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IDENTIFICATION AND CHARACTERIZATION OF INTERACTIONS BETWEEN ITK AND Gα13

A Thesis in

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by

Weishan Huang

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The thesis of Weishan Huang was reviewed and approved* by the following:

Avery August
Distinguished Professor of Immunology
Thesis adviser

Zhi-Chun Lai
Professor of Biology, Biochemistry and Molecular Biology
Chair, Intercollege Graduate Degree Program in Cell and Developmental Biology

Pamela A. Hankey
Professor of Immunology

* Signatures are on file in the Graduate School.

ABSTRACT

The Tec family kinase, Itk has been shown to be critical in T cell activation and development downstream of the T cell receptor (TCR). Other than TCR ligands, migratory responses to chemokines such as CCL11/Eotaxin and CXCL12/SDF-1α that act through G protein coupled receptors (GPCRs), require Itk. However, the mechanism by which chemokine receptor signals connect to Itk is not clear.

We hypothesize that the G-protein Gα13 may serve to couple GPCRs, Itk and downstream signals. Using a Bimolecular Fluorescence Complementation system, we show that Itk and Gα13 are in close physical proximity, less than 80Å.

We extended the investigation to test the properties of Itk and Gα13 that are important for this interaction. The interaction requires the membrane localization of both parties, as a membrane targeting deficient mutant of Itk (R29C), lost its association with WT Gα13, and a membrane targeting deficient mutant of Gα13 (C14S, C18S) lost its interaction with WT Itk. However, this interaction is Itk kinase activity independent (shown by Itk K391M mutant). We also show that both GTP bound and GDP bound forms of Gα13 can interact with Itk. Interestingly, we found that mutations in the Zn^{2+} binding Bruton's tyrosine kinase (Btk) homology (BH) motif (C132GC133G, CCGG for further reference), disrupted the interaction between Itk and Gα13, even though this Itk mutant was still able to get recruited by WT Itk and form membrane associated clusters. The striking finding is that co-expression of Itk and Gα13 resulted in tyrosine phosphorylation of Gα13. Moreover, we found that the interaction between Itk and Gα13 is transient upon stimulation against TCR signaling axis.

Taken together, we propose that interactions between Gα13 and Itk may connect chemokine/chemokine receptor stimulation and downstream responses regulated by Itk. Itk may also modulate the function of Gα13 by tyrosine phosphorylation. These data provide evidence for better understanding of the mechanism by which chemokines regulate immune cell responses.

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CHAPTER 1

INTRODUCTION

1.1 Role of Itk in signal transduction upon T cell receptor activation

IL-2 inducible T cell kinase (Itk) belongs to the Tec-family of non-receptor protein tyrosine kinase, and it contains an N-terminal pleckstrin homology (PH) domain, a Tec homology (TH) domain (containing a Zn^{2+}-binding BH [Bruton's tyrosine kinase (Btk) homology] motif, and a proline-rich region (PRR)), a Src homology 3 (SH3) domain, a SH2 domain, and a C-terminal kinase domain (Fig. 1.1).

Fig. 1.1: Scheme of Itk structure

Itk is composed of five main domains: from N-terminus to C-terminus, the PH, TH (further divided into BH and PRR), SH3, SH2, and Kinase domains.

Tec family kinases, including Itk, have been shown to be involved in various membrane receptor signaling transductions, such as T cell receptor, B cell receptor, FcR, c-Kit, CD28, CD2, CD32, and the erythropoietin receptor (August et al., 2002; August et al., 1994; King et al., 1996; Oda et al., 2000; Smith et al., 2001; Yamashita et al., 1998). In particular, Itk is essential in the signaling regulatory network for T cell activation and function during immune responses, acting downstream of T cell receptor (TCR) signaling, and controls increased intracellular calcium concentration ($[Ca^{2+}]_i$). This calcium signaling further remodels the cellular profile, either by new gene expression, such as cell type-specific transcriptional factor induction, or cell structure change, such as those based on actin remodeling (August et al., 2002; Qi

and August, 2007). The interaction between the TCR and antigen, leading to Itk activation, which could be kinase dependent or independent, would be reflected by molecular and cellular responsiveness, which are observable and detectable through experimental methods. Itk knockout mice have been generated and their phenotypes are being characterized.

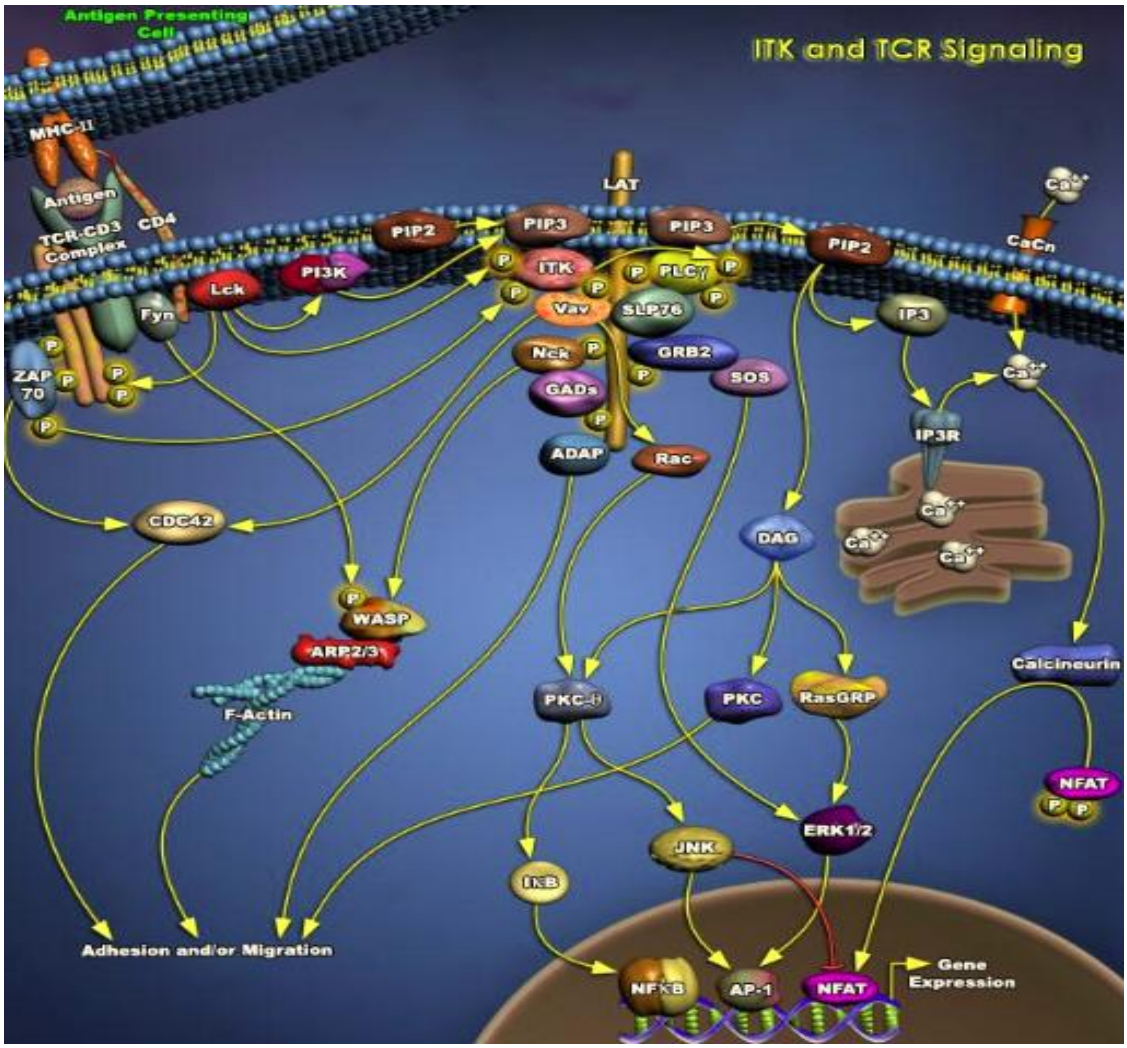


Fig. 1.2: Overview of TCR-Itk signaling.

Upon stimulation of the TCR, Itk is recruited and activated on the membrane, leading to downstream signal transduction, and information flow for cell differentiation and function (http://gspd.biocompare.com/Gene/Pathway.aspx?gp=193).

