). There was no fluorescence in control transfections missing Itk and/or Gα13.

In these experiments, we noted that the fluorescence signal indicating interaction was primarily at the membrane of the transfected cells (Fig. 1E). To determine whether membrane localization is important for this interaction, we examined mutants of Itk that could not interact with the plasma membrane (PH domain mutant R29C (Woods et al., 2001)), along with mutants of Gα13 that cannot be palmitoylated and thus are unable to interact with the plasma membrane (C14S and C18S, (Bhattacharyya and Wedegaertner, 2000)). We found that the ability of Itk to interact with the membrane via its PH domain is important for the interaction with Gα13 (Fig. 2A–C). Analogously, the C14S and C18S mutants of Gα13 that cannot interact with the plasma membrane were unable to interact with Itk (Fig. 2B, C). We quantified the fluorescence of the complementation partners, comparing the fluorescence generated by the mutants to the WT proteins, confirming the microscopy analysis. This demonstrated that the interaction is significantly reduced when either Itk or Gα13 is unable to interact with the plasma membrane (Fig. 2B,C). These experiments indicate that Itk interacts with Gα13, and that membrane localization of both proteins is required for the interaction.

3.2. Gα13 interaction with Itk is partially dependent on GDP- and GTP- bound conformation

Gα is normally bound to GDP in its inactive state, and with GTP in its active state (Holinstat et al., 2006). The GTP-bound Gα13 can interact with downstream effectors to regulate downstream signaling (Hart et al., 1998, Meigs et al., 2002, Yamaguchi et al., 2002, Niu et al., 2001, Gong et al., 2010). To determine whether Itk is a downstream mediator of Gα13, we examined whether Gα13 could interact with Itk when locked in the GDP-bound or GTP-bound state. Analysis of HEK293T cells transfected with plasmid encoding YFP1-tagged Itk along with a YFP2-tagged constitutively active mutant of Gα13 that is locked in the GTP-bound state (Q226L, (Masters et al., 1989, Vara Prasad et al., 1994)), revealed interaction with Itk, although reduced compared to WT Gα13 (Fig. 3A, B & E). Similarly, analysis of co-transfectants expressing Itk with a dominant negative mutant of Gα13 that is locked in the GDP-bound state (G225A, (Gohla et al., 1999)), revealed that this mutant was also able to interact with Itk, although at a reduced level compared to WT Gα13. However, the fluorescence resulting from expression of either mutant with Itk was higher than that seen with Gα13 co-expressed with the Itk mutant that cannot interact with the membrane (R29C, PH domain mutant (negative control) (Fig. 3A, B & E). These results suggest that the interaction between Gα13 and Itk may not be regulated by the GTPase activity of Gα13.

3.3. Residues in Gα13 that regulate interaction with Itk

In order to determine if other residues are important for the interaction between Gα13 and Itk, we examined other mutants of Gα13. T²⁰³ has been shown to be critical for the

interaction with and phosphorylation of Gα13 by Protein Kinase A (PKA) (Manganello et al., 2003). We therefore examined whether this residue is also important for the interaction between Gα13 and Itk. We found that changing T²⁰³ to a proline (see proposed structures in Fig. 7) abolished the interaction between Gα13 and Itk (Fig. 3C–E). In our initial experiments, we observed that co-expression of a control GCN4-leucine zipper (LZ)-Linker-YFP2 protein along with YFP1-Itk led to reconstitution of YFP signals, which could be reduced by removing the leucine zipper (see Fig. 1). Sequence analysis of Gα13 revealed two leucines in a spacing pattern resembling the GCN4-LZ motif (Landschulz et al., 1988). To determine whether these leucines in Gα13 were important for the interaction with Itk, these leucines, L²⁹⁵L²⁹⁶, were mutated to alanines and the resulting mutant examined for ability to interact with Itk. We found that the LLAA Gα13 mutant exhibited significantly reduced ability to interact with Itk (Fig. 3C–E). These data indicate that the conformation and/or specific residues within Gα13 are important for its ability to interact with Itk.

