Role of the Lck Src Homology 2 and 3 Domains in Protein Tyrosine Phosphorylation*

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Many protein tyrosine phosphorylation events that occur as a result of T cell receptor (TCR) stimulation are enhanced when CD4 is co-cross-linked with the TCR, and this increased phosphorylation is thought to be a mechanism by which T cell functions are augmented by CD4. Such enhanced tyrosine phosphorylation was originally attributed to the kinase activity of the CD4-associated tyrosine kinase Lck. However, it has been shown that CD4-associated Lck lacking the catalytic domain can enhance T cell functions, suggesting that the non-catalytic domains of Lck are also important in CD4 signaling. Using T cells expressing various CD4-Lck chimeric molecules, we assessed the role of different Lck domains in early T cell signaling. Following TCR-CD4 co-cross-linking, cells expressing a CD4-Lck full-length chimera showed enhanced tyrosine phosphorylation of many cellular proteins in a CD4-dependent manner. Surprisingly, cells expressing a CD4-Lck chimera lacking the catalytic domain (termed CD4-N32) also showed enhanced phosphorylation. This enhancement of phosphorylation required both the Src homology 2 (SH2) and SH3 domains of Lck. Lck has been postulated to dimerize through the SH2 and SH3 domains. In this way, CD4-N32 may interact with endogenous Lck, and although it lacks intrinsic kinase activity, it may be capable of enhancing phosphorylation through the associated full-length Lck. Consistent with this model, when CD4-Lck chimeric molecules were expressed in J.CaM1.6 cells lacking endogenous Lck, CD4-N32 failed to enhance tyrosine phosphorylation. Moreover, a Lck SH2 and SH3 domain fragment expressed as a glutathione S-transferase fusion protein associated with Lck when incubated with activated Jurkat T cell lysates, suggesting that the SH2 and SH3 domains of Lck can associate with endogenous full-length Lck upon activation. Thus, our data suggest that dimerization is an important mechanism of Lck function in T cell activation.

Lck is a 56-kDa protein tyrosine kinase that is predominantly expressed in T lymphocytes. A member of the Src kinase family, it has a unique N-terminal region followed by Src homology (SH)3,1 SH2, and catalytic domains (1). Lck is an important protein tyrosine kinase in lymphocytes; its overexpression renders T cells hypersensitive to antigen stimulation (2), and an Lck-deficient T cell line, J.CaM1, exhibits dramatically reduced protein tyrosine phosphorylation following T cell receptor (TCR) cross-linking (3). Furthermore, genetic experiments have shown that mice deficient in Lck or expressing a dominant-negative mutant form of Lck exhibit a severe defect in T cell maturation (4, 5). Lck is localized to the membrane through myristylation (6) and palmitylation (7–9), and a portion of cellular Lck associates with the cytoplasmic tail of CD4 via cysteine residues (10–13). CD4 has been termed a T cell co-receptor because engagement of CD4 together with the TCR enhances T cell functions such as interleukin 2 production and proliferation (14–20). CD4 binds to class II major histocompatibility complex molecules on antigen-presenting cells, and this interaction between CD4 and major histocompatibility complex activates Lck, perhaps through conformational changes. The Lck associated with CD4 propagates key biochemical signals in CD4 co-receptor function (21, 22).

In T cells, tyrosine phosphorylation of intracellular proteins is one of the earliest events that occur following activation (23–26), and Lck is one of the crucial kinases involved in this early tyrosine phosphorylation (3). Although many substrates become tyrosine phosphorylated in T cells following TCR triggering alone, co-activating CD4 with the TCR results in the augmented phosphorylation of many of these proteins (27). This enhanced phosphorylation is thought to be one biochemical mechanism by which T cell functions are augmented by co-receptors such as CD4. Increased tyrosine phosphorylation results in the enhancement of phosphotyrosine-dependent protein interactions, which results in the augmentation of signaling cascades. Since its association with Lck has been shown to be crucial for the co-receptor functions of CD4 (21, 22), this CD4-dependent enhancement of protein tyrosine phosphorylation has been assumed to be due to the kinase activity of Lck. However, Xu and Litman (28) have shown that a chimeric molecule, composed of the CD4 extracellular and transmembrane domains ligated to a catalytic domain-deleted form of Lck, functions as well as wild type CD4 associated with endogenous Lck in enhancing interleukin 2 production in an antigen-specific, CD4-dependent manner. Consistent with this observation, we (29) have shown that T cell hybridomas overexpressing a kinase-deleted Lck exhibit normal CD4-dependent function despite a greater than 90% diminution of CD4-associated Lck kinase activity. These data suggest that other components of Lck besides its catalytic domain are important for mediating CD4 co-receptor activity.

