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Pertussis Toxin Utilizes Proximal Components of the T-Cell Receptor Complex To Initiate Signal Transduction Events in T Cells[▽]

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Pertussis toxin (PTx) is an AB_5 toxin produced by the human pathogen *Bordetella pertussis*. Previous work demonstrates that the five binding (B) subunits of PTx can have profound effects on T lymphocytes independent of the enzymatic activity of the A subunit. Stimulation of T cells with holotoxin (PTx) or the B subunit alone (PTxB) rapidly induces signaling events resulting in inositol phosphate accumulation, Ca^{2+} mobilization, interleukin-2 (IL-2) production, and mitogenic cell growth. Although previous reports suggest the presence of PTx signaling receptors expressed on T cells, to date, the receptor(s) and membrane proximal signaling events utilized by PTx remain unknown. Here we genetically and biochemically define the membrane proximal components utilized by PTx to initiate signal transduction in T cells. Using mutants of the Jurkat T-cell line deficient for key components of the T-cell receptor (TCR) pathway, we have compared stimulation with PTx to that of anti-CD3 monoclonal antibody (MAb), which directly interacts with and activates the TCR complex. Our genetic data in combination with biochemical analysis show that PTx (via the B subunit) activates TCR signaling similar to that of anti-CD3 MAb, including activation of key signaling intermediates such as Lck, ZAP-70, and phospholipase C-γ1. Moreover, the data indicate that costimulatory activity, as provided by CD28 ligation, is required for PTx to fully stimulate downstream indicators of T-cell activation such as IL-2 gene expression. By illuminating the signaling pathways that PTx activates in T cells, we provide a mechanistic understanding for how these signals deregulate immune system functions during *B. pertussis* infection.

Pertussis toxin (PTx) is a complex AB_5 bacterial toxin produced and secreted by the human pathogen *Bordetella pertussis*. The A subunit (S1) has catalytic activity and functions to ADP ribosylate and inhibit cellular Gα proteins, while the five B subunits (S2, S3, two copies of S4, and S5) are responsible for binding and delivery of the A subunit to mammalian cells (34). Traditionally, the biological effects of PTx have been attributed to the enzymatic activity of the A subunit; however, recent work demonstrates that PTx has biological activities that cannot be accounted for by the enzymatic activity of the A subunit alone (11, 28, 39, 43). The nonenzymatic activities of PTx have been attributed to the B subunit and have been largely defined using preparations of PTx that contain the B subunit but are devoid of the A subunit. PTx preparations that contain only the B subunit have been termed PTxB, or B oligomer. One function of the PTx B subunit appears to be the interaction with and activation of cell surface signaling receptors. However, the identity of the receptor(s) engaged by the PTx B subunit and the molecular details of the signaling pathway(s) activated following receptor engagement remain undefined.

In T lymphocytes, PTx and PTxB induce the rapid activation of second messenger signaling pathways leading to inositol phosphate (InsP) accumulation and release of intracellular Ca^{2+} (11, 39, 43). The activation of these signaling pathways in turn results in T-cell proliferation as well as the production of numerous cytokines, including interleukin-2 (IL-2), gamma interferon, and tumor necrosis factor alpha (39, 40, 45, 46). Due to the speed of these events, it has been hypothesized that a receptor specific to T cells, possibly the T-cell receptor (TCR) itself, is engaged and activated by the B subunits of PTx (11, 37, 52). This hypothesis is supported by data showing that InsP accumulation and Ca^{2+} release fail to be induced by PTx in a mutant Jurkat cell line, termed Jurkat-RT3, which lacks expression of the TCR and CD28 (37, 38). Although these studies suggest that PTx utilizes the TCR to promote signal transduction, there have been no molecular data demonstrating that PTx activates specific downstream components of the TCR signaling pathway. Moreover, it is not clear if PTx utilizes the canonical TCR pathway to stimulate InsP and Ca^{2+} signaling or if PTx has evolved a novel signaling mechanism that ultimately leads to a similar response. For example, TCR activation mediated by bacterial superantigens requires the TCR, but utilizes an alternative, noncanonical signaling pathway to trigger Ca^{2+} release (6). Without defining the specific components required for PTx signaling and subsequently examining the activation state of these components, it is impossible to assign a mechanism for PTx action.

TCR signaling is initiated when antigen presented by major histocompatibility complex (MHC) molecules binds to the TCR complex (α and β chains) (49, 51). Signaling via the TCR has been extensively studied using anti-TCR or anti-CD3 monoclonal antibodies (MAbs), which bind to and activate the receptor similar to MHC-presented antigen (1, 57). Engage-

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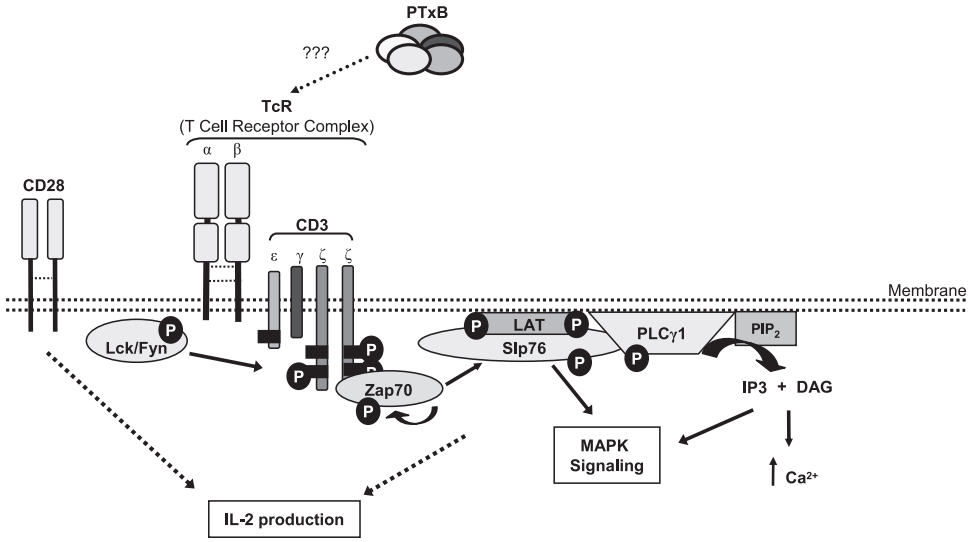

FIG. 1. Model depicting potential role of TCR signaling proteins in PTx action. Activation of the TCR leads to the tyrosine phosphorylation (and hence activation) of several signaling components that are ultimately responsible for accumulation of InsPs, such as IP_3, activation of MAP kinases (MAPK), and production of IL-2. The TCR is indicated by the α and β chains, while CD3 is indicated by the ζ, ε, and γ chains. DAG, diacylglycerol.

ment of the TCR leads to activation of Src family kinases Lck and Fyn, via a poorly understood mechanism (Fig. 1) (31, 56). Lck then phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAM) contained within the TCR chains (31). Subsequent recruitment of ZAP-70 to the phosphorylated ITAM initiates a cascade of tyrosine phosphorylation events that ultimately promote the activation of the major signaling nodes involved in TCR signaling (20). Two targets of ZAP-70-induced tyrosine phosphorylation include the phospholipase C-γ1 (PLC-γ1) and Slp76 proteins, both of which play major roles in signal transmission. Activated PLC-γ1 catalytically cleaves phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol and inositol triphosphate (IP_3), while Slp76 functions as a scaffolding protein to intensify signal propagation (8, 19, 23, 27, 54). Together, the activity of the TCR signaling pathway culminates in the activation of numerous transcription factors including NF-κB, NFAT, and AP-1 (32). Ultimately, this signal results in an "activated" T-cell phenotype, marked by proliferation, expression of CD45, and production of IL-2 (1, 49, 50).

