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# Tec Kinase Signaling in T Cells Is Regulated by Phosphatidylinositol 3-Kinase and the Tec Pleckstrin Homology Domain<sup>1</sup>

Wen-Chin Yang,\* Keith A. Ching,<sup>†</sup> Constantine D. Tsoukas,<sup>†</sup> and Leslie J. Berg<sup>2\*</sup>

**Tec, the prototypical member of the Tec family of tyrosine kinases, is abundantly expressed in T cells and other hemopoietic cell types. Although the functions of Itk and Txk have recently been investigated, little is known about the role of Tec in T cells. Using antisense oligonucleotide treatment to deplete Tec protein from primary T cells, we demonstrate that Tec plays a role in TCR signaling leading to *IL-2* gene induction. Interestingly, Tec kinases are the only known family of tyrosine kinases containing a pleckstrin homology (PH) domain. Using several PH domain mutants overexpressed in Jurkat T cells, we show that the Tec PH domain is required for Tec-mediated *IL-2* gene induction and TCR-mediated Tec tyrosine phosphorylation. Furthermore, we show that Tec colocalizes with the TCR after TCR cross-linking, and that both the Tec PH and Src homology (SH) 2 domains play a role in this association. Wortmannin, a phosphatidylinositol 3-kinase inhibitor, abolishes Tec-mediated *IL-2* gene induction and Tec tyrosine phosphorylation, and partially suppresses Tec colocalization with the activated TCR. Thus, our data implicate the Tec kinase PH domain and phosphatidylinositol 3-kinase in Tec signaling downstream of the TCR. *The Journal of Immunology*, 2001, 166: 387–395.**

Stimulation of the TCR on mature T cells induces a series of biochemical events resulting in the induction of new gene transcription in the activated cell. The initiating signals are the activation of protein tyrosine kinases, including members of the Src family, Syk/Zap-70 family, and Tec family. Subsequent events are the phosphorylation of substrates for these tyrosine kinases, and the modification or metabolism of membrane phospholipids mediated by enzymes such as phosphatidylinositol 3-kinase (PI3K)<sup>3</sup> and phospholipase C (PLC)- $\gamma$ 1. These signals eventually lead to the production of second messengers, such as diacylglycerol and inositol trisphosphate, and to the activation of small GTP-binding proteins, such as p21<sup>ras</sup> (1, 2). The ultimate outcome of TCR signaling is the activation of transcription factors leading to new gene expression. In primary resting T cells, a hallmark of activation is the induction of the *IL-2* gene (3).

The Tec family of tyrosine kinases is now the second largest subgroup of cytoplasmic protein tyrosine kinases, consisting of Tec, Btk/Bpk/Atk/Emb, Itk/Tsk/Emt, Txk/Rlk, and Bmx/Etk in mammalian cells (4, 5). These kinases share a similar overall structure. At their amino terminus, all members of this family except

Txk contain a pleckstrin homology (PH) domain; for Btk, this domain has been shown to bind inositol phosphates in vitro (6–8) and to be responsible for membrane localization in vivo (9). Tec kinases also share a Tec homology domain containing a region homologous to the GTPase-activating protein 1 family of Ras GTPases at its amino-terminal end and one or two proline-rich motifs at its carboxyl-terminal end (10). In addition, all Tec kinases have a single Src homology (SH) 3 domain, a single SH2 domain, and a kinase domain.

Only three of the Tec kinases are known to be expressed in T cells. Two of them, Itk and Txk, are predominantly expressed in T cells, whereas Tec is more broadly distributed among all hemopoietic cell types, including T cells (11–13). Despite many biochemical studies linking Tec kinases with numerous cell surface receptors (14), the precise role(s) of Tec kinases in T cell signal transduction are not well understood. Gene-targeting studies first demonstrated that Itk plays a role in TCR signaling leading to *IL-2* gene induction, and indicated a role for Itk in the activation of PLC- $\gamma$ 1 leading to calcium mobilization (15, 16). More recent studies have suggested that Txk may have a similar function to Itk in T cells (17, 18). Additional studies have also suggested a specific role for Itk in the differentiation and function of Th2 effector cells (19) and for Txk in Th1 effector cells (20). Although Tec has been implicated in cytokine receptor signaling in nonlymphoid cells (21), little is known about the role of Tec in T cells. The data that has been generated, implicating Tec in both TCR and CD28 signaling, has predominantly relied on transient transfections and overexpression systems (13, 22).

The Tec family of tyrosine kinases is the only known family of kinases containing a PH domain. Numerous functions of this domain have been proposed, involving binding to both protein and phospholipid ligands. For instance, the PH domain of Btk has been shown to interact with protein kinase C (23) and actin (24), that of Bmx binds to the protein tyrosine phosphatase D1 (25), and both Itk and Btk PH domains bind to the  $\beta\gamma$  subunits of heterotrimeric G proteins (26, 27). In addition, the Itk and Btk PH domains have been shown to preferentially interact with inositol compounds phosphorylated at the D-3 position, such as phosphatidylinositol

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<sup>3</sup> Abbreviations used in this paper: PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; PLC, phospholipase C; SH, Src homology; PtdInsP<sub>3</sub>, phosphatidylinositol 3,4,5 trisphosphate; GAM, goat anti-mouse; GFP, green fluorescence protein; Jtag, SV40 T Ag-transfected Jurkat; MCC, moth cytochrome c.

3,4,5 trisphosphate (PtdInsP<sub>3</sub>) and inositol 1,3,4,5 tetrakisphosphate (InsP<sub>4</sub>) (6–9, 28, 29).

In particular, the phospholipid interactions of the PH domain are thought to play an important role in recruiting Tec family kinases to the plasma membrane, thus facilitating their proximity to upstream activators (e.g., Src family kinases) and downstream substrates (5). Supporting the importance of the PH domain in Tec family kinase function, point mutations in the Btk PH domain lead to immunodeficiency diseases in both mouse and human (*xid* and X-linked agammaglobulinemia, respectively; Refs. 30, 31). In particular, substitution of the conserved arginine at residue 28 of the Btk PH domain, resulting in reduced PH domain binding to phospholipids, leads to a loss of Btk function in B cells (8, 28, 32). Unexpectedly, a gain-of-function Btk mutant with transforming activity in fibroblast cells also results from a point mutation in the Btk PH domain (E41K); interestingly, this substitution leads to increased binding of the PH domain to phosphoinositides, and to increased Btk kinase activity in NIH 3T3 cells (9). In contrast to these data on the Btk PH domain, a point mutation in the Bmx PH domain (E42K, comparable to E41K in Btk) has been shown to result in decreased Bmx kinase activity in vivo (33). These results suggest that the PH domains of individual Tec kinase family members may differ in their function and/or binding specificities.