3.4. Itk phosphorylates Gα13 on tyrosine, but kinase activity is not required for interaction with Gα13

In our previous experiments, we noticed that expression of Itk led to tyrosine phosphorylation of the co-transfected Gα13 (Fig. 4A). This ability of Itk to phosphorylate Gα13 was abolished in Itk mutants R29C (no longer able to interact with the membrane, and thus unable to interact with Gα13) and K391M (no kinase activity (Hao et al., 2006)) (Fig. 4A, B). This suggested that Itk may phosphorylate Gα13, allowing it to interact with this protein. However, the K391M mutant of Itk retained its ability to interact with Gα13 when co-expressed, as indicated by co-immunoprecipitation (Fig. 4B), and ability to reconstitute YFP at the plasma membrane (Fig. 4C–E), indicating that the ability of Itk to phosphorylate substrates, including Gα13, was not required for its interaction with Gα13.

3.5. The zinc-binding motif within the TH domain of Itk is required for its interaction with Gα13

Since tyrosine phosphorylation of Gα13 is not required for the interaction with Itk, we examined whether other domains of Itk were required for this interaction. We transfected an Itk mutant in the BH module of the TH domain of Itk, C132GC133G (CCGG), which disrupted this domain by preventing its interaction with zinc (Qi and August, 2009), along with WT Gα13 into HEK293T cells. We found that this BH region mutant of Itk could no longer interact with Gα13, with fluorescence complementation values similar to the Itk mutant that can no longer interact with the membrane and thus is unable to interact with Gα13 (Fig. 5). Note that we have shown that this BH mutant of Itk is still able to interact with the membrane, and be activated and phosphorylate membrane receptor targets ((Hao et al., 2006), data not shown).

3.6. Itk regulates the ability of Gα13 to activate SRF transcriptional activity

Gα13 is able to activate the transcription factor SRF (Mao et al., 1998a), and the structurally similar Tec family kinases Tec and Bmx have been shown to enhance SRF in synergy with G! 13 (Mao et al., 1998a). To determine whether Itk regulates the ability of Gα13 to do this, we transfected a SRF-luciferase reporter plasmid, along with Gα13 alone or with mutants of Itk (PH, BH) that abolish its ability to interact with Gα13, or the kinase inactive mutant, and

examined SRF activity. We found that similar to Tec, Itk is able to enhance serum induced SRF transcriptional activity in synergy with G β 13 (Fig. 6A). Abolishing interaction of Itk with the membrane (PH domain mutant) and thus with G α 13, abolished its ability to enhance SRF transcriptional activity. Similarly, the BH mutant of Itk, which abolishes its ability to interact with G α 13, was also unable to enhance SRF transcriptional activity. Furthermore, mutants of G α 13 that are unable to, or only weakly interact with Itk, are also unable to enhance SRF activity (Fig. 6B). Thus G α 13 mutants G225A and Q226L, while able to interact with Itk, have weaker interactions compared to WT G α 13 (as measured by the bi-fluorescence complementation assay, see Fig. 3), are unable to enhance SRF activity. However, the kinase inactive mutant of Itk retained the ability to enhance SRF transcriptional activity in synergy with G β 13. This result suggests that although Itk can phosphorylate G α 13, this is neither required for the interaction, nor for the ability to synergize with G β 13 in enhancing SRF transcriptional activity. More importantly, while the ability of Itk to synergize with G α 13 to activate SRF is kinase independent, it is dependent on their ability to interact. We next examined Itk and G β 13 regulation of SRF activation following TcR stimulation using the human Jurkat T cell line with overexpression or knockdown of Itk and/or G β 13. While expression of either Itk or G β 13 alone did not change SRF transcriptional activity induced though TcR activation, co-expression of Itk and G β 13 significantly enhanced this activity, which was not observed when the zinc binding domain mutant of Itk (CCGG) was used (Fig. 6C). Confirming these results, shRNA mediated knockdown of Itk or G β 13 expression in Jurkat T cells led to a failure to efficiently activate SRF transcriptional activity following TcR stimulation (Fig. 6D). This data indicates that Itk and G β 13 play critical roles in the activation of SRF downstream of the TcR in human T cells.