The signaling network regulated by Itk, downstream of the TCR, regulates T cell development and differentiation is shown in Fig. 1.2.

In this TCR signaling axis, Itk interacts with upstream kinases Src-family tyrosine kinases, predominantly Lck and Fyn (Liao et al., 1997), products of the lipid kinase phosphoinositide 3-kinase (PI3K) and the Syk-family tyrosine kinase member ζ chain-associated protein kinase of 70kD (ZAP70), in order to be activated (Perez-Villar et al., 2002). The whole activation process for Itk, includes translocation of Itk, and interaction between Itk and its activators. Itk interacts with SLP-76, an adaptor protein, which helps Itk maintain a stable localization, hence a close proximity to the activator in order to get activated (Wange, 2000). Activation of PI3 kinase, as well as Src kinase are required for Itk to get fully active (Gibson et al., 1996; Lu et al., 1998; Mano et al., 1996; Yang et al., 2001).

Previous studies showed that the PH domain of Itk allows it to be recruited to the plasma membrane by an activated PI3 kinase (Smith et al., 2001). The Arg29Cys (R29C) mutant within the PH domain, has been shown to impair membrane localization as well as the membrane clustering of Itk (Qi et al., 2006). The BH motif of Tec family kinases is conserved, with Cys involved as Zn^{2+} binding site, the zinc finger motif. Cys-155 is one of the Zn^{2+} ligands and mutation of this amino acid disrupted the BH motif in Btk (Hyvonen and Saraste, 1997; Okoh and Vihinen, 1999). In Itk, Cys-132 and Cys-133 are two corresponding conserved amino acids in the BH motif. An Itk mutant carrying the C132GC133G motif is no longer folded in an intramolecular head to tail fold in the cytosol and has enhanced activity (Qi and

August, 2009). These critical mutants are used for the investigation of the interaction between Itk and Gα13 (Table 1.1).

Table 1.1: Summary of critical Itk mutants used in this thesis.

Domain	Mutant	Functional Defect
PH	R29C	Membrane localization↓, Kinase ↓
BH	CCGG	Zinc Binding ↓, Kinase↑
Kinase	K391M	Kinase Dead, Kinase ↓

1.2 The role of Gα13 in GPCR signaling

Heterotrimeric G-proteins mediate numerous G-protein couple receptor signal transduction processes. Three subunits, α, β and γ, form the G protein complex. Gα proteins are divided into 4 major families: G_s, G_i, G_q and G_{12}. Two members of the G_{12} family, Gα12 and Gα13 (Strathmann and Simon, 1990), are involved in a broad range of signaling networks (Fig. 1.3, cited from Applied Biosystems). To date, Despite the technical limitations, in the last decade information about the G_{12} family is gradually being revealed, although at a slower pace compared to the other families. Gα13 is highly conserved among many vertebrate species (Wilkie and Yokoyama, 1994). Even in *D. melanogaster* , there is a counterpart playing similar function as Gα12/Gα13 , regulating cell morphology and migration (Parks and Wieschaus, 1991). In mammals, Gα12/Gα13 directly regulate a series of Rho guanine nucleotide exchange factors (RhoGEFs),. PDZ-RhoGEF and leukaemia-associated RhoGEF (LARG) are found to be the targets of both Gα12 and Gα13, while p115-GEF (also

called Lsc, lymphoid blast crisis-like-2) is exclusively used by Gα13 (Fukuhara et al., 2001; Hart et al., 1998; Kozasa et al., 1998). These RhoGEFs mediate small GTPase RhoA activation regulated by Gα12/Gα13 (Buhl et al., 1995). Interestingly, this subfamily of GEFs interacts with the active form of Gα through its regulator of G-protein signaling (RGS) domain, which is in a highly conserved pattern among all members.

Along with RhoGEF mediated RhoA activation, Gα12/Gα13 also interferes with other cell signals, through direct or indirect interactions with its partners. In many cases, the G_{12} family works with other G protein families to secure regulatory control, for example, Gα12 and Gα13 interact with protein phosphatase type 5, which leads to the inhibition of G_i (Yamaguchi et al., 2002). Gα12/Gα13 binding to the cytoplasmic motif of class I and II classic cadherin leads to the release of β-catenin, and inhibits the adhesive function of cadherin (Meigs et al., 2002; Meigs et al., 2001). The interactions of Gα12/Gα13 with A-kinase anchoring protein (AKAP) direct the proper translocation of PKA. Specifically, Gα12 binds AKAP-Lbc in the heart, while Gα13 binds AKAP110 in the testis, which might implicate a novel Gα12/Gα13-PKA signaling transduction pathway, highlight the tissue specific signaling (Diviani et al., 2001; Niu et al., 2001).

We are interested in tyrosine kinase related interactions through chemokine/chemokine receptor stimulation. Gene regulation responses to Gα12/Gα13 activation, include *c-fos*, serum response element (SRE), nuclear factor-κB (NF- κB) and series of downstream signals mediated by tyrosine kinase PYK2 in muscarinic receptor type 1(Shi et al., 2000). The expression of herpesvirus sequence resembling

chemokine receptor CXCR1/CXCR2 in Hela cells induces Gα13-RhoA dependent IL-8 secretion and NF-κB activation (Shepard et al., 2001). Gα13 is critical, regulating signals through ezrin, radixin, and moesin, which form a protein family that connects numerous cellular surface proteins (such as receptors, ion channels and integrins) and intracellular microfilaments. The cooperation of Gα13 and Rho is indispensible for the activating process, to modulate cellular morphology and migration (Vaiskunaite et al., 2000). Another GPCR signaling axis, lysophospholipids and their receptors, have recently been shown to be modulate inflammatory infiltration through the blood brain barrier, and the lysophosphatidic acid (LPA) receptor requires Gα12/Gα13 to control axonal morphology in hippocampal neurons. Importantly, the Gα12/Gα13-RhoA signaling pathway plays an essential role in LPA stimulated cell migration (An et al., 1998; Bian et al., 2006; Graler and Goetzl, 2002; Yamazaki et al., 2008). G2A, which is predominantly expressed in T and B cells, is also shown to be coupled to Gα13, and to skew RhoA-dependent actin rearrangement (Kabarowski et al., 2000). Gα12/Gα13 also functions through controlling $CD4^+$ T cells adhesiveness and motility to shape T cell immune responsiveness (Herroeder et al., 2009). Interestingly, Gα13 could directly interact with the aryl hydrocarbon receptor (AhR) – interacting protein (AIP), and inhibit the interaction between AhR and AIP, to negatively regulate ligand induced AhR activation (Nakata et al., 2009). More recent reports demonstrate that Gα13 binds to integrin $\alpha_{IIb}\beta_3$, mediating integrin "outside-in" signaling, showing that integrins are noncanonical Gα13 coupled receptor for RhoA regulatio (Gong et al., 2010).

Purified Gα12 is able to stimulate the kinase activity of the related Tec kinase Bruton's tyrosine kinase (Btk) *in vitro*, but not Src tyrosine kinase (Kurose, 2003). Gα12 couples thromboxane A_2 receptor and intracellular signaling through its interaction with Btk and Ras GTPase-activating protein (rasGAP) (Jiang et al., 1998; Ma et al., 2000). However, it remains as a question whether Gα13 is involved in non-receptor tyrosine kinase interaction, and what role the Btk related Itk plays in GPCR signaling.

In this thesis we have investigated the interaction between Itk and Gα12/Gα13. We used specific defective mutants to investigate potential protein-protein interactions. Specifically, we used a Gα13G225A mutant which is reported to be locked in the GDP bound conformation and acts as a dominant negative mutant. This substitution of glycine for alanine of Gαs in its nucleotide binding pocket blocks Gαs activation induced by receptor-mediated guanine nucleotide exchange (Gohla et al., 1999) . Meanwhile, replacement of a consensus glutamine with a leucine inhibits the GTPase activity of Gα, generating a constitutively active mutant (Dhanasekaran et al., 1994; Masters et al., 1989) . The other critical property of Gα is its membrane-cytosol translocation ability. Upon covalent modification by palmitate attached to cysteine residue(s) in N-terminus, Gα relocates to the plasma membrane. Mutation of either cysteines (C14 and C18) results in a defect in Gα13 membrane targeting. Although direct interaction between Gα13 and p115GEF is not disrupted, this complex is not longer able to locate to the plasma membrane, and can no longer activate Rho-dependent serum response factor- mediated transcription and actin stress fiber formation (Bhattacharyya and Wedegaertner, 2000).