We have used CD4-Lck chimeric molecules to study the role of noncatalytic domains of Lck in the biochemical events that occur during early T cell activation. Using T cells expressing chimeric molecules with the CD4 extracellular and transmembrane domains fused to various domains of Lck, we examined protein tyrosine phosphorylation following TCR and CD4-Lck...
chimera co-activation. We present data showing that a CD4-Lck chimera lacking the catalytic domain (CD4-N32) can enhance tyrosine phosphorylation upon TCR-CD4 co-cross-linking. We also show that endogenous, full-length Lck can associate with the SH2 and SH3 domains of Lck and is required for enhanced tyrosine phosphorylation by CD4-N32. These findings suggest that dimerization may be an important part of the mechanism for Lck function in CD4 co-receptor activity.

**EXPERIMENTAL PROCEDURES**

**Constructs**—CD4-Lck chimeric constructs were generated by ligating a CD4 cDNA fragment containing the extracellular and transmembrane domains of human CD4 to different fragments of murine Lck cDNA. The CD4 fragment was generated by polymerase chain reaction using a 5′-primer (spanning nucleotides 45 to 31 relative to the ATG start codon) containing a SalI site and a 3′-primer (nucleotides 1245–1256) containing an EcoRI site. The Lck portions of the CD4-FL, CD4-N2, CD4-N3, and CD4-N constructs were generated by polymerase chain reaction using Lck 5′-primer (nucleotides 12 to 1 relative to the start codon) containing an EcoRI site, Lck FL 3′-primer (nucleotides 1552–1569) with an XbaI site, Lck N32 3′-primer (nucleotides 727–744) with an XbaI site, Lck N33 3′-primer (nucleotides 288–308) with a BamHI site, and Lck N 3′-primer (nucleotides 180–198) with a BamHI site. The CD4 fragment was cloned into pBluescript at SalI and EcoRI sites, and Lck fragments were introduced into pBluescript via EcoRI and XbaI (for CD4-FL and CD4-N32) or EcoRI or BamHI (for CD4-N and CD4-N3) sites in pBluescript. CD4 and Lck portions were ligated together at the EcoRI site in pBluescript. The CD4-N2 construct was generated by ligating a polymerase chain reaction-generated Lck SH2 fragment to the 3′-end of the CD4-N construct via BamHI (3′-end of CD4-N) and XbaI sites. The Lck SH2 fragment was generated using a 5′-primer with a BglII site (nucleotides 331–345) and the Lck N32 3′-primer (nucleotides 727–744). The final CD4-Lck constructs were moved to the pFneo mammalian expression vector using the 5′-SalI and 3′-XbaI or BamHI sites for expression of the chimeric proteins in BY155.16 cells. For expression of the chimeric molecules in J.CaM1.6 cells, the pB-A mammalian expression vector was used. At the junction of CD4 and Lck, two amino acids in Lck were substituted; instead of the starting Met and Gly at the beginning of Lck, it is Glu and Phe. The nucleotide sequences of all constructs were verified by sequencing.

**Cell Lines**—BY155.16, a CD4+ murine T cell hybridoma line (14), was transfected with the chimeric constructs by electroporation (Hoeffer Scientific Instruments) at 213 V and 1200 microfarads for 11 ms. J.CaM1.6 is a Jurkat T cell line derived by transfection of wild type Lck (3), was transfected with the chimeric constructs by electroporation (Bio-Rad) at 250 V and 800 microfarads. The cells were allowed to recover for 2 days and then plated in G418 selection medium (2 mg/ml) 10^4 cells/well in 48-well plates (Nunc). Neomycin-resistant clones were screened for surface expression of CD4 by flow cytometry analysis using a FACScan (Becton Dickinson). 16T4 is a BY155.16 cell line stably transfected with wild type human CD4, which has been previously described (14).

**Antibodies, Fusion Proteins, and Peptides**—P23.1 anti-mouse TCR V8.8 antibody (Ab), OKT3 anti-human CD3ε Ab, and OKT4D anti-human CD4 Ab were purified from ascites. Rabbit anti-mouse IgG + IgM Ab was purchased from Fisher. 4G10 antiphosphotyrosine Ab and anti-Lck polyclonal Ab against the N-terminal region of Lck were obtained from Upstate Biotechnology, Inc. G3 anti-ζ Ab culture supernatant was a kind gift from Dr. H. S. Teh (University of British Columbia, Vancouver, British Columbia, Canada). The anti-Shc rabbit polyclonal Ab, anti-Crk mouse monoclonal Ab, and anti-Lck monoclonal Ab were purchased from Transduction Laboratories. Anti-Grb2 rabbit polyclonal Ab was obtained from Santa Cruz. The glutathione S-transferase (GST) fusion protein of Lck SH2 and SH3 domains (GST-SH2SH3) was purchased from Santa Cruz. The sequence for the phosphorhage S family SH2 domain binding peptide is EEQYIEEPYIL, and that for the nonphosphorylated control peptide is AEEEYYYEEAKKK. and these were kind gifts from Dr. L. C. Cantley (Harvard Medical School, Boston, MA).