4. DISCUSSION

Understanding the intersection between components of signaling pathways provides an opportunity to better determine how they may be manipulated to control cell function. We have shown here that the Tec kinase Itk directly interacts with the heterotrimeric G protein β 13 subunit G β 13, and that this interaction occurs at the plasma membrane. Plasma membrane localization of both Itk and G β 13 are critical for their function and for their interaction, and this interaction is not dependent on the GTPase activity of G β 13, although nucleotide-binding mutants reduce the interaction. Although the Itk and G β 13 interaction induces tyrosine phosphorylation of G β 13, Itk kinase activity is not required for the interaction. We find that this interaction is however, dependent on the zinc-binding region of Itk, providing a function for this region of the Tec homology domain of Itk. Finally, we show that the interaction between Itk and G α 13 leads to synergistic activation of SRF in an Itk kinase independent fashion.

The TH domain of Tec kinases includes two identifiable motifs, a Btk Homology (BH) domain which includes a zinc binding region, and a proline rich region (Smith et al., 2001). The functions of these regions remains unclear, but the proline rich region is suggested to be involved in inter- and intra-molecular interactions either with other Itk molecules, or other proteins involved in Tec kinase signaling (Andreotti et al., 2010). We have recently shown that the same zinc binding region of Itk is required to maintain a “closed” or inactive

structure of Itk (Qi and August, 2009), suggesting that this region is used to regulate Itk activity as well as interaction with other proteins. It is likely that by binding to Gα13 via this zinc-binding region, Itk is activated. Indeed, Ma and colleagues have reported that G12 as well as G1q can interact with and activate the related Tec kinase Btk (Jiang et al., 1998, Ma and Huang, 1998). G1q can also activate SRF and it is likely that Btk can enhance SRF activity driven by G1q. Interestingly, they found that Btk interacts with these G1 proteins via its PH and TH domain, the same zinc-containing region (Jiang et al., 1998), suggesting that Itk may behave similarly to Btk in this regard. These data also suggest that perhaps Gα13 lies upstream of Itk, and that receptors which activate Gα13 may be able to activate Itk.

Of note is that Tec and Bmx, the related Tec kinases, can also activate SRF and functionally interact with G12/13 (Mao et al., 1998a). However, while the TH domain was demonstrated to be important for the ability of Tec and Bmx to activate SRF, these investigators did not examine whether these kinases interacted with G12/13, and which subdomains of the TH domain was critical. They did argue that G12/13 is an upstream activator of Bmx (i.e. co-expression led to increased tyrosine phosphorylation of Bmx), as well as a downstream mediator of Bmx (i.e. mutant forms of Bmx, including kinase deleted forms, can block SRF activator downstream of G12/13) (Mao et al., 1998a). However, our results suggest that while Itk may act in synergy with Gα13 in activating SRF transcriptional activity, kinase activity was not required for this event, unlike the case for Bmx, since the kinase inactive mutant of Itk can still cooperate with Gα13 to enhance SRF activity. By contrast, mutants of Itk that do not interact with Gα13, do not cooperate with Gα13 to enhance SRF activity. Mao and colleagues also found that Bmx did not require its PH domain to act in concert with Gα13, while we find that Itk requires its PH domain in order to interact with and cooperate with Gα13. Perhaps these differences are due to the fact that Bmx is the most atypical of the Tec kinases (Smith et al., 2001). However, the fact that the TH domain was important for the ability of Tec and Bmx to enhance SRF activation by Gα13 suggests that the zinc-binding region might be critical for these interactions with Gα13.

Our data indicates that although Itk and Gα13 interact, and Gα13 is tyrosine phosphorylated by Itk, phosphorylation is not required for Gα13 mediated activation of SRF since the kinase inactive form of Itk that cannot phosphorylate Gα13 can still cooperate with Gα13 to activate SRF. It remains to be determined whether tyrosine phosphorylation of Gα13 by Itk alters other functions of this protein. Our attempts to date to map the tyrosine-phosphorylated residue(s) have been unsuccessful, as each single mutation of tyrosine to phenylalanine failed to abolish G13 phosphorylation by WT Itk (data not shown), suggesting that Itk may phosphorylate multiple tyrosine residues on G13.

