The lymphoid-specific Src family protein tyrosine kinase p56<sup>Lck</sup> (Lck) is non-covalently associated with the cytoplasmic tail of CD4 and has an essential role in T cell activation. Engagement of ligand by the T cell antigen receptor (TCR) is followed by rapid tyrosine phosphorylation of several cellular proteins, including phospholipase Cγ1 (PLC) and the TCR-associated CD3ζ polypeptides. Tyrosine phosphorylation of PLC-γ1 results in activation of PLC and subsequent phosphatidylinositol turnover. We have studied the effects of the CD4-associated Lck molecule on TCR-mediated activation of the protein tyrosine kinase (PTK) pathway in a murine T cell hybridoma. Antibodies against CD3 elicited the expected PTK activation, which was enhanced upon co-cross-linking of CD4. In contrast, anti-TCR-α β antibodies had no effect on the PTK pathway unless CD4 was co-cross-linked. Antibody cross-linking of CD4 alone failed to induce the same pattern of tyrosine phosphorylation. Similar results were obtained when a chimeric protein consisting of the extracellular and transmembrane domains of CD4 linked to the intracellular Lck molecule was used in place of CD4. The tyrosine kinase activity of Lck was essential for the activity of the chimeric protein. Cross-linking of the CD4/Lck chimera to a CD8/γ chimeric molecule also facilitated induction of the PTK pathway with anti-CD8 antibodies. Moreover, the interaction of the two chimeric proteins, either in vitro or in vivo, resulted in tyrosine phosphorylation of CD8/γ. The effects of CD4/Lck on tyrosine phosphorylation and activation of PLC correlated well with the effects on PTK activation. Our results suggest that the Lck molecule positively regulates the TCR-coupled PTK pathway by phosphorylating tyrosines on the TCR-associated CD3ζ polypeptides.

Signal transduction in T lymphocytes is initiated by the simultaneous contact of T cell antigen receptor (TCR) and CD4 or CD8 glycoproteins with peptide antigen-containing MHC molecules on antigen-presenting cells (1-4). Subsequent coupling of the TCR complex to intracellular signaling pathways can result in lymphokine production, cell differentiation, proliferation, or cell death (1-5). The TCR is a multicomponent complex consisting of an antigen-specific disulfide-linked α β heterodimer and non-covalently associated CD3 invariant chains (γ, δ, ε, ζ, and η) (1-4, 6). The α β heterodimer binds to the antigen-MHC complex, and the associated CD3 polypeptides transduce signals (1, 6-10). After ligand binding, the earliest signal transduction events detected are increased protein tyrosine kinase (PTK) activity and protein tyrosine phosphorylation (11, 12). Tyrosine kinase inhibitors block TCR-mediated signaling, indicating that PTK activation is critical in this process.

Several PTKs have been reported to be associated with the TCR-CD3 complex, which alone has no intrinsic enzymatic activity (1, 6). The Src family tyrosine kinase p56<sup>Lck</sup> has been co-immunoprecipitated with the TCR-CD3 complex in a murine T cell hybridoma and human T cells (13, 14). Recently, another PTK, ZAP70, was shown to be associated with the CD3ζ chain in activated Jurkat cells (15, 16). The lymphoid-specific Src family PTK, p56<sup>Lck</sup> (Lck), is non-covalently associated with the cytoplasmic domains of the CD4 and CD8 coreceptors (17-19). This association is required for optimal activation of murine T cell hybridomas by antigen and for effective positive selection of mature T cells during thymopoiesis (20-24). The interaction of CD4 and CD8 with the nonpolymorphic regions of MHC class II and class I molecules, respectively, is thought to contribute to the recruitment of CD4-Lck and CD8-Lck complexes adjacent to ligand-occupied TCR-CD3 complexes. In addition, Lck expression appears to be essential for signal transduction even in T cells whose activation is independent of CD4 or CD8. For example, in mutant T cell tumor lines lacking Lck expression, PTK activation with anti-TCR antibodies was severely impaired, and responses were restored upon introduction of a functional lck gene (25, 26). Overexpression of an activated form of Lck in a CD4<sup>+</sup> T cell hybridoma increased the sensitivity of the response to antigen (27). It is not yet understood how Lck interacts with the TCR-CD3 complex in the absence of coreceptor. Lck also has an essential function in early thymocyte differentiation, as its absence results in a profound block in maturation of CD4<sup>+</sup>CD8<sup>+</sup> cells to the CD4<sup>+</sup>CD8<sup>+</sup> phenotype (28, 29). It is likely that this effect also reflects an interaction of Lck with a distinct early developmental form of the TCR-CD3 complex (30, 31).