In addition to the biochemical data addressing the binding interactions of Tec kinase PH domains, functional data also support the importance of the PH domain in regulating Tec-kinase activity in cells. As mentioned, PH domain mutations can cause the loss of Btk function in B cells. Furthermore, the PH domain of Itk recently has been shown to be important for colocalization of Itk with the TCR complex (34). The phospholipid modifying enzyme, PI3K, has also been implicated in the regulation of Tec family kinases. PI3K is capable of phosphorylating membrane phospholipids at the D-3 position of the inositol ring, thus creating the preferred phospholipid ligand for the Btk PH domain (35). In this regard, activation of Itk by a Src family kinase has been shown to require the Itk PH domain and PI3K activity (32). In addition, overexpression of PI3K $\gamma$ , together with a Src family kinase, can directly activate Btk in fibroblast cells (36). Together, these data suggest that PI3K plays a role in regulating Tec-kinase activity downstream of an activated receptor.

To determine the role of Tec in primary T cells, we examined the consequences of Tec protein depletion on TCR-mediated IL-2 production. We show that reduced Tec leads to a diminished response to TCR stimulation. We also show that overexpression of Tec in Jurkat T cells leads to enhanced TCR signaling, and furthermore that Tec colocalizes with the activated TCR. This activity of Tec requires an intact Tec PH domain and depends on PI3K function. These data implicate Tec in the TCR signaling pathway and emphasize the role of the Tec PH domain in this functional activity.

## Materials and Methods

### *Abs and cell culture*

The PY20 anti-phosphotyrosine mAb and anti-Txk antiserum (M20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tec polyclonal antiserum and the 4G10 anti-phosphotyrosine mAb were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-Itk mAb (2F12) has been reported previously (36). Anti-human CD3 mAb UCHT1, anti-mouse IL-2 mAbs, and peroxidase-conjugated streptavidin were purchased from PharMingen (San Diego, CA). The C305 Ab-producing hybridoma was a gift from Dr. A. Weiss (37). The OKT3 Ab-producing hybridoma was obtained from American Type Culture Collection (Manassas, VA). PE-conjugated goat-anti-rabbit IgG, FITC-conjugated streptavidin, and Texas Red-conjugated goat anti-mouse (GAM) IgG were purchased from Molecular Probes (Eugene, OR). Anti-FLAG and GAM antiserum were purchased from Sigma (St. Louis, MO). Murine splenocytes, human leukemic Jurkat cells, and SV40 T Ag-transfected Jurkat

(Jtag) cells were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 2-ME (50  $\mu$ M), sodium pyruvate (1 mM), and glutamate (292  $\mu$ g/ml). COS-7 cells were maintained in DMEM medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and glutamate (292  $\mu$ g/ml).

### *Antisense oligonucleotide*

Synthetic oligonucleotides (phosphorothioate-2'-O-methyl RNA chimera) were purchased from Oligos Etc. (Wilsonville, OR). The Tec anti-sense sequence is 5'-TGAAATTCATCTCGGTCT-3', and the control Tec sense sequence is 5'-AGACCGAGATGAATTTCA-3'. In addition to the sequence complementary to the ATG (common to all mRNAs), the Tec anti-sense oligonucleotide shares only 4 and 5 of 18 nucleotides in common with the sequences complementary to the Itk and Txk mRNAs, respectively.

### *Tec constructs*

The full-length murine Tec cDNA was amplified by PCR using the following primers; forward, 5'-CCAGAAGACCGAGATGAATTTCAAC3'; backward, 3'-AAACTGAGGCCACCATTCATCTTCC-3'. A Flag-epitope tag (DYKDDDDK) was added to the 5' end of the Tec cDNA sequence, and the clone was introduced into the PME18S vector, producing pME18S-WT-Tec (34). pME18S- $\Delta$ PHTec, containing the Tec cDNA lacking the PH domain, has a deletion of aa 1–110 of the Tec protein sequence, and was derived from PME18S-WT-Tec by PCR. The plasmid pME18S- $\Delta$ PHSH2\**Tec* encodes a Tec protein lacking the PH domain, and including a substitution of Ala for Arg at residue 272 of the SH2 domain. pME18S-E42K*Tec* encodes a Tec protein with a single amino acid substitution at residue 42 in the PH domain (EgK), whereas PME18S-R29C*Tec* has a substitution at residue 29 in the PH domain (RgC). Point mutations were generated with the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. green fluorescence protein (GFP) fusion protein expression vectors, pWTTec-GFP, p $\Delta$ PHTec-GFP, pE42K*Tec*-GFP, pR29C*Tec*-GFP, and p $\Delta$ PHSH2\**Tec*-GFP, were generated by excising the Tec cDNAs from pME18S-WT-Tec, pME18S- $\Delta$ PHTec, pME18SE42K-Tec, pME18S-Tec, and pME18S- $\Delta$ PHSH2\**Tec*, respectively, and introducing them into the vector, pEGFP-N3 (Clontech, Palo Alto, CA). GST fusion protein expression vectors, pWTBtkPH, pR28CBtkPH, and p41KPH, contain wild-type or mutant versions of the murine Btk PH domain (aa 6–217) in pGEX (Amersham Pharmacia Biotech, Piscataway, NJ) and have been described previously (8). GST fusion protein expression vectors, pWTTecPH, pR29C*Tec*PH, and pE42KPH, contain the murine Tec PH domain (aa 5–187) in pGEX. The sequences of all Tec cDNAs generated by PCR or site-directed mutagenesis were verified by DNA sequencing. Plasmid pIL-2-Luc is composed of 2 kb of the murine IL-2 promoter region driving expression of the firefly luciferase reporter gene, and was a gift from Dr. T. Yokota (38). The pRL-TK vector is composed of the Herpes simplex virus thymidine kinase promoter driving expression of the *Renilla* luciferase reporter gene (Promega, Madison, WI).

### *Stimulation of primary T cells*

Splenocytes from 5C.C7 TCR-transgenic mice (39) were isolated and subjected to RBC lysis. Cells were preincubated in medium for 2 h and then incubated in the presence or absence of 10  $\mu$ M of synthetic oligonucleotide (anti-sense Tec or sense Tec) for 8 h. Cells were then stimulated with 1  $\mu$ M moth cytochrome *c* (MCC) peptide (residues 92–103, the cognate Ag for the 5C.C7 TCR; Ref. 39) for an additional 24 h. The quantity of IL-2 present in the supernatants was measured by ELISA.

### *Jurkat cell stimulations, immunoprecipitations, and immunoblots*

Jtag cells were stimulated at a concentration of 10<sup>7</sup> cells/ml at 37°C for 2 min with 10  $\mu$ g of UCHT1 (anti-human CD3e) followed by 15  $\mu$ g of GAM antiserum for 5 min at 37°C. For unstimulated controls, 10<sup>7</sup> cells/ml were incubated in medium alone at 37°C for 2 min, followed by 30  $\mu$ g of GAM antiserum for 5 min at 37°C. Postnuclear lysates were immunoprecipitated and/or immunoblotted as previously described (13).