Gα13 interacts with a number of effectors, including upstream activators, as well as downstream effectors (Hart et al., 1998, Meigs et al., 2001, Yamaguchi et al., 2002, Niu et al., 2001, Gong et al., 2010). Our data indicates that Gα13 interacts with Itk independent of the ability of Gα13 to bind to GDP or GTP, however this is probably weaker than with WT Gα13. This is unlike the case with other effector proteins that interact with Gα13, generally in its activated GTP-bound state. However, there is likely to be a threshold for the strength

of the interaction between the two proteins that lead to synergistic SRF activation since on its own, the GTP-locked Gα13 (the Q226L mutant) is still able to enhance SRF activity as expected, but is unable to synergize with Itk to further activate SRF. In addition, our analysis of residues that affect the interaction between Gα13 and Itk suggest that conformational changes may be important since these sensitive residues in Gα13 are not found on the surface of the protein as predicted by the crystal structure (Fig. 7). Mutation of T²⁰³ in Gα13 was previously shown to disrupt its functional interaction with PKA (Manganello et al., 2003). Although a role for the dileucine motif in Gα13 has not been previously described, we show here that this motif is important for the ability of Gα13 to interact with Itk. This motif may act in a similar fashion to what has been described for other interactions using such motifs (e.g. the dileucine in GCN4) (Kouzarides and Ziff, 1989). Alternatively the loss of this dileucine motif may disrupt the structure of Gα13 such that it can no longer interact with Itk, but there may not be direct interaction with the dileucine motifs in Gα13.

In conclusion, our work supports the idea of important crosstalk between the Tec kinase Itk and the heterotrimeric G-protein subunit Gα13. This opens up new pathways for analysis of signaling downstream of this kinase and its function in T cells.

Acknowledgments

We thank Nicole Bem and Susan Magargee at Penn State and Rod Getchell at Cornell for technical support with flow cytometry and confocal microscopy, and Victor Tse at Cornell for help with luciferase activity assay. We also thank Dr. Maurine E. Linder in Department of Molecular Medicine at Cornell University for critical comments on the manuscript and members in August lab for comments and feedback. This work was supported by grant from the National Institutes of Health (AI051626 & AI065566 to A.A.).

Abbreviations

G-protein	guanine nucleotide binding protein
GFP	Green Fluorescent Protein
Itk	Inducible T cell Kinase
PH	Pleckstrin Homology domain
PRR	Proline Rich Region
TcR	T cell Receptor
TH	Tec Homology domain
SH2	Src Homology domain 2
SH3	Src Homology domain 3
YFP	Yellow Fluorescent Protein

References

- Andreotti A, Schwartzberg P, Joseph R, Berg L. T-cell signaling regulated by the Tec family kinase, Itk. *Cold Spring Harb Perspect Biol.* 2010; 2:a002287. Epub 002010 Jun 002282. [PubMed: 20519342]