**Flow Cytometric Analysis**—Approximately 3 × 10^6 cells were incubated on ice for 30 min with 50 μl fluorescein isothiocyanate-conjugated anti-CD3ε Ab (Boehringer Mannheim), diluted 1:1000, or with 50 μl (1 μg/ml) Leu-3a-PE, an anti-CD4 Ab conjugated with phycoerythrin (Becton Dickinson). The control cells were incubated without Ab or with mouse IgG-phycoerythrin control Ab (Becton Dickinson). The cells were washed, fixed with 2% paraformaldehyde, and analyzed by fluorescence-activated cell sorting.

**Expression of Chimeric Molecules**—Cells (10^6) were washed in phosphate-buffered saline, pH 7.4, and incubated at 4 °C in 1 mg/ml N-hydroxysuccinimidyl-6-(biotinamido)hexonate (NHS-LC-biotin) (Pierce) in phosphate-buffered saline with constant rocking for 45 min. The cells were washed with 0.15% glycine solution in phosphate-buffered saline, and lysis, immunoprecipitation, and Western blotting were carried out as described below.

**Immunoprecipitation and Immunoblotting**—The cells (10^6) were incubated in RPMI 1640 medium in microfuge tubes with mouse anti-TCR Ab (1 μg/ml) and/or OKT4D mouse anti-CD4 Ab (1 μg/ml) for 10 min on ice with intermittent mixing, followed by incubation with rabbit anti-mouse IgG + IgM Ab (RAM) (10 μg/ml) for 10 min on ice. The cells were then activated by incubating at 37 °C for 2 min. They were quickly centrifuged, washed once with RPMI 1640 medium, centrifuged, and lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 10 μg/ml each of leupeptin and aprotinin, 1 mM Na3VO4, 1% Nonidet P-40, 2 μM phenylmethylsulfonyl fluoride, and 10 μg/ml NaF). These lysates were immunoprecipitated with 2 μg of the indicated purified antibody or 200 μl of culture supernatant along with 50 μl of protein A-Sepharose beads (Pharmacia Biotech Inc.) at 4 °C for 1–2 h. In cases in which precleavage of stimulating antibodies was required, the lysates were first incubated with 50 μl of protein A-Sepharose beads for 1 h at 4 °C, and the supernatant was used for subsequent immunoprecipitation. The beads were washed four times with immunoprecipitation wash buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 μg/ml each of leupeptin and aprotinin, 0.1% Negfect P-40, and 4% glycerol), boiled in 50 μl of SDS-polyacrylamide gel electrophoresis loading buffer, and separated by SDS-polyacrylamide gel electrophoresis. In assays in which GST fusion proteins were used, the lysates were incubated with 2 μg of the protein-conjugated glutathione beads for 1–2 h, and 40 μl of glutathione beads were added at the end of incubation before washes.

**Western Blot Analysis**—Proteins were transferred to polyvinylidene difluoride membranes (Millipore), and the membranes were blocked with 4% bovine serum albumin (Boehringer Mannheim). For antiphosphothreonine immunoblotting, membranes were incubated with a horseradish peroxidase-conjugated recombinant antiphosphotyrosine antibody, RC20H (Transduction Laboratories). For anti-Lck immunoblotting, anti-Lck monoclonal Ab (Transduction Laboratories) was used. In surface biotinylation experiments, membranes were incubated with streptavidin-horseradish peroxidase (Amersham). The blots were developed by enhanced chemiluminescence (ECL; Amersham).