To determine the mechanism by which p56<sup>Lck</sup> affects TCR-mediated signal transduction, we examined PTK activation upon antibody cross-linking of CD4-associated Lck to the TCR-CD3 complex. Using a CD4-dependent murine T cell hybridoma, we found that PTK activation and PLC phosphorylation/activation were significantly enhanced when either CD4 or a CD4/Lck fusion protein were co-cross-linked with the TCR-CD3 complex. Similar results were obtained when a CD8/γ chimeric molecule and anti-CD8 antibodies were used to transduce the TCR-coupled activation signal. The observation that the CD4/Lck chimera phosphorylated CD8/γ on tyrosines both in vitro and in vivo suggests that CD4-associated Lck facilitates coupling of a TCR-mediated signal to the downstream tyrosine kinase pathway by tyrosine-phosphorylating the TCR-associated CD3 polypeptides.

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The abbreviations used are: TCR, T cell antigen receptor; MHC, major histocompatibility complex; PTK, protein tyrosine kinase; PLC, phospholipase Cγ1; APC, antigen presenting cell.
EXPERIMENTAL PROCEDURES

Cell and Antibodies—The CD4-dependent antigen-specific T cell hybrids 171.3 and derivate cell lines were grown as described previously (21). The 171.3 CD4/1ck cell line was generated by introducing a chimeric molecule consisting of the extracellular and transmembrane domains of murine CD4 and the full-length murine Lck molecule into 171.3 cells by retroviral transduction as described previously (21, 32). 171.3 CmL273A cells were similarly prepared and express a CD4/Lck chimera mutated at the ATP-binding site (Lys-273 to Ala) and deficient in kinase function. The rabbit chimeric molecule expressed in COS cells had no detectable kinase activity (32). 171.3 CD8/3 expresses a chimera of the extracellular and transmembrane domains of human CD8 fused to the cytoplasmic domain of the human z chain (7) in the 171.3 CD4/1ck background. Cell lines were used for virus-infected cells and were screened for comparable surface expression of various forms of CD4 and TCR-CD3 complexes by FACSscan (Becton-Dickinson) analysis as described previously (21, 32).

145-2C11 (2C11) and H57.97 (Pharmingen) are hamster monoclonal antibodies and bind to the murine CD3e chain and the murine TCR-a/b heterodimer, respectively. The purified L3T4 rat monoclonal antibody (GK1.5, anti-murine CD4) was purchased from Becton-Dickinson. The OKT8 mouse monoclonal antibody (Ortho) is specific for human CD8. The rabbit anti-hamster affinity purified antibody (cross-reacting with human, mouse, and rat Ig) was used as secondary cross-linking anti-OKT8 mouse monoclonal antibody (Ortho) is specific for human CD8.

Cell Activation and Immunoblotting—Cells were activated by antibody cross-linking as described previously (33) with some modification. In summary, 2 ¥ 10^5 cells in 100 ml of RPMI 1640 (3% fetal calf serum) were incubated with 0.5 pg of 2C11, H57.97, or OKT8 with or without GK1.5 at 4°C for 10 min, washed with phosphate-buffered saline (PBS), and cross-linked by adding 2 yg of rabbit anti-hamster Ig at 37°C for an appropriate time, and lysed by adding an equal amount of 2 ¥ lysis buffer (40 mM Tris, pH 8.0, 274 mM NaCl, 2% Triton X-100, and phenylmethylsulfonyl fluoride). The lysates were analyzed by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with 4G10 antibody as described previously (16). Immunoblots were developed using the Enhanced Chemiluminescence method (Amersham Corp.). Usually, 10% of the cellular proteins of 171.3 CD4 Ack cells, is required for up-regulation of the PTK activation process generated through CD3 complexes. When CD4 was cross-linked alone in 171.3 CD4 Ack cells, the most heavily phosphorylated protein had an electrophoretic mobility corresponding to that of ~56"~ (33, 36, 37).

