

The proline rich region of the Tec homology domain of ITK regulates its activity

Shengli Hao^{a,b}, Avery August^{a,b,*}

^a*Immunology Research Laboratories and Department of Veterinary Science, The Pennsylvania State University, 115 Henning Building, University Park, PA 16802, USA*

^b*Life Science Consortium Graduate Program in Cell and Developmental Biology, The Pennsylvania State University, University Park, PA 16802, USA*

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Abstract Inducible T-cell kinase (ITK) is a member of the Tec family of tyrosine kinases that are involved in signals emanating from cytokine receptors, antigen receptors and other lymphoid cell surface receptors. Stimulation of tyrosine phosphorylation and activation of ITK by the T-cell antigen receptor, CD28 and CD2 requires the presence of the Src family kinase Lck in T-cells. We have previously demonstrated that the activation of ITK by Src family kinases uses a phosphatidylinositol 3-kinase pathway, which recruits ITK to the membrane via its pleckstrin homology (PH) domain where it is acted upon by Src. We have further explored the mechanism of this requirement for Src family kinases in the activation of ITK. We found that deletion of the proline rich sequence found in the Tec homology domain of ITK results in reduced basal activity of ITK approximately 50%. These differences in the basal activity of ITK were observed when the PH domain was deleted or when the kinase was membrane targeted. Furthermore, this deletion reduces the ability of the Src family kinase Lck to activate ITK, as well as to induce the ITK mediated tyrosine phosphorylation of its substrate PLC γ 1. By contrast, deletion of the SH3 domain of ITK resulted in a two-fold increase in the basal activity of ITK, and allowed this mutant to have an enhanced response to Lck. These results suggest that the proline rich region within the Tec homology domain of ITK regulates its basal activity and its response to Src family kinase signals. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Inducible T-cell kinase; Src; Tyrosine kinase; CD28; T-cell receptor

1. Introduction

The Tec family of non-receptor protein tyrosine kinases includes inducible T-cell kinase (ITK) [1–3], Tec I and II [4,5], Bmx [6], Txk/Rlk [7], and BTK [8]. Expression of ITK is predominantly detected in T-cells and NK cells [1–3]. Mutations in BTK are responsible for human X-linked agammaglobulinemia and the murine X-linked immunodeficiency,

where maturation of the B-cell compartment is impaired [8,9]. ITK has an analogous function in T-cells as mice lacking ITK have reduced numbers of mature thymocytes, with reduced proliferative responses following T-cell receptor (TcR) crosslinking of their mature T-cells, and deficient T_H2 cell development [10,11]. ITK and BTK have been proposed to act as amplifiers of signals from TcR and the B-cell receptor [12,13].

ITK, like most of the other members of the Tec family of kinases, has an Src homology 3 (SH3) and an Src homology 2 (SH2) domain. In addition, ITK has a pleckstrin homology (PH) domain and a Tec homology (TH) domain that has a GAP homology region and a proline rich region (PRR). This PRR has been demonstrated to interact with the SH3 domains of Src family kinase in vitro and in the yeast two-hybrid system [14–16]; however, no interaction between ITK and Src family kinases in vivo has been reported and the significance of these findings is unclear.

Tec kinases have been shown to be regulated by Src family kinases by acting as direct substrates of these kinases [17–23]. We and others have determined that ITK, BTK, and BMX are regulated by the actions of phosphatidylinositol 3-kinase (PI 3-kinase) [18,24–26]. Cumulatively, these data suggest that Src family kinases activate PI 3-kinase, which recruit Tec family kinases to the membrane, where they are acted upon by Src family kinases.

ITK is activated as a result of ligation of the T-cell antigen receptor (TcR), CD28 and CD2, cell surface receptors found on T-cells, and the Fc ϵ R found on mast cells [27–30]. In the case of the TcR, CD28 and CD2, this activation event requires the presence of the Src family kinase Lck [28,30,31]. Similarly, BTK is activated by the B-cell antigen receptor and the Fc ϵ R and requires Src kinase activity [32,33]. In addition, the G α subgroup of heterotrimeric G proteins can also activate BTK and other Tec family kinases, the mechanism of which may involve the N-terminal region of the TH domain [34]. As the PRR of the TH domain of Tec family kinases has been reported to interact with Src family kinase in yeast and in cells, as well as with the SH3 domain of ITK in an intramolecular fashion, we examined this region for a possible role in the activation of ITK by Src family kinases. We report here that the PRR of ITK is required for both normal basal activity and activation by Src family kinases. These data, along with recently published findings on the activation of BTK by G α , suggest that the TH domain is the target of regulation of Tec family kinases by both tyrosine kinases (Src family kinases) and G proteins.

