The Tec family tyrosine kinase Itk has been implicated in T cell receptor (TCR) signaling, yet its precise role and mechanism of activation remain undefined. To investigate these issues, we examined the biochemical response of Itk to TCR stimulation. We found that Itk is tyrosine-phosphorylated after TCR cross-linking and that this phosphorylation depends on the presence of functional Lck. To determine if this Lck dependence results from direct phosphorylation of Itk by Lck, we generated recombinant Itk and Lck using a baculovirus expression system and used these proteins in subsequent biochemical analyses. We found that Lck phosphorylates Itk upon co-expression in insect cells and, further, that this phosphorylation of Itk results in increased Itk in vitro kinase activity. The major site of Lck phosphorylation on Itk was mapped to the conserved tyrosine (Tyr511) in the activation loop of the Itk kinase domain. Substitution of this tyrosine with phenylalanine abolishes Itk kinase activity in insect cells, indicating that phosphorylation at this site plays a critical role in regulating Itk function.

Engagement of the T cell antigen receptor (TCR) by physiological ligands triggers multiple signal transduction cascades (for review, see Ref. 1). The initiation of these cascades depends on the activation of Lck and Fyn, cytoplasmic tyrosine kinases associated with the activated antigen receptor. These Src-family kinases phosphorylate activation motifs (ITAMs) in the CD3e and CD3ζ subunits of the TCR. The tyrosine-phosphorylated ITAMs recruit additional Src homology-2 domain-containing signaling molecules, including the tyrosine kinase Zap-70, which binds doubly phosphorylated ITAMs via tandem Src homology-2 domains. This recruitment of Zap-70 facilitates both its tyrosine phosphorylation and activation. Zap-70 then contributes to the activation of downstream signaling pathways, leading to the eventual phosphorylation and activation of PLCγ. The resulting cleavage of phosphatidylinositol 4,5-bisphosphate by activated PLCγ generates the second messengers diacylglycerol and inositol-1,4,5-trisphosphate, thus activating protein kinase C and mobilizing intracellular calcium, respectively. TCR stimulation also leads to the activation, recruitment, and/or phosphorylation of additional downstream signaling molecules, including both widely expressed proteins, such as phosphatidylinositol-3-kinase, Ras, extracellular signal-regulated kinase, and Jun N-terminal kinase, and hematopoietic-specific proteins such as Vav, SLP-76, and pp36. In most cases, the precise mechanisms by which Zap-70, Lck, and Fyn activate these proteins have not been fully elucidated. The roles of additional tyrosine kinases, such as Itk (2–5), RAFTK/Pyk2 (6, 7), and others in signaling pathways immediately downstream of the TCR are even less well defined. It is likely that these tyrosine kinases will be found to synergize with Zap-70, Lck, and Fyn to facilitate the activation of the signaling proteins described above.

Geneic evidence has implicated the Tec family tyrosine kinase Itk in signaling through the TCR. T cells from Itk-deficient mice have a defect in TCR-induced proliferation that can be overcome by bypassing the TCR with phorbol ester and calcium ionophore treatment (8). In addition, Itk-deficient T cells produce virtually no interleukin-2 and fail to generate a calcium flux in response to TCR stimulation. Finally, Itk-deficient mice have a defect in T cell development consistent with decreased TCR signaling during positive selection in the thymus (8). Further support for the involvement of Tec family kinases in antigen receptor signaling pathways comes from studies of Btk, a Tec family member expressed primarily in B cells and mast cells. Btk was first identified as the gene defective in the human and murine immunodeficiencies X-linked agammaglobulinemia (9, 10) and X-linked immunodeficiency (11, 12), respectively. Biochemical studies have demonstrated that Btk is activated by signaling through the immunoglobulin receptor on B cells and the FcεR on mast cells (13–16). In addition, Btk-deficient B cells isolated from X-linked immunodeficiency mice have defects in calcium mobilization and proliferative responses to antigen receptor stimulation (17).

Despite these genetic data, biochemical evidence clarifying the role of Itk in TCR signaling is sparse. One study has shown that Itk is tyrosine-phosphorylated in response to TCR stimulation of Jurkat cells but not of Jurkat cells lacking the Src family kinase, Lck (18). We confirm these findings and, further, using recombinant proteins expressed in insect cells, show that Lck directly phosphorylates Itk. We also demonstrate that the phosphorylation of Itk by Lck leads to enhanced Itk in vitro kinase activity. Finally, we map the major site of tyrosine phosphorylation by Lck and show that a mutant Itk protein with a tyrosine to phenylalanine substitution at this position has dramatically reduced kinase activity in vitro.

**EXPERIMENTAL PROCEDURES**

* T Cell Lines—Jurkat clone E6–1 and J.CaM1.6 cells (19), obtained from ATCC, were cultured as described (20) and were shown to have equivalent levels of surface TCR/CD3 expression (data not shown).