In Vitro PLC Assay of 4G10 Immunoprecipitates—Briefly, in the in vitro PLC assay was performed by mixing anti-phosphotyrosine-immunoprecipitated material with [3H]inositol trisphosphate bisphosphate (Sigma) at 37°C for 15 min, and measuring the amount of aqueous phase [3H]-inositol trisphosphate generated by phospholipase C. Aliquots of the precipitated material were prepared by first incubating 1 ml of 50% protein A-Sepharose bead suspension with 10 ml of 4G10 hybridoma cell supernatant at 4°C for 1 h with constant rotation and washing twice with 1 ¥ lysis buffer/5% glycerol plus protease inhibitors. The washed beads were divided into 12 samples and incubated with cell lysates (200 pg of protein/sample) at 4°C for 2 h. The beads were then used for PLC assay.

Immunoprecipitation of CD4, CD8, and CD4-CD8 and in Vitro Kinase Assay—171.3 CD3 b/c cells (4 ¥ 10^5 cells/sample) were cross-linked with appropriate antibodies as described above. Two min after addition of the appropriate second antibodies, the cells were washed with ice-cold PBS, resuspended in Ca^2+-free, Mg^2+-free PBS and lysed as described above. The cell lysates were incubated with appropriate antibodies (500 ng of GK1.5 for CD4 IP, 1 yg of 1C3 for CD8, and 1 yg of OKT8 for CD8) at 4°C for 1 h and with protein A-Sepharose 4B (Pharmacia LKB Instruments Inc.) at antibody concentration used specifically for immunoprecipitation of CD4 (Sigma) for another hour. All the immunoprecipitates were done in the presence of phosphatase inhibitor (sodium orthovanadate), kinase inhibitor (ETDA), and proteinase inhibitor (phenylmethylsulfonyl fluoride). The beads were washed, and 75% of the 1C3/1GK1.5 blots and 25% for the 4G10 kinase assay as described previously (21). Briefly, the immunoprecipitates were incubated with 10 yCi of [32P]ATP (adenosine triphosphate) at room temperature for 20 min, washed three times with water, and separated on 10% SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed to x-ray film as described (21).

RESULTS

Up-regulation of CD3-mediated PTK Activation by 4G10-associated Lck—Tyrosine phosphorylation of cellular proteins was analyzed by anti-phosphotyrosine immunoblotting of extracts from 171.3 T cell hybrids, expressing various forms of CD4, following anti-CD3 and anti-CD4 antibody cross-linking. A number of proteins became phosphorylated on tyrosine (p145, p129, p110, p100, p95, p80, p70–72, p63–65, and p57) at 30 s after anti-CD3 cross-linking (Fig. 1). The phosphorylation reaction peaked at 2 min and persisted for 10 min after cross-linking. The same kinetics were obtained in 171.3 cells regardless of whether or not surface CD4 was expressed (Figs. 1, A and B, and data not shown). This correlates well with previous findings that antibody cross-linking of CD3 can induce interleukin-2 secretion independent of CD4 expression in this cell and is consistent with previous studies demonstrating activation of a PTK pathway upon treatment with anti-CD3 (1–4, 21).

When CD3 and CD4 were co-cross-linked in 171.3 CD4/1ck and 171.3 CD4 Ack cells, the kinetics and magnitude of protein tyrosine phosphorylation were altered, although the pattern of tyrosine phosphoproteins was similar to that induced by CD3 cross-linking alone (Fig. 1, B and C). The tyrosine phosphoproteins appeared earlier, peaked at 30 s after co-cross-linking, and persisted longer (15 min after co-cross-linking). This indicates that CD4 can positively affect PTK activation through CD3 and thus change the kinetics of the PTK activation process when it is brought into proximity of the CD3 complex.