*Corresponding author. Fax: (1)-814-863 6140.

E-mail address: axa45@psu.edu (A. August).

Abbreviations: ITK, inducible T-cell kinase; PH, pleckstrin homology domain; PRR, proline rich region; SH3, Src homology domain 3; SH2, Src homology domain 2; TcR, T-cell receptor; TH, Tec homology domain; PI 3-kinase, phosphatidylinositol 3-kinase

2. Materials and methods

2.1. Construction of expression plasmids

Wild-type (WT) ITK and mutants Δ PH and Kit/ Δ PH have been previously published [18]. The v-Src kinase inactive cDNA was a gift of Dr. J. Michael Bishop (UCSF, San Francisco), and was cloned into the mammalian expression vector pMEXneo. ITK mutants Δ PH PR, Kit/ Δ PH PR, Δ PR, and Δ SH3 ITK were generated by PCR or recombination with previous mutants and cloned into the expression vector pMEXHAneo or pG (A. August, unpublished). All ITK constructs were tagged at the N-terminus with the HA tag.

2.2. Cell culture and transfections

293 cells were grown in DMEM containing 10% fetal calf serum and antibiotics at 37°C with 5% CO₂. Cells were transiently transfected with calcium phosphate/DNA complexes as described [18].

2.3. Cell lysis, immunoprecipitation and kinase assays

Cell lysis, immunoprecipitation, SDS-PAGE, and Western blotting were performed exactly as described [18]. Kinase assays were performed exactly as described either with Sam68 as a substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or without exogenous substrate [18]. Tyrosine phosphorylation of substrates and the kinase autophosphorylation was quantified by phosphor imager and corrected for protein expression following probing the blots with anti-HA antibodies. Western blots were quantified using the phosphor imager in fluorescence mode and ECL+ (Amersham Pharmacia).

3. Results and discussion

3.1. The PR region of ITK regulates its basal activity

The PRR within the TH domain of Tec family kinases has been demonstrated to interact with the SH3 domains of Src family kinases in yeast [14]. In addition, this region was shown to interact in an intramolecular fashion with ITK's own SH3 domain, suggesting a mechanism of regulation of these kinases [16]. These investigators suggested that ITK was regulated in an intramolecular fashion, with the SH3 domain of ITK interacting with its PRR, leading to inhibition of ITK. This model suggests that removal of either the PRR or the SH3 domain of ITK should be sufficient to activate its kinase activity. We therefore wanted to determine the behavior of a mutant of ITK that lack the PRR or the SH3 domain, or both (Fig. 1) and their regulation by the Src family kinase Lck. We transfected 293 cells either with WT ITK, ITK lacking its PRR (Δ PRR ITK), or ITK lacking its SH3 domain (Δ SH3 ITK), and determined their activity in vitro in autophosphor-

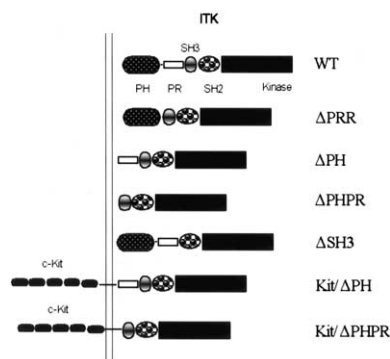


Fig. 1. Structure of the different ITK mutants used in this study. Δ PRR ITK: PRR of TH domain deleted ITK; Δ PH ITK: PH domain deleted ITK; Δ PH PRR ITK: PH and PRR of TH domain deleted ITK; Δ SH3 ITK: SH3 domain deleted ITK; Kit/ Δ PH ITK: murine c-Kit/ Δ PH ITK fusion; Kit/ Δ PH PR ITK: murine c-Kit/ Δ PH Δ PRR ITK fusion.