### *Transfections and luciferase assays*

Jtag cells (10<sup>7</sup>) were electroporated at 960  $\mu$ F and 260 V (Bio-Rad Gene Pulser) with 10  $\mu$ g of pIL-2-Luc, 1  $\mu$ g of pRL-TK, and 10  $\mu$ g of additional DNA expressing Tec or Tec mutants. After a 2-h recovery, the cells were cultured in medium alone or stimulated for 6 h with C305 mAb (anti-TCR, 1:100 dilution of ascites) plus PMA (50 ng/ml). Cells were then washed and lysed, and the protein concentration of the lysate was quantitated with the Bradford reagent (Bio-Rad, Hercules, CA). Ten micrograms of cell lysate was subjected to the dual luciferase reporter assay (Promega), according to the manufacturer's instructions. The efficiency of transfection,

as determined by *Renilla* luciferase activity in the lysate, was used to normalize the activity of firefly luciferase. The normalized firefly luciferase activities are presented in arbitrary units.

### Confocal microscopy

To examine the colocalization of the TCR/CD3 complex with the Tec-GFP fusion proteins, Jtag cells ( $2 \times 10^7$ ) were electroporated with Tec-GFP expression vectors (20  $\mu$ g) at 960  $\mu$ F and 260 V. After 48 h, cells were incubated for 30 min on ice in 250  $\mu$ l of culture medium containing 40  $\mu$ g/ml OKT3 mAb. After washing, the cells were incubated for 30 min on ice in medium containing 20  $\mu$ g/ml Texas Red-conjugated GAM IgG. The cells were then incubated at 37°C (stimulated) or on ice (nonstimulated) for 10 min. Following several washes, the cells were placed on poly-L-lysine-coated glass slides and fixed with 2% paraformaldehyde in PBS. The slides were then mounted with Prolong anti-Fade kit (Molecular Probes), according to the manufacturer's instructions. Confocal images were generated as previously described (34).

### In vitro binding to [ $^3$ H]dioctanoyl PtdIns $P_3$

GST fusion proteins were produced according to the manufacturer's instructions (Amersham Pharmacia Biotech). For in vitro binding assays, 20  $\mu$ g of GST fusion proteins bound to glutathione beads were incubated with 20,000 cpm of [ $^3$ H]dioctanoyl PtdIns  $P_3$  ( $\sim 0.4$  nmol) for 1 h at room temperature (8). Following several washes with HNE buffer (30 mM HEPES, pH 7.0, 100 mM NaCl, 1 mM EDTA) containing 0.5% Nonidet P-40, the beads and supernatants were separated by centrifugation and counted in a Beckman scintillation counter in scintillation cocktail. The relative binding activity was obtained by normalizing the cpm bound to the beads to GST fusion protein quantitation as determined by Coomassie Blue staining.

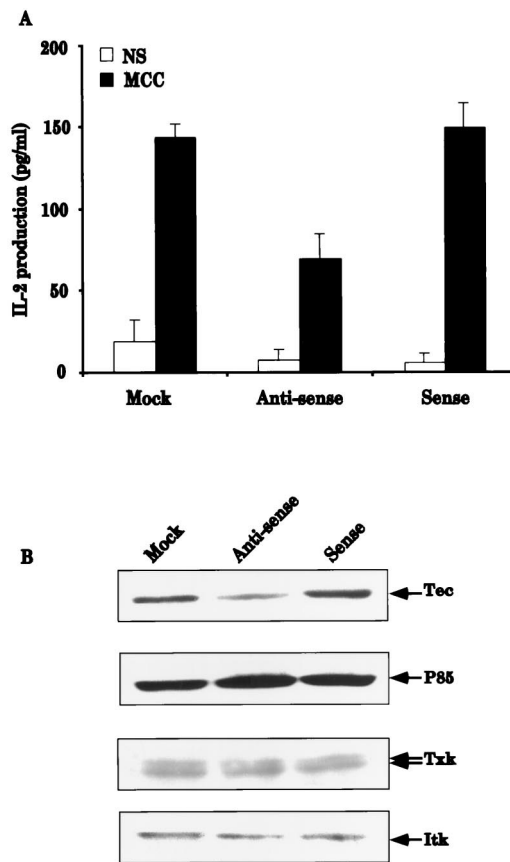
## Results

### The Tec kinase is involved in TCR-mediated IL-2 production in primary T cells

Three Tec family tyrosine kinase members are expressed in T cells: Itk, Txk, and Tec. Although the roles of Itk and Txk in T cells have been addressed (15, 17–19, 40–42), no data on the role of Tec in primary T cells has been reported. Based on the known roles of Itk and Txk in TCR signaling leading to IL-2 production, we examined whether depletion of Tec protein from primary T cells would affect this signaling pathway. For these experiments, we used T cells isolated from the 5C.C7 TCR-transgenic line (43). The 5C.C7 TCR is specific for a peptide (92–103) of MCC bound to the MHC class II molecule I-E<sup>k</sup>. Total splenocytes isolated from H-2<sup>k</sup> 5C.C7-transgenic mice include both T cells as well as Ag-presenting cells capable of binding and presenting the MCC peptide.

To deplete the Tec protein, splenocytes from 5C.C7 TCR-transgenic mice were incubated with a Tec anti-sense oligonucleotide complementary to the 5' end of the mRNA. This sequence is quite specific for Tec, as it shares only 7 and 8 of 18 nucleotides in common with the sequences complementary to the Itk and Txk mRNAs, respectively (three of which are the ATG, common to all mRNAs). As controls, cells were incubated in medium alone, or in the presence of a Tec sense oligonucleotide. Following oligonucleotide incubation, splenocytes were stimulated by addition of the MCC peptide at a concentration of 1  $\mu$ M, and IL-2 secreted into the supernatants was measured 24 h later. As shown in Fig. 1A, Tec anti-sense oligonucleotide treatment reduced IL-2 production by activated splenic T cells in comparison to untreated cells, or to cells treated with the Tec sense oligonucleotide. This reduced IL-2 production was not due to diminished cell viability after anti-sense oligonucleotide treatment (data not shown), and instead, correlated with reduced levels of the Tec protein (Fig. 1B). In contrast, no decrease was observed in the levels of the related family members Itk and Txk (Fig. 1B).