**RESULTS**

**Expression of CD4-Lck Chimeric Molecules in a T Cell Hybridoma**—BY155.16 cells (14), a murine T cell hybridoma line that lacks CD4, was transfected with chimeric CD4-Lck molecules containing the extracellular and transmembrane domains of CD4 ligated to the full-length Lck (CD4-FL); the N, SH3, and SH2 domains of Lck (CD4-N32); the N and SH3 domains (CD4-N3); the N and SH2 domains (CD4-N2); or the N domain of Lck (CD4-N) (Fig. 1A). After screening for surface expression of CD4-Lck chimeric molecules by anti-CD4 antibody staining, several clones expressing each chimeric molecule with similar amounts of expression were used for the studies (Fig. 1B). Expressing chimeric CD4-Lck in CD4-negative cells ensured that, upon manipulation of CD4, only the Lck fused to CD4 was directly recruited to the TCR complex in a CD4-dependent manner. Cell surface biotinylation followed by anti-CD4 antibody immunoprecipitation and blotting with streptavidin showed that proteins of the expected size were expressed in these cell lines (Fig. 1C). Protein tyrosine kinase activity was observed when CD4-FL was immunoprecipitated from cells and an in vitro kinase assay was performed, whereas the CD4-Lck chimeras lacking the catalytic domain did not demonstrate kinase activity as measured by autophosphorylation (data not shown).

**Tyrosine Phosphorylation following Activation of Cells Expressing Different CD4-Lck Chimeras**—Wild type CD4, when co-cross-linked with the TCR, enhances phosphorylation of several cellular proteins (Fig. 2). We assessed the role of various domains of Lck in CD4-dependent phosphorylation by studying
the tyrosine phosphorylation of intracellular proteins. Cells were activated by cross-linking the TCR alone or by co-cross-linking the TCR with wild type CD4 or CD4-Lck chimeras, and either whole cell lysates or immunoprecipitates of tyrosine-phosphorylated cellular substrates were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antiphosphotyrosine antibody. Comparison of tyrosine phosphorylation in cells expressing wild type CD4 (16T4) and CD4-FL showed...
that the CD4-FL chimeric molecule was capable of functioning in the same manner as wild type CD4 in stimulating early tyrosine phosphorylation events (Fig. 2). Like 16T4 cells (Fig. 2) and untransfected BY155.16 parent cells (data not shown), cross-linking of the TCR alone in cells expressing CD4-Lck chimeras resulted in phosphorylation of several proteins, notably of molecular masses of approximately 140, 110, 85, 60, 50–55, and 36 kDa (Fig. 3A). This indicated that the cellular machinery for TCR-mediated tyrosine phosphorylation remained intact and was not affected by expression of CD4-Lck chimeras. Co-cross-linking of the CD4-FL chimera with the TCR complex enhanced the level of phosphorylation of these proteins (Fig. 3A). Phosphorylation of p140, p110, p50–55, and p36 was observed when CD4-FL was cross-linked without the TCR, indicating that TCR-independent phosphorylation of these proteins can occur. Following co-cross-linking of the TCR with CD4 in cells expressing CD4-N32, enhanced phosphorylation of p140, p110, p85, p70, p50–55, and p36 was also observed (Fig. 3A), although the degree of enhancement was less than in cells expressing CD4-FL. Thus, CD4-N32 was capable of enhancing tyrosine phosphorylation of several substrates in early T cell activation even though it lacked the catalytic domain.

To further assess the structural requirements for this enhanced tyrosine phosphorylation, we examined CD4-induced tyrosine phosphorylation in cells expressing CD4-Lck chimeras lacking the SH3 and/or SH2 domains. None of the cell lines expressing CD4-N, CD4-N3, or CD4-N2 showed enhanced tyrosine phosphorylation of any of the cellular substrates upon TCR and CD4-Lck chimera co-cross-linking (Fig. 3, B–D). Several clones expressing varying levels of CD4 and each of the CD4-N, -N3, and -N2 chimeras were tested, and none of the clones showed enhanced phosphorylation after TCR and CD4-Lck chimera co-cross-linking (data not shown), indicating that the inability of these CD4-Lck chimeras to enhance tyrosine phosphorylation was not due to insufficient expression of the receptors. These results suggest that both the SH3 and the SH2 domains of Lck are necessary for enhancement of tyrosine phosphorylation mediated by CD4-N32.

**Enhancement of Shc Phosphorylation Does Not Depend on the Catalytic Domain of CD4-Lck**—To identify the individual proteins with phosphorylation that is enhanced in a CD4/Lck-dependent manner, proteins that are known to be phosphorylated following T cell activation and are involved in early T cell signaling were immunoprecipitated, and their phosphorylation states were assessed. Shc is one phosphoprotein that migrates near 50–55 kDa. Rapidly phosphorylated on T cell activation, Shc associates with Grb2 and mSos-Ras nucleotide exchange factor and therefore may link TCR activation to the Ras pathway (31–34). We have previously shown that Shc associates with the phosphorylated \( \zeta \) chain upon T cell activation (35). To examine the phosphorylation of Shc, cells expressing various CD4-Lck chimeras were stimulated in the same manner as described above, and Shc was immunoprecipitated and immunoblotted using an antiphosphotyrosine antibody (Fig. 4). Shc was phosphorylated after TCR cross-linking alone in all cell lines, but its phosphorylation was markedly enhanced by TCR and CD4 co-cross-linking in cells expressing CD4-FL as well as in cells expressing CD4-N32 (Fig. 4A). Cells expressing CD4-N, CD4-N3, or CD4-N2 did not show enhanced Shc phosphorylation (Fig. 4, B–D). This suggests that upon