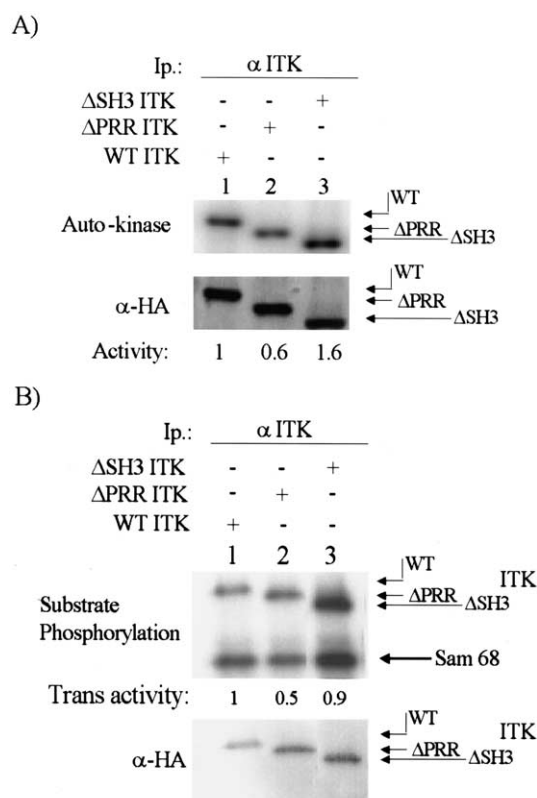


Fig. 2. The PR region of ITK regulates its basal autophosphorylation and transphosphorylation activity. A: 293 cells were transfected with WT, Δ PRR or Δ SH3 mutants of ITK, lysed 2 days later and ITK immunoprecipitated using anti-HA antibodies and analyzed for kinase activity by in vitro kinase assay. Lane 1: WT ITK, lane 2: Δ PRR ITK, lane 3: Δ SH3 ITK. Top panel: Labeling of ITK during autophosphorylation. Bottom panel: The blot was probed with an anti-HA antibody. Note that activity was determined after correcting for expression of the respective mutants. B: 293 cells were transfected with WT, Δ PRR or Δ SH3 mutants of ITK, lysed 2 days later and ITK immunoprecipitated using anti-HA antibodies and analyzed for transphosphorylating activity in the presence of SAM68 by in vitro kinase assay. Lane 1: WT ITK, lane 2: Δ PRR ITK, lane 3: Δ SH3 ITK. Top panel: Labeling of ITK and SAM68 during kinase reaction. Bottom panel: The blot was probed with an anti-HA antibody. Note that the blots were treated with KOH before exposure and that the activity was determined after correcting for expression of the respective mutants. Arrows indicate the position of the ITK mutants.

ylation (Fig. 2A) or in transphosphorylation of a substrate, SAM68, a putative substrate of ITK (Fig. 2B, [16]). Note that based on experimental data as well as homology with BTK, ITK has two autophosphorylation sites, one in the catalytic domain and the other in the SH3 domain, and removal of the SH3 domain may have removed one of these putative autophosphorylation sites [35]. It is therefore possible that if the Δ SH3 mutant's activity is unaffected by the deletion of the SH3 domain, it should have an apparent 50% reduced autophosphorylation activity, but WT activity in the transphosphorylation assay. Examining autophosphorylation of the different mutants showed that removal of the PRR of ITK results in an approximately 50% reduction in ability to autophosphorylate (average of three experiments. Note that removal of the PRR does not remove any tyrosine residues). By contrast, removal of the SH3 domain results in an apparent 1.5-fold increase in its ability to autophosphorylate (aver-

age of three experiments. Note that removal of the SH3 domain may have removed one of the two tyrosine residues that can be autophosphorylated by ITK, and this may be an underestimate by two-fold of its ability to autophosphorylate, i.e. true autophosphorylation activity of this mutant may be closer to three-fold). Examining transphosphorylation of an exogenous substrate, SAM68, confirms these findings and assumptions. The mutant lacking its PRR had an approximately 50% reduction in its ability to phosphorylate SAM68 (Fig. 2B). Surprisingly, the Δ SH3 mutant did not exhibit an increase in its ability to phosphorylate SAM68, suggesting that perhaps the SH3 domain of ITK was important for phosphorylating this substrate in vitro, a fact supported by the fact that the SH3 domain of ITK interacts with SAM68 in vitro [17]. These data suggest that in the basal state, the PRR positively regulates the activity of ITK both to autophosphorylate, as well as in phosphorylating specific substrates. By contrast, deletion of the SH3 domain of ITK increased its ability to autophosphorylate without apparently increasing its ability to phosphorylate an exogenous substrate in vitro, similar to