- August A, Gibson S, Kawakami Y, Kawakami T, Mills GB, Dupont B. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc Nat Acad Sci (USA)*. 1994; 91:9347–9351. [PubMed: 7524075]
- August A, Sadra A, Dupont B, Hanafusa H. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc Nat Acad Sci (USA)*. 1997; 94:11227–11232. [PubMed: 9326591]
- Berg L, Finkelstein L, Lucas J, Schwartzberg P. Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol*. 2005; 23:549–600. [PubMed: 15771581]
- Bhattacharyya R, Wedegaertner PB. Galpha 13 requires palmitoylation for plasma membrane localization, Rho-dependent signaling, and promotion of p115-RhoGEF membrane binding. *J Biol Chem*. 2000; 275:14992–14999. [PubMed: 10747909]
- Ching K, Kawakami Y, Kawakami T, Tsoukas C. Emt/Itk associates with activated TCR complexes: role of the pleckstrin homology domain. *J Immunol*. 1999; 163:6006–6013. [PubMed: 10570288]
- DeLano, WL. The PyMOL Molecular Graphics System on World Wide Web. 2002. <http://www.pymol.org>
- Dolmetsch R, Lewis R, Goodnow C, Healy J. Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature*. 1997; 386:855–858. [PubMed: 9126747]
- Dolmetsch R, Xu K, Lewis R. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*. 1998; 392:933–936. [PubMed: 9582075]
- Dombroski D, Houghtling RA, Labno CM, Precht P, Takesono A, Caplen NJ, Billadeau DD, Wange RL, Burkhardt JK, Schwartzberg PL. Kinase-independent functions for Itk in TCR-induced regulation of Vav and the actin cytoskeleton. *J Immunol*. 2005; 174:1385–1392. [PubMed: 15661896]
- Fowell D, Shinkai K, Liao X, Beebe A, Coffman R, Littman D, Locksley R. Impaired NFATc Translocation and Failure of Th2 Development in Itk Deficient CD4 T Cells. *Immunity*. 1999; 11:399–409. [PubMed: 10549622]
- Gohla A, Offermanns S, Wilkie TM, Schultz G. Differential involvement of Galpha12 and Galpha13 in receptor-mediated stress fiber formation. *The J Biol Chem*. 1999; 274:17901–17907.
- Gong H, Shen B, Flevaris P, Chow C, Lam SC, Voyno-Yasenetskaya TA, Kozasa T, Du X. G protein subunit Galpha13 binds to integrin alphaIIb beta3 and mediates integrin “outside-in” signaling. *Science*. 2010; 327:340–343. [PubMed: 20075254]
- Grasis J, Browne C, Tsoukas C. Inducible T cell tyrosine kinase regulates actin-dependent cytoskeletal events induced by the T cell antigen receptor. *J Immunol*. 2003; 170:3971–3976. [PubMed: 12682224]
- Hajicek N, Kukimoto-Niino M, Mishima-Tsumagari C, Chow CR, Shirouzu M, Terada T, Patel M, Yokoyama S, Kozasa T. Identification of critical residues in G(alpha)13 for stimulation of p115RhoGEF activity and the structure of the G(alpha)13-p115RhoGEF regulator of G protein signaling homology (RH) domain complex. *J Biol Chem*. 2011; 286:20625–20636. [PubMed: 21507947]
- Hao S, Qi Q, Hu J, August A. A kinase independent function for Tec kinase ITK in regulating antigen receptor induced serum response factor activation. *FEBS Lett*. 2006; 580:2691–2697. [PubMed: 16631752]
- Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G. Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science*. 1998; 280:2112–2114. [PubMed: 9641916]
- He Y, Linder ME. Differential palmitoylation of the endosomal SNAREs syntaxin 7 and syntaxin 8. *J Lipid Res*. 2009; 50:398–404. [PubMed: 18980942]
- Holinstat M, Oldham WM, Hamm HE. G-protein-coupled receptors: evolving views on physiological signalling: symposium on G-protein-coupled receptors: evolving concepts and new techniques. *EMBO reports*. 2006; 7:866–869. [PubMed: 16906127]
- Iyer A, Morales J, Huang W, Ojo F, Ning G, Wills E, Baines J, AA. Absence of Tec family kinases interleukin-2 inducible T cell kinase (Itk) and Bruton’s tyrosine kinase (Btk) severely impairs Fc