![Fig. 2. Enhancement of protein tyrosine phosphorylation following co-cross-linking of TCR and CD4 in cells expressing wild type CD4 or CD4-FL. Cells expressing wild type CD4 or CD4-FL were stimulated with the indicated antibodies, and the lysates were prepared as described under “Experimental Procedures.” Whole cell lysates (WCL; 10^6 cell equivalents) were immunoblotted with RC20H antiphosphotyrosine Ab.](image)

![Fig. 3. Enhancement of tyrosine phosphorylation following TCR and CD4-Lck chimera co-cross-linking is seen in cells expressing CD4-FL or CD4-N32 but not in cells expressing CD4-N, CD4-N3, or CD4-N2. Cells expressing various CD4-Lck chimeras were stimulated as indicated, and the lysates were immunoprecipitated (IP) with antiphosphotyrosine Ab (4G10) as described under “Experimental Procedures.” Antiphosphotyrosine blots using RC20H for cells expressing CD4-FL or CD4-N32 (A), CD4-N3 (B), CD4-N2 (C), or CD4-N (D) are shown.](image)
TCR and CD4 co-cross-linking the SH2 and SH3 domains of the CD4-Lck chimeric molecule can mediate CD4-dependent enhancement of Shc phosphorylation.

**Enhanced Phosphorylation of p36 Does Not Require the Catalytic Domain of CD4-Lck**—The 36-kDa protein that becomes prominently phosphorylated upon T cell activation may be the recently cloned Lnk protein (36), which, like Shc, associates with Grb2 following activation (37). Although it is a major phosphorylation target in early T cell activation, the function of p36 in T cell signaling has yet to be determined. Grb2 was immunoprecipitated following activation from cells expressing various chimeras, and phosphoproteins associated with Grb2 were examined by antiphosphotyrosine immunoblotting (Fig. 5). In all cell lines both p36 and Shc were precipitated with Grb2 following TCR cross-linking, but only in cells expressing CD4-FL or CD4-N32 did co-cross-linking of the TCR with the CD4 chimera enhance phosphorylation of p36 and Shc and, therefore, their association with Grb2 (37, 38). The phosphorylation levels of Shc and p36 (Figs. 4A and 5A) following TCR cross-linking alone in cells expressing CD4-FL were lower than those in cells expressing CD4-N32 due to the lower expression level of TCR in CD4-FL* cells. In cells expressing CD4-N, CD4-N3, or CD4-N2, no enhancement of p36 or Shc phosphorylation was observed. This indicates that noncatalytic regions of Lck can mediate the increase in phosphorylation of p36 and Shc in a CD4-dependent manner.

**Significant Enhancement of Cbl Phosphorylation Is Mediated by CD4-FL but not by CD4-N, CD4-N3, CD4-N2, or CD4-N32**—In antiphosphotyrosine immunoblots, proteins migrating in the region of 110–120 kDa are heavily phosphorylated following TCR cross-linking. Although the precise identities of all the proteins in this region have not been determined, experiments have suggested that p110 may contain Cbl (39, 40) and/or a p130 isoform (41, 42). We have found that Cbl is a significant component of phosphorylated p110 in T cells (43). It has also been observed that phosphorylated Cbl associates with Crk upon T cell activation, via the SH2 domain of Crk (40, 44). Crk may be involved in Ras functions, since it also associates with C3G, a guanine nucleotide exchange factor for Ras and/or Ras-related molecules (45, 46). We therefore assessed the phosphorylation state of Cbl and its association with Crk following TCR and CD4-Lck chimera co-cross-linking. Crk immunoprecipitation, using an antibody against all three known isoforms of Crk (Crk-I, Crk-II, and Crk-L), showed that Cbl became phosphorylated and associated with Crk following TCR stimulation (Fig. 6). This phosphorylation and association with Crk was enhanced following cross-linking of CD4-Lck chimeras with the TCR in cells expressing CD4-FL. Such enhancement was not seen in cells expressing CD4-N3, CD4-N2, or CD4-N and was minimal in CD4-N32 cells, suggesting that the catalytic domain of CD4-Lck is required for the optimal enhanced phosphorylation of Cbl.