Using a combination of biochemical, genetic, and pharmacological approaches, we examine the membrane proximal events involved in PTx signaling in T cells. In these studies, we demonstrate that PTx activates the Src family kinase Lck or Fyn, leading to the phosphotyrosine-dependent assembly of a signaling cascade at the TCR, including activation of ZAP-70 and PLC-γ1. The activation of this signaling complex further transmits the PTx signal through intermediates InsP and the mitogen-activated protein (MAP) kinases Erk1 and -2. Moreover, our work demonstrates that PTx requires the presence of a costimulatory signal from CD28 to promote maximal activation of downstream events such as IL-2 gene expression. Taken together, our results demonstrate that PTx utilizes the canonical TCR signaling pathway to induce InsP and MAP kinase signaling and suggest that the complex interaction of PTx with T-cell signaling networks may have broad implications for disease and immune system regulation.

MATERIALS AND METHODS

Cell lines and reagents. Jurkat cell lines and derivatives, E6-1 (wild type), J.gamma1 (PLC-γ1$^-$), and J14 (SLP76$^-$) were purchased from the ATCC, while OKT3.3 (TCR$^-$) was the kind gift of A. Weiss (University of California, San Francisco) (51). Jurkat cells were maintained in RPMI containing 10% fetal clone III (HyClone) and 5% penicillin–streptomycin (Mediatech) at a cell density of between 2×10^5 and 1.5×10^6 cells/ml. PTx and PTxB were purchased from List Biological Laboratories. The presence of contaminating PTx holotoxin in PTxB preparations was assessed using the CHO cell-clustering assay (17). The concentration of residual PTx holotoxin in PTxB preparations was found to be less than 2%. No effect was observed in experiments using the residual PTx holotoxin concentrations, and we therefore conclude that the observed effects are due to PTxB and not contaminating catalytic subunit activity. To control for endotoxin in PTx preparations, PTx was heat inactivated by boiling for 30 min (39). Stimulating antibodies against the epsilon chain of CD3 (HIT3a) and CD28 (CD28.2) were purchased from BD Pharmingen. Immunoprecipitating antibodies to PLC-γ1 (B-6-4) and ZAP-70 (2F3.2) and goat anti-mouse immunoglobulin G (IgG) used for cross-linking CD28.2 were purchased from Upstate Biotechnology (10, 30). Western blotting antibodies for phosphorylated and total p44/42 Erk MAP kinases were obtained from Cell Signaling Technologies, and the antiphosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (10). Ionomycin and the Src family kinase inhibitor PP2 were purchased from Sigma. IL-2p (RE/AP elements) and IL-2p (−326 to +46) promoter luciferase constructs were kind gifts from V. Shapiro (University of Pennsylvania) and Chi-Wing Chow (Albert Einstein University), respectively (14, 29, 41).

InsP assays. Jurkat cells (5×10^5/ml) were labeled with 1.5 μCi/ml [*myo*-^{3}H]inositol (Perkin-Elmer) for 18 h in RMPI containing 10% serum. The cells were washed with cold phosphate-buffered saline (PBS) and suspended at 10^7 cells/ml in serum-free RMPI containing 20 mM LiCl. Cells (10^6/treatment) were stimulated with designated amounts of PTx, PTxB, or 10 μg/ml anti-CD3 MAb for 2 h at 37°C. Costimulated cells were treated with PTx or anti-CD3 MAb in combination with 2 μg/ml anti-CD28 MAb cross-linked by the addition of 20 μg/ml anti-mouse IgG. The addition of anti-mouse IgG clusters the anti-CD28 antibody, resulting in additional CD28 receptor clustering. For PP2 experiments, cells were preincubated for 5 min with 10 μM PP2. After stimulation, the cells were lysed with 0.4 M perchloric acid at 4°C for 15 min and neutralized with 0.72 M KOH and 0.6 M $KHCO_3$. Total InsPs were isolated with Dowex resin (Bio-Rad), washed with water, and eluted with 1 M ammonium formate–0.1 M formic

acid. The eluted samples were counted with a liquid scintillation counter and InsP accumulation was calculated as increase (fold) over basal levels in unstimulated cells. Data were graphed and analyzed using GraphPad Prism 4 software.

Measurement of Erk activity. Cells (1×10^6)/treatment were serum starved when indicated and stimulated for various times with 2 μg/ml anti-CD3 MAb or 19 nM PTx in the absence or presence of cross-linked anti-CD28 MAb, as stated above. After the indicated treatment, cells were lysed directly in Laemmli sample buffer and analyzed for phosphorylated Erk1 and -2 (pErk) and total Erk by Western blotting.

Immunoprecipitations and Western blotting. Jurkat cells (2.5×10^7) were washed once with PBS and suspended in serum-free RPMI. The samples were slowly warmed to 37°C and then stimulated with 10 μg/ml anti-CD3 MAb or 95 nM PTx for 5 min. The increased cell number needed for these immunoprecipitation experiments required us to increase the PTx concentration to 95 nM to maintain a PTx/cell ratio equivalent to that used in other experiments. Costimulated samples were additionally incubated with 2 μg/ml anti-CD28 MAb cross-linked by adding 20 μg/ml anti-mouse IgG. Samples were washed once with ice-cold PBS and lysed in lysis buffer (1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholate, 150 mM NaCl, 50 mM Tris, Na_3VO_4, and Complete protease inhibitor tablets [Roche]). Lysates were clarified by centrifugation, and supernatants containing 1 μg of immunoprecipitating antibody per 100 μg total protein were incubated overnight at 4°C with 30 μl of 60% protein A/G bead slurry (Calbiochem). Beads were washed four times with 1 ml lysis buffer, suspended in 3× Laemmli sample buffer, boiled for 10 min, and electrophoresed in 8% SDS–polyacrylamide gel electrophoresis (PAGE) gels. The gels were transferred to nitrocellulose, probed with the appropriate primary and secondary antibodies, and visualized via chemiluminescence (Super Signal; Pierce).

Luciferase assays. Jurkat cells (5×10^6) were electroporated (Bio-Rad Cell Porator) with either 20 μg of the IL-2p (RE/AP elements) luciferase reporter or the IL-2p (−326 to +46) luciferase reporter. IL-2p (RE/AP elements) is a synthetic reporter construct containing four copies of the CD28 and NF-IL-2B AP-1 response elements from the IL-2 promoter (41). IL-2p (−326 to +46) contains the IL-2 promoter region from −326 to +46 relative to the transcription start site (55). Each transfection also included 1 μg of *Renilla* luciferase as an internal control. All cells were stimulated in serum-free media containing 1 μM ionomycin with 2 μg/ml anti-CD3 MAb or 19 nM PTx ± 2 μg/ml anti-CD28 MAb cross-linked by adding 20 μg/ml anti-mouse IgG for 6 h. The cells were lysed, and luciferase activity was quantitated for relative light units using a luminescence assay kit (Promega).