2 K. Liu, S. C. Bunnell, and L. J. Berg, unpublished observations.
Monoclonal Antibodies (mAbs)—Anti-CD28 antibody 9.3 was kindly provided by Craig Thompson (University of Chicago, Chicago, IL). Anti-CD8 and anti-CD3 antibody-producing hybridomas, OKT8 and OKT3, respectively, were purchased from ATCC; antibodies were purified from tissue culture supernatants on Protein A-Sepharose (Sigma). The cross-linking reagent, rabbit anti-mouse Ig, was purchased from Sigma.

Anti-Lck antibody 3A5 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine monoclonal antibody 4G.10 was kindly provided by Brian Druker (Oregon Health Science University, Portland, OR). Anti-Itk monoclonal antibodies 2F12 and 7F10 were generated by injecting Balb/C mice with a glutathione S-transferase fusion protein of Itk amino acids 1–26; monoclonal antibody 10B2 was generated against a glutathione S-transferase fusion protein of Itk amino acids 97–174. Splenocytes from the immunized mice were fused with the P3.AG myeloma line. 2F12 and 7F10 are both IgG1, and 10B2 is IgG2a. All baculovirus Itk immunoprecipitations and kinase assays were done using 2F12 ascites; for all other experiments, ascites fluid containing each antibody was used interchangeably.

Cell Transfection/Flow Cytometry—The CDB-CD28 chimera was generated by subcloning a PCR product encoding the carboxyl-terminal 42 amino acids of CD28 into PCRII/CD8, which was then transferred into expression vector EMCV-SRs (gifts of Ian MacNeil, ARIAD, Cambridge, MA). To generate stable lines, the expression vector was linearized and transfected into Jurkat T cells (15 μg of expression vector and 10–20 μl of media, electroporated at 960 microfarads at 250-V GenePulser, Bio-Rad) and selected in 1 mg/ml G418 (Life Technologies, Inc.). Neomycin-resistant lines were screened for surface expression of CD8 by flow cytometry. Sublines expressing high levels of the CD8-CD28 chimera were isolated by fluorescence-activated cell sorting. To assess CD8 and CD3 surface expression, cells were stained with OKT8 or OKT3, respectively, followed by goat anti-mouse Ig-FTTC (Cappel). T Cell Stimulation, Preparation of Lysates, Immunoprecipitations, and Western Blotting—Jurkat and J.CaM1.6 cells were stimulated, and lysates were prepared as described previously (20). Cell lysates were preclad and then subjected to immunoprecipitation with anti-Itk mAb or anti-Lck mAb prebound to protein G-agarose beads (Life Technologies, Inc.). Immunoprecipitates were extensively washed with either lysis buffer or lysis buffer supplemented with 1% deoxycholate and 0.1% SDS (where indicated). Immunoprecipitates were then either subjected to an in vitro kinase assay or directly resolved by 10% SDS-polyacrylamide gel electrophoresis. For Western blotting, proteins were transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.), blocked and probed as described previously (20).

Sf9/Baculovirus Expression System—Sf9 insect cells were obtained from ATCC and maintained in Sf9 insect cell culture fluid containing each antibody were used interchangeably.

In Vitro Kinase Assays—Immunoprecipitates were rinsed once with 20 mM Tris, pH 7.4, and resuspended at room temperature in 25 μl of kinase assay buffer. Buffer and kinase assay conditions were as described (21). For autophosphorylation assays, samples were resolved on a 10% SDS-polyacrylamide gel, blotted onto polyvinylidene difluoride membrane, and subjected to autoradiography. Quantitation was determined by densitometry using a pdi (Huntington Station, New York) machine and software. To assess phosphorylation of the SR-src peptide (22), samples were spotted onto p81 phosphocellulose filter paper discs (Whatman) and analyzed by liquid scintillation counting as described (21).

Phosphopeptide Mapping—Sf9 cells were co-infected with baculoviruses expressing kinase-inactive Itk (Itk-KR, 100 μl/100-mm dish) and Lck (10 μl/100-mm dish) for 72 h. Itk was affinity-purified with Itk mAb bound to protein G-Sepharose and loaded onto a 10% SDS-polyacrylamide gel electrophoresis preparative gel. The Itk band, containing approximately 5 μg of protein, was excised and subjected to in-gel tryptic digestion as described by Hellman et al. (23), but without the addition of 0.02% Tween. The resulting peptide mixture was separated by microbore high performance liquid chromatography using a Zorbax C18 1.0 × 150-mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Monitoring peaks of absorbance at 205 nm, 12 peptide fractions were collected over 90 min. Fraction 55 was collected at 41 min, and fraction

FIG. 1. Itk phosphorylation is rapidly induced in response to TCR cross-linking or pervanadate treatment in an Lck-dependent manner. Jurkat cells and Lck-negative Jurkat derivative cells (J.CaM1) were stimulated by anti-CD3 cross-linking (A) or pervanadate (B) for the indicated times. Stimulated cell lysates were immunoprecipitated with anti-Itk antibody and immunoblotted with anti-phosphotyrosine (p-Tyr) antibody (top parts) or anti-Itk antibody (bottom parts).