- epsilonRI-dependent mast cell responses. *J Biol Chem.* 2011; 286:9503–9513. Epub 2011 Jan 9506. [PubMed: 21212279]
- Jiang Y, Ma W, Wan Y, Kozasa T, Hattori S, Huang XY. The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. *Nature.* 1998; 395:808–813. [PubMed: 9796816]
- Kouzarides T, Ziff E. Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. *Nature.* 1989; 340:568–571. [PubMed: 2505081]
- Kreut B, Hajicek N, Yau D, Nakamura S, Kozasa T. Distinct regions of Galpha13 participate in its regulatory interactions with RGS homology domain-containing RhoGEFs. *Cell Signal.* 2007; 19:1681–1689. [PubMed: 17449226]
- Labno C, Lewis C, You D, Leung D, Takesono A, Kamberos N, Seth A, Finkelstein L, Rosen M, Schwartzberg P, Burkhardt J. Itk functions to control actin polymerization at the immune synapse through localized activation of Cdc42 and WASP. *Curr Biol.* 2003; 13:1619–1624. [PubMed: 13678593]
- Landschulz WH, Johnson PF, McKnight SL. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science.* 1988; 240:1759–1764. [PubMed: 3289117]
- Liu K, Bunnell S, Gurniak C, Berg L. T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *J Exp Med.* 1998; 187:1721–1727. [PubMed: 9584150]
- Ma YC, Huang XY. Identification of the binding site for Gqalpha on its effector Bruton's tyrosine kinase. *Proc Nat Acad Sci (USA).* 1998; 95:12197–12201. [PubMed: 9770463]
- Manganello JM, Huang JS, Kozasa T, Voyno-Yasenetskaya TA, Le Breton GC. Protein kinase A-mediated phosphorylation of the Galpha13 switch I region alters the Galphabeta gamma13-G protein-coupled receptor complex and inhibits Rho activation. *J Biol Chem.* 2003; 278:124–130. [PubMed: 12399457]
- Mao J, Xie W, Yuan H, Simon MI, Mano H, Wu D. Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Galpha12/13. *EMBO J.* 1998a; 17:5638–5646. [PubMed: 9755164]
- Mao J, Yuan H, Xie W, Simon M, Wu D. Specific involvement of G proteins in regulation of serum response factor-mediated gene transcription by different receptors. *J Biol Chem.* 1998b; 273:27118–27123. [PubMed: 9765229]
- Masters SB, Miller RT, Chi MH, Chang FH, Beiderman B, Lopez NG, Bourne HR. Mutations in the GTP-binding site of GS alpha alter stimulation of adenylyl cyclase. *J Biol Chem.* 1989; 264:15467–15474. [PubMed: 2549064]
- Meigs TE, Fedor-Chaiken M, Kaplan DD, Brackenbury R, Casey PJ. Galpha12 and Galpha13 negatively regulate the adhesive functions of cadherin. *J Biol Chem.* 2002; 277:24594–24600. [PubMed: 11976333]
- Meigs TE, Fields TA, McKee DD, Casey PJ. Interaction of Galpha 12 and Galpha 13 with the cytoplasmic domain of cadherin provides a mechanism for beta -catenin release. *Proc Nat Acad Sci (USA).* 2001; 98:519–524. [PubMed: 11136230]
- Miller AT, Berg LJ. Defective Fas Ligand Expression and Activation-Induced Cell Death in the Absence of IL-2-Inducible T Cell Kinase. *J Immunol.* 2002; 168:2163–2172. [PubMed: 11859102]
- Niu J, Vaiskunaite R, Suzuki N, Kozasa T, Carr DW, Dulin N, Voyno-Yasenetskaya TA. Interaction of heterotrimeric G13 protein with an A-kinase-anchoring protein 110 (AKAP110) mediates cAMP-independent PKA activation. *Curr Biol.* 2001; 11:1686–1690. [PubMed: 11696326]
- Olson E, Nordheim A. Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol.* 2010; 11:353–365. [PubMed: 20414257]
- Qi Q, August A. The Tec family kinase Itk exists as a folded monomer in vivo. *J Biol Chem.* 2009; 284:29882–29892. [PubMed: 19717557]
- Qi Q, Sahu N, August A. Tec kinase Itk forms membrane clusters specifically in the vicinity of recruiting receptors. *J Biol Chem.* 2006; 281:38529–38534. [PubMed: 17060314]
- Schaeffer E, Debnath J, Yap G, McVicar D, Liao X, Littman D, Sher A, Varmus H, Lenardo M, Schwartzberg P. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. *Science.* 1999; 284:638–641. [PubMed: 10213685]