RESULTS

PTx induces InsP accumulation. PTx has been previously reported to rapidly trigger an increase in the level of total InsP and promote the release of intracellular Ca^{2+} in T cells (11, 39). This rapid activation of classical signal transduction pathways is similar to that observed following ligation of the TCR complex in antigen-stimulated cells (38, 43, 49, 50). To begin to investigate the mechanism by which PTx induces signaling in T cells and to evaluate the contributions of the B oligomer (PTxB), we examined the relative abilities of PTx and PTxB to induce InsP accumulation in the human T-cell line Jurkat (38). InsP accumulation induced by various concentrations of PTx was compared to InsP accumulation induced by equivalent amounts of PTxB. Data are presented as accumulation (fold) in stimulated samples over basal levels observed in unstimulated samples. Treatment of Jurkat cells with PTx results in InsP accumulation in a dose-dependent manner. Stimulated levels of InsP reach a maximum of approximately sixfold over basal at 19 nM (2 μg/ml) PTx stimulation (Fig. 2A). The concentrations utilized in these experiments are within the physiological range produced by cultured *Bordetella pertussis* and are in agreement with that previously reported for PTx-induced Ca^{2+} release (11, 36, 39, 43). Figure 2A also shows that PTxB retains similar InsP-stimulating capacity to PTx at maximum stimulation, with no significant difference between equivalent molar doses at 19 nM (2 μg/ml) PTx and 19 nM (1.47 μg/ml) PTxB. To ensure that the effect observed in Fig. 2A was not due to contaminating endotoxin or other factors possibly present at low levels in PTx or PTxB preparations, we compared levels of InsP accumulation in response to PTx or heat-inactivated PTx (39). As shown in Fig. 2B, InsP accumulation is dependent on intact PTx as heat inactivation abrogates the PTx response. Moreover, no InsP accumulation was seen in response to stimulation with concentrations of lipopolysaccharide (1, 10, or 100 μg/ml) known to activate signaling in T cells (58; data not shown).

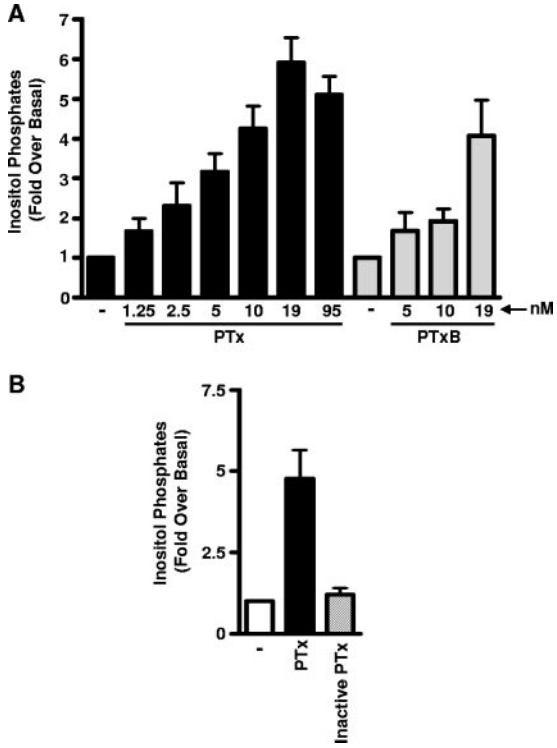

FIG. 2. PTx induces InsP accumulation in Jurkat T cells. (A) Cells were left unstimulated (−) or stimulated with PTx (1.25, 2.5, 5, 10, 19, and 95 nM) or PTxB (5, 10, and 19 nM) for 2 h. Total InsPs were collected and graphed as increases (fold) over basal. Data shown are the means ± standard errors of four to six experiments done in duplicate. The PTx- or PTxB-induced InsP accumulation was significantly greater than that in unstimulated controls at all concentrations tested. The InsP accumulation in mock-treated cells represents 0.2% conversion of input [*myo*-^{3}H]inositol, and accumulation in 19 nM PTx-treated cells represents 1.2% conversion. (B) Total InsPs were measured in response to 19 nM PTx or 19 nM PTx that was boiled for 30 min. The data shown are the means ± standard errors of three experiments done in duplicate.

PTx activates the MAP kinases Erk1 and -2. To determine if PTx can induce other signaling events typically observed in antigen-stimulated T cells, PTx and PTxB were examined for their ability to activate the MAP kinases Erk1 and -2 (25). As shown in Fig. 3, Erk activity, as assessed by evaluation of pErk, increases in response to increasing concentrations of PTx. This activity is dependent on the B subunit alone and is not due to effects of PTx holotoxin on G-protein inhibition, as molar equivalent concentrations of PTxB induce similar levels of Erk activation. In addition, the data in Fig. 2 and 3 show that concentrations greater than or equal to 19 nM are maximal for signaling.

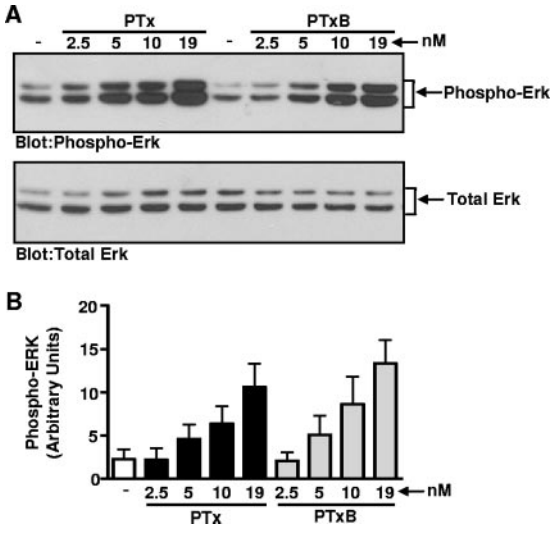

FIG. 3. PTx induces activation of the MAP kinases Erk1 and Erk2. (A) Cells were left unstimulated (−) or were stimulated with PTx (2.5, 5.0, 10, and 19 nM) or PTxB (2.5, 5, 10, and 19 nM) for 15 min. Five micrograms of whole-cell extracts was analyzed by Western blotting for pErk and total Erk. (B) Quantification of pErk levels. Arbitrary values for pErk were obtained using ImageQuant and equivalent Western blot exposures. For these values, total Erk was normalized to wild-type total Erk and then used to calculate arbitrary pErk values relative to total Erk protein. The graph represents the means ± standard errors of three individual experiments.