We thus focus on these two aspects, the GDP-/GTP- bound structures and membrane localization of Gα13, to further examine the potential interaction with Itk. A brief summary of critical Gα13 mutants used in this work are lined out in table 1.2.

Table 1.2 Summary of critical Gα13 mutants used in this work.

Domain	Mutant	Functional Defect
N terminal	C14S, C18S	Membrane localization↓ Ability to activate RhoA↓
GTP binding site	G225A	GTP binding ↓
	Q226L	GTPase activity ↓

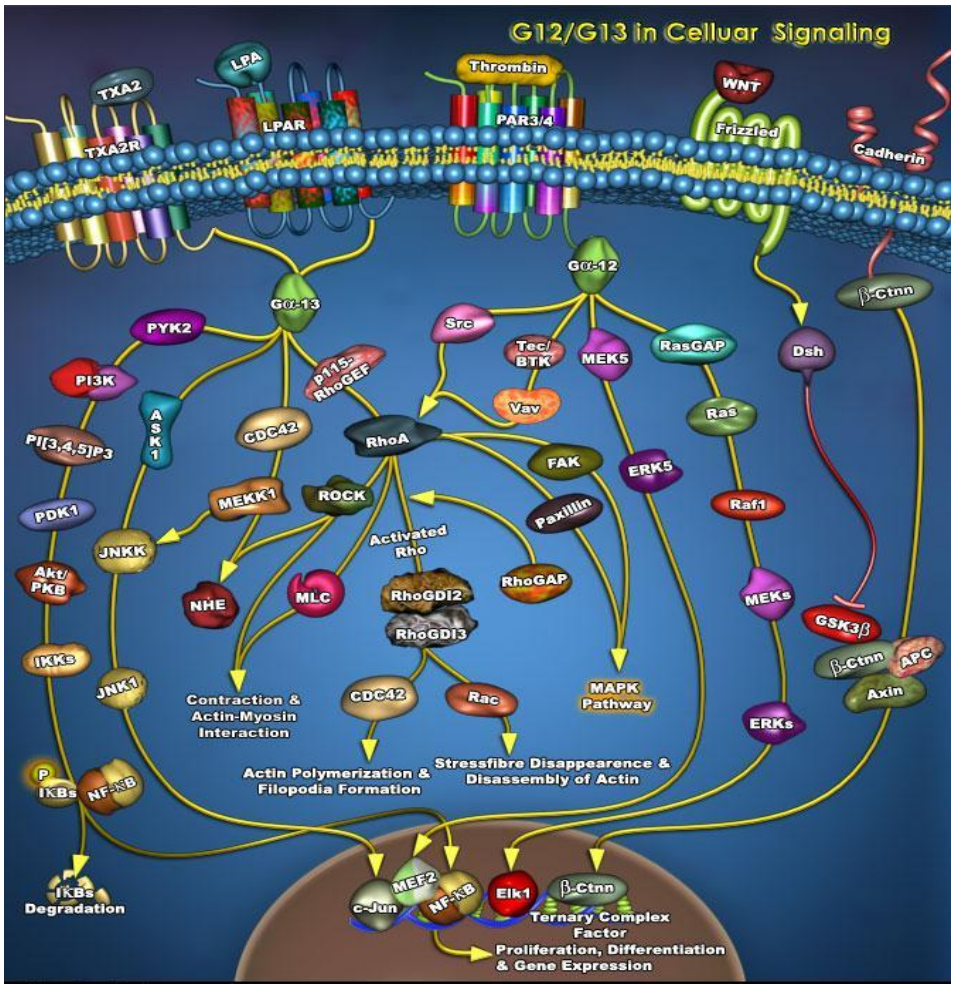


Fig. 1.3: Gα12/Gα13 is involved in numerous cellular signaling pathways

Gα12/Gα13 directly couples related receptors to their downstream mediators, leading to changes in gene transcriptional profiles, which are responsible for the regulation of cell morphology and motion.

(http://gspd.biocompare.com/Gene/Pathway.aspx?gp=144)

1.3 Potential interaction between Itk and Gα13: bridging the gap

Itk and Btk have both been shown to interact with certain βγ subunits to increase their tyrosine kinase activities (Langhans-Rajasekaran et al., 1995). Although the specificity of this activation regarding Gα is unclear, it is highly promising that Itk

and Btk are the effectors of heterotrimeric G proteins (Langhans-Rajasekaran et al., 1995). The two domains of Tec family kinases, that are structurally and functionally conserved in Itk and Btk: PH and BH domains, have revealed some clues that they might be critical in heterotrimetic G protein signaling. A mutant Btk lacking the N terminal domain TH Btk fails to get activated by Gαq, compared to the WT Btk, which might be due to the disruption of Gαq and Btk interaction (Ma and Huang, 1998).

Tec/Bmx non-receptor tyrosine kinases were reported to be involved in the regulation of Rho and serum response factor by G_{12} family (Mao et al., 1998). Itk is also activated by chemokine receptors SDF1α/CXCR4 and CCL11/CCR3, which are igniters for G protein coupled receptors signaling pathways, able to lead to cytoskeleton change and activation of migration, reflected by actin and integrin signaling axis (Fischer et al., 2004; Sahu et al., 2008; Takesono et al., 2004). TCR signaling could also lead to Itk mediated actin cytoskeleton modulation and β1 integrin activation (Woods et al., 2001). Also, Itk interacts with T cell specific adaptor protein to promote SDF1α induced T cell migration in both human and murine models (Berge et al., 2010). Markedly, the Gα13-Rho signaling axis is required for the SDF1α/CXCR4 induced cell migration (Tan et al., 2006). These clues induce our great interest in the relationship of chemokine and antigen on T cell stimulation, to see whether they have any connection with each other.

TCR and GPCR stimulation and related intracellular signaling pathways regulated by Itk and Gα13 might lead to shared downstream outputs by pathway cross-talk. This

might be bridged by certain protein-protein interaction between key mediators. We investigated Itk and Gα13 to determine if they represent such cross talk.

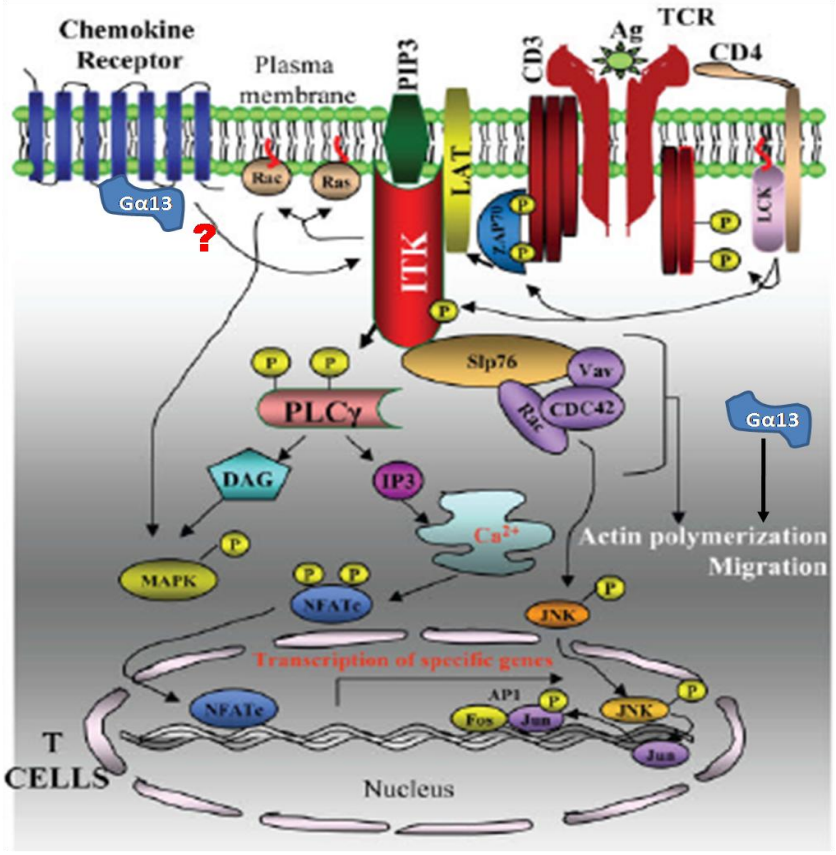


Fig. 1.4 Gα13 potentially couples the GPCR and TCR through Itk signaling

This figure is modified from (Sahu and August, 2009), to show the common downstream output regarding actin cytoskeleton modulation and cell migration, regulated by both Itk and Gα13. The involvement of Itk in actin and integrin signaling, bring up the potential role of Gα13 dependent chemokine receptor stimulation, through Itk activity.