To further assess the role of the Tec protein in TCR-mediated IL-2 gene induction, we also transiently overexpressed Tec in hu-



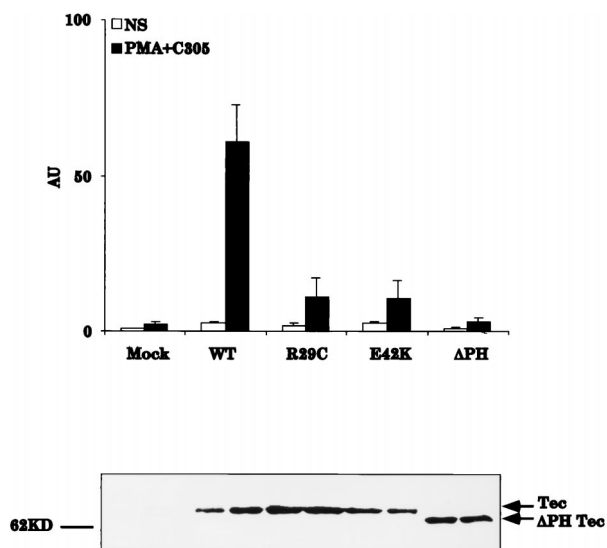
**FIGURE 1.** Tec depletion leads to a decrease in TCR-mediated IL-2 production in primary T cells. **A**, Total 5C.C7 splenocytes were incubated with medium containing 10  $\mu$ M anti-sense Tec (Anti-sense) or sense Tec (Sense) oligonucleotides or in medium alone (Mock) for 8 h. Cells were then stimulated in the presence (MCC) or absence (NS) of 1  $\mu$ M MCC peptide for another 24 h, and IL-2 in the supernatants was measured by ELISA. **B**, Postnuclear lysates from the cultured splenocytes were fractionated and immunoblotted with the anti-Tec Ab. Following stripping, the membrane was probed with anti-p85 (PI3K) Ab as a loading control. Equivalent amounts of total lysate were fractionated and immunoblotted with Abs to the related family members, Txk and Itk. Data are representative of three experiments.

man Jtag cells (a derivative of Jurkat cells expressing SV-40 large T Ag). IL-2 promoter activity was assessed by cotransfection of an IL-2 promoter-reporter construct driving luciferase activity. As shown in Fig. 2, treatment of Jtag cells with anti-TCR Ab plus PMA leads to a modest increase in IL-2 promoter activity compared with unstimulated cells. Interestingly, overexpression of Tec results in a 30-fold increase in TCR-mediated IL-2 promoter activity compared with cells transfected with the reporter construct alone. Together, these data strongly support a role for Tec in TCR-mediated IL-2 gene induction.

### The Tec PH domain is required for enhanced TCR signaling

It is well known that point mutations in the PH domain of Btk lead to immunodeficiency diseases in humans and mice, thus demonstrating the important role of the Btk PH domain (44). However, several studies have indicated differences in the functional properties of PH domains among the Tec kinase family members, suggesting that PH domains of individual Tec kinases may have distinct roles in signaling (6, 9, 33). To determine the role of the Tec PH domain in TCR signaling, we generated mutant forms of the Tec protein containing alterations in the PH domain. In addition to





**FIGURE 2.** Wild-type Tec, but not PH domain mutants, enhance TCR-mediated IL-2 promoter activity in Jurkat cells. Jtag cells were electroporated with vector alone or the indicated Tec constructs as follows: pME18S (Mock), pME18S-WTTec (WT), pME18S-R29CTec (R29C), pME18S-E42KTec (E42K), or pME18S-ΔPHTec (ΔPH). Cells were cotransfected with pIL-2-Luc and pRL-TK. After a 2-h recovery, the cells were left unstimulated (NS) or were stimulated for 6 h with PMA plus the C305 mAb (PMA+C305). Ten micrograms of cell lysate was subjected to the dual luciferase reporter assay. IL-2 promoter activity (*top*) is indicated in arbitrary units (AU) obtained from the ratio of firefly luciferase activity to *Renilla* luciferase activity in the lysates. Lysates were fractionated and immunoblotted with the anti-FLAG mAb (*bottom*). Data are representative of three experiments.

a total deletion of the PH domain (Δ1–110), Tec proteins were generated with single amino acid substitutions in the PH domain. One mutation, R29C, is based on the mutation in the Btk PH domain found in *xid* mice. Substitutions at this position abolish the binding of the Btk PH domain to phosphoinositides (7, 8). A second mutation, E42K, is also based on a mutation first described in the Btk PH domain. In this latter case, substitution of lysine for glutamic acid at position 41 of Btk results in increased binding of the PH domain to phosphoinositides and to increased Btk kinase activity (9).

Each of these mutant forms of Tec was overexpressed in Jtag cells together with the IL-2 promoter reporter construct. As shown in Fig. 2, each of the mutations in the Tec PH domain interfered with the ability of Tec overexpression to dramatically enhance TCR-mediated IL-2 promoter activity. Although point mutations in the Tec PH domain (R29C or E42K) led to a severe reduction in Tec function, deletion of the PH domain (ΔPH) totally abolished Tec kinase-mediated IL-2 promoter activity following TCR stimulation. Of note, the E42K substitution in the Tec PH domain did not appear to increase Tec function as might have been expected based on the known activity of this mutant form of Btk. Overall, these results indicate the important role of the Tec PH domain in enhancing Tec-mediated IL-2 promoter activity in response to TCR signaling.

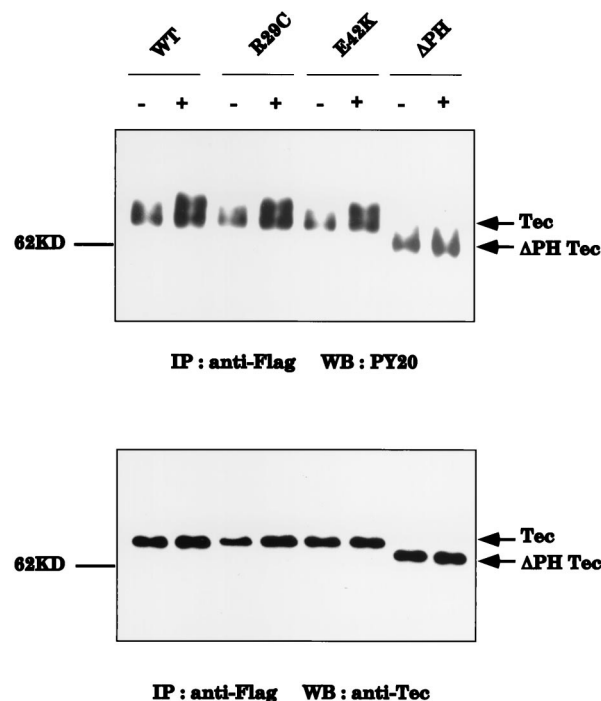
#### *The Tec PH domain is required for TCR-induced tyrosine phosphorylation of Tec*

As described above, mutation of the Tec PH domain interferes with Tec function downstream of the TCR. To determine whether this loss of function is associated with a lack of Tec activation following TCR stimulation, we examined TCR-induced tyrosine

phosphorylation of Tec. Wild-type or mutant forms of Tec were expressed in Jtag cells following transient transfection. Cells were then stimulated by anti-TCR cross-linking, and the transfected Tec protein was immunoprecipitated using an Ab specific for the epitope tag (anti-FLAG). As shown in Fig. 3, the tyrosine phosphorylation of wild-type Tec is substantially increased after TCR stimulation. A similar increase in tyrosine phosphorylation is also observed for Tec proteins carrying single amino acid substitutions in the PH domain (R29C and E42K). In contrast, complete deletion of the Tec PH domain abolishes the TCR-induced increase in tyrosine phosphorylation.