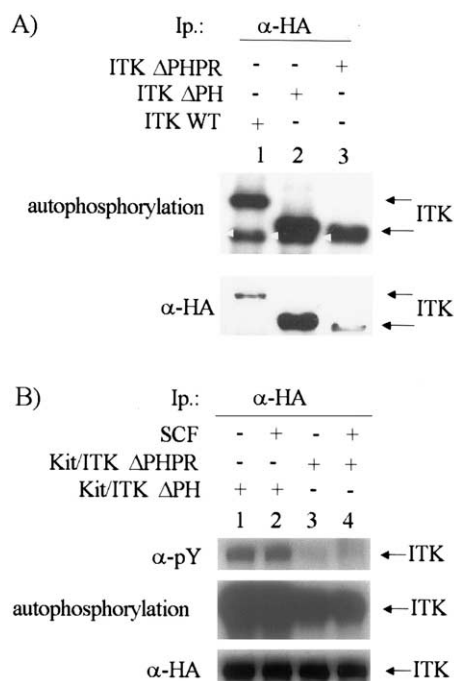


Fig. 3. Neither membrane targeting nor the PH domain affects the regulation of the basal activity by the PR region. A: 293 cells were transfected with WT, Δ PH or Δ PH PRR mutants of ITK, lysed 2 days later and ITK immunoprecipitated using anti-HA antibodies and analyzed by kinase activity by in vitro kinase assay using SAM68 as an exogenous substrate. Lane 1: WT ITK, lane 2: Δ PH ITK, lane 3: Δ PH PRR ITK. Top panel: Labeling of ITK during autophosphorylation (the blot was treated with KOH before exposure). Bottom panel: The blot was probed with an anti-HA antibody. White arrowheads indicate the phosphorylated SAM68. B: 293 cells were transfected with Kit/ Δ PH or Kit/ Δ PHPRR mutants of ITK. Two days later, cells were treated with SCF or vehicle (RPMI) as indicated for 30 min, lysed, and ITK immunoprecipitated using anti-HA antibodies and analyzed for kinase activity by in vitro kinase assay and for the presence of phosphotyrosine by anti-phosphotyrosine antibody. Lanes 1 and 2: Kit/ Δ PH ITK, lanes 3 and 4: Kit/ Δ PHPRR ITK. Top panel: The blot was probed with an anti-phosphotyrosine antibody. Middle panel: Labeling of ITK during autophosphorylation (the blot was treated with KOH before exposure). Bottom panel: The blot was probed with an anti-HA antibody. Arrows indicate the position of the ITK fusions.

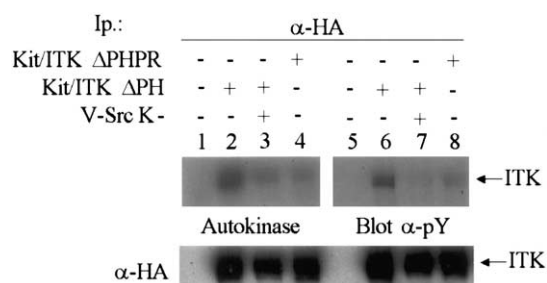


Fig. 4. Kinase inactive Src reduces the basal activity of membrane targeted Δ PH ITK to that seen in membrane targeted Δ PH PRR ITK. 293 cells were transfected with Kit/ Δ PH ITK in the presence of absence of kinase inactive v-Src, or with Kit/ Δ PHPRR mutant of ITK. Two days later, cells were lysed, and ITK immunoprecipitated using anti-HA antibodies and either analyzed for kinase activity by in vitro kinase assay, and for the presence of phosphotyrosine by anti-phosphotyrosine antibody. Lanes 1 and 5: mock transfections, lanes 2 and 6: Kit/ Δ PH ITK alone, lanes 3 and 7: Kit/ Δ PH ITK along with kinase inactive v-Src, lanes 4 and 8: Kit/ Δ PHPRR ITK alone. Lanes 1–4: Labeling of ITK during autophosphorylation (the blot was treated with KOH before exposure), lanes 5–8: the blot was probed with an anti-phosphotyrosine antibody. Bottom panel: The blot was probed with an anti-HA antibody. Arrows indicate the position of the ITK fusions. Expression of v-Src was confirmed using anti-Src antibody 327 (data not shown).