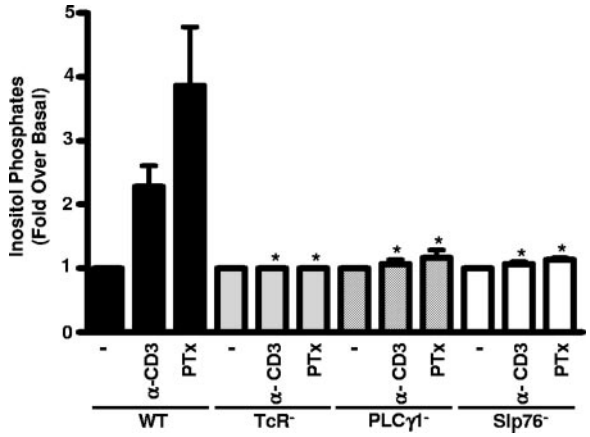

FIG. 4. TCR, Slp76, and PLC-γ1 are required for PTx-induced InsP accumulation. Wild-type Jurkat cells or the indicated Jurkat derivatives were left untreated (−) or stimulated with 10 μg/ml of anti-CD3 (α-CD3) MAb or 19 nM PTx for 2 h. InsPs were collected by column chromatography, and the data were graphed as accumulation (fold) over basal. The graph represents the means ± standard errors of four to six individual experiments performed in duplicate. *, $P < 0.05$ comparing each mutant sample to the equivalent wild-type sample.

PTx utilizes multiple components of the TCR signaling pathway to induce InsP accumulation. It has been previously reported that a mutant Jurkat derivative, termed Jurkat-RT3, which is defective in both TCR and CD28 expression, fails to induce a Ca^{2+} flux in response to PTx (37, 50). To confirm the specific requirement for the TCR in PTx signaling, we obtained a mutant Jurkat cell line deficient for the TCR (OKT3.3), which fails to express the TCR but retains CD28 expression (50). Wild-type and TCR^- cells were stimulated with PTx or anti-CD3 MAb, and InsP accumulation was assessed as described above. Figure 4 shows that the TCR^- cells completely fail to respond to PTx stimulation, confirming that the TCR itself is required for PTx signaling. As expected, these cells also fail to respond to TCR engagement via anti-CD3 MAb stimulation (1, 21, 51).

To further examine the requirement for additional components of the TCR signaling pathway, mutant Jurkat cell lines deficient for PLC-γ1 (Jgamma.1) or Slp76 (J14) were tested for their ability to induce InsP accumulation in response to PTx (1, 19, 54). PLC-γ1 is the predominate isoform of PLC expressed in T cells and is responsible for the increase in InsP induced by TCR engagement (19, 54). Slp76 is another key protein in the TCR pathway that is thought to serve as scaffolding or a docking protein to support propagation of signaling to downstream proteins (13, 23, 27). As seen in Fig. 4, disruption of PLC-γ1 or Slp76 expression prevents InsP accumulation in response to anti-CD3 MAb or PTx. These data genetically implicate multiple downstream components of the TCR signaling pathway in PTx-induced InsP accumulation.

PTx utilizes multiple components of the TCR signaling pathway to induce Erk activation. To study the TCR pathway's role in a PTx-induced signaling event separate from PLC activation, the same Jurkat clones lacking key components of the TCR pathway used above were used to examine activation of the MAP kinases Erk1 and -2 in response to PTx. Prior to analysis, cells were serum starved for 1 h to reduce basal amounts of activated Erk. Cells were then stimulated and lysed, and equal amounts of total protein were assessed for total or pErk by Western blotting (Fig. 5A). The data are also expressed graphically following normalization based on levels of total cellular Erk protein (Fig. 5B). As seen in Fig. 5, wild-type Jurkat cells stimulated with either PTx or anti-CD3 MAb exhibit similar levels of activated Erk. Furthermore, PTx or anti-CD3 MAb fails to activate Erk in TCR^- Jurkat cells. Thus, similar to observations with InsP accumulation, PTx requires the TCR to induce activation of Erk.

To further explore the requirement of additional components of the TCR signaling pathway, we analyzed the ability of PTx or anti-CD3 MAb to induce Erk activity in mutant Jurkat cell lines deficient for PLC-γ1 or Slp76 (Fig. 5). The ability of anti-CD3 MAb to activate Erk in PLC-$\gamma1^-$ and $Slp76^-$ cells is decreased by approximately 50% compared to wild-type Jurkat cells. Surprisingly, the ability of PTx to activate Erk in PLC-$\gamma1^-$ or $Slp76^-$ cells is decreased by 80 to 90% compared to wild-type Jurkat cells. These data suggest that PTx-induced Erk-signaling is heavily dependent on multiple components of the TCR complex, including TCR, PLC-γ1, and Slp76.

PTx activates the TCR proximal proteins ZAP-70 and PLC-γ1. Since our genetic studies indicate that components of the TCR signaling cascade are important for PTx-stimulated events such as InsP accumulation and MAP kinase activation, we next sought to biochemically investigate the activation status of the critical TCR proximal effectors ZAP-70 and PLC-γ1. ZAP-70 is a nonreceptor tyrosine kinase that associates with and is activated by the CD3ζ chain following TCR engagement (1, 7, 10). Activation of ZAP-70 then leads to the activation of additional molecules, including PLC-γ1, which is a key enzyme

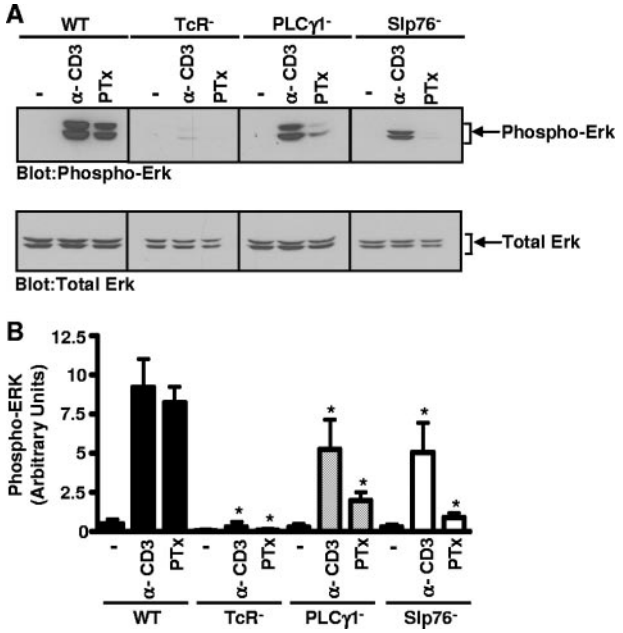

FIG. 5. TCR, Slp76, and PLC-γ1 are required for PTx-stimulated Erk activation. (A) Jurkat cells or the indicated Jurkat derivatives were left untreated (−) or were stimulated with 2 μg/ml anti-CD3 (α-CD3) MAb or 19 nM PTx for 15 min. Whole-cell extract (2.5 μg) was subjected to Western blotting to detect pErk and total Erk. (B) Quantification of pErk levels. Arbitrary values for pErk were obtained using ImageQuant and equivalent Western blot exposures. For these values, total Erk was normalized to wild-type total Erk and then used to calculate arbitrary pErk values relative to total Erk protein. The graph represents the means ± standard errors of three individual experiments. *, $P < 0.05$ comparing each mutant sample to equivalent wild-type sample.