**Enhanced Phosphorylation of the ζ Chain of the TCR-CD3 Complex Does Not Require the Catalytic Domain of CD4-Lck**—The ζ chain of the TCR-CD3 complex is phosphorylated on T cell activation (47–49). The identity of the kinase that phosphorylates ζ has not been firmly established, although there are data to suggest that Lck may serve this function (50). To elucidate the role of different domains of Lck in ζ phosphorylation, we assessed the phosphorylation state of ζ after stimulation (Fig. 7). In all cell lines the ζ chain became phosphorylated upon TCR stimulation alone, showing that TCR cross-linking can result in ζ chain phosphorylation in a CD4-independent manner, perhaps through TCR-CD3-associated Fyn or Lck. The ζ phosphorylation was enhanced following cross-linking of the TCR with CD4-Lck chimeras in cells expressing CD4-FL or CD4-N32 but not in cells expressing CD4-N, CD4-N2, or CD4-N3, similar to the results observed with Shc and p36.

Both CD4-FL and CD4-N32 Can Affect the Phosphorylation of Endogenous Lck—Since it has been shown that the level of phosphorylation of Lck following activation correlates with its kinase activity (51), we examined the phosphorylation state of endogenous Lck to assess its activation state following cross-linking of various CD4-Lck chimeras with the TCR. Lck was immunoprecipitated from activated cells, and its phosphorylation state was examined by immunoblotting. Endogenous Lck was phosphorylated following cross-linking of the TCR alone in all cells, but its phosphorylation was enhanced only in cells expressing CD4-FL or CD4-N32 and not in cells expressing CD4-N3, CD4-N2, or CD4-N.
molecules (Fig. 8). The CD4-FL chimera itself was also phosphorylated following CD4 cross-linking and TCR and CD4 co-cross-linking (Fig. 6A), whereas other CD4-Lck chimeric molecules failed to become phosphorylated, since they lack the major phosphorylation site Y394, which lies in the catalytic domain of Lck (data not shown). This suggests there is a cross-talk between the CD4-bound Lck and endogenous Lck.

Expression of CD4-FL and CD4-N32 in J.CaM1.6 Cells Shows the Requirement of Endogenous Lck for Enhancement of Phosphorylation by CD4-N32—The data presented above suggested that Lck lacking the catalytic domain can nevertheless mediate enhancement of tyrosine phosphorylation of many cellular substrates. Via its SH2 and SH3 domains, CD4-N32 may recruit other signaling molecules containing intrinsic or associated kinase activity that functions in CD4-dependent phosphorylation. Many signaling molecules have been shown to associate with the SH2 and SH3 domains of Lck, including Lck itself; it has been postulated that Lck molecules can dimerize through the SH2 and SH3 domains (52, 53). Given the data showing that CD4-N32 can enhance the tyrosine phosphorylation of endogenous Lck, and given the requirement for both the SH2 and SH3 domains of Lck for enhanced phosphorylation, we considered endogenous Lck to be a strong candidate for such an Lck SH2-SH3 domain-associating kinase. As a first step to testing this hypothesis, we determined whether CD4-N32-dependent phosphorylation is mediated through endogenous Lck. J.CaM1.6 cells, a Jurkat variant that lacks the Lck kinase, were transfected with CD4-FL, CD4-N32, or the empty expression vector. The expression level was tested by fluorescence-activated cell sorting analysis (data not shown), and several clones with different amounts of TCR and CD4-Lck chimera were used for experiments. Cells were stimulated by TCR and/or CD4 cross-linking, and phosphoproteins were immunoprecipitated and immunoblotted using antiphosphotyrosine antibody. In untransfected J.CaM1.6 cells (data not shown) and vector transfectants, weak tyrosine phosphorylation was observed following TCR stimulation (Fig. 9). Protein tyrosine phosphorylation was increased in cells expressing CD4-FL when this chimera was co-cross-linked with TCR. However, no enhancement of phosphorylation on TCR and CD4-N32 chimera co-cross-linking was observed in two different J.CaM1.6 clones expressing CD4-N32. This lack of enhancement was observed in all four CD4-N32-expressing cell lines that were tested (data not shown). This suggests that endogenous Lck plays a crucial role in CD4-N32-mediated tyrosine phosphorylation.

SH3 plus SH2 Fragment of Lck Associates with Full-Length Lck following Activation—To assess whether the isolated SH2 and SH3 domains of Lck can interact with full-length Lck, Jurkat T cells were activated by TCR cross-linking, and lysates were incubated with various domains of Lck expressed as GST fusion proteins. Proteins associating with the GST protein-conjugated glutathione beads were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using an anti-Crk Ab and immunoblotted with RC20H as described under “Experimental Procedures.”