107 was collected at 74 min. One-half the volume of each fraction was spotted onto high retention polyvinylidene difluoride membrane (Bio-Rad) and probed with anti-phosphotyrosine antibody, 4G.10. Approximately 50 pmol of synthetic tyrosine-phosphorylated and nonphosphorylated peptides (Tufts University Peptide Facility) were spotted as controls. The polyvinylidene difluoride membrane was fitted into the manifold and wetted with 100% methanol. Samples, diluted to 50% methanol in a 96-well plate, were rapidly loaded using a multichannel pipetter. The membrane was allowed to dry overnight in the manifold. The filter was then blotted with anti-phosphotyrosine antibody as described above. Phosphotyrosine-positive fractions were screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Finnigan Lasermat 2000 (Hemel, United Kingdom). Strategies for peak selection, reverse-phase separation, and Edman microsequencing have been previously described (24). Amino acid sequences of tryptic peptides in fraction 55 were obtained by automated Edman degradation on an Applied Biosystems 477A protein sequencer (Foster City, CA). Trypsin digestion, HPLC fractionation, mass spectrometry, and peptide sequencing were performed by the Harvard Microchemistry Facility.

RESULTS

Itk Phosphorylation Is Induced in Response to TCR Cross-linking—Due to similarity between Itk and Blk and to the genetic evidence implicating Itk as a proximal signaling molecule downstream of the TCR (8), we were interested in examining the biochemical responses of Itk to TCR stimulation. For these experiments, we used a T cell tumor line, Jurkat. Jurkat cells were stimulated by TCR cross-linking, and the Itk protein was immunoprecipitated using the Itk monoclonal antibody 2F12. Itk immunoprecipitates were probed with the anti-phosphotyrosine antibody, 4G.10. As shown in Fig. 1A, Itk becomes highly phosphorylated within 2 min of TCR stimulation; this phosphorylation is maintained for approximately 5 min and diminishes by 15 min poststimulation. Given the rapid kinetics of Itk tyrosine phosphorylation after TCR cross-linking, it seemed likely that this phosphorylation was mediated by an Src family kinase activated by TCR signaling. We therefore tested whether Itk tyrosine phosphorylation would still occur in a variant line of Jurkat cells, J.CaM1, which lacks functional Lck (19). When the J.CaM1 cells were TCR-stimulated by cross-linking CD3e, we were unable to detect tyrosine phosphorylation of Itk (Fig. 1A). These results indicate that Itk is downstream of Lck in the TCR signal transduction pathway, as has previously been reported (18).

To further address the dependence of Itk tyrosine phosphorylation on Lck, we also examined Jurkat and J.CaM1 cells
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that were treated with pervanadate, a potent inhibitor of phosphotyrosine phosphatases. Pervanadate treatment shifts the balance between the competing activities of kinases and phosphatases, allowing tyrosine phosphorylation to accumulate on a variety of cellular proteins. This experiment, therefore, addresses the possibility that the lack of Itk tyrosine phosphorylation results from the increased activity of a tyrosine phosphatase in the J.CaM1 cells. As can be seen in Fig. 1B, pervanadate treatment of Jurkat cells for 2 min leads to the accumulation of tyrosine phosphorylation on Itk; increased phosphorylation is observed with longer treatment times (up to 20 min). In contrast, pervanadate treatment of J.CaM1 cells, which lack the tyrosine kinase Lck, leads to barely detectable tyrosine phosphorylation of Itk, even after 20 min. These data confirm that the presence of a functional Lck kinase is essential for the tyrosine phosphorylation of Itk in Jurkat cells.