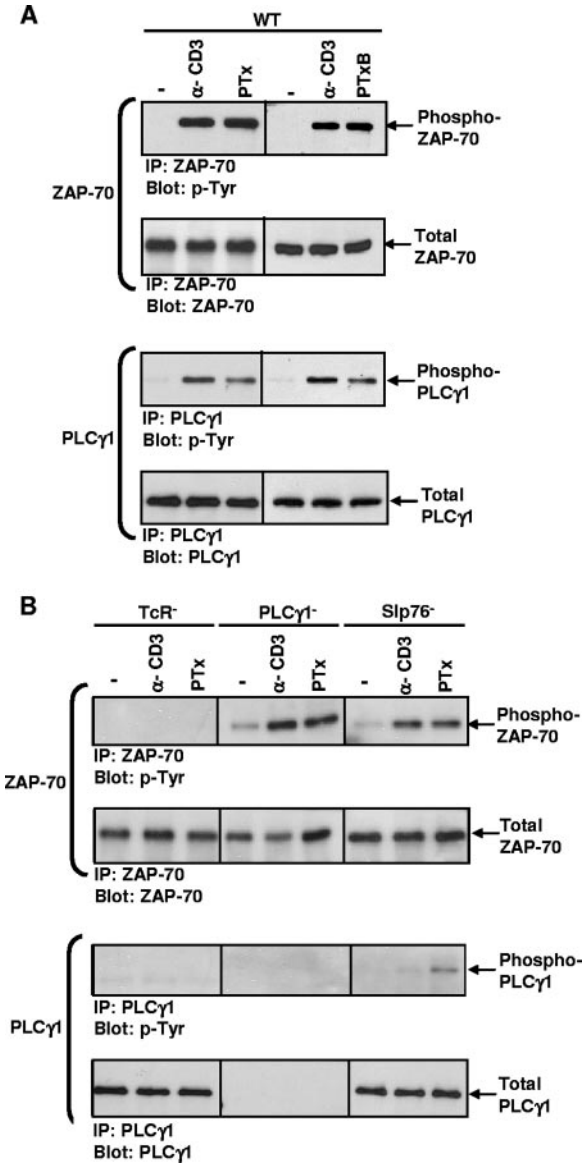

FIG. 6. PTx induces tyrosine phosphorylation of the proximal TCR signaling molecules ZAP-70 and PLC-γ1. (A) Wild-type Jurkat cells were left untreated (−) or stimulated with 10 μg/ml anti-CD3 (α-CD3) MAb, 95 nM PTx, or 95 nM PTxB for 5 min. The cells were washed with cold PBS and lysed with NP-40 lysis buffer. The indicated immunoprecipitating antibody (IP) was incubated with equivalent amounts of cell lysate. Washed immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with either an antiphosphotyrosine-specific antibody (pTyr) or an antibody specific for the immunoprecipitated protein (ZAP-70 or PLC-γ1). (B) Jurkat cells deficient for the indicated molecules were left untreated (−) or stimulated with 10 μg/ml anti-CD3 antibody or 95 nM PTx for 5 min. Activation of ZAP-70 and PLC-γ1 was assessed as described for panel A.

involved in converting PIP_2 into diacylglycerol and IP_3 (19, 54). Activation of the TcR rapidly induces tyrosine phosphorylation of both ZAP-70 and PLC-γ1, and thus assessment of the phosphotyrosine content of these molecules serves as a direct measure of their activation status (8, 10, 22). To assess ZAP-70 and PLC-γ1 activation following treatment with anti-CD3 MAb, PTx, or PTxB, ZAP-70 and PLC-γ1 were immunoprecipitated and analyzed by Western blotting for phosphotyrosine content with the phosphotyrosine-specific antibody 4G10. Total ZAP-70 and PLC-γ1 protein levels were also analyzed by Western blotting to ensure equal loading. Stimulation of wild-type Jurkat cells with anti-CD3 MAb, PTx, or PTxB leads to rapid tyrosine phosphorylation of both ZAP-70 and PLC-γ1 (Fig. 6A). As in earlier experiments, Jurkat cells lacking the TCR do not respond to PTx or anti-CD3 MAb, as shown by the lack of detectable phospho-ZAP-70 or phospho-PLC-γ1 from either treatment (Fig. 6B, left panels). Interestingly, tyrosine phosphorylation of ZAP-70 was detected in both PLC-γ1- and Slp76-deficient Jurkat cells following stimulation with either PTx or anti-CD3 MAb, while PLC-γ1 tyrosine phosphorylation was absent or markedly reduced in the PLC-γ1$^-$ and SLP76$^-$ cell lines, respectively (Fig. 6B, middle and right panels). ZAP-70 is upstream of both PLC-γ1 and Slp76, so it is not surprising that PLC-γ1$^-$ and Slp76$^-$ Jurkat cells support ZAP-70 phosphorylation when treated with PTx. As expected, PLC-γ1 mutants have no detectable phospho- or total PLC-γ1, confirming the mutant genotype (1, 54). Analysis of the Slp76 mutant nicely illustrates that activation of upstream proteins like ZAP-70 is intact in response to PTx stimulation, while signaling to downstream proteins such as PLC-γ1 is dramatically disrupted compared to that in wild-type cells (54). These data show that PTx activation of ZAP-70 and PLC-γ1 is reliant on an intact TCR signaling cascade.