#### *Both the R29C and E42K substitutions in the Tec PH domain decrease binding to PtdInsP<sub>3</sub>*

Previous studies have examined the specificity and affinity of PH domain interactions with inositol lipids and soluble inositol phosphates. For Tec family kinases, a strong correlation has been established between protein function and PH domain binding to phosphatidylinositides phosphorylated at the D-3 position of the inositol ring (6–9, 28, 29). These studies have strongly implicated the PH domain in membrane targeting of Tec kinases, thus establishing the importance of Tec kinase recruitment to the site of an activated receptor, and indicating the role of PI3K in Tec kinase activation (32, 36). The vast majority of these studies have been performed on Btk, owing in part to the fact that a three-dimensional structure of the Btk PH domain has been determined (45). These data have demonstrated that substitutions at residue R28 in Btk (comparable to R29 in Tec) dramatically reduce binding of the Btk PH domain to D3 phosphoinositides, whereas substitution of



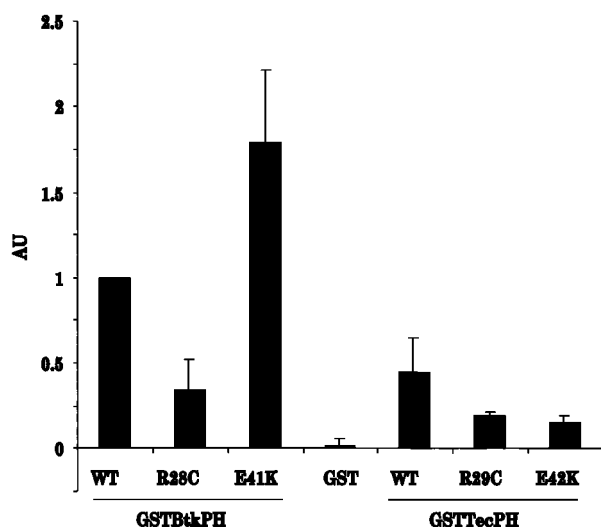
**FIGURE 3.** The PH domain is important for Tec tyrosine phosphorylation after TCR/CD3 stimulation. Jtag cells were electroporated with pME18S-WTTec (WT), pME18S-R29CTec (R29C), pME18S-E42KTec (E42K), or pME18S-ΔPHTec (ΔPH). After 24 h, cells were left unstimulated (–) or were stimulated (+) with UCHT1 mAb for 3 min followed by cross-linking with GAM for 5 min. Total lysates were immunoprecipitated with anti-FLAG mAb and blotted with anti-phosphotyrosine mAb, PY20 (*top*). Following stripping, the membrane was reprobed with anti-Tec Ab (*bottom*). Data are representative of two experiments.

lysine at residue E41 (comparable to E42 in Tec) increases binding. In a recent report, Okoh and Vihinen have presented models of the other Tec kinase PH domains based on the structure of the Btk PH domain (46). Interestingly, these models predict striking differences in the binding pockets between different Tec family PH domains. Nonetheless, these authors predicted that substitutions at residue E41(E42) would also generate gain-of-function mutants in other Tec family kinases as a result of increased PH domain binding to the membrane. Given our functional data indicating a loss of Tec activity with both the R29C and E42K substitutions, we chose to directly assess binding of the wild-type and mutated Tec PH domains to phosphoinositides.

For these experiments we used a radioactively labeled water-soluble form of  $\text{PtdInsP}_3$ , as described previously (8). This compound was incubated with GST fusion proteins containing wild-type or mutated Tec kinase PH domains. To directly compare the Tec PH domain binding to that of Btk PH domains, we also tested three GST fusion proteins containing wild-type Btk PH, Btk PH (R28C), or Btk PH (E42K). After incubation, the immobilized GST fusion proteins were washed, and the amount of labeled compound binding to each fusion protein was determined. Fig. 4 shows the results of a representative experiment indicating binding activities relative to the wild-type Btk PH domain. As has previously been reported, substitution of R28C in the Btk PH domain reduces binding to  $\text{PtdInsP}_3$  (8), whereas substitution of E41K dramatically increases binding. The wild-type Tec PH domain binds  $\text{PtdInsP}_3$  but to a lesser degree than the wild-type Btk PH domain, as was previously reported (6). Interestingly, both the R29C and E42K substitutions in the Tec PH domain substantially reduce binding to  $\text{PtdInsP}_3$ . These data correlate well with the functional data and indicate that both of these mutations are likely to reduce membrane localization of the Tec kinase.

#### *Tec colocalizes with the TCR/CD3 complex after TCR stimulation*

To further assess the role of the Tec kinase in TCR signaling, we examined whether the subcellular localization of Tec is altered by



**FIGURE 4.** Mutations in the Tec PH domain decrease binding to  $\text{PtdInsP}_3$ . Twenty micrograms of Btk or Tec PH domain fusion proteins bound to glutathione-agarose beads were incubated with 20,000 cpm of  $[\text{H}^3]$ diocanoyl  $\text{PtdInsP}_3$  ( $\sim 0.4$  nmol) for 1 h at room temperature. After washing, the beads were counted in a scintillation counter. The relative activity (AU) was calculated as the ratio of cpm bound to the beads containing PH domains to cpm bound to 20  $\mu\text{g}$  of GST fusion protein alone. Data represent the means of three independent experiments.

TCR stimulation. Furthermore, because previous studies had indicated that Itk colocalizes with the TCR/CD3 complex after activation, we were interested to determine whether Tec is also recruited to the activated TCR. For these studies, wild-type Tec or individual Tec mutants were fused to GFP to allow monitoring of the subcellular localization of the protein by confocal microscopy. We first determined that fusion of Tec to GFP did not interfere with Tec function by confirming that tyrosine phosphorylation of the Tec-GFP fusion protein was induced normally after TCR stimulation (data not shown).