that seen with BTK [35], although this may also reflect a requirement for the SH3 domain in efficiently phosphorylating SAM68.

We then tested whether this difference we saw would also be seen in kinase mutants lacking the PH domain, as it is possible that the PH domain could be targeting these mutants to the membrane and allowing for low level kinase activation, with the PRR regulating this step. We have demonstrated that the PH domain of ITK is sufficient to target a heterologous protein (GFP) to the membrane in the absence of the TH domain, and that there are no differences in membrane targeting of PH vs. PHTH containing GFP fusion proteins [36], A. August, unpublished). In addition, the Δ PR mutant of ITK had WT activity in phosphorylating the membrane bound receptor CD28 (A. August, unpublished). However, to address this, we therefore expressed WT ITK, PH domain deleted ITK (Δ PH ITK), or PH and PRR deleted ITK (Δ PH PR ITK) in 293 cells and determined their ability to autophosphorylate and transphosphorylate SAM68 (Fig. 3A). Expression of the Δ PH mutant resulted in WT activity, while the Δ PH PR mutant had more than 50% reduction in both its autophosphorylation activity and its transphosphorylating activity, suggesting that membrane localization was not a controlling factor in the difference in basal activity of the Δ PR mutant of ITK (the white arrowheads indicate the position of the phosphorylated SAM68, which runs just below the Δ PHPRR ITK mutant on gels). To confirm this, we fused the two mutants Δ PH and Δ PHPR to a membrane receptor (c-Kit) so that both mutants had equal access to the membrane, and measured their basal activity, as well as following dimerization with the ligand for c-kit, SCF (Fig. 3B). In the basal state, the Δ PHPR membrane localized ITK had approximately 50% reduced activity compared to the Δ PH membrane localized ITK, agreeing with our results with the WT and Δ PH kinases. Interestingly, dimerization of the receptor chimeras did not result in increased activity, however, these re-

sults agree with our analysis of the other mutants that the PRR regulates the basal activity of ITK.

These data support the model of SH3/PRR interaction in ITK, but suggest that the PRR may play another role in the regulation of ITK activity, and that mere deletion of this region is not sufficient for deregulation of its activity. Thus our data suggest that the SH3 domain does play a role in down-regulating the activity of ITK, probably by interacting with the PRR, but that the PRR is also important in regulating this activity independently of the SH3 domain.

3.2. Src kinases regulate the basal activity of ITK

Previous analysis of the TH domain of Tec kinases has indicated that they bind to the SH3 domain of a number of other proteins, including Src family kinases [14,37]. As Src kinases also serve to activate ITK, we decided to find out if Src kinases can also regulate the basal activity of ITK. We therefore transfected 293 cells (which express endogenous c-Src) with the membrane localized Δ PH mutant of ITK in the absence or presence of kinase inactive v-Src, and compared its ability to autophosphorylate with that of the membrane localized Δ PHPR mutant (Fig. 4) (we used v-Src kinase inactive mutant because this is a more effective Src dominant negative than a c-Src kinase inactive due to the lack of C-terminal tyrosine in v-Src, and therefore a constitutively open configuration). Expression of kinase inactive Src along with the membrane localized Δ PH ITK mutant (c-Kit/ Δ PHPR ITK) resulted in a reduction in autophosphorylation activity to that seen in the membrane localized Δ PHPR ITK mutant, suggesting that endogenous Src kinases may be interacting with the PRR of ITK to regulate its basal activity, and that removing that region prevents this regulation (Fig. 4). The kinase inactive Src accomplishes the same purpose, interacting with the PRR, without being able to phosphorylate ITK. These data suggest either that Src kinases or some other molecule(s) regulated by Src kinases regulate ITK's basal activity by interacting with its PRR.