FIG. 6. Maximal phosphorylation of Cbl and its association with Crk is enhanced in cells expressing CD4-FL but not in cells expressing CD4-N32, CD4-N, CD4-N3, or CD4-N2. Cells expressing CD4-FL or CD4-N32 (A), CD4-N3 (B), CD4-N2 (C), or CD4-N (D) were stimulated as indicated, and cell lysates were immunoprecipitated (IP) with anti-Crk Ab and immunoblotted with RC20H as described under “Experimental procedures.” Arrow on right, CD4-FL chimera that becomes phosphorylated following cross-linking and is immunoprecipitated by the residual stimulating anti-CD4 Ab. CD4-FL was not observed in experiments in which the lysates were precleared of stimulating antibodies, were immunoprecipitated (IP) with anti-Lck Ab and immunoblotted with RC20H as described under “Experimental Procedures.”

FIG. 7. Enhancement of ζ phosphorylation following TCR and CD4-Lck chimera co-cross-linking in cells expressing CD4-FL or CD4-N32 but not in cells expressing CD4-N, CD4-N3, or CD4-N2. Cells expressing CD4-FL or CD4-N32 (A), CD4-N3 (B), CD4-N2 (C), or CD4-N (D) were stimulated as indicated, and the lysates were immunoprecipitated (IP) with anti-ζ chain Ab culture supernatant and immunoblotted with RC20H as described under “Experimental Procedures.” Arrow, the two phosphorylated forms of the ζ chain.
anti-Lck monoclonal Ab. GST-SH3SH2, which contains the SH3 and SH2 domains of Lck, associated with full-length Lck upon activation of T cells, whereas GST-SH3, GST-SH2, and GST proteins did not (Fig. 10A). There are two ways in which Lck-Lck interactions are thought to occur: by the Lck SH2 domain binding to the phosphorylated Y505 (54, 55) via the classic SH2-phosphotyrosine interaction, and by the Lck SH2 domain binding to the Lck SH3 domain (52, 53). To distinguish between these possibilities, we tested for competitive inhibition by the phosphopeptide pYEEI. Although this peptide binds with high affinity to the SH2 domains of Src family kinases, it did not block the interaction between Lck SH3-SH2 and full-length Lck (Fig. 10B) at a concentration that inhibited association of other SH2-binding phosphoproteins (data not shown). These data indicate that this interaction is not through the SH2 phosphotyrosine binding pocket.

**DISCUSSION**

We have shown that activation of T cells by co-cross-linking the TCR with the CD4-FL chimera results in enhanced tyrosine phosphorylation of several intracellular substrates such as Shc, Crk-associated Cbl, Grb2-associated p36 (Lnk), the ζ chain of the TCR complex, and endogenous Lck. We have also shown that enhanced phosphorylation of Shc, p36, ζ, and endogenous Lck can also be mediated by CD4-N32. In contrast, Cbl behaved differently from Shc, p36, ζ, or Lck in that its phosphorylation by CD4-N32 was only minimally increased on TCR and CD4 co-cross-linking. Cbl phosphorylation does not seem to depend as much on Lck as other molecules; Cbl was phosphorylated following TCR activation in J.CaM1.6 cells, whereas Shc phosphorylation required co-cross-linking of TCR with CD4-FL (data not shown). The SH3 and SH2 domain-dependent enhancement of phosphorylation by CD4-N32 was best observed in CD4-N32+ cells expressing lower levels of TCR. In CD4-N32− cells expressing high levels of TCR, titration of the anti-TCR antibody concentration was necessary to observe maximal phosphorylation enhancement via CD4 (data not shown). The degree of phosphorylation on cross-linking TCR alone was determined largely by the TCR expression levels in a given cell line, as seen in CD4-FL+ cells, which expressed lower levels of TCR than CD4-N32+ cells.

How can CD4-N32 mediate tyrosine phosphorylation when it lacks the catalytic domain? Our data show that both the SH2 and SH3 domains of Lck have an important role in CD4-dependent tyrosine phosphorylation. The SH2 and SH3 domains of Lck may act as an “adapter” molecule to bring in a signaling molecule(s) that is a tyrosine kinase itself or has associated kinase activity. Our data demonstrate that this kinase activity has similar substrate specificity as Lck. We propose that endogenous Lck is this kinase, and that Lck dimerization through the SH2 and SH3 domains upon T cell activation is an important part of Lck function in T cells.