- Schaeffer E, Yap G, Lewis C, Czar M, McVicar D, Cheever A, Sher A, Schwartzberg P. Mutation of Tec family kinases alters T helper cell differentiation. *Nat Immunol.* 2001; 2:1183–1188. [PubMed: 11702066]
- Seedorf M, Damelin M, Kahana J, Taura T, Silver PA. Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase. *Mol Cell Biol.* 1999; 19:1547–1557. [PubMed: 9891088]
- Smith CI, Islam TC, Mattsson PT, Mohamed AJ, Nore BF, Vihinen M. The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. *Bioessays.* 2001; 23:436–446. [PubMed: 11340625]
- Smith-Garvin J, Koretzky G, Jordan M. T cell activation. *Annu Rev Immunol.* 2009; 27:591–619. [PubMed: 19132916]
- Vara Prasad M, Shore SK, Dhanasekaran N. Activated mutant of G alpha 13 induces Egr-1, c-fos, and transformation in NIH 3T3 cells. *Oncogene.* 1994; 9:2425–2429. [PubMed: 8036026]
- Woods ML, Kivens WJ, Adelman MA, Qiu Y, August A, Shimizu Y. A novel function for the Tec family tyrosine kinase Itk in activation of beta 1 integrins by the T-cell receptor. *EMBO J.* 2001; 20:1232–1244. [PubMed: 11250890]
- Yamaguchi Y, Katoh H, Mori K, Negishi M. Galpha(12) and Galpha(13) interact with Ser/Thr protein phosphatase type 5 and stimulate its phosphatase activity. *Curr Biol.* 2002; 12:1353–1358. [PubMed: 12176367]

Fig 1. Itk interacts with Gα13

(A) Jurkat T cells were stimulated with anti-CD3ε antibody for indicated time. Cells were lysed and subjected to immunoprecipitation (IP) with anti-Itk antibody prior to SDS-PAGE. Immunoprecipitations and total cell lysate were probed with anti-Itk (top) or anti-Gα13 antibody (bottom). Data represents two independent experiments. (B) Schematic of bimolecular fluorescence complementation approach. (C-E) HEK293T cells were transfected with YFP1-Linker and Linker-YFP2, YFP1-Linker-Itk (abbreviated YFP1-Itk) and Linker-YFP2, Gα13-Linker-YFP2 (abbreviated Gα13-YFP2) and YFP1-Linker, or

YFP1-Itk and Ga13-YFP2. Cells were analyzed by western blot for expression of proteins on the same blot (C). Complemented YFP fluorescence was analyzed by flow cytometry (D) and confocal imaging (E). Flow cytometry plots and fluorescent microscopy images are representative of two independent experiments. Bar indicates 50 ! m.

Fig 2. Membrane localization of both Itk and Gα13 is required for interaction

HEK293T cells were transfected as follows: YFP1-Itk + Gα13-YFP2, YFP1-R29C/Itk + Gα13-YFP2, YFP1-Itk + C14S/Gα13-YFP2 and YFP1-Itk + C18S/Gα13-YFP2. (A) Total cell lysates were probed to confirm Itk (top panel) and Gα13 (bottom panel) expression. (B) YFP fluorescence ratios from flow cytometric analysis normalized to that of YFP1-WT/Itk + WT/Gα13-YFP2 transfected live cells. **p* < 0.05, compared to Y1-Itk + Gα13-Y2 group. NS = Not Significant across the connected groups. n = 3. (C) Fluorescent confocal images of

live HEK293T cells transfected as indicated. Fluorescent microscopy images are representative of at least three independent experiments. Bar indicates 50 ! m.

Fig 3. Interaction between Gα13 and Itk is partially dependent on nucleotide-bound conformation, but requires residues T203 and L295L296 within Gα13

(A–B) HEK293T cells were transfected as follows: YFP1-Itk + Gα13-YFP2, YFP1-R29C/Itk + Gα13-YFP2, YFP1-Itk + G225A/Gα13-YFP2 and YFP1-Itk + Q226L/Gα13-YFP2. (A) Total cell lysates were probed for Itk or Gα13 on the same blot. (B) YFP fluorescence ratios from flow cytometric analysis normalized to that of YFP1-WT/Itk + WT/Gα13-YFP2 transfected live cells. **p* < 0.05, compared to Y1-Itk + Gα13-Y2 group; ***p* < 0.05 between groups connected. n = 3. (C–D) HEK293T cells were transfected as follows: YFP1-Itk + Gα13-YFP2, YFP1-R29C/Itk + Gα13-YFP2, YFP1-Itk + T203P/Gα13-YFP2 and YFP1-Itk + LLAA/Gα13-YFP2. (C) Total cell lysates were probed for Itk or Gα13 (on the same blot). (D) YFP fluorescence ratios from flow cytometric analysis of cells transfected as in (C). **p* < 0.05, compared to Y1-Itk + Gα13-Y2 group; NS = Not Significant between groups connected. n = 3. (E) Fluorescent confocal images of live