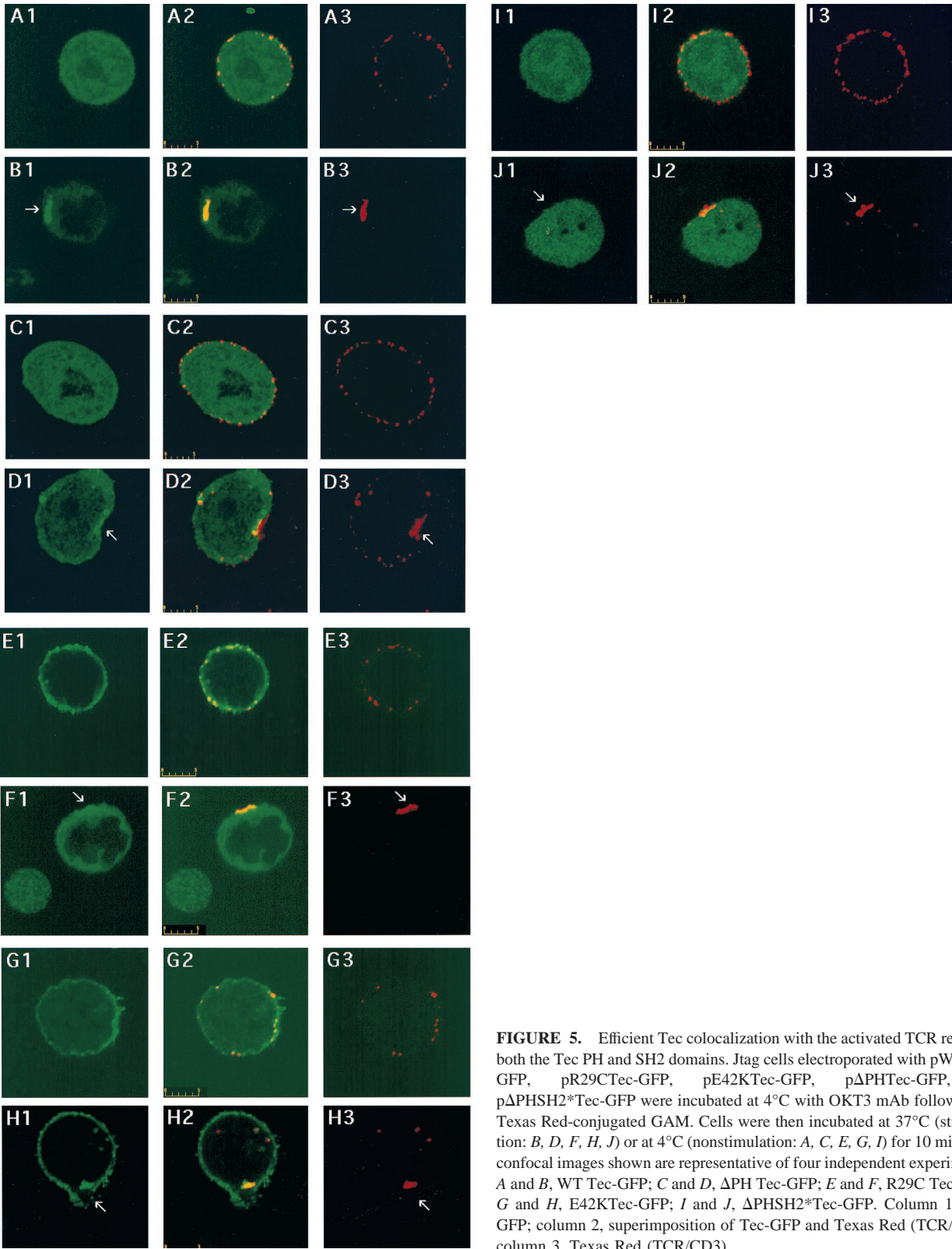
As shown in Fig. 5, wild-type Tec is clearly recruited to the capped TCR/CD3 complex after TCR cross-linking (Fig. 5, A and B). Interestingly, deletion of the Tec PH domain impaired the colocalization of Tec-GFP with the activated TCR, but did not completely abolish Tec recruitment (Fig. 5, C and D). The Tec R29C and E42K mutants were also somewhat reduced in their ability to colocalize with the activated TCR compared with wild-type Tec (Fig. 5, E–H). These results indicate that while the PH domain is playing some role in Tec recruitment to the activated TCR, an additional domain(s) of Tec must also be contributing to TCR colocalization.

Another domain of the Tec kinase that might contribute to colocalization with the activated TCR is the Tec SH2 domain, as this domain is expected to interact with tyrosine-phosphorylated proteins. Thus, the vicinity of the activated TCR may recruit Tec via the high concentration of potential binding partners for the Tec SH2 domain. To test this possibility, we generated a double mutant of Tec, completely lacking the PH domain, and, in addition, carrying a point mutation in the SH2 domain (R272A). Specifically, the conserved arginine in the SH2 domain was substituted with alanine; in other SH2 domains, a comparable substitution completely abolishes binding to phosphorylated tyrosine residues (47, 48). This PH/SH2 domain double mutant of Tec was also fused to GFP ( $\Delta\text{PHSH2}^*\text{Tec-GFP}$ ). When introduced into Jtag cells, the PH/SH2 domain double mutant completely failed to colocalize with the activated TCR (Fig. 5, I and J). These results indicate that the Tec PH domain, as well as the SH2 domain, is involved in the recruitment of Tec to the activated TCR/CD3 complex.

#### *Inhibition of PI3K abolishes Tec signaling in response to TCR stimulation*

PI3K has been implicated in TCR-mediated *IL-2* gene induction (49, 50) and in the activation of Tec family kinases such as Btk, Itk, and Bmx (32, 33, 36). Our functional and biochemical data indicated that the Tec PH domain is crucial for Tec function, and that this domain may regulate Tec activity by mediating binding to phosphoinositides in the plasma membrane. For these reasons, we chose to investigate the role of PI3K in regulating Tec kinase signaling. Wortmannin, a specific inhibitor of PI3K, was used for these studies. As shown in Fig. 6, treatment of Jtag cells with wortmannin completely abolished the Tec-mediated induction of the *IL-2* promoter in response to TCR stimulation. These results suggested that PI3K plays an important role in regulating Tec kinase activity after TCR cross-linking.

To further investigate the role of PI3K in regulating Tec, we assessed whether wortmannin treatment affects the tyrosine phosphorylation of Tec. For these studies, wild-type Tec was expressed in COS cells. In these cells, Tec is constitutively phosphorylated on tyrosine, presumably as a function of Tec kinase autophosphorylation activity. Wortmannin treatment of the COS cells resulted in a substantial decrease in Tec tyrosine phosphorylation (Fig. 7). Finally, we examined whether wortmannin treatment would interfere with Tec colocalization with the activated TCR in Jtag cells



**FIGURE 5.** Efficient Tec colocalization with the activated TCR requires both the Tec PH and SH2 domains. Jtag cells electroporated with pWTTec-GFP, pR29CTec-GFP, pE42KTec-GFP, pΔPHTec-GFP, or pΔPHSH2\**Tec*-GFP were incubated at 4°C with OKT3 mAb followed by Texas Red-conjugated GAM. Cells were then incubated at 37°C (stimulation: *B, D, F, H, J*) or at 4°C (nonstimulation: *A, C, E, G, I*) for 10 min. The confocal images shown are representative of four independent experiments. *A* and *B*, WT *Tec*-GFP; *C* and *D*, ΔPH *Tec*-GFP; *E* and *F*, R29C *Tec*-GFP; *G* and *H*, E42K *Tec*-GFP; *I* and *J*, ΔPHSH2\**Tec*-GFP. Column 1, *Tec*-GFP; column 2, superimposition of *Tec*-GFP and Texas Red (TCR/CD3); column 3, Texas Red (TCR/CD3).

because *Tec* recruitment is partially dependent on the *Tec* PH domain. As shown in Fig. 8, we found that wortmannin treatment substantially inhibited *Tec* colocalization with the activated TCR/CD3 complex. Taken together, these three lines of evidence strongly support the conclusion that *Tec* is regulated by PI3K during TCR signaling.