When CD4 was cross-linked alone in 171.3 CD4 Ack cells, the most heavily phosphorylated protein had an electrophoretic mobility corresponding to that of p56" (Fig. 1B, and data not shown). It was previously shown that CD4 cross-linking results in increased tyrosine phosphorylation of p56" (35, 36, 37). There were other less prominent tyrosine phosphoproteins induced by CD4 cross-linking alone (Fig. 1B). In comparison with cellular protein tyrosine phosphorylation after CD3 cross-linking, the most obvious difference was that no cellular proteins of approximately 70–72 kDa molecular mass were detected in the anti-phosphotyrosine blot after CD4 cross-linking alone (Fig. 1B). This suggests that the CD4 molecule itself is not directly coupled to the PTK pathway in the same way as the CD3 complex, to which the 70–72-kDa molecules appear to be specifically coupled.

To determine whether the alteration in kinetics was due to p56" associated with CD4, similar co-cross-linking experiments were carried out in 171.3 CD4 Ack and 171.3 CmL273A cells, which express, respectively, a tailless form of CD4 that cannot bind to Lck and a kinase-deficient CD4/1ck chimeric molecule at levels equivalent to those of CD4 in 171.3 CD4 Ack cells. In these cell lines, co-cross-linking of CD4 and CD3 failed to alter the extent or kinetics of PTK activation compared with treatment with anti-CD3 alone (Fig. 1D, and data not shown). Therefore, a catalytically active Lck molecule, noncovalently linked to the cytoplasmic tail of CD4 in 171.3 CD4 Ack cells or covalently fused to the transmembrane domain of tailless CD4 in 171.3 CD4/1ck cells, is required for up-regulation of the PTK activation process generated through CD3 complexes.

The PTK Pathway Cannot be Activated by Cross-linking TCR-a/b Alone, but Requires Co-cross-linking of CD4-associated Lck—Binding of TCR-a/b heterodimers on the T cell surface to peptide antigen plus MHC molecules on the APC surface results in signal transduction through CD3 complexes during normal T cell activation. To study the effect of CD4-associated Lck on TCR-a/b-initiated signaling, cross-linking was performed with antibodies against TCR-a/b heterodimers instead
of CD3. Surprisingly, the PTK pathway could not be activated by antibody cross-linking of TCR-αβ alone in 171.3 cells, regardless of whether CD4 was expressed (Fig. 1, A, E, and F). However, co-cross-linking of CD4 and TCR-αβ in 171.3 CD4/ltk cells induced the same pattern of protein tyrosine phosphorylation as that observed after CD4 and CD3 co-cross-linking (Fig. 1E). The PTK activation could not be achieved by co-cross-linking CD4 and TCR-αβ in 171.3 CtmL273A cells (Fig. 1F). Similar results were obtained with anti-TCR antibodies specific for TCR-αβ (H57.97) or for V,3 (KJ25), which is expressed on 171.3 cells (data not shown). A series of antibody titration experiments were done without successful PTK activation upon cross-linking of TCR-αβ alone (data not shown). Therefore, in contrast to antibodies against CD3ε, those binding to TCR-αβ, studied here, are strictly dependent on CD4-associated p56lck kinase activity for initiation of the PTK pathway upon cross-linking in 171.3 cells.

To determine whether the antibodies used for TCR-αβ cross-linking are able to activate other T cells under the same conditions, another murine hybridoma line (DO11.10) and a T cell tumor line (EL4) were subjected to similar treatment. Both cell lines could be activated by TCR-αβ cross-linking alone (data not shown). These cell lines both expressed approximately 4-5-fold higher levels of TCR and CD3 than the 171.3 cells on cytofluorometric analysis (data not shown), possibly accounting for some of the difference in activation.