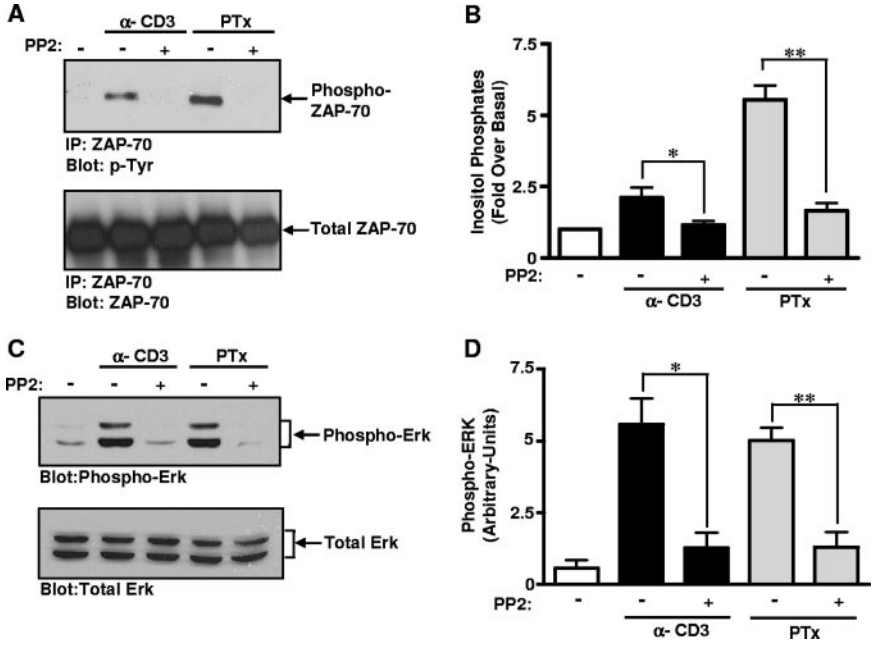

FIG. 7. PTx activation of Src family kinases leads to ZAP-70 activation, InsP accumulation, and MAP kinase activation. In all experiments, wild-type Jurkat cells were pretreated with or without 10 μM PP2, a Src family kinase inhibitor, for 5 min prior to stimulation or with equal concentrations of vehicle (dimethyl sulfoxide). (A) After PP2 treatment, cells were either left unstimulated (−) or were stimulated with 10 μg/ml anti-CD3 (α-CD3) MAb or 95 nM PTx. Cells were lysed with NP-40 lysis buffer, and ZAP-70 was immunoprecipitated (IP) from equivalent amounts of lysate using anti-ZAP-70 antibody. Washed immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with an antiphosphotyrosine antibody (pTyr) or an anti-ZAP-70 antibody. (B) Cells were pretreated with or without PP2 and then stimulated with 10 μg/ml anti-CD3 MAb or 19 nM PTx for 2 h. InsPs were collected via column chromatography, and the data are expressed as accumulation (fold) over basal. The graph represents the means ± standard errors of six experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$. (C) After PP2 pretreatment, cells were treated with 2 μg/ml anti-CD3 MAb or 19 nM PTx for 15 min. Whole-cell lysate (5 μg) was separated by SDS-PAGE and Western blotted with a phospho-specific Erk antibody (pErk) or total Erk antibody. (D) Quantification of pErk levels. Arbitrary values for pErk were obtained using ImageQuant and equivalent Western blot exposures. For these values, total Erk was normalized to wild-type total Erk and then used to calculate arbitrary pErk values relative to total Erk protein. The graph represents the means ± standard errors of three individual experiments. *, $P < 0.05$; **, $P < 0.01$.

The Src inhibitor PP2 blocks PTx signaling. Following TCR engagement, activation of the Src family kinases Lck and Fyn is thought to be a primary initiating event in TCR signaling (31, 56). Lck and Fyn are activated when a phosphate group is removed from the C-terminal autoregulatory domain followed by phosphorylation of a tyrosine residue in the active site of the respective kinase (24). Once activated, Lck is then the predominant kinase responsible for phosphorylating the intracellular ITAM of CD3, which in turn leads to recruitment and activation of ZAP-70 (44). PP2, a low-molecular-weight compound, inhibits Src family protein tyrosine kinases such as Lck with high specificity (50% inhibitory concentration of 4 nM), but not ZAP-70 (50% inhibitory concentration of >100 μM) (14). Since our data strongly implicate the TCR signaling complex in PTx-induced signaling, we next sought to determine if Src family kinases such as Lck and Fyn play an important role in enabling T cells to respond to PTx. The phosphorylation status of ZAP-70 was assessed when cells were stimulated with PTx or anti-CD3 MAb ± PP2 pretreatment. As shown in Fig. 7A, PP2 pretreatment strongly inhibits ZAP-70 phosphorylation induced by either PTx or anti-CD3 MAb. To continue these analyses, we investigated if PP2 has the capacity to block additional PTx-induced signaling events, including InsP accumulation and Erk activation. Figure 7B clearly shows that increases in InsP seen after PTx stimulation are significantly inhibited when Jurkat cells were treated with PP2 prior to stimulation. Lastly, the effects of PP2 on PTx-induced Erk activation were examined. As shown in Fig. 7C and D, inhibition of Src family kinases with PP2 prevents the ability of PTx to activate Erk. As expected, the data also show that PP2 inhibits TCR signaling initiated by anti-CD3 MAb. Taken together, these data strongly support that Lck activation is an essential step in the initiation of PTx signaling in T cells.

CD28 costimulation does not augment PTX stimulation of ZAP-70, PLC-γ1, Erk, or InsP accumulation. CD28 is a coreceptor required for maximal TCR signaling during antigen recognition. When CD28 and the TCR are simultaneously engaged by their cognate ligands, T-cell activation is strongly enhanced (2, 18, 22, 26, 41, 48, 50). Typically, the effects of CD28 costimulation are modest (1.5- to 2-fold) when analyzing signaling events proximal to the membrane, while the effects of CD28 costimulation on downstream events such as IL-2 gene expression are dramatic (>10-fold) (22, 26, 41, 50, 55). Since PTx signals via a pathway that strongly resembles that of the TCR, we sought to determine what role, if any, CD28 costimulation might have on PTx signaling. Several key PTx-activated signaling arms were investigated for augmentation by anti-CD28 MAb costimulation, including induction of ZAP-70 and PLC-γ1 tyrosine phosphorylation, InsP accumulation, and Erk activation (Fig. 8). We observed that anti-CD3 MAb-mediated