It has been proposed by several groups that Lck molecules may form dimers. The x-ray crystallographic analysis of the SH2 and SH3 domains of Lck suggests that Lck exists as a dimer, with the SH2 and the SH3 domains of one molecule associating with the SH3 and SH2 domains of another in a “head-to-toe” fashion (52). Panchamoorthy et al. (53) provided in vitro biochemical evidence that the isolated SH2 domain of Lck can interact with the SH3 domain. In our model the SH2 and SH3 region of CD4-FL, following activation via TCR and CD4 co-cross-linking, would associate with the SH2 and SH3 region of another CD4-FL or endogenous Lck, and this higher degree of oligomerization achieved by Lck dimerization and CD4 clustering would function to amplify the signaling cascade. The CD4-N32 chimera contains both the SH2 and SH3 domains of Lck and, therefore, is capable of forming such dimers with endogenous Lck, which could then phosphorylate cellular substrates in a CD4-dependent manner. The CD4-N, CD4-N2, and CD4-N3 molecules did not show enhancement of phosphorylation, consistent with the requirement for both of the SH2 and SH3 domains in forming dimers. In further support of this model, our data demonstrate that enhancement of tyrosine phosphorylation via CD4-N32 requires the presence of endogenous Lck, since CD4-N32 in J.CaM1.6 cells did not augment phosphorylation. CD4-FL was capable of enhancing tyrosine phosphorylation in J.CaM1.6 cells, since it retains the catalytic domain. We also showed that the GST fusion protein containing the SH2 and SH3 domains of Lck associates with full-length Lck, and that this association requires T cell activation. This association is not through the SH2 domain of one Lck binding to Y505 of another, since: 1) the phosphorylated YEEI peptide did not block this interaction; and 2) the SH2 domain alone did not associate with full-length Lck. Although the data do not exclude the possibility of other proteins also associating with the Lck SH2 and SH3 domains, they never

**FIG. 9.** CD4-N32 fails to enhance tyrosine phosphorylation following TCR and CD4-Lck chimera co-cross-linking when expressed in J.CaM1.6 cells that lack endogenous Lck. J.CaM1.6 cells transfected with the vector alone (JC.SRa-4), CD4-N32 (JC.N32.3 and JC.N32.13), or with CD4-FL (JC.FL.12) were stimulated with OKT3 anti-TCR Ab and/or OKT4D anti-CD4 Ab, and the lysates were immunoprecipitated (IP) with 4G10 antiphosphotyrosine antibody and immunoblotted with RC20H as described under “Experimental Procedures.”

**FIG. 10.** SH3-SH2 region of Lck associates with full-length Lck in activated lysates in a manner independent of the Lck SH2 binding pocket. A, J77 Jurkat cells were stimulated with OKT3 anti-TCR Ab, and the lysates were incubated with glutathione beads conjugated with the indicated Lck domains expressed as the GST fusion protein (2 μg). Proteins were immunoblotted with anti-Lck monoclonal Ab. B, J77 cells were activated as described above, and 100 μl of the lysates (106 cell equivalent) was incubated with GST-SH3SH2-conjugated glutathione beads (2 μg) that had been incubated with 100 μl of 20 μg phosphorylated YEEI peptide (pYEEI) or nonphosphorylated YEEI peptide (YEEI) to bring the final peptide concentration to 10 μM. The proteins were immunoblotted with anti-Lck monoclonal Ab.
Signaling Through Noncatalytic Domains of Lck

thereby lend support to Lck dimerization. Further experiments with point mutations in the SH2 and SH3 domains, based on crystallographic analysis of the SH2-SH3 interaction, will shed light on the mechanism of Lck dimerization.

Does Lck dimerization require prior activation of Lck, or is dimerization a mechanism by which Lck becomes activated? Our data suggest that Lck dimerization may occur following Lck activation, since the interaction of GST-SH3SH2 with full-length Lck occurred only following TCR stimulation. Lck has been shown to become activated when the TCR or CD4 is cross-linked (23, 51, 56, 57); this activation may induce certain conformational changes, perhaps from dephosphorylation of Y505 and/or autophosphorylation of Y394. In cells expressing CD4-N32, activation of endogenous Lck via TCR cross-linking seems to be necessary for enhancing tyrosine phosphorylation of cellular proteins when the chimera was expressed in J.CaM1.6 cells, suggesting that endogenous Lck is an important component of tyrosine phosphorylation by cross-linking of CD4 alone.

There are other proteins besides Lck that have been shown to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinositol-3-kinase, SYK, ZAP-70, or SH2 domain alone, the other SH domain is still needed to associate with the noncatalytic regions of Lck, such as ras-GAP, p85 of phosphatidylinosito