1.4 Overview: goals of this thesis

This project aims to identify and characterize the interaction between Itk and Gα13, based on the hypothesis that: TCR and GPCR signaling pathways crosstalk through interactions of key mediators.

CHAPTER 2

MATERIALS AND METHODS

2.1 Site-directed mutagenesis

The pCDNA3.1- YFP1- Itk (Y1-I) and pCDNA3.1-Itk-YFP2 (I-Y2) were generated by Dr. Qian Qi in the August Lab (Qi et al., 2006). The Gα13-Y2 and constitutively active Gα13(CA)-Y2 constructs were made by replacing the Itk sequence in I-Y2, with the cDNA sequence of Gα13. In this thesis, Itk and mutants were tagged with YFP1 at the N terminus, while Gα13 and mutants were tagged with YFP2 at the C terminus. Mutants, including Y1-R29C, Y1-CCGG, C14S-Y2, C18S-Y2, DN-Y2 were generated by site-directed mutagenesis (Stratagene, Santa Clara, California). All DNA primers were from Integrated DNA Technologies.

2.2 HEK 293T cell transfection and Jurkat T cell stimulation:

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The plasmids were transfected into HEK-293T cells by using TransIT-293T (Mirus). Human Jurkat T cells were cultured in complete RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS). Stimulation was performed with indicated reagents and for the indicated time frame. Anti human CD3ε antibody (OKT3) was from eBioscience.

2.3 Imaging and Flow cytometry: Bimolecular Fluorescence Complementation (BiFC)

The basis of the protein-protein interaction assay for this project is Bimolecular Fluorescence Complementation (BiFC). Briefly, yellow fluorescence protein (YFP) is split into two parts (Y1 and Y2), then are separately tagged onto target proteins for assay. If the two targets are in a proximity close enough (80Å in this thesis), Y1/Y2

could pair with each other and recover the fluorescence of YFP. In this model, we transfected HEK 293T cells with Y1 and/or Y2 tagged protein plasmids. 24 h after HEK 293T cell transfection, the cells were switched from 37°C to 30°C for overnight incubation to promote fluorophore maturation. Live cells on glass bottom 6-well plates were directly imaged using a FV1000 confocal microscopy (Olympus America Inc.). 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 system (Beckman). YFP fluorescence intensities were obtained from positive cell subsets. Itk and Gα13 expression were confirmed by western blot and intracellular staining. Values of YFP intensities were then compared with cells cotransfected with Y1-Itk and Gα13-Y2, which was set at 1. The change in YFP intensity was used as the primary parameter to determine differences in protein-protein interaction, due to various point mutants of either Itk or Gα13. All combinations of cotransfections were repeated more than 3 times for statistical analysis.

This molecular method is the main technique we used to applied to verify protein-protein interaction.

2.4 Intracellular staining for protein expression and localization

To check protein expression in HEK 293T cells, we used cells after live imaging and flow cytometry. Cells were fixed using 4% paraformaldehyde for 20–30 minutes at room temperature. The fixed cells were then permeabilized with blocking buffer (2% FBS and 0.25% Saponin in PBS) for 30 minutes, followed by primary antibody

(rabbit/mouse anti-Itk and/or mouse anti-GFP) staining using the same buffer for 1 hour. After washing three times with PBS, cells were then stained with PE-conjugated anti mouse IgG, Alexa Fluor 532-conjugated anti mouse IgG or Alexa Fluor 647-conjugated anti rabbit IgG secondary antibody for 30 min in the dark. The unbound antibody was removed by washing three times with blocking buffer. The cells were then analyzed by confocal microscopy or flow cytometry.

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

Lysates of transfected HEK 293T cells were used to confirm protein expression and phosphotyrosine status. Stimulated Jurkat T cells were used to perform co-immunoprecipitation. Cells were lysed in lysis buffer (10mM Tri-HCI buffer containing 150mM sodium chloride, 1mM EDTA, 2mM sodium vanadate, 10mM sodium fluoride, 10mM sodium pyrophosphate, 1% NP-40 detergent, 1mM phenylmethylsufonylfluoride, and 2μg/ml pepstatin, pH 7.4). For co-IP, lysis buffer was supplemented with phosphatase inhibitors (Roche Applied Science).
Mouse anti-human monoclonal antibodies against Itk (Cell Signaling) and GFP (Roche Applied Science) were used for western blotting. Rabbit anti-human Itk polyclonal antibody was a gift from Dr. Gordon Mills (M. D. Anderson Cancer Center) (August et al., 1997). Rabbit anti-human Gα13 polyclonal antibody was purchased from Santa Cruz. Horseradish peroxidase (HRP) conjugated anti mouse rabbit IgG secondary antibodies were applied and detected by ECL system (Amersham).

2.6 Statistical Analysis

Data were analyzed by Student's *t* test, using Microsoft Excel and GraphPad Prism. A probability value of $p < 0.05$ was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Itk and Gα13 interact with close proximity

As we described for BiFC, when Y1-Itk and Gα13-Y2 both present and are in a proximity equal to or less than 80Å, YFP fluorescence would be recovered, indicating an Itk and Gα13 interaction (Fig. 3.1 A). To test whether Itk and Gα13 interact *in vivo*, HEK 293T cells were transfected with Y1-Itk and Gα13-Y2. We found that the co-expression of Itk and Gα13 rescued the fluorescence of YFP, shown in Fig. 3.1 B. Compared to the non-transfected HEK 293T cells, and cells with Itk single transfection, the shift of the fluorescence intensity (FI) curve clearly shows the YFP signal. This indicates that Itk and Gα13 interact in cells.

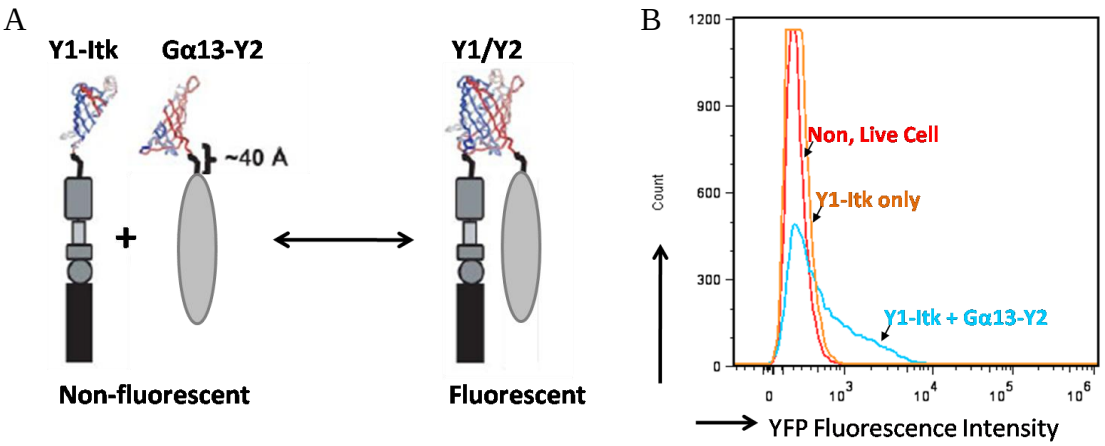


Fig. 3.1: Itk interacts with Gα13, recovering the fluorescent ability of YFP.