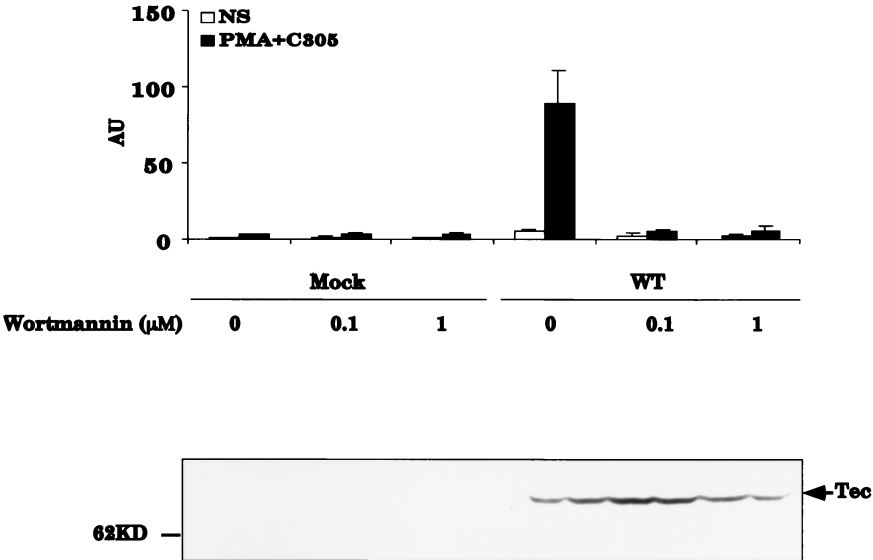
**Discussion**

*The function of the *Tec* kinase in *T* cells*

In this report, we demonstrate that the *Tec* kinase plays a role in TCR-mediated stimulation of IL-2 production in primary *T* cells. This result is consistent with previous studies that showed that



**FIGURE 6.** Wortmannin treatment abolishes Tec-mediated induction of IL-2 promoter activity after TCR stimulation. Jtag cells were electroporated with pME18S (Mock) or pME18S-WTtec (WT) plus pIL-2-Luc and pRL-TK. After a 2-h recovery, the cells were left unstimulated (NS) or were stimulated for 6 h with PMA plus C305 mAb (PMA+C305) in the presence of the indicated concentrations of wortmannin. Cell lysate (10  $\mu$ g) was subjected to dual luciferase reporter assay (*top*). Cell lysates were fractionated and immunoblotted with the anti-FLAG mAb (*bottom*). Data are representative of three experiments.

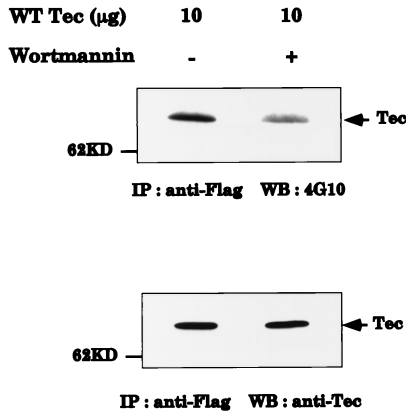


overexpression of Tec in Jurkat T cells could enhance TCR-mediated IL-2 gene induction (13, 22). Furthermore, we show that the Tec PH domain is important for Tec signaling leading to IL-2 gene induction, Tec tyrosine phosphorylation, and colocalization of Tec with the activated TCR. Finally, we provide evidence implicating PI3K in regulating Tec signaling downstream of the TCR. Thus, to date, three Tec kinase family members, Itk, Txk, and Tec, have been shown to play a role in TCR signaling.

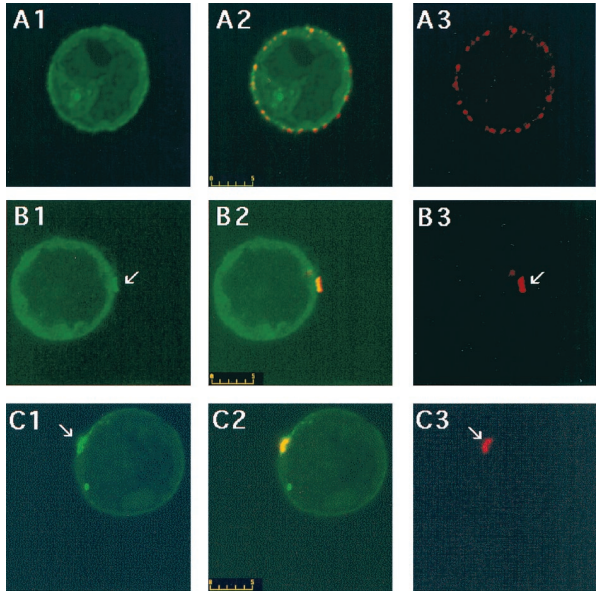
Despite these data, the precise role of Tec family kinases in T cells is still not well understood. Itk and Txk have both been shown to be downstream of the TCR, and to play a role in  $\text{Ca}^{2+}$  mobilization, PLC- $\gamma$ 1 phosphorylation and activation, and cytokine (IL-2 and IFN- $\gamma$ ) production (15, 16, 18). Interestingly, Itk appears to play a more dominant role in these signaling pathways than Txk, as *Txk*<sup>-/-</sup> T cells showed more modest defects than those observed in T cells from *Itk*<sup>-/-</sup> or *Itk*<sup>-/-</sup>/*Txk*<sup>-/-</sup> mice (17, 18). Additional data have suggested that Itk and Txk have different functions in T cell signaling. For example, when *Itk*<sup>-/-</sup> mice are backcrossed to the BALB/c background, Itk-deficient T cells from these mice are unable to produce IL-4 but make normal levels of

IFN- $\gamma$  when stimulated (19). In contrast, Txk was found to be present in differentiated Th1, but not Th2 cells, and is involved in regulating TCR-mediated IFN- $\gamma$  production (20).

It still remains possible that additional Tec family kinases are important in T cell signaling, as even T cells from *Itk*<sup>-/-</sup>/*Txk*<sup>-/-</sup> doubly deficient mice show residual TCR signaling; furthermore, T cell development is only modestly affected in *Itk*<sup>-/-</sup>/*Txk*<sup>-/-</sup> mice (18). Because the comparison of *Itk*<sup>-/-</sup>, *Txk*<sup>-/-</sup>, and *Itk*<sup>-/-</sup>/*Txk*<sup>-/-</sup> indicates that these two Tec family members can compensate for each other's absence in T cells, it is not difficult to imagine that Tec, the third member of this family in T cells, may also be compensating for the



**FIGURE 7.** Wortmannin treatment can inhibit Tec tyrosine phosphorylation in COS cells. COS-7 cells transfected with pME18S-WTtec were grown for 48 h. The cells were incubated with (+) or without (-) wortmannin for 1 h. Following harvest and lysis, total lysates were immunoprecipitated with anti-FLAG mAb and immunoblotted with the 4G10 anti-phosphotyrosine mAb (*top*). The membrane was stripped and reblotted with the anti-Tec Ab (*bottom*).