3.3. The PRR of ITK regulates the ability of Src kinase Lck to activate ITK

The experiments described above show that the PRR of ITK regulates its basal activity, probably by interacting with Src kinases. As we and others have shown that ITK is activated by Src kinases, we decided to test if the PRR also regulates the ability of Lck (a Src family kinases expressed in T cells) to activate ITK. Fig. 5 shows the results of experiments where we transfected WT ITK or the mutants analyzed in Fig. 2 in the absence or presence of Lck, and analyzed the activation of ITK. These experiments confirm our data in Fig. 2 that the basal activity of the Δ PR mutant of ITK is reduced approximately two-fold compared to the WT (lanes without Lck). In addition, they show that in the presence of Lck, the WT ITK becomes activated, while the Δ PR mutant is not activated, and the Δ SH3 domain mutant was 'superactivatable'. These data suggest that removal of the PRR of ITK affected the ability of Lck to interact with it and therefore activate its enzymatic activity, whereas removal of the SH3 domain resulted in an increased ability of Lck to interact with ITK, allowing for 'superactivation' (note that all fold activation values are corrected for the expression level of the particular mutant as determined by densitometry, and compared to the WT ITK).

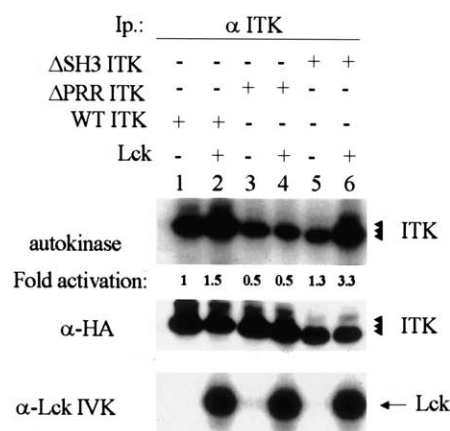


Fig. 5. The PR region of ITK regulates the ability of ITK to be activated by Lck. 293 cells were transfected with either the WT or the indicated mutants of ITK in the presence or absence of Lck. Kinase activity was determined as described. Lanes 1, 3 and 5: ITK or mutant without Lck, lanes 2, 4 and 6: ITK or mutant with Lck. Top panel: The blot was treated with KOH then exposed for autoradiography. Activity was corrected for the expression level of the different mutants and expressed as fold increase or decrease compared to WT ITK alone. Note that the Δ PRR mutant has reduced basal activity and is not activated by Lck. Middle panel: The blot in the top panel was probed with an anti-HA antibody. Bottom panel: Lck kinase expression in the respective cells.

3.4. The PR region affects the ability of ITK to phosphorylate substrates in cells

All of the experiments described above indicate that the basal activity of ITK and ability to be activated by Lck are controlled by its PRR. However, we wanted to determine if it affected the ability of ITK to phosphorylate exogenous substrates in cells. We therefore took advantage of the fact that one substrate for ITK is the enzyme PLC γ [38]. Transfection of PLC γ along with either LCK alone or ITK alone resulted in a low level of tyrosine phosphorylation of PLC γ . However, cotransfecting PLC γ along with LCK and WT ITK, or LCK with Δ SH3 ITK resulted in increased tyrosine phosphorylation of PLC γ (Fig. 6A). By contrast, transfecting PLC γ with LCK and Δ PR ITK resulted in no increases in tyrosine phosphorylation of PLC γ (Fig. 6B). We noted that the Δ SH3 mutant of ITK did not have increased ability to phosphorylate PLC γ , similar to our results in vitro with the SAM68 substrate. These data suggest that perhaps the SH3 domain of ITK is important for its ability to phosphorylate substrates in vitro and in vivo. Indeed, ITK has been reported to interact with PLC γ via its SH3 domain in vivo [39].