(A) Schematic mechanism for BiFC assay modified from (Qi et al., 2006): Y1 and Y2 were tagged to Itk and Gα13, respectively. When the two proteins are coexpressed, the pairing of Y1/Y2 would only happen if the two tagged proteins can interact with each other. The linkers will allow interaction if the proximity is 80Å or less. (B) HEK 293T cells were transfected as indicated. 24 hours later, plates were moved from 37℃ into 30℃ for overnight incubation to promote fluorophore maturation before analysis by flow cytometry. Red curve indicates the non-transfected HEK 293T cells, gated on the live cell based on proper forward scatter and side scatter. Orange curve represents Y1-Itk single transfected cells, while blue curve is for the Itk and Gα13 co-transfected cells. Unless specifically indicated, all Itk plasmid constructs in this study were with Y1 in the N-terminus, all Gα13 plasmid were with Y2 to the C-terminus.

3.2 Itk and Gα13 interaction requires membrane localization of both parties

To determine the sub-cellular localization of the interaction between Itk and Gα13, we further analyzed the adherent HEK 293T cell by confocal fluorescence microscopy.

Both Itk and Gα13 are recruited to the plasma membrane for further activation, as discussed in chapter 1 of this thesis. Mutation of R29 to C in Itk abolished its ability to be localized to the even with the presence of upstream activators. Similarly, C14S, C18S have been shown to disrupt the Gα13 membrane targeting process and fails to activate Rho-dependent serum response factor mediated transcription and actin stress fiber formation (Bhattacharyya and Wedegaertner, 2000). We therefore used these membrane localization disrupting mutants to investigate the role of plasma membrane translocation in the interaction between Itk and Gα13. As shown in Fig. 3.2 A, YFP fluorescence recovery was impaired in the cells transfected with the mutants. Confocal microscopy imaging clearly shows the sub-cellular localization of the interaction at the plasma membrane, while defects in membrane targeting of either parties, Itk or Gα13, diminished the YFP fluorescence at the membrane (Fig. 3.2, panel B). All combinations including R29C + Gα13, Itk + C14S, and Itk + C18S, exhibited weak ability to reconstitute the YFP fluorescence. Protein expression was confirmed by western blotting (Fig. 3.2, panel C) and intracellular staining (data not shown).

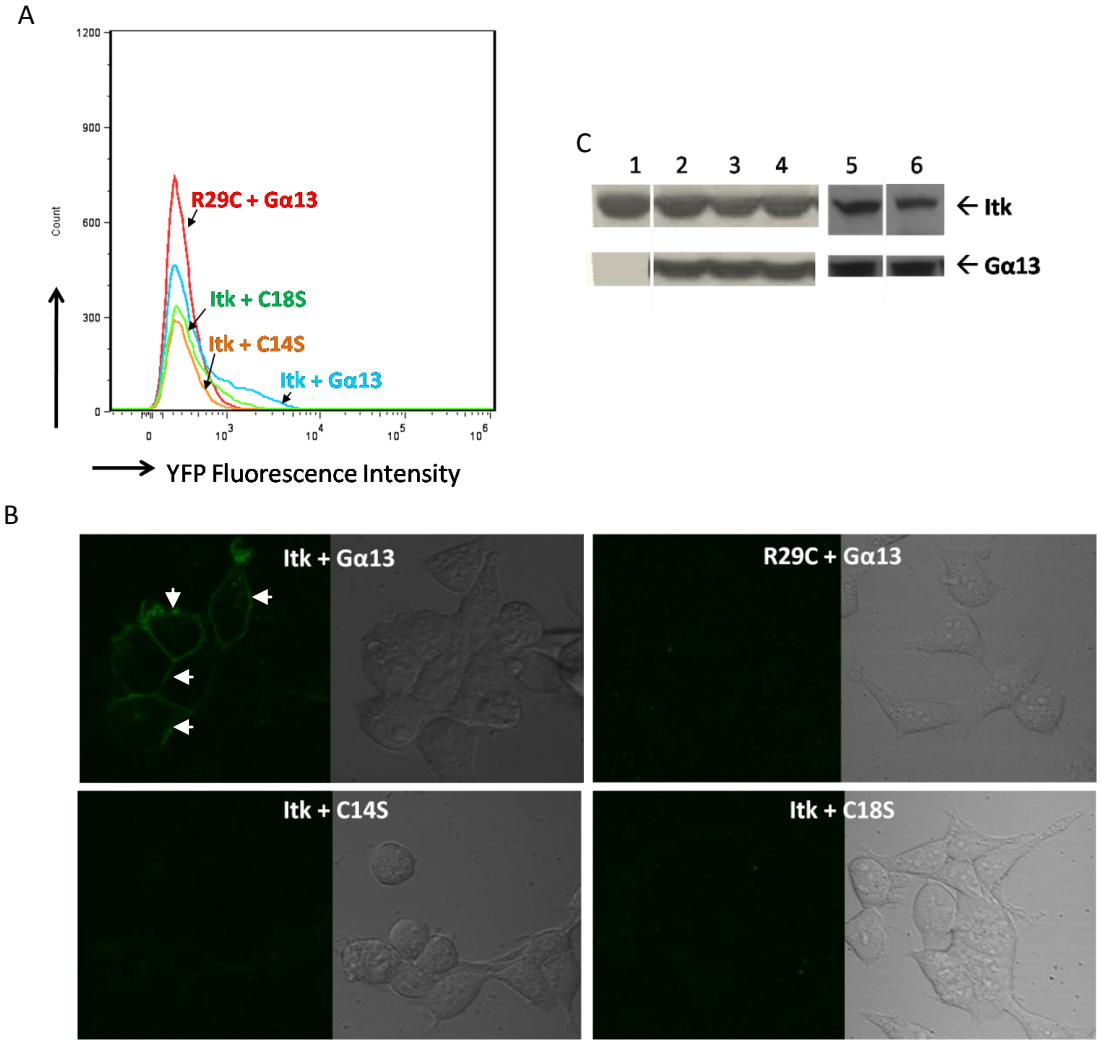


Fig. 3.2: Itk interacts with Gα13 at the membrane, and requires membrane localization of both parties.

HEK 293T cells were cotransfected with Y1/2 tagged protein plasmids, and treated as described, before analysis by (A) flow cytometry and (B) confocal imaging. (C) Protein expression was confirmed by western blotting (Lane 1: Itk only; 2, 5: Itk + Gα13; 3: Itk + C14S; 4: Itk + C18S; 6: R29C + Gα13). In figure C, note that lanes 1-4 were run on the same gel, while lane 5 and 6 were on the same one, and lanes 1, 5 and 6 moved so that they could be juxtaposed beside the other lanes.

3.3 Itk kinase activity is not required for its interaction with Gα13

One of the essential functions of Itk is its kinase activity upon stimulation. So it is of our great interest to determine the role of Itk kinase activity in the ability of Itk and Gα13 to interact. The K391M mutant of Itk has been shown to be deficient in its kinase activity (August et al., 1997; Hao and August, 2002). Hence, we transfected HEK 293T cells with K391M Itk along with Gα13, to determine if the interaction is affected. Surprisingly, as Fig. 3.3 shows, Itk mutant that lacks kinase activity maintains its ability to interact with Gα13.