**FIGURE 8.** Wortmannin treatment can partially inhibit Tec colocalization with the activated TCR. Jtag cells were electroporated with pWTtec-GFP and then treated in the presence (A and B) or absence (C) of 1 mM wortmannin at 37°C for 1 h. Cells were then incubated at 4°C with OKT3 mAb followed by Texas Red-conjugated GAM. Cells were then incubated at 37°C (stimulation: B and C) or at 4°C (nonstimulation: A) for 10 min. The confocal images shown are representative of three independent experiments. Column 1, Tec-GFP; column 2, superimposition of Tec-GFP and Texas Red (TCR/CD3); column 3, Texas Red (TCR/CD3).



absence of Itk, Txk, or both. For these reasons, we felt it was important to assess whether Tec was involved in TCR signaling. On the basis of our data, both dependent on Tec depletion in primary T cells, and on Tec overexpression in Jurkat cells, we conclude that Tec plays a role in IL-2 gene induction downstream of the TCR.

#### *The importance of the PH domain in Tec family kinases*

The first evidence supporting the importance of PH domains in Tec kinases came from the identification of the *xid* defect as a single amino acid substitution in the Btk PH domain (30). Subsequent studies have shed some light on the function of the Tec kinase PH domains. Biochemical experiments have demonstrated the preference of Tec kinase PH domains for phosphoinositides phosphorylated at the D-3 position of the inositol ring (6–8), thus implicating the PH domain in recruiting Tec kinases to the plasma membrane. This *in vitro* binding activity correlates well with the functional data available. For Btk, Itk, and Bmx, an intact PH domain is essential for activation-induced tyrosine phosphorylation of the kinase, for kinase activity, as well as for recruitment of the kinase to the plasma membrane (32–34, 51, 52). However, a PH domain is clearly not essential for all Tec kinase functions. Txk, one of the Tec kinases present in T cells, possesses no PH domain, yet can almost completely restore Btk signaling in Btk-deficient DT40 B cells (53). In addition, Wen et al. demonstrated that a chimeric protein containing the extracellular domain of the estrogen receptor fused to PH-deleted Bmx could activate the downstream STAT1/5 pathway following estrogen stimulation (25). These results indicate that Tec kinase PH domains play an important role in recruiting the kinase to the site of an activated receptor, but are not essential for kinase activity *per se*. Our results showed that the Tec PH domain, like that of Itk and Btk, is important for its membrane localization and tyrosine phosphorylation.

One surprising finding in this study was the loss of Tec functional activity with the PH domain mutant, E42K. This amino acid substitution was first described for Btk, where it increases Btk activity and Btk recruitment to the plasma membrane (52). Furthermore, *in vitro* binding experiments indicate that the Btk PH domain mutant, E41K, has increased binding to phosphoinositides. Our own *in vitro* binding data correlate with our functional data and indicate that the Tec PH domain mutant, E42K, has reduced binding activity for phosphatidylinositol-3,4,5-trisphosphate (PtdInsP<sub>3</sub>). These results with Tec are also consistent with a previous study of Bmx that found that the E42K mutant of Bmx has reduced kinase activity in fibroblasts (33). One possible explanation for the discrepancy between Btk and Tec/Bmx is apparent from models of other Tec kinase PH domain structures (46) based on the x-ray crystal structure of the Btk PH domain (54). Specifically, the glutamic acid residue at position 41 in Btk (42 in other Tec kinases) is thought to be too far away from the inositol-binding pocket to directly interact with the inositol phosphate moiety of phosphatidylinositol-3,4,5-trisphosphate (PtdInsP<sub>3</sub>). Instead, this residue may enhance Btk binding to the membrane by binding other polar head groups of phospholipids in the membrane or, alternatively, may stabilize a putative dimerization of the PH domain by creating a more favorable interaction with the glutamic acid residue at position 45 in the dimer interface (45, 55). Interestingly, the other Tec family PH domains, including Tec and Bmx, have an arginine or lysine residue at position 44 or 45; thus, substitution of E42 with lysine may, in fact, destabilize the putative dimer structure. This interpretation is consistent with our observation that the E42K substitution in the Tec PH domain decreases phosphoinositide binding and nearly eliminates Tec functional activity.

Another surprising finding of this study was the observation that mutation of, or complete deletion of, the Tec PH domain did not

abolish Tec colocalization with the activated TCR. This is in direct contrast to the results with Itk, where mutation of the PH domain totally eliminated Itk recruitment to the TCR/CD3 cap in activated Jurkat cells (data not shown; Ref. 34). Interestingly, we also observed a difference in the subcellular distribution of Tec vs Itk in Jurkat cells. Whereas wild-type Tec is predominantly cytosolic (Fig. 5A, 1), with no visible partitioning to the plasma membrane in resting T cells, Itk is predominantly membrane associated before TCR cross-linking (34, 51, 56). These distinctions provide further evidence highlighting the differences between the PH domains of the individual Tec family members, and support the conclusions from structural modeling studies (46).

We also observed a discordance between the tyrosine phosphorylation of Tec mutants (R29C and E42K) in response to TCR stimulation, their membrane localization, and their ability to enhance IL-2 promoter activity. Specifically, we could detect no decrease in tyrosine phosphorylation of Tec(R29C) or Tec(E42K) after TCR cross-linking in transfected Jurkat cells, whereas each of these mutations substantially reduced the ability of Tec to enhance IL-2 promoter activity. The failure of these point mutations in the Tec PH domain to abolish Tec colocalization with the activated TCR may, in part, account for this discrepancy. The anti-phosphotyrosine immunoblotting may be sufficiently sensitive to detect the increased tyrosine phosphorylation of a small fraction of the mutant Tec proteins that still colocalize with the activated TCR; however, activation of this small fraction of Tec may not be sufficient to generate robust downstream signals leading to substantial enhancement of IL-2 promoter activity.

#### *The role of PI3K in regulating Tec kinase function*

Phosphoinositides have recently been identified as second messengers with distinct binding properties for different PH domains. Several studies have documented the preference of Tec family PH domains for binding phosphoinositides phosphorylated at the D-3 position of the inositol ring (6–8). These observations suggested that Tec kinases would be regulated by the activity of PI3K, an enzyme that converts phosphatidylinositol 4,5 bisphosphate to PtdInsP<sub>3</sub>, thus creating the optimum ligand for the Tec family PH domains (35). Previous studies have indicated that several members of the Tec family, including Btk, Itk, and Bmx, but not Txk (which lacks a PH domain), are regulated by PI3K. Here we present evidence strongly supporting a role for PI3K in regulating Tec activity in T cells. These findings also suggest the intriguing possibility that Tec kinases will also be negatively regulated by the phospholipid phosphatase, the tumor suppressor, PTEN, which has a preference for dephosphorylating PtdInsP<sub>3</sub> at the D-3 position of the inositol ring (57). This possibility could relate to the observation that PTEN<sup>+/-</sup> mice exhibit T cell hyperresponsiveness, and eventually succumb to a fatal T cell-mediated autoimmune disease (58).

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