Several recent studies have reported an association between Itk and the cytoplasmic tail of the T cell co-stimulatory molecule CD28 and the phosphorylation and activation of Itk in response to CD28 stimulation (25–27). We attempted to confirm the phosphorylation of Itk in Jurkat cells after CD28 stimulation with anti-CD28 monoclonal antibody 9.3, but were unsuccessful at doing so (data not shown). Due to limited access to this antibody, we chose to pursue this issue by generating a chimeric protein containing the extracellular and transmembrane domains of human CD8α fused to the cytoplasmic domain of human CD28 (CD8/CD28). This chimeric protein was transfected into Jurkat cells, and cell lines were generated that stably expressed the CD8/CD28 chimeric protein. Fig. 2A shows the flow cytometry analysis confirming the expression of CD8 on the transfected cells, but not on the parental Jurkat cells. This analysis also indicated that the transfected cells express a slightly lower level of the TCR-CD3 complex compared with the parental Jurkat cells. Using these transfected cells, we compared the level of tyrosine phosphorylation on Itk induced by TCR (anti-CD3 cross-linking) versus CD28 (anti-CD8 cross-linking) stimulation. As can be seen in Fig. 2B, cross-linking of the TCR, but not of the CD8/CD28 chimera, led to rapid and substantial phosphorylation of Itk. While some weak phosphorylation of Itk could be detected after CD8/CD28 stimulation, the overall magnitude of this response was minor compared with the response induced after TCR stimulation and was similar to that seen in the absence of primary antibody. Furthermore, co-cross-linking the CD8/CD28 chimera with the TCR did not enhance the phosphorylation of Itk relative to TCR cross-linking (data not shown). It should be noted that we observed only very weak changes in overall tyrosine phosphorylation in total lysates of CD28- or CD8/CD28-stimulated cells (data not shown); thus, our failure to observe significant tyrosine phosphorylation of Itk may correlate with this poor induction of tyrosine phosphorylation.

Itk Is Directly Phosphorylated by Lck in Insect Cells—The data described above indicate that Itk is downstream of the TCR and that TCR-induced Itk phosphorylation is dependent on the Lck kinase. To test the role of Lck in Itk phosphorylation more directly, we generated recombinant Itk and Lck proteins in Sf9 insect cells using the baculovirus expression system. Baculoviruses encoding Itk and Lck were generated. We also generated a mutant form of the Itk protein, containing a single amino acid substitution of a conserved lysine in the kinase domain (Itk K390R, referred to as Itk-KR). In many other kinases, substitution of this lysine with arginine generates a kinase-inactive form of the protein (28–33). Finally, a baculovirus was generated that encodes a kinase-inactive form of the Lck protein (Lck K273R, referred to as Lck-KR). These baculoviruses were used to infect Sf9 insect cells; subsequently, the tyrosine phosphorylation of Itk was analyzed.

As shown in Fig. 3, our anti-Itk antibodies are highly specific; following expression of Lck alone, neither Itk nor tyrosine phosphoproteins were detected in Itk immunoprecipitates. However, when Itk was expressed alone, the immunoprecipitated Itk protein was found to be tyrosine-phosphorylated. Co-infection of the insect cells with baculoviruses encoding Itk and increasing amounts of Lck led to increased tyrosine phosphorylation on Itk. In contrast, co-infection of Itk with kinase-inactive Lck (Lck-KR) had no effect on Itk tyrosine phosphorylation. These results suggested that Lck could directly phosphorylate Itk; however, we could not rule out an indirect
effect of the Lck kinase on Itk autophosphorylation activity. We therefore examined this issue using the kinase-inactive form of Itk, Itk-KR. When expressed alone in Sf9 cells, Itk-KR has severely reduced autophosphorylation activity, as indicated by the unphosphorylated state of the immunoprecipitated Itk protein. Co-infection of Itk-KR with Lck led to substantial tyrosine phosphorylation of Itk; in contrast, co-infection with Lck-KR did not. Together, these data indicate that co-expression of Itk with kinase-active Lck leads to tyrosine phosphorylation of Itk by Lck, most likely due to direct phosphorylation.

Itk in Vitro Autophosphorylation Activity Is Enhanced by Co-expression with Lck—Since we observed that Itk is phosphorylated by Lck when the two proteins are co-expressed in insect cells, we were interested in determining whether this phosphorylation had any effect on Itk kinase activity. At the time of these studies, only one substrate had been reported for the Itk kinase, the RR-src peptide derived from the kinase domain of Src (18, 27). However, in our system we could not detect Itk phosphorylation of this substrate in in vitro kinase assays (see below). Therefore, we examined instead Itk autophosphorylation activity. We performed these experiments using the recombinant Itk protein generated by baculovirus infection of insect cells, allowing the use of a kinase-inactive Itk mutant as a control. To confirm that the K390R mutation in the Itk kinase domain did abolish kinase activity, we examined autophosphorylation activity of Itk and Itk-KR. Itk was isolated from infected cells and subjected to in vitro kinase assays. As can be seen in Fig. 4A, duplicate samples of wild-type Itk show strong in vitro kinase activity, with a major phosphorylated band present at 72 kDa; in contrast, Itk-KR has virtually no in vitro kinase activity in this assay. The two minor phosphorylated bands visible at molecular weights lower than full-length Itk in the wild-type Itk kinase assay are likely to be breakdown products of Itk. This experiment indicates that the K390R mutation abolishes Itk in vitro kinase activity and demonstrates that the in vitro kinase activity observed with wild-type Itk is not due to a contaminating kinase activity present in the immunoprecipitates.