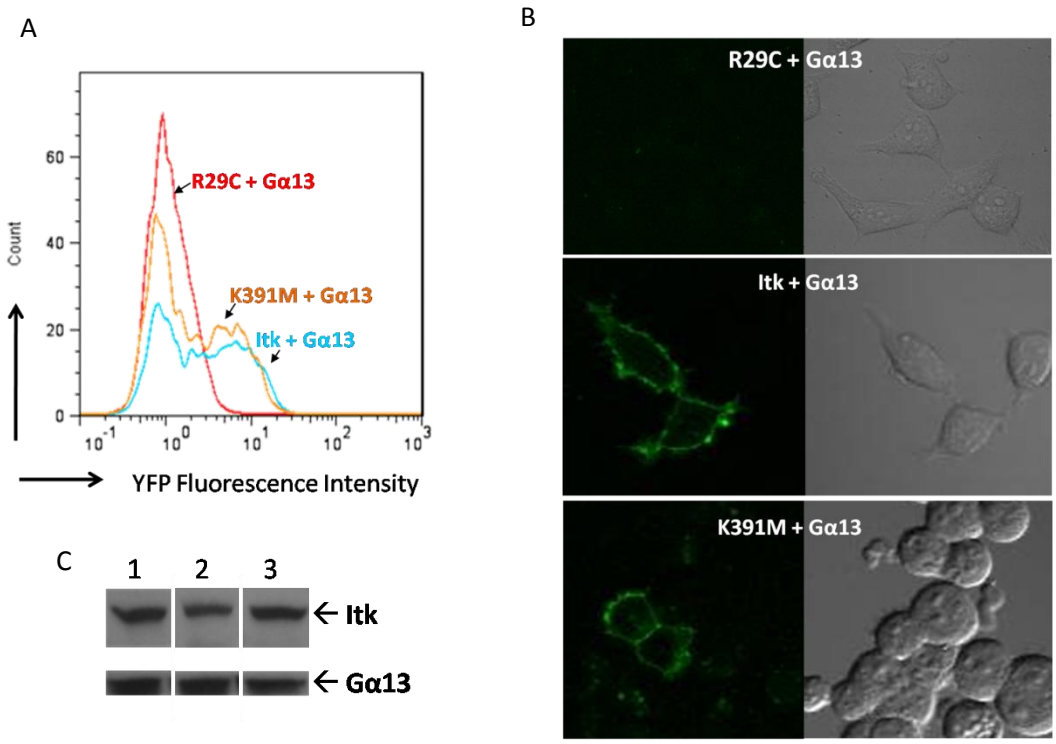


Fig. 3.3: Itk and Gα13 interaction is independent of Itk kinase activity

HEK 293T cells were transfected with the indicated plasmids as indicated. (A) YFP fluorescence intensity determined based on individual cells; (B): confocal microscopy showing the sub-cellular localization of protein-protein interaction; (C): protein expression confirmed by western blotting (Lane 1: Itk + Gα13; 2: R29C + Gα13; 3: K391M + Gα13). In figure C, note that all lanes were run on the same gel, but were moved so that they could be juxtaposed beside each other.

3.4 Itk and Gα13 interaction is independent of Gα13 binding to GDP or GTP

Gα13 has the ability to switch between GDP bound and GTP bound conformers, in order to perform proper functions during signaling transduction. The Q226L mutant of Gα13 has been determined to be a constitutively active form, as it has defective GTPase activity and is constitutively bound to GTP, while G225A is dominantly negative due to a defect in GDP exchange for GTP. To test the role of the switchable structure of Gα13 in the interaction between Itk and Gα13, HEK 293T cells were transfected with Itk and Gα13 plasmids, and WT and related mutants were combined to see any possible defects. Surprisingly, neither the constitutively active or dominantly negative mutant affected the interaction, compared to the WT-WT combination, using R29C+Gα13 as the negative control (Fig. 3.4). This reveals that, Gα13 does not rely on its GDP-/GTP- binding to interact with Itk, and there is something else critical for this interaction that remains to be investigated.

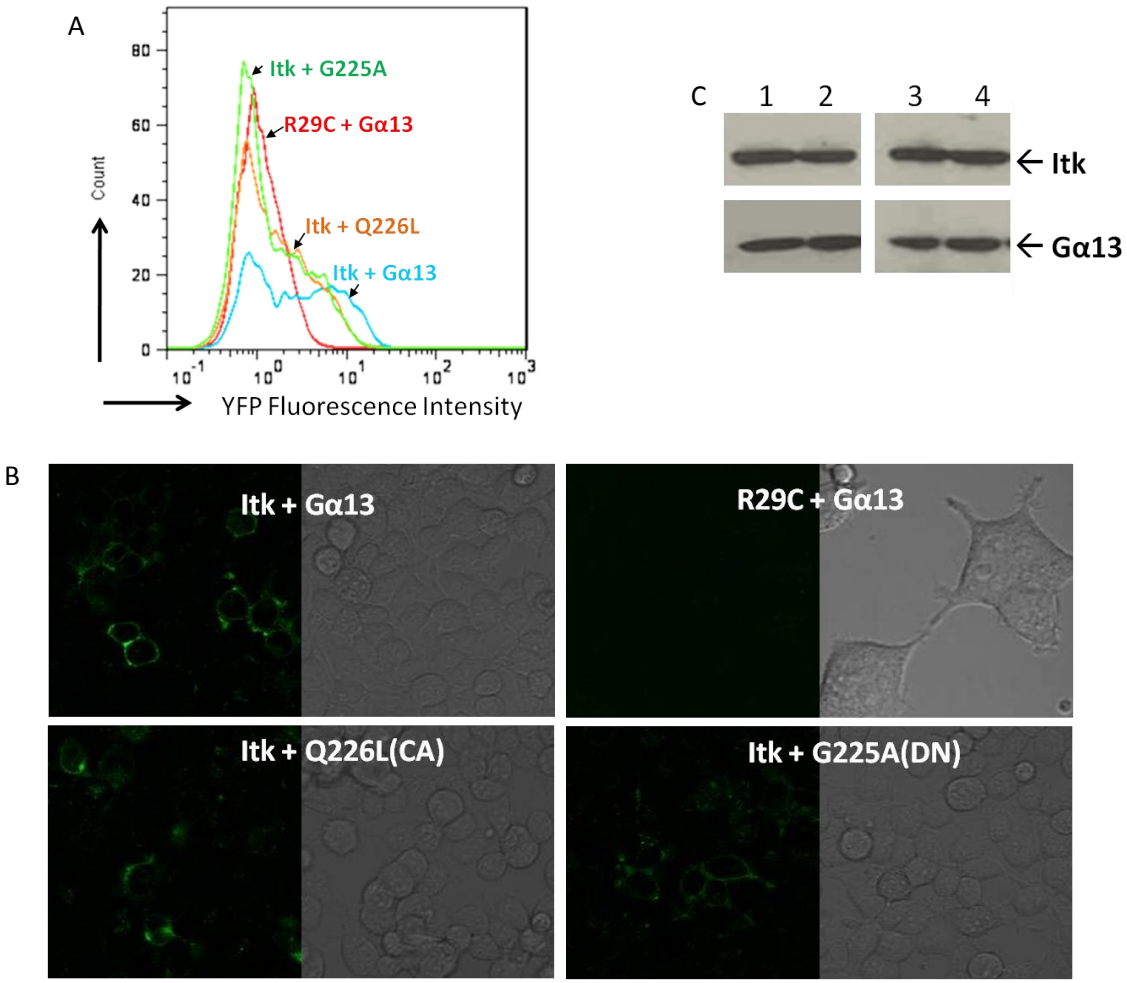


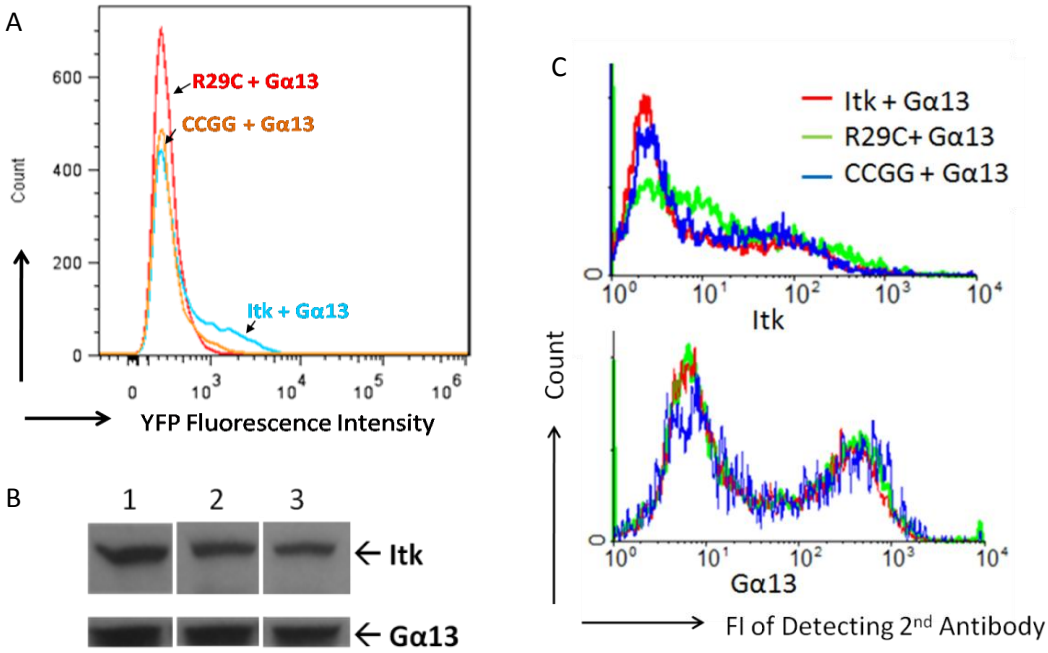
Fig. 3.4: Itk and Gα13 interaction is not dependent on GDP or GTP binding by Gα13