**CD4-associated Lck Facilitates PTK Activation When Co-cross-linked to a CD8/ζ Chimeric Protein—**Previous studies have shown that the cytoplasmic domains of TCR-associated CD3 and ζ chains are sufficient to deliver activating signals when expressed as chimeric molecules at the cell surface (7-10). For example, CD8/ζ, a fusion molecule consisting of the extracellular and transmembrane domains of human CD8α and the cytoplasmic domain of the ζ chain, reconstitutes the signal transducing properties of the CD3 complex upon anti-CD8 cross-linking in Jurkat cells (7). The same CD8/ζ chimera was introduced into 171.3 CD4/ltk cells (giving rise to the 171.3 CD8/ζ cell line) in order to simplify our study of the role of CD4-associated Lck on ζ-mediated signaling. The PTK pathway could not be activated by cross-linking of CD8/ζ alone at a low antibody concentration (0.5 µg of OKT8/ζ x10³ cells) (Fig. 2A). Interestingly, at this concentration of OKT8 (0.5 µg/100 µl), co-cross-linking of CD4/ltk and CD8/ζ on the cell surface resulted in the same pattern of protein tyrosine phosphorylation as that obtained after CD3 cross-linking (Fig. 2A).

**CD4-associated Lck Can Phosphorylate Tyrosines of CD8/ζ**—CD4/ltk may help to initiate the PTK pathway by phosphorylating the tyrosine(s) of ζ and thus providing binding site(s) for ζ-associated PTK(s), such as ZAP70 and Syk. To determine whether CD4-associated Lck influences the tyrosine phosphorylation state of CD8/ζ, cell lysates prepared after antibody cross-linking were subjected to 4G10 immunoblotting, to show successful tyrosine kinase activation (Fig. 2B), and to immunoprecipitation with various antibodies, followed by immunoblotting and in vitro kinase assays (Fig. 2C and D). After co-cross-linking of CD4 and CD8 in 171.3CD8/ζ cells, increased tyrosine-phosphorylation of the 35-kDa CD8/ζ protein was detected in immunoprecipitates (Fig. 2B, C, and F). CD8/ζ phosphorylation was observed at 30 s and reached a peak at 2 min after CD8 cross-linking alone; after CD4 and CD8 co-cross-linking, the phosphorylation of the CD8/ζ chain was much more intense at both 30 s and 2 min (Fig. 2F).

To confirm that CD8/ζ can be phosphorylated by CD4/ltk directly, in vitro kinase assays were performed after immunoprecipitation of CD4 and/or CD8 molecules. After CD8 cross-linking alone, no detectable kinase activity was co-precipitated by OKT8 in an in vitro kinase assay (Fig. 2D). CD8/ζ molecules were phosphorylated in the in vitro kinase assay in the presence of CD4/ltk immunoprecipitates (Fig. 2D). In anti-CD4 immunoprecipitates alone, the CD4/ltk chimeric molecule was both autophosphorylated in the in vitro kinase assay and tyrosine-phosphorylated in vivo, as shown by anti-phosphotyrosine (4G10) immunoblotting after CD4 cross-linking alone (Fig. 2C and D). There was no other protein detected on immunoprecipitation of CD4/ltk or CD8/ζ in both in vitro kinase assays and in the 4G10 blot of lysates after in vivo antibody cross-linking. These results indicate that CD8/ζ is a good in vitro substrate for CD4-associated Lck. The increased tyrosine phosphorylation of

**Fig. 1. Effects of CD4/ltk on CD3 and TCR-αβ cross-linking.** Anti-phosphotyrosine (4G10) immunoblots of extracts from cells incubated with various combinations of anti-CD3ε (2C11), anti-CD4 (GK1.5) and anti-TCR-αβ (H57.97) at 4°C for 15 min, washed with phosphate-buffered saline, and then cross-linked with a secondary antibody at 37°C. Cells were lysed at various time points as indicated in the figures, and equal amounts of whole cell lysates were used for electrophoresis in non-reducing SDS-polyacrylamide gel electrophoresis.

CD8ζ upon co-cross-linking of CD4/lck and CD8ζ in vivo might hence be the consequence of p56lck being brought into the proximity of ζ chain.