To determine whether Lck phosphorylation of Itk affects Itk kinase activity, insect cells were co-infected with baculoviruses encoding Lck and either wild-type Itk or kinase-inactive Itk. Itk was then immunoprecipitated and subjected to in vitro kinase assays. Insect cells infected with empty baculovirus or expressing Lck alone show no detectable in vitro kinase activity in Itk immune complexes (Fig. 4B). When Itk is expressed alone and immunoprecipitated, kinase activity is observed. After co-expression with increasing amounts of Lck, the Itk in vitro kinase activity increases markedly; quantitation of this increase in three independent experiments indicates that Itk is 2.5 ± 0.1-fold more active after phosphorylation by Lck. To ensure that this increase in phosphorylation is due to increased Itk kinase activity, and not to contaminating Lck in the Itk immunoprecipitates, this analysis was also performed with the kinase-inactive Itk control. As can be seen in Fig. 4B, Itk-KR expressed alone has no detectable kinase activity; after co-expression with increasing amounts of Lck, a small amount of kinase activity was observed in the Itk immunoprecipitates. This modest phosphorylation is most likely due to Lck contaminating the Itk immunoprecipitates. However, this weak background is unlikely to account for the increased kinase activity observed with wild-type Itk upon co-expression of Lck. Interestingly, this background activity probably results from weak binding between Itk and Lck, since no such background is seen in Itk immunoprecipitates from cells infected with Lck alone (Fig. 4B).

Although a number of measures were taken to eliminate Lck contamination from the Itk immunoprecipitates, including stringent wash conditions (RIPA buffer: lysis buffer supplemented with 1% deoxycholate, 0.1% SDS), a small amount of Lck apparently remained associated with Itk in the in vitro kinase assays. To further assess the level of Lck contamination, we subjected the Itk immune complexes to kinase assays using an exogenous substrate of the Lck kinase. This substrate, RR-src, is a peptide derived from the sequence flanking the auto-phosphorylation site in the Src kinase domain (22). Interestingly, this substrate has been used by others as an exogenous substrate for assessing Lck in vitro kinase activity. When in vitro kinase assays were performed, low levels of activity on the RR-src peptide were observed in Itk immune complexes from cells infected with either empty baculovirus or Itk baculovirus (Fig. 5). A similar low background was seen in Itk immune complexes from cells infected with the Itk-KR baculovirus. Together, these data indicate that Itk does not detectably phosphorylate the RR-src peptide. When Itk immune complexes from cells co-infected with Lck and either Itk or Itk-KR were tested, RR-src phosphorylation increased slightly above background (Fig. 5A and B). This level of Lck activity is relatively minor, representing <2% of the Lck activity present in an anti-Lck immune complex kinase assay (Fig. 5B). These experiments demonstrate two important findings. First, we conclude that the amount of Lck contaminating the Itk immunoprecipitates is small, indicating that the kinase responsible for Itk phosphorylation in these kinase assays is Itk itself and not Lck.
Second, we conclude that the RR-src peptide is not a substrate for Itk expressed in insect cells.

Mapping the Lck Phosphorylation Site on Itk—The experiments described above indicated that Lck can phosphorylate Itk, enhancing Itk kinase activity. We were therefore interested in identifying the Lck phosphorylation site(s) on Itk. To accomplish this, insect cells were co-infected with Lck and Itk-KR. Itk was then purified and subjected to trypsin digestion. The resulting peptides were separated by HPLC. Each of the 74 HPLC fractions was Western blotted for phosphotyrosine (Fig. 6A). Two fractions, 55 and 107, were reactive with the anti-phosphotyrosine antibody. The extremely late elution time of fraction 107 indicated that this fraction contained sequences of very high hydrophobicity, probably partially digested Itk. Consistent with this possibility, no small peptides were detected in fraction 107 (data not shown). To determine the identity of the Itk peptides in fraction 55, a portion of this fraction was subjected to matrix-assisted laser desorption time-of-flight mass spectrometry (Fig. 6B). Two ions were detected in this fraction, one of m/z 755 and one of m/z 2113. Sequence analysis by Edman degradation allowed the identification of these peptides as residues 30–35 (FFVLTK) and residues 505–522 (FVDQYTSSTGTKFPVK). Assuming that the single tyrosine residue in the larger peptide was phosphorylated, the predicted mass of each peptide was within the expected error of the mass spectrometry measurements (±0.1%). These results unambiguously identified tyrosine 511 of Itk as the major site of Lck phosphorylation.