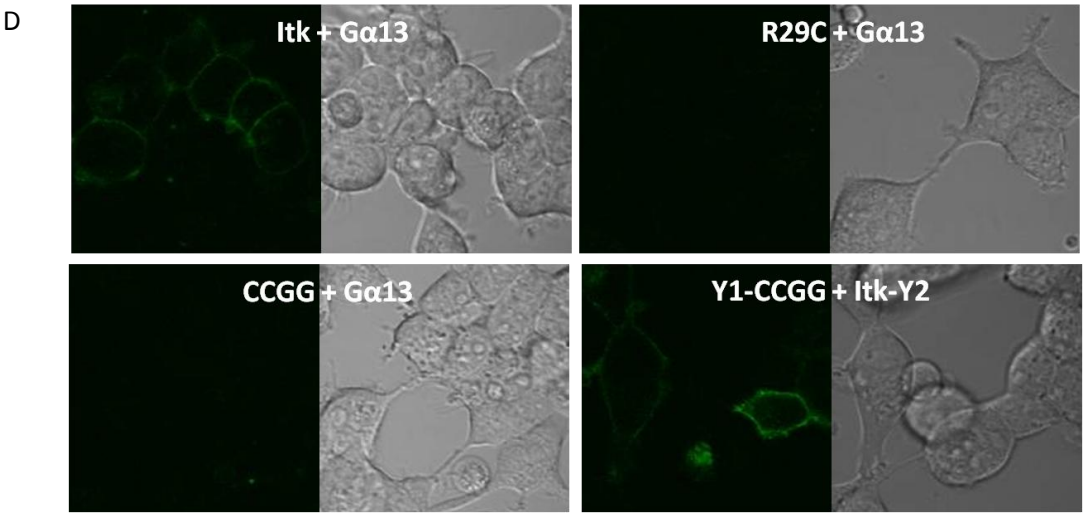
Cells were transfected as indicated , followed by (A) flow and (B) confocal analysis. (C) Western blotting detects protein expression. (Lane 1: Itk + Gα13; 2: R29C + Gα13; 3: Itk + G225A; 4: Itk + Q226L).

3.5 The zinc binding site within the BH domain of Itk is critical for Itk and Gα13interaction

As discussed earlier, the N terminal of TH domain, where the BH motif is located, in Btk, has been shown to be essential for Btk activation by Gαq, which might implicate a critical role of BH domain between Btk and Gα interaction (Ma and Huang, 1998). We therefore examined the conserved BH zinc binding finger motif to determine if it has a role in the interaction between Itk and Gα13. These amino acids are C132C133 in BH domain of Itk, highly homologous to the C155 in Btk BH domain. They were mutated simultaneously to Gly, forming the C132GC133G (CCGG) mutant of Itk. When co-expressed with Gα13, CCGG doesn't recover the YFP, suggesting that this motif may be important for the ability of Itk to interact with Gα13.

As previously discussed, the R29C mutant of Itk does not interact with Gα13 due to its defect in membrane targeting, and it is possible that this zinc binding motif may have defects in membrane localization. We therefore determined whether this mutant could get to the membrane by co-expressing it along with WT Itk.. However, the CCGG, is able to be recruited to the membrane in the presence of WT Itk, revealing that the zinc binding site might be the motif that allows the physical interaction between Itk and Gα13, because it is still able to be recruited to the membrane, but disrupts the interaction.


A
R29C + Gα13
CCGG + Gα13
Itk + Gα13
Count
YFP Fluorescence Intensity
B
1
2
3
Itk
Gα13
C
Itk + Gα13
R29C+ Gα13
CCGG + Gα13
Itk
Count
Gα13
FI of Detecting 2nd Antibody


D
Itk + Gα13
R29C + Gα13
CCGG + Gα13
Y1-CCGG + Itk-Y2

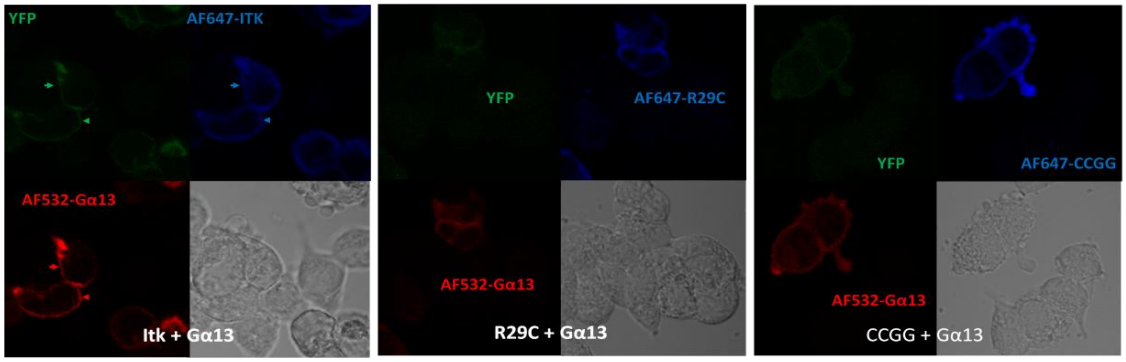

E
YFP
AF647-ITK
AF532-Gα13
Itk + Gα13
YFP
AF647-R29C
AF532-Gα13
R29C + Gα13
YFP
AF647-CCGG
AF532-Gα13
CCGG + Gα13

Fig. 3.5: Zinc binding site within BH domain of Itk is critical for Itk and Gα13 interaction

HEK 293T cells were transfected as indicated and analyzed as described with (A) flow cytometry and (D) live cell confocal microscopy imaging. Cells were then either (B) lysed and run through SDS-PAGE for western blotting (Lane 1: Itk + Gα13; 2: R29C + Gα13; 3: CCGG + Gα13), or (C) resuspended for intracellular staining to determine the protein expression (Mean fluorescence intensity (MFI) of detecting secondary antibodies were shown on figures). (E) Adherent cells were fixed, permeabilized and stained by Itk or Gα13 specific antibodies, followed by fluorescent secondary antibodies, for confocal imaging to determine the sub-cellular localization of expressed proteins and protein-protein interaction. In figure B, note that all lanes were run on the same gel, but were moved so that they could be juxtaposed beside each other.

3.6 Over expression of Itk induces tyrosine phosphorylation of Gα13

Although we have shown in the previous sections that Itk kinase activity is not required for its interaction with Gα13, we were still very interested in whether the interaction between Itk and Gα13 resulted in altered tyrosine phosphorylation due to the kinase activity of Itk. To investigate this, we compared lysate from non-transfected cells with Itk single-transfected and Itk/Gα13 co-transfected cells for levels of phosphotyrosine. Remarkably, only lysates from cells expressing WT Itk and Gα13 had an extra phosphorylated band at around the size of Gα13 (size of Gα13-Y2 is 54kD), suggesting that Itk is able to induce tyrosine phosphorylation of Gα13 or a third party. Moreover, in the presence of the membrane targeting deficient mutant of Itk (R29CItk), the extra phosphorylated band is gone, along with the interaction between Itk and Gα13 (Fig. 3.6).

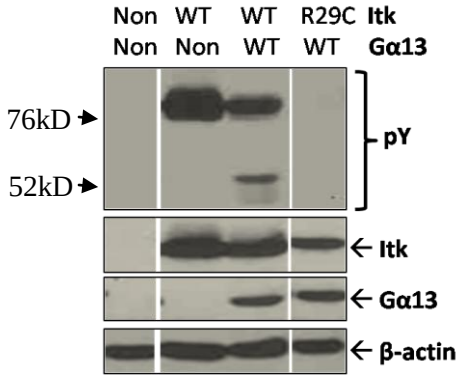


Fig. 3.6: Itk induces tyrosine phosphorylation of Gα13 or potential third protein when over-expressed with Gα13

The indicated transfected HEK 293T cell lysates were analyzed for phosphotyrosine (pY, top panel), Itk (second panel), Gα13 (third panel) or β-actin as a loading control (bottom panel). Note that all lanes were run on the same gel, but were moved so that they could be juxtaposed beside each other.