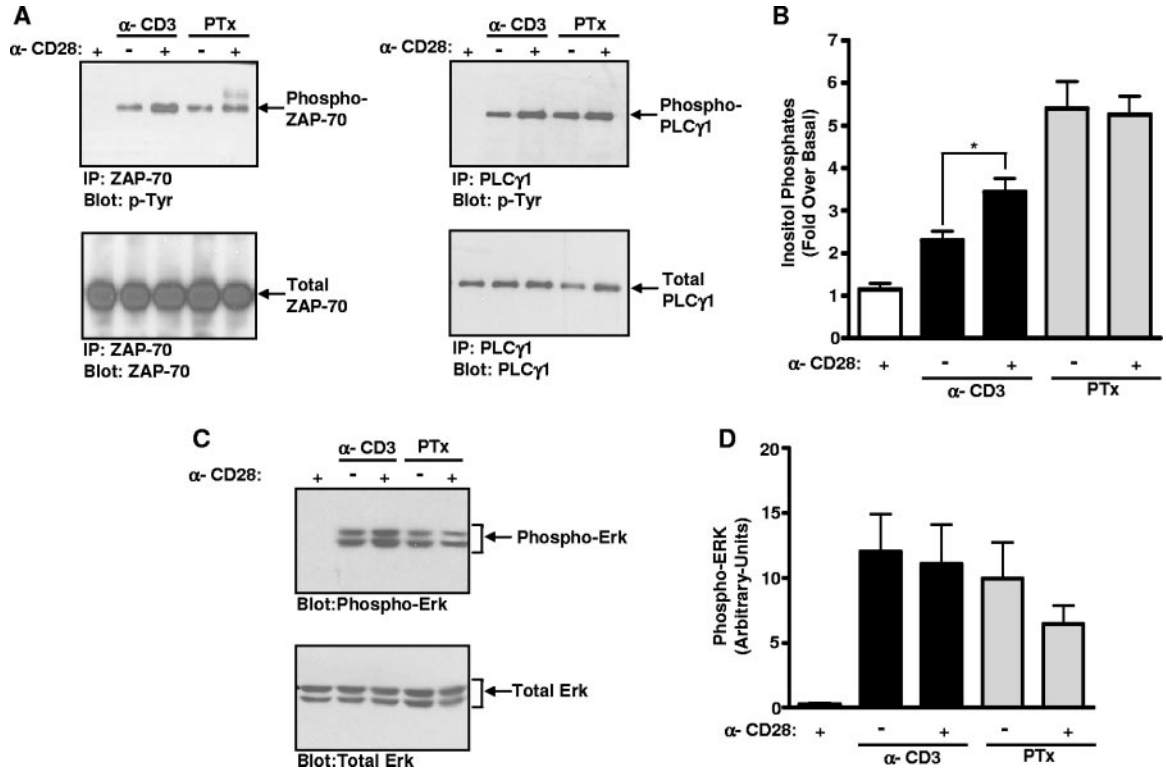

FIG. 8. CD28 costimulation does not effect proximal PTx signaling. (A) Cells were stimulated with 10 μg/ml anti-CD3 (α-CD3) MAb or 95 nM PTx for 5 min in the absence or presence of cross-linked anti-CD28 (α-CD28) MAb. The cells were lysed with NP-40 lysis buffer, and ZAP-70 or PLC-γ1 was immunoprecipitated from equivalent amounts of lysate using the appropriate antibody. Washed immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting with either an antiphosphotyrosine-specific antibody (pTyr) or an antibody specific for the immunoprecipitated protein (ZAP-70 or PLC-γ1). (B) Cells were stimulated with 10 μg/ml anti-CD3 MAb or 19 nM PTx for 2 h in the absence or presence of cross-linked anti-CD28 MAb. InsPs were collected via column chromatography, and the data are expressed as accumulation (fold) over basal. The graph represents the means ± standard errors of five experiments done in duplicate. *, $P < 0.05$. (C) Cells were treated with 2 μg/ml anti-CD3 MAb or 19 nM PTx for 15 min in the absence or presence of cross-linked anti-CD28 MAb. Whole-cell lysate (5 μg) was separated by SDS-PAGE and Western blotted with a phospho-specific Erk antibody (pErk) or total Erk antibody. (D) Quantification of pErk levels. Arbitrary values for pErk were obtained using ImageQuant and equivalent Western blot exposures. For these values, total Erk was normalized to wild-type total Erk and then used to calculate arbitrary pErk values relative to total Erk protein. The graph represents the means ± standard errors of three individual experiments.

activation of ZAP-70, PLC-γ1, InsP, and Erk was slightly, but reproducibly, enhanced by anti-CD28 MAb costimulation (Fig. 8) (19, 22). This slight augmentation of signal by anti-CD28 MAb was not seen in PTx-treated cells when analyzing ZAP-70, PLC-γ1, InsP, or Erk. In addition, no augmentation of PTx signal was observed by the addition of anti-CD28 MAb to cells treated with submaximal concentrations of PTx (2.5, 5, or 10 nM), confirming that the lack of CD28 costimulatory effect observed in PTx-treated cells is not due to an already maximal PTx signal (data not shown).

CD28 costimulation enhances PTx stimulation of IL-2 promoter activity. In contrast to the proximal TCR signaling events described above, downstream indicators of TCR activation are strongly enhanced by CD28 costimulation (50). One example is IL-2 production, a classic indicator of T-cell activation (18, 26, 55, 57). To determine if PTx can simultaneously engage both TCR and CD28, and thus mimic costimulatory conditions, PTx and PTxB were tested for their ability to activate an IL-2 reporter construct, IL-2p (RE/AP elements) (41). Figure 9A shows that anti-CD3 MAb, PTx, or PTxB alone does not significantly activate the IL-2p (RE/AP elements) reporter, but anti-CD3 MAb (4-fold), PTx (14-fold) and PTxB (10-fold) rapidly activate the reporter construct when anti-CD28 MAb is included in the stimulation conditions. The slight difference between the levels of stimulation observed for PTx and PTxB is not statistically significant. These data therefore indicate that PTx does not appear to simultaneously engage both the CD28 and TCR pathways, but like TCR engagement by anti-CD3 MAb, PTx is able to act synergistically with CD28 costimulation.

These experiments were confirmed using a second luciferase construct driven by the authentic IL-2 promoter (18, 29). Data in Fig. 9B show that PTx alone weakly induces the IL-2 promoter, in agreement with published reports (45), but this reporter activity is strongly enhanced under conditions of CD28 costimulation using the anti-CD28 MAb. Anti-CD3 MAb alone also weakly activates the IL-2 promoter but is greatly increased with CD28 costimulation. These data show that IL-2 promoter activity induced by PTx mimics the activity observed with TCR engagement by anti-CD3 MAb. Collectively, Fig. 8 and 9 demonstrate that if PTx does engage both the CD28 and TCR pathways, it does so only weakly. Instead, PTx stimulation is likely to result in abortive activation of T cells, typical of

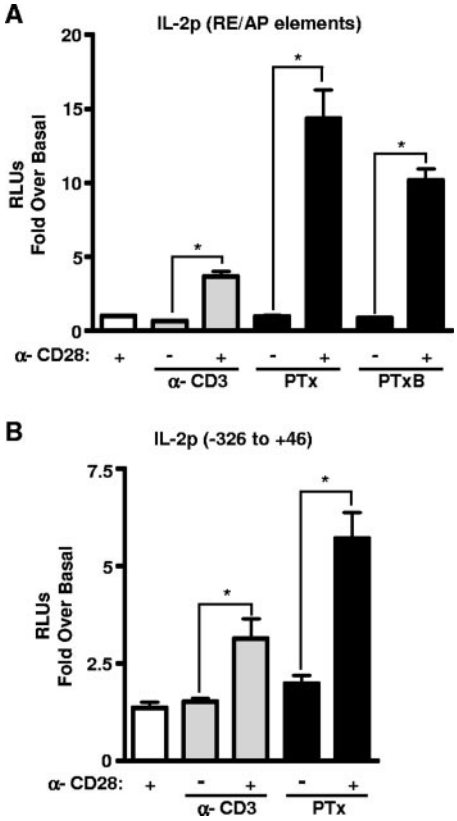

FIG. 9. CD28 costimulation promotes PTx-stimulated IL-2 expression. (A) Cells were transfected with the IL-2p (RE/AP elements) luciferase reporter construct in combination with an internal control *Renilla* construct. Cells were stimulated with 2 μg/ml anti-CD3 (α-CD3) MAb, 19 nM PTx, or 19 nM PTxB in the absence or presence of cross-linked anti-CD28 (α-CD28) MAb. (B) Cells were transfected with the IL-2p (−326 to +46) luciferase reporter construct in combination with an internal control *Renilla* construct. Cells were stimulated with 2 μg/ml anti-CD3 MAb or 19 nM PTx in the absence or presence of cross-linked anti-CD28 MAb. Luciferase activity was normalized and is graphed as relative light units (RLUs) over basal. The data represent the means ± standard errors of four (A) to six (B) experiments performed in duplicate. *, $P < 0.05$.

TCR ligation alone (2–4, 48). In the presence of costimulatory activity, the PTx-engaged TCR signaling pathway then results in a response similar to that of T-cell activation, including the expression of markers such as IL-2.