Analysis of an Itk Mutant Lacking the Major Lck Phosphorylation Site—The experiments described above indicated that tyrosine 511 of Itk is the major site of phosphorylation by Lck. We therefore generated a point mutation in Itk, altering the tyrosine at 511 to phenylalanine. This Itk mutant (Itk-Y511F) was expressed in Sf9 insect cells using the baculovirus expression system, as above. Strikingly, Itk-Y511F protein was not ty-
Rosine-phosphorylated, suggesting that this mutation abolishes kinase activity in the insect cells (Fig. 7A, lane 7). The lack of phosphorylation on Itk-Y511F in insect cells is comparable with that observed with the kinase-inactive Itk mutant, Itk-KR (Fig. 7A, lane 3) and is in direct contrast to the strong phosphorylation observed with wild-type Itk (Fig. 7A, lane 2). To confirm the lack of kinase activity exhibited by the Itk-Y511F mutant in the insect cells, we also examined total lysates from the infected cells. As can be seen in Fig. 7B, a variety of insect cell proteins become tyrosine-phosphorylated after the expression of wild-type Itk; in contrast, virtually no phosphorylated bands are visible in lysates from insect cells infected with an empty baculovirus or a baculovirus encoding kinase-inactive Itk (Itk-KR). By these criteria, Itk-Y511F appears to have extremely weak kinase activity in insect cells.

To determine whether the Y511F mutation eliminated phosphorylation of Itk by Lck, Itk-Y511F was co-expressed with Lck and assessed for tyrosine phosphorylation. As can be seen in Fig. 7A, lanes 8–10, Lck induces much less phosphorylation of Itk-Y511F than Itk-KR (Fig. 7A, lanes 4–6); however, the Y511F mutation does not abolish Lck-induced phosphorylation of Itk. These results indicate that Itk contains an additional site for Lck-induced tyrosine phosphorylation or that Itk autophosphorylation activity is indirectly induced by co-expression with Lck in the absence of direct phosphorylation by Lck. To distinguish between these two possibilities, we generated kinase-inactive Itk containing the tyrosine-to-phenylalanine substitution (Itk-KR/Y511F). The phosphorylation of Itk-KR/Y511F following co-expression with Lck was similar to that of Itk-Y511F (Fig. 7C). Therefore, the residual Lck-induced tyrosine phosphorylation of Itk-Y511F does not result from the indirect activation of Itk autophosphorylation activity but results instead from Lck phosphorylation at an alternative site in Itk.

In Vitro Kinase Activity of Itk-Y511F—We considered the possibility that wild-type Itk expressed alone in insect cells is activated by autophosphorylation, inter- or intramolecularly on Tyr511, thus accounting for its activity in an in vitro kinase assay. If this were the case, the Itk-Y511F mutant would be expected to have severely defective in vitro kinase activity. To test this possibility, wild-type Itk, Itk-KR, and Itk-Y511F were each expressed in insect cells and tested for in vitro autophosphorylation activity. As shown in Fig. 7D, Itk-Y511F has substantial in vitro kinase activity compared with kinase-inactive Itk-KR mutant. However, a slight reduction in kinase activity (76 ± 24% of wild type) is seen when Itk-Y511F is compared with wild-type Itk. These results demonstrate that phosphorylation of Tyr511 in Itk is not essential for Itk kinase activity in encoding Itk, Itk-KR, or Itk-Y511F either alone (−) or together with increasing amounts of Lck baculovirus (+Lck). A control of S9 cells infected with empty baculovirus (be) is included. In panel A, cell lysates were immunoprecipitated with anti-Itk antibody and immunoblotted with anti-phosphotyrosine (p-Tyr) antibody (top) or anti-Itk antibody (bottom). In panel B, total insect cell lysates were analyzed by anti-phosphotyrosine (left) or anti-Itk (right) immunoblotting. Positions of molecular mass standards in kDa are indicated. C, S9 cells were infected with baculovirus encoding Itk-Y511F, Itk-K390R/Y551F, or Itk-KR alone (−) or together with Lck (+Lck; 1 or 10 μl/100-mm dish). Cell lysates were immunoprecipitated with anti-Itk antibody and immunoblotted with anti-phosphotyrosine antibody (top) or anti-Itk antibody (bottom). An immunoprecipitate of wild-type Itk (Itk) was analyzed for comparison. D, S9 cells were infected with baculovirus encoding Itk-Y511F, Itk-KR, or Itk (10 μl/100-mm dish). Cell lysates were immunoprecipitated with anti-Itk antibody; immunoprecipitates were washed extensively in lysis buffer and subjected to an in vitro kinase assay (left). Itk protein was detected by immunoblotting the same filter with anti-Itk antibody (right). The arrow indicates the full-length Itk protein.