3.7 Gα13 transiently interacts with Itk during T cell stimulation

We next extended our study to determine if the Itk:Gα13 interaction could be observed in non-transfected cells. We used Jurkat T cells for this analysis so that we could examine any potential interaction during T cell signaling. We speculate that Gα13 requires outside signals to guide its interaction with Itk, rather than being constitutively bound to it. Starting with the well studied ability of the TCR to activate Itk model, we stimulated Jurkat T cells with OKT3, an anti-human CD3ε antibody. Afterward, cell lysates were subjected to co-IP assay, using anti-Itk as immuno-precipitating antibody, and detected for Itk and Gα13 composition in precipitated complexes. We found that Gα13 is pulled down with Itk, but surprisingly, in a transient pattern during the T cell activation time course, peaking at around 2 minutes post-stimulation (Fig. 3.7).

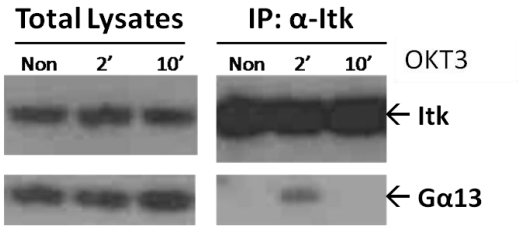


Fig. 3.7: Gα13 transiently binds Itk upon TCR stimulation in Jurkat T cells

Jurkat T cells are stimulated using an anti human CD3ε antibody, OTK3, for indicated time or "Non" as non-stimulated control. Cells were lysed and used for immuno-precipitation. Total cell lysates and IP products were analyzed for Itk and Gα13 levels.

3.8 Summary of Itk and Gα13 interaction and effects of mutants

BiFC has served as a primary tool in this thesis, providing a method for quick identification of protein–protein interaction, and screening of the effects of disruptive mutants on the Itk and Gα13 interaction. In addition to the "yes or no" results we showed, we quantitated and compared the intensities of recovered YFP (Fig. 3.8). The YFP intensity from WTItk-WTGα13 cotransfected HEK 293T cells were set to be "1", and all other results were normalized and compared to this level. As Fig. 3.8 exhibits, C14S and C18S mutants significantly diminished the interaction of Gα13 with WT Itk, while R29C and CCGG mutants disrupted the interaction of Itk with WT Gα13. Also note that Q226L and G225A mutants of Gα13, and K391M mutant of Itk did not cause significant change in the Itk: Gα13 interaction.

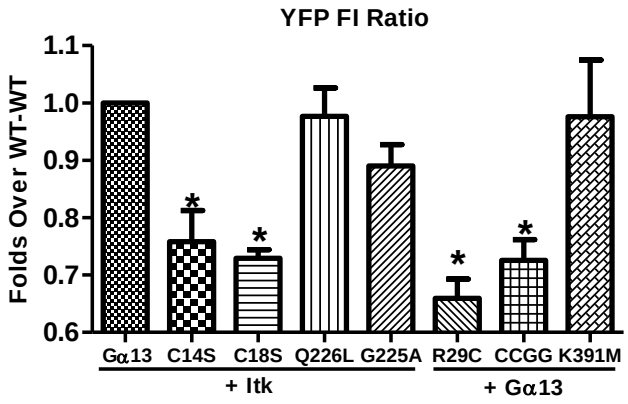


Fig. 3.8: Summary of Itk and Gα13 interaction and effects of mutants

Based on the BiFC system, cells with positive yellow fluorescence are gated, setting the R29CItk-Gα13 as the "negative→positive" threshold. We normalized YFP intensities to the one in Itk-Gα13 WT-WT combination. All combinations were repeated more than three times, and only groups with equivalent levels of Itk and Gα13 expression were pooled in this statistical analysis. *: $p<0.05$ by student test.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

4.1 Discussion

As shown by both over-expression and endogenous proteins, Itk and Gα13 interact with each other. Remarkably, protein translocation of both parties to the plasma membrane was required to ensure this interaction, as evidenced by the impaired recovery of YFP intensity when the C14S and C18S mutant of Gα13, and R29C mutant of Itk were used. Since the recovery of YFP fluorescence by WT Itk and Gα13 co-expression was executively on the plasma membrane, it was our expectation that defects in membrane targeting of either party would block proper sub-cellular localization, therefore abolished the interaction.

In the case of the CCGG mutant of Itk with WT Gα13, the zinc binding motif (CC motif) of Itk might be directly involved in physical interaction with Gα13, as CCGG Itk was still able to localize at the plasma membrane.

These results, related to the Itk PH and BH domains, are consistent to previous findings that PH domain and N-terminal of TH domain are critical for Btk to interact with Gαq (Ma and Huang, 1998).

Notably, the Itk/Gα13 interaction is independent of the kinase activity of Itk or Gα13 GDP-/GTP- bound conformation, since kinase dead Itk mutant, as well as Gα13 dominant negative and constitutively active mutants did not significantly affect the interaction.

Itk might perform differently after the association with Gα13, and this interaction might be through novel motif(s) in Gα13 instead of its well studied GTP binding related structure.

Transient interaction of Itk and Gα13, stimulated through TCR-CD3 signaling pathway, shows the inducible interaction was transient. This means, this interaction is a messenger to spread the incoming of outside signals. Combined with the results from the phosphotyrosine assay, it is likely that Itk and Gα13 interaction induces tyrosine phophorylation on Gα13 or related third party, to modulate signal transduction. The signaling activation by TCR, might mark its steps on the field of GPCR axis, through the connection of Gα13 and Itk. The physical proximity of Itk and Gα13, essential to molecular modulation, also reveal the role of Gα13, to TCR and GPCR pathways cross-talking. Therefore, investigation of this interaction, will lead to a comprehensive understanding how immune cells respond to pathogen and migrate to targeted sites.

4.2 Future directions

Most of the work done in thesis so far focused on the characterization of protein-protein interaction and looking for motifs for physically interaction. The nexty steps would be to examine the potential function of this interaction. I have attempted to examine this by looking at activation of serum response factor (using a luciferase reporter system), since SRF activation is the common downstream effect of both Itk and Gα13 (Bhattacharyya and Wedegaertner, 2000; Hao et al., 2006). Actin stress fiber formation is also another potential downstream effect, because they both involve Itk and Gα13 signaling regulations (Bhattacharyya and Wedegaertner, 2000; Dombroski et al., 2005; Gohla et al., 1999; Tsoukas et al., 2006). To emphasize, both of these two pathways are Itk kinase independent, however, they depend on RhoA

signaling. Meanwhile, the interaction between Itk and Gα13 is also Itk kinase independent. It is possible that Itk and Gα13 cooperate by a novel mechanism to regulate actin rearrangement and SRF related gene transcription. Analysis of these two pathways has provided some hints, however, no clear or solid conclusions can yet be reached.

Another potential direction to follow up is integrin signaling, which also involves both Itk and Gα13 signaling (Finkelstein et al., 2005; Gong et al., 2010; Woods et al., 2001). Although we only examined TCR-CD3 stimulation, chemokine stimulation is also of significant interest. For example, investigation of SDF1α stimulation and its effect on Itk and Gα13 interaction may help in better understanding the role of Gα13, and relationship of TCR and GPCR signaling pathways.

Promisingly, information revealed from the sequence and structure will be followed up, according to homology analysis. The similarity in sequence and structure, for example, between Gα13 and proteins that interact with Itk, or between Itk and proteins that interact with Gα13, might be a good source to unravel the mystery.

Finally, the best model to examine, will be a potential animal model, and transgenic mice with related mutants which abolish Itk and Gα13 interaction will further allow us to determine the significance of Itk and Gα13 interactions. Since Gα13 knockout is fetal lethal (Offermanns et al., 1997), finding a Gα13 mutant that is viable and critical in interacting with Itk will be helpful, although challenging. Alternatively, bone marrow from Gα13 knockout fetuses could be obtained and transferred into viable recipients, to allow the analysis of the role of Gα13 in immune cell development.

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