We next determined whether CD4-associated Lck can tyrosine-phosphorylate CD8ζ in vivo in the absence of other T cell-specific tyrosine kinases. Anti-phosphotyrosine immunoblotting was performed on lysates of COS cells transiently transfected with constructs encoding the different proteins. There was significantly increased CD8ζ tyrosine phosphorylation when either CD4/lck or the Lck protein were co-expressed (Fig. 2E, lanes 6 and 7). Surface expression of murine CD4 and human CD8 were equivalent, as determined by FACS analysis, in the different transfections (data not shown).

When the amount of lysate used for CD4 and CD8 immunoprecipitations from the T cell hybridoma was increased from 2 μg (as used above) to 15 μg of protein, a tyrosine-phosphorylated protein of approximately 70 kDa was co-precipitated with CD8 from lysates of cells co-cross-linked with anti-CD4 and anti-CD8 but not from lysates of cells cross-linked with anti-CD8 alone (Fig. 2F). This co-precipitation was transient, as it was observed at 30 s after co-cross-linking, reached a peak at 2 min, and disappeared at later time points. It correlated very well with the extent of CD8ζ chain phosphorylation as shown in Fig. 2F. This result thus supports the notion that CD4/lck facilitates ζ chain association with ZAP70, possibly through the direct tyrosine-phosphorylation of the ζ chain.

To determine if the 70-kDa tyrosine-phosphoprotein associated with CD8ζ is the mouse homologue of human ZAP70, the immunoprecipitates were probed with an anti-ZAP70 antiserum (Fig. 2G). The co-precipitated 70-kDa protein observed after co-cross-linking of CD8ζ and CD4/lck was weakly cross-reactive with the anti-ZAP70 antibody and had the same electrophoretic mobility as that of ZAP70 in a Jurkat cell lysate (Fig. 2G). This suggests that the 70-kDa phosphoprotein may be the mouse homologue of ZAP70.

CD4/lck Increases Tyrosine Phosphorylation and the Activity of PLC—As a result of PTK pathway activation during TCR-mediated signal transduction, PLCγ1 is phosphorylated on tyrosines and is activated, resulting in PI turnover (34, 35). To determine whether CD4/lck modulates this process, the effects
on tyrosine phosphorylation and activation of PLC by antibody cross-linking of CD4/\(\text{lck}\) to TCR-\(\alpha\beta\), CD3 and CD8/\(\zeta\) were studied. Cell lysates were analyzed for PTK activation by 4G10 immunoblotting (Fig. 1, A-F, 2A, and data not shown) and were also subjected to immunoprecipitation with the anti-phosphotyrosine antibody 4G10 and assayed for PLC activity in vitro (Fig. 3A). A rapid increase in PLC activity was observed in 4G10 immunoprecipitates from both 171.3 CD4\(\text{lck}\) and 171.3 CD4/\(\text{lck}\) cells after CD3 cross-linking alone (Fig. 3A). The increases were detected 30 s after antibody cross-linking, peaked at 2 min, and returned to base levels at 10 min. As was observed with PTK activation, co-cross-linking of CD4 and CD3 changed the kinetics of PLC activation in 4G10 immunoprecipitates from 171.3 CD4/\(\text{lck}\) but not from 171.3 CD4\(\text{lck}\) cell lysates. The PLC activation peaked at a higher level and persisted longer (15 min after antibody cross-linking). There was no detectable change in PLC activity when only CD4 was cross-linked in 171.3 CD4/\(\text{lck}\) cells (Fig. 3B). This suggests that CD4/
Kinase Requirement of CD4-associated Lck

Lck is coupled to the PLC pathway in a fashion that requires interaction with TCR components, even though cross-linking of CD4-associated Lck alone results in its autophosphorylation and increased kinase activity (36).