FIG. 7. Tyrosine 511 is the major, but not exclusive, site of Lck phosphorylation on Itk. A, S9 cells were infected with baculovirus encoding Itk, Itk-KR, or Itk-Y511F either alone (−) or together with increasing amounts of Lck baculovirus (+Lck). A control of S9 cells infected with empty baculovirus (be) is included. In panel A, cell lysates were immunoprecipitated with anti-Itk antibody and immunoblotted with anti-phosphotyrosine (p-Tyr) antibody (top) or anti-Itk antibody (bottom). In panel B, total insect cell lysates were analyzed by anti-phosphotyrosine (left) or anti-Itk (right) immunoblotting. Positions of molecular mass standards in kDa are indicated. C, S9 cells were infected with baculovirus encoding Itk-Y511F, Itk-K390R/Y551F, or Itk-KR alone (−) or together with Lck (+Lck; 1 or 10 μl/100-mm dish). Cell lysates were immunoprecipitated with anti-Itk antibody and immunoblotted with anti-phosphotyrosine antibody (top) or anti-Itk antibody (bottom). An immunoprecipitate of wild-type Itk (Itk) was analyzed for comparison. D, S9 cells were infected with baculovirus encoding Itk-Y511F, Itk-KR, or Itk (10 μl/100-mm dish). Cell lysates were immunoprecipitated with anti-Itk antibody; immunoprecipitates were washed extensively in lysis buffer and subjected to an in vitro kinase assay (left). Itk protein was detected by immunoblotting the same filter with anti-Itk antibody (right). The arrow indicates the full-length Itk protein.
vitro and indicate that Tyr$^{511}$ is not the major, or only, site of Itk autophosphorylation.

**DISCUSSION**

Genetic evidence implicates the Tec family tyrosine kinase, Itk, as having an important role in TCR signaling \( ^{(8)} \), yet little biochemical data describing the mechanism of Itk involvement has been generated. Here we report that Itk is tyrosine-phosphorylated in response to TCR cross-linking of Jurkat cells and that this phosphorylation depends on the presence of Lck. Experiments using recombinant proteins expressed in insect cells indicate that Lck directly phosphorylates Itk and that this phosphorylation enhances Itk kinase activity. Finally, we have mapped the major site of Lck phosphorylation site in Itk to Tyr$^{511}$, a conserved tyrosine in the activation loop (for review, see Ref. 34) of the Itk kinase domain. A tyrosine to phenylalanine substitution at this position \( \text{Y511F} \) abolishes Itk kinase activity in insect cells and reduces, but does not prevent, Lck phosphorylation of Itk.

Previous reports from other groups have shown that tyrosine phosphorylation of Itk is induced in Jurkat cells in response to stimulation through a number of T cells surface molecules, including CD28, the TCR-CD3 complex, and CD2 (18, 25, 27, 35). Here we confirm that Itk phosphorylation increases after CD3 cross-linking and that this phosphorylation is dependent on the presence of a functional Lck kinase. In contrast to previous reports, we do not observe significant tyrosine phosphorylation of Itk after CD28 stimulation. One possible explanation for this discrepancy is that the state of our cells and/or our stimulation conditions may differ markedly from those used previously. In support of this possibility, we observed extremely weak increases in overall tyrosine phosphorylation in total lysates of CD8/CD28-stimulated cells. Nonetheless, our experiments do strongly suggest that the magnitude of Itk phosphorylation induced by TCR stimulation far exceeds that induced by CD28 stimulation.

Using a baculovirus expression system to produce recombinant Itk protein, we observe that wild-type Itk autoprophosphorylates strongly on tyrosine residues; no phosphorylation is observed after expression of kinase-inactive Itk. In contrast, Itk immunoprecipitated from resting Jurkat cells or primary T cells\(^{5,6}\) is not tyrosine-phosphorylated. We also observe that Itk immunoprecipitated from Jurkat cells has relatively weak \textit{in vitro} kinase activity compared with baculovirally produced Itk protein,\(^{3}\) suggesting that the increased phosphorylation of the Itk produced in insect cells causes, or at least correlates with, increased kinase activity. We have not yet identified the site(s) that are autophosphorylated by baculovirally produced Itk; however, a point mutation at tyrosine 511 abolishes this autophosphorylation in insect cells. It is possible that under conditions of overexpression, as seen in the baculovirus system, Itk may undergo trans-autophosphorylation at tyrosine 511. This autophosphorylation may be relatively inefficient compared with the phosphorylation of this site by Lck yet may be sufficient for the induction of kinase activity. Under normal conditions in T cells, the concentration of Itk is likely to be too low to mediate this autophosphorylation at tyrosine 511, leading to undetectable levels of constitutive Itk phosphorylation and weak Itk kinase activity. Alternatively, T cells may contain a specific tyrosine phosphatase that removes this phosphate, thereby resulting in a net low level of Itk phosphorylation in Jurkat cells or primary T cells.