HEK293T cells transfected as indicated. Fluorescent microscopy images are representative of at least three independent experiments. Bar indicates 50 μ m.

Fig 4. Itk phosphorylates Gα13, but kinase activity of Itk is not required for interaction
(A) HEK293T cells were transfected as indicated and whole cell lysates analyzed for tyrosine phosphorylation, expression of Itk, Gα13 and β-actin (all on the same blot, representative of more than three independent experiments). **(B)** HEK293T cells were transfected as indicated and Gα13 was immunoprecipitated to analyze tyrosine phosphorylation, the presence of Itk and Gα13 (all on the same blot, representative of two independent experiments). **(C)** Whole cell lysates from HEK293T cells transfected as indicated were examined for protein expression for Gα13 and Itk (all on the same blot). **(D)**

YFP fluorescence ratios from flow cytometric analysis of cells from experiment in (C) normalized to that of YFP1-WT/Itk + WT/Gα13-YFP2 transfected live cells. * $p < 0.05$, compared to YFP1-Itk + Gα13-YFP2 group; ** $p < 0.05$ between groups connected. NS = Not Significant compared to YFP1-Itk + Gα13-YFP2 group. $n = 3$. (E) Fluorescent confocal images of live HEK293T cells transfected as indicated. Fluorescent microscopy images are representative of at least three independent experiments. Bar indicates 20 μm.

Fig 5. The zinc-binding motif within the BH region of the Tec Homology domain of Itk is required for interacting with Gα13

(A) HEK293T cells were transfected as indicated, and cell lysates probed for Itk and Gα13

(all on the same blot). (B) YFP fluorescence ratios was determined by flow cytometry and

normalized to that of YFP1-WT/Itk + WT/Gα13-YFP2 transfected live cells. **p* < 0.05,

compared to Y1-Itk + Gα13-Y2 group. NS = Not Significant between groups connected. n

3. (C) Fluorescent confocal images of live HEK293T cells transfected as indicated.

Fluorescent microscopy images are representative of at least three independent experiments.

Bar indicates 50 μm.

Fig 6. Itk synergistically enhances Gα13-induced SRF activation

(A–B) HEK293T cells were transfected with SRF-luciferase reporter plasmid and indicated plasmids. Cells were serum starved for overnight, then stimulated with 20% FBS in DMEM for 8 hours prior to luciferase activity assay. Values were normalized to the level in the “pEGFP only” transfected group, and shown as fold induction. (A) WT Gα13 transfected with the indicated Itk mutants, (B) WT Itk transfected with the indicated Gα13 mutants. (C–D) Jurkat T cells were either transfected with the indicated plasmids, or infected with lentivirus expressing indicated shRNA, along with transfection of SRF-luciferase reporter plasmid, followed by stimulation as indicated for 12 hours. Values were normalized to the “pEGFP only” PBS group in (C) or “control shRNA” PBS treated group in (D). (C) Jurkat T cells overexpressing Itk and/or Gα13 (WT or mutant as indicated). (D) Knock down of Itk or Gα13 expression in Jurkat T cells. * $p < 0.05$, compared to Gα13 group or between groups connected; ** $p < 0.05$, compared to Gα13 + Itk group. $n = 3$.

Fig 7. Structure of G! 13 and location of residues required for interaction with Itk
 (A) Model of WT G! 13 structure with amino acids T²⁰³ and L²⁹⁵L²⁹⁶ indicated in red. (B) Model indicating the positions of the T203P and L295A/L296A mutants in the G! 13 structure.