Small increases (2-3-fold) in PLC activity were observed 2 min after TCR-αβ cross-linking alone in 171.3 CD4/lek and 171.3 CD4/Ti cells (Fig. 3, C and D). These reproducible increases were observed in the same cell lysates in which no protein tyrosine phosphorylation events were detected by immunoblotting (Fig. 1, A, E, and F). This discrepancy may be explained by differences in sensitivity between the PLC assay and 4G10 immunoblotting. Upon co-cross-linking of CD4 and TCR-αβ, there was enhanced and sustained PLC activation (6-fold) in 171.3 CD4/lek cells (Fig. 3C) but not in 171.3 CD4/Ti cells (Fig. 3D). In cells expressing the CD4/lek chimera, PLC activity could still be precipitated with 4G10 at 10 min after co-cross-linking, whereas activity returned to basal level after CD3 cross-linking (Fig. 3C).

Similar results were obtained when CD8 was cross-linked alone or with the CD4/lek chimera in 171.3 CD8/β cells. Co-cross-linking of CD4 and CD8 altered the kinetics of PLC tyrosine phosphorylation and activation as shown in Fig. 3E. Overall, CD4-associated Lck enhanced tyrosine phosphorylation and activation of PLC when co-cross-linked to CD3, TCR-αβ, and CD8/β.

**DISCUSSION**

PTK activity is critical for T cell activation, as PTK inhibitors block early signaling events such as Ca²⁺ influx and subsequent T cell activation (12). There are a number of tyrosine kinases involved in T cell activation, including p56lck, p55, and a 70-kDa zeta-associated PTK (ZAP70) (16-19). Lck has been shown to be essential for both thymocyte development and T cell activation (25, 26, 29). In mice genetically ablated for exons 1-5 of Lck, T cell activation is by phosphorylating tyrosine(s) of the CD3ζ chain (30) and of DP TCR-αβ (31) thymocytes employed.

One possible mechanism by which CD4/lek may function in T cell activation is by phosphorylating tyrosine(s) of the CD3ζ chain, thus facilitating the association of ζ chain with downstream signal transducing molecules. It has been reported that phosphorylation of CD3ζ chain follows the ligand binding of TCR during T cell activation and thymocyte maturation (15). There are 6 tyrosines in the cytoplasmic domain of ζ chain, all located in the well-defined "signaling motifs," which are conserved among a number of putative signaling molecules (6). Mutagenesis studies have shown that the tyrosines are absolutely required for signaling (50). ZAP70 has been shown to associate with ζ after activation of Jurkat cells (15, 16). The binding of ZAP70 to CD3ζ chain through the interaction of its SH2 domains with phosphotyrosyl motifs may be a critical step for activation of the PTK pathway.

In COS cells, the association of ZAP70 with the ζ chain required the presence of p56lck or p55 (16). This co-expression also resulted in increased tyrosine phosphorylation of both ζ chain and ZAP70. In T cells, we found that co-cross-linking of the CD4/lek and CD8/β chimeric proteins at suboptimal anti-CD8 concentration activated the PTK pathway and was accompanied by increased tyrosine phosphorylation of CD8/β and association of CD8/β with a 70-kDa phosphoprotein, potentially ZAP70. From our experiments, it is not clear if the TCR-CD3 complex is required for this effect on CD8/β. However, the earlier demonstration that the CD8/β chimera functions in cells lacking surface TCR/CD3 expression (7) suggests that the interaction of the intracellular domains of Lck and ζ is sufficient to trigger the relevant PTK pathway. In COS cells, co-expres-
Taken together, these data are consistent with the notion that CD4-associated Lck phosphorylates tyrosines within the TCR-associated \( \zeta \) chain, permitting subsequent association of ZAP70 with the TCR complex.

The fact that cross-linking of CD4 alone did not result in PLC tyrosine phosphorylation and activation indicates that CD4-associated p56\( ^{la} \) is not directly linked to the PLC pathway during T cell activation (Fig. 3B). However, PLC activation can be achieved through the interaction of CD4-associated p56\( ^{la} \) with the TCR-CD3 complex. The most obvious difference between CD4 cross-linking alone and CD4 cross-linking alone in 4G10 blots was the absence of tyrosine phosphoproteins in the PLCs associated with the TCR complex.

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