Our mapping data identify tyrosine 511 as the major site of Itk phosphorylation by Lck. In our analysis, no other phosphorylated peptide was detected. However, biochemical analysis using the Itk-Y511F and Itk-KR/Y511F mutants indicated that at least one other site of Lck phosphorylation must exist on Itk. Specifically, co-expression of either of these Itk mutants with Lck resulted in reduced, but detectable, phosphorylation on Itk. We have not yet identified this additional Lck phosphorylation site. Using the predicted motif for optimal Src kinase phosphorylation sites (see Ref. 36 and references therein), one candidate tyrosine can be identified, Tyr$^{199}$. Based on the homology of Itk to Btk, a second candidate tyrosine should also be considered. Witte and colleagues have identified Tyr$^{225}$ in Btk as the Btk autophosphorylation site \( (37) \). The corresponding tyrosine in Itk \( \text{Tyr}^{180} \) may, under conditions of protein overexpression, be weakly phosphorylated by Lck, a situation that is unlikely to occur in T cells. Experiments are in progress to map the second site of Itk phosphorylation by Lck in the insect cell system.

Positive regulation of Itk by phosphorylation of a tyrosine in the activation loop of the kinase domain is consistent with the mechanism regulating a large number of protein kinases \( (34) \). For instance, in the cAMP-dependent kinase, phosphorylation on a residue located in the activation segment of the kinase domain leads to a conformational change in the protein, relieving steric hindrance and allowing access of substrates to the catalytic site \( (38) \). Itk is most likely regulated by a comparable mechanism. In addition, the regulation of Itk activity by a Src kinase-induced phosphorylation on tyrosine 511 is compatible with reports of Btk regulation \( (32, 37, 39, 40) \). Specifically, published data indicate that Btk is phosphorylated and activated upon co-expression with an active Src family kinase. Src phosphorylation of Btk on the activation loop tyrosine \( \text{Tyr}^{551} \) leads to Btk autophosphorylation at a site in the binding groove of the SH3 domain \( \text{Tyr}^{225} \); Ref. 37). Such a phosphorylation may ultimately result in an alteration in Btk conformation caused by release of an intramolecular interaction between the Btk SH3 domain and its own proline motif, as predicted for Itk \( (41) \). Alternatively, phosphorylation of the Btk SH3 domain could result in the disruption of intermolecular SH3 interactions, leading to the relocalization of Btk within the cell.

One unexpected finding concerning the regulation of Itk kinase activity was the nearly normal \textit{in vitro} kinase activity of Itk-Y511F. Since we observed that Itk-Y511F was inactive as a kinase in the insect cells, it was surprising to discover that this Itk mutant had relatively normal kinase activity in the immune complex kinase assay. One explanation for this finding is that binding Itk-Y511F to the anti-Itk antibody directed at the amino-terminal 25 amino acids of Itk stabilizes the protein in an active conformation, despite the lack of phosphorylation at Tyr$^{511}$ and elsewhere. Alternatively, the \textit{in vitro} kinase assay, which depends on autophosphorylation, may not adequately represent Itk kinase activity. For instance, if wild-type Itk is substantially more active as a kinase than is Itk-Y511F but lacks sites to phosphorylate (since a large fraction of them have already been phosphorylated \textit{in vivo} in the insect cells), we may be grossly underestimating the kinase activity of wild-type Itk. In this case, the Itk mutant Y511F may appear to have similar activity due to the greater availability of free phosphorylation sites within the protein. To resolve this issue it will be necessary to reassess Itk kinase activity with an exogenous Itk expression, be weakly phosphorylated by Lck, a situation that is unlikely to occur in T cells. Experiments are in progress to map the second site of Itk phosphorylation by Lck in the insect cell system.

Overall, these experiments have placed Itk downstream of Lck in the TCR signaling cascade, in a position similar to that determined for Btk downstream of the B cell antigen receptor \( (14, 16, 42) \). However, the precise role of Itk in TCR signaling remains to be determined. For instance, one important issue is to determine whether Itk is downstream of, or parallel to,
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Zap-70 after TCR stimulation. In addition, the range of Itk substrates needs to be identified and characterized, particularly in light of recent reports that Itk phosphorylates the cytoplasmic tails of the TCR β-chain (43) and CD28 (44). Our preliminary data analyzing Itk-deficient T cells indicate that these cells have a defective calcium flux response after TCR cross-linking, suggesting a role for Itk in the activation of PLCg, as has been observed for Btk (42). Biochemical analyses of T cells from Itk-deficient mice should provide important information about these issues and the precise function of Itk in T cell activation and TCR signaling.

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