DISCUSSION

Although for several years it has been evident that the TCR plays an important role in PTx signaling in T cells, the molecular details involved in the initiation and propagation of PTx signal transmission have been unclear (11, 37, 38). Identification of a bona fide PTx signaling receptor has been particularly challenging because PTx has been shown to bind to numerous cell surface molecules, including sialic acid (16). Since many cell surface proteins are heavily glycosylated, some PTx binding proteins may initiate signaling while others may not (9). Receptor binding studies in T cells identified proteins of 70 kDa and 43 kDa to be PTx binding proteins; however, neither of these proteins appear to be peptide chains comprising the TCR, as PTx binding was preserved in Jurkat cells that fail to express the TCR (5, 37). Considering the requirement for the TCR in PTx signaling, the failure to demonstrate PTx binding to components of the TCR suggests that PTx may be binding to a structure closely associated with the TCR and not the TCR itself (37). Witvliet and colleagues demonstrated that PTx binding to T cells caused a reduction in the cell surface expression of the TCR, an observation consistent with a direct interaction between PTx and the TCR itself (52). Based on these published observations, it remains unclear if PTx binds to the TCR itself or a structure associated with the TCR. The failure of classical receptor binding experiments to identify the signaling receptor for PTx on T cells highlights the fact that a better understanding of events proximal to PTx-induced receptor signaling is necessary before the relevant PTx signaling receptor(s) can be identified.

We hypothesized that a molecular analyses of the membrane proximal signaling events involved in PTx signaling would provide a framework from which one can work to identify the membrane protein(s) involved in both PTx binding and initiation of signaling. We used genetic and biochemical approaches to examine the membrane proximal events involved in PTx-induced InsP accumulation and MAP kinase activation. As depicted in Fig. 1, PTx (via the action of the B subunit) engages a cell surface receptor closely associated with the TCR complex, perhaps the TCR itself, triggering a series of molecular events very similar to the canonical signal transduction events initiated by anti-CD3 MAb activation of the TCR. Briefly, the data indicate that exposure of Jurkat T cells to purified PTx leads to the activation of an Src family tyrosine kinase (Lck), which in turn promotes the activation of the CD3ζ-associated protein kinase ZAP-70. Tyrosine-phosphorylated ZAP-70 then induces the assembly of a phosphotyrosine-based protein complex including PLC-γ1, Slp76, and others. Once PLC-γ1 is activated by phosphorylation, it cleaves PIP_2, resulting in diacylglycerol and IP_3 accumulation. In conjunction with other events, accumulation of these activated molecules results in activation of the MAP kinases Erk1 and -2. Finally, as observed for canonical signal transduction initiated by anti-CD3 stimulation of the TCR, downstream cellular responses such as changes in IL-2 transcription are strongly enhanced when PTx-treated cells also receive a costimulatory signal such as that provided by CD28 ligation.

The identification of Src family tyrosine kinase Lck as a junction point enabling PTx to induce InsP accumulation in T cells is important, as activation of Lck is thought to initiate signaling via the CD3ζ/ZAP-70 pathway (31). Currently there is no evidence indicating that PTx directly binds to one of the chains of the TCR, the TCR/CD3 complex, or whether PTx engages a novel membrane protein, which in turn activates Lck. Therefore, the identification of the plasma membrane-bound molecules connecting PTx to Lck will provide solid evidence regarding the nature of the PTx signaling receptor in T cells.

The ability of PTx to activate signaling in T cells is similar to that observed with bacterial superantigens (SAgs), but two key differences suggest that it is unlikely that PTx functions as an SAg (6, 33, 35). First, the SAgs bind simultaneously to MHC class II on antigen-presenting cells and to TCRs on T cells (33). The experiments in the present study utilize clonal cul-

tures of the Jurkat T-cell line, and thus the PTx activity we observed is independent of MHC class II binding. Second, SAgs have been shown to bypass Lck-dependent TCR signaling to activate T cells, whereas we show that Lck activity is required for TCR signaling with PTx stimulation (6). Reflective of the very different systemic effects typical of PTx or SAg exposure, these findings suggest that the mechanism of PTx signaling in T cells is very different from that of the SAgs (35, 43, 57).

Regardless of the receptor utilized by PTx to initiate signaling in T cells, there are major biological implications for this aberrant TCR signaling. Moreover, the consequence of this signaling will depend on the differentiation state of the T cell and whether or not the PTx-interacting cell receives a costimulatory signal (50, 51). PTx stimulation of naïve T cells in the absence of costimulatory antigen-presenting cells will only deliver one signal for activation, which has been shown to promote apoptosis or drive T cells into anergy (4, 15). On the contrary, if a naïve T cell encounters PTx when a costimulatory signal is present, the T cell will receive both signals and progress to an activated state (4, 12). Likewise, memory T cells that only require one activation signal will likely be activated upon encountering PTx in the absence of antigen. By activating TCR signaling pathways, PTx will inappropriately induce activation, anergy, or apoptosis of T cells in an antigen-independent manner. This disruption of normal T-cell function and regulation is likely to serve an important function in the context of *B. pertussis* pathogenesis.

It is clear that PTx can mediate toxicity by interfering with at least two very different signaling pathways. First, the mobilization of the catalytic A subunit into recipient cells leads to the ADP ribosylation of G-protein alpha subunits, which robustly inactivates signaling via G_i and G_o proteins (34, 43). Second, the binding of PTx (via its B subunit) to the cell surface of T lymphocytes initiates a series of rapid signaling events analogous to that of TCR activation. PTx also rapidly activates receptor-mediated signaling pathways in a number of other cell types, including platelets, monocytes, and dendritic cells (25, 42, 47, 53). Thus, the PTx B subunit appears to be activating signal transduction in a diverse array of cell types, indicating that PTx can have specialized biological consequences depending on the combination of signaling pathways affected (25).

During pertussis, the bacteria remain localized to the respiratory tract. However, toxicity mediated by catalytic activity of the PTx holotoxin occurs at very low toxin concentrations and appears to alter the function of tissues and organs very distal from the site of bacterial growth. In contrast, PTx toxicity mediated by B subunit interaction with cell surface receptors requires higher doses of toxin. These doses are achievable during in vitro culture but likely sustainable only near the site of bacterial growth in vivo. Thus, the biological consequences of PTx intoxication would vary depending on the cell type encountered and the proximity to the bacteria. It appears that most cells would be susceptible to altered G-protein signaling as a result of ADP ribosylation of G alpha subunits and that PTx holotoxin-mediated toxicity could occur at many sites in the body (34). Our data predict that T lymphocytes will not only exhibit inhibited G-protein signaling but will also exhibit strong activation of a TCR-"like" pathway: however, only T cells in close proximity to the site of bacterial infection will be targeted.

In conclusion, we have defined major components of the PTx signaling pathway in T cells and demonstrate that PTx signaling differs dramatically depending on whether the PTx-treated cells receive a costimulatory signal such as that provided by CD28 ligation. Our studies provide important evidence regarding the membrane proximal events in PTx action and constitute the basis for future investigations aimed at identifying a PTx binding protein or "receptor" that couples PTx to Src family kinase Lck or Fyn. Finally, our work provides a mechanistic analysis of the signaling pathways involved in PTx perturbation of T-cell function during *B. pertussis* infection in vivo.

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