These data suggest that the PRR within the TH domain of ITK is important not only in the regulation of the basal activity of ITK, but also for the activation of ITK by Src family kinases, and thus its activation of downstream targets. Indeed, Berg and colleagues recently reported that the PR of ITK was important in its ability to inhibit the activation of NFAT in transfected Jurkat cells [17]. The PRR of BTK has previously been shown to interact with Src kinases in vitro and in the yeast two-hybrid system [14,40]. However, while Yang et al. have analyzed a BTK PRR mutant and showed that a kinase inactive mutant carrying a PRR mutation was defective in being transphosphorylated by an unknown kinase or kinases stimulated by pervanadate, the tyrosine phosphorylation of the WT BTK kinase carrying the mutant PRR was unaffected [40]. In addition, when Fluckiger et al. analyzed the role of the

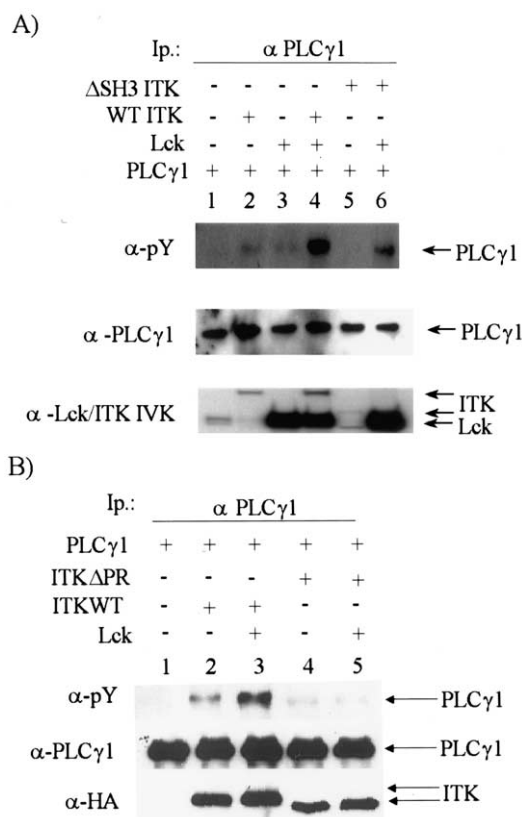


Fig. 6. The PR domain affects Src induced ITK tyrosine phosphorylation of PLC γ 1. A: 293 cells were transfected with PLC γ 1 alone, or with Lck, WT or Δ SH3 mutant of ITK in the presence or absence of Lck. Cells were then lysed 2 days later, PLC γ 1 immunoprecipitated and tested for the presence of phosphotyrosine. Top panel: Lane 1: PLC γ 1 alone, lane 2: PLC γ 1 plus WT ITK, lane 3: PLC γ 1 plus Lck, lane 4: PLC γ 1 plus Lck and WT ITK, lane 5: PLC γ 1 plus Δ SH3 ITK, lane 6: PLC γ 1 plus Δ SH3 ITK and Lck. Blot probed with anti-phosphotyrosine. Middle panel: Blot in top panel probed with anti-PLC γ 1 antibodies. Bottom panel: Expression of Lck and ITK mutants in the respective cells. B: 293 cells were transfected with PLC γ 1 alone, or with WT ITK in the presence or absence of Lck, or Δ PRR mutant of ITK in the presence or absence of Lck. Cells were then lysed 2 days later, PLC γ 1 immunoprecipitated and tested for the presence of phosphotyrosine. Top panel: Lane 1: PLC γ 1 alone, lane 2: PLC γ 1 plus WT ITK, lane 3: PLC γ 1 plus Δ PRR ITK and Lck, lane 4: PLC γ 1 plus Δ PRR ITK, lane 5: PLC γ 1 plus Δ PRR ITK and Lck. Blot probed with anti-phosphotyrosine antibody. Middle panel: Blot in top panel probed with anti-PLC γ 1 antibodies. Bottom panel: Expression of ITK mutants in the respective cells. Lck expression was confirmed by specific Lck antibodies (data not shown).

PRR of BTK in phosphorylating being activated and phosphorylating PLC γ 2, they found that this mutant was not defective in being activated by Lyn or in phosphorylating PLC γ 2 in vivo, suggesting that BTK may be less dependent than ITK on the PRR for its activation and function as a kinase [41]. We also note that the TH domain is also the target of the G α regulation of BTK, although the region involved is the N-terminal region of the TH domain and Δ PR mutants retain the ability to be activated by G α [42]. We propose that the TH domain of ITK and other Tec family kinases may serve as an important module for the regulation of both the basal activity as well as the ability to be activated by upstream effectors.

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