Lck is a lymphoid-specific, Src family protein-tyrosine kinase that is known to interact with the T-cell coreceptors, CD4 and CD8. This interaction, which is critical for proper T-cell function, is mediated by the N-terminal unique region of Lck and the C-terminal cytoplasmic tail of the coreceptors. A pair of cysteines on each molecule is essential for association, suggesting that CD4 or CD8 may interact with Lck by jointly coordinating a metal ion. We describe here experiments in which a maltose-binding protein fusion protein bearing the CD4 tail has been coexpressed in *Escherichia coli* with an N-terminal fragment of Lck. The proteins associate in the expressing cells, forming a complex that can be affinity-purified. Formation of this complex, like the in *vivo* interaction, depends upon the two pairs of cysteines. Biochemical and biophysical experiments show that the complex dissociates in the presence of EDTA and that it contains a single Zn$^{2+}$ ion. These results are consistent with the proposal that Lck and CD4 associate by thiol-mediated co-coordination of zinc.

Signal transduction through the T-cell antigen receptor activates a number of cytoplasmic protein-tyrosine kinases (PTKs), including Lck, Fyn, Itk, and ZAP-70 (see Refs. 1–3 for review). Activation of Lck (p56$^{lck}$) is one of the earliest and most central events in this signaling cascade. Lck is a Src family PTK found primarily in T-cells, and it is recruited to the activated T-cell receptor via its association with the cytoplasmic domain of the CD4 or CD8 coreceptors (4–9). Genetic and mutational analyses demonstrate that Lck plays an indispensable role in antigen-dependent T-cell activation (10–13) and in T-cell development in the thymus (14–16). Lck-deficient T-cells fail to respond to antigen stimulation, and lose cytotoxic potential (11, 17, 18).

Like other Src family PTKs, Lck contains regulatory SH3 and SH2 domains, followed C-terminally by a conserved tyrosine kinase domain (19). Each family member has a unique N-terminal domain, which lacks the homology prevalent in the rest of the protein. The role of the unique domain remains unclear for most proteins in the superfamily, but in the case of Lck it has been shown to mediate association with the T-cell surface coreceptors CD4 and CD8 (20–24). This association is essential for normal T-cell function (8). Structure-function analysis of deletion mutants in cell culture has shown that the cytoplasmic tail of either CD4 or CD8α (Lck binds the α variant only) and the first 32 residues of the Lck unique region are sufficient for *in vivo* association (21). The interaction mediated by these regions appears to be quite specific. When the extracellular and transmembrane domains of platelet-derived growth factor receptor are fused to the cytoplasmic domain of CD4, the fusion protein complexes with Lck and transduces signals through it (25). Similarly, when the regulatory and kinase domains of p60$^{src}$ are joined to the unique region of Lck, CD4 activation produces Src kinase activity (20). Analysis of point mutations has demonstrated that the coreceptor-Lck interaction is mediated by cysteine residues. More specifically, the sequence CXCP in the cytoplasmic tails of both CD4 and CD8α appears to interact with the sequence CXXC in the N-terminal domain of Lck. When these cysteines are mutated to alanine, serine, or even histidine, the complex, which is detectable by immunoprecipitation in wild type cells, does not form (20, 22). This evidence has been corroborated by antibody induced capping and immunofluorescence microscopy of the CD4-Lck and CD8-Lck complexes *in vivo* (26, 27). Lck appears to be the only Src-related PTK that utilizes cysteines to bind surface receptors.

There are at least three known cysteine-mediated receptor/kinase interactions, and all of them involve Lck. CD4 and CD8α represent two of these. In addition, 4–1BB, a T-cell-specific antigen whose structure resembles members of the nerve growth factor superfamily, also bears a CXCP motif and interacts with Lck (28). The dependence of these non-covalent interactions on four cysteines suggests that receptor and kinase may be held together by a metal ion coordinated by thiol groups from both molecules. This notion is supported by the observation that 1,10-ortho-phenanthroline, a metal chelator, disrupts *in vitro* reconstitution of a CD4-Lck complex from detergent lysates (20). Metal-mediated association has been previously demonstrated with the human immunodeficiency virus Tat protein, which has been shown to form zinc- and cadmium-linked homodimers (29). A direct demonstration of the participation of a metal ion has hitherto been lacking, however. This paper describes experiments in which fragments of the two proteins have been coexpressed as distinct fusion proteins in bacteria and their complex purified. The 1:1 complex of MBP-CD4-(396–433) and Lck-(7–226) that we can isolate is cysteine-dependent and EDTA-sensitive, and it contains one equivalent of Zn$^{2+}$. These results are consistent with the
model that the association is mediated by a co-ordinated zinc ion.

**EXPERIMENTAL PROCEDURES**

**Cloning of CD4 and Lck Constructs**—In order to coexpress two proteins in bacteria from two different plasmid vectors, it is necessary that those plasmids bear different origins of replication. A coexpression plasmid was constructed using pACYC184 (New England Biolabs), a low copy number cloning vector. The T7 expression cassette from a pRSET vector (Invitrogen) was amplified by the polymerase chain reaction (PCR), and the resultant fragment ligated into pACYC184. The cloning process replaced more than half of the pACYC184 tetracycline resistance gene, but left the chloramphenicol resistance gene intact. The resultant vector (pACYC-T7) was used for Lck expression. The LckN32 construct was made by PCR amplification of the appropriate DNA fragment, followed by ligation into pACYC-T7. DNA required for the Lck32 construct was excised from large quantities of pRSET vector bearing the Lck32 DNA. The fragment was then ligated into pACYC-T7. DNA fragments coding for different CD4 constructs were amplified by PCR using appropriate primers. Cys to Ala mutations were introduced by long 3' primers, which hybridized over the codons to be mutated. The various CD4 tail fragments were ligated into pMAL-c2, an MBP fusion protein expression vector (New England Biolabs).

**Coexpression of CD4 and Lck**—pMAL-c2 and pACYC-T7 vectors bearing CD4 and Lck constructs were used to transform *Escherichia coli* BL21. Cells were grown in the presence of 150 μg/ml ampicillin and 75 μg/ml chloramphenicol to select for cells bearing both plasmids. Expression was induced with 0.5 mM isopropyl-1-thio-b-D-galactopyranoside during log phase growth. Suitable induction of the MBP-CD4 fusion proteins and the Lck32 fragment was usually achieved in 2 h. The CD4 fusions express as arrays of bands between 45 and 48 kDa, indicating either proteolytic cleavage or premature termination. Expression of the N32 fragment was low and relatively non-inducible, but the yield was adequate for the studies described here.

**Phosphotyrosine Affinity Purification**—Isopropyl-1-thio-b-D-galactopyranoside-induced cells were lysed by sonication in a standard physiological buffer consisting of 50 mM Tris, pH 7.5, 200 mM NaCl, 0.2% β-ME, and 2 mM benzanamide (the MBP-CD4 fusions are slowly degraded in the absence of benzanamide). The lysate was subjected to ultracentrifugation at 40–60 K for at least 2 h to divide the soluble and insoluble components. The supernatant from this step was loaded onto a phosphotyrosine affinity column that had been washed at high and low pH, and then equilibrated in 50 mM Tris, pH 7.5, 200 mM NaCl, 0.2% β-ME, 2 mM benzanamide (hereafter called standard buffer). After loading, the column was washed with 10 column volumes of standard buffer, and then eluted with standard buffer plus 100 μM phenylphosphosphate. Yields generally range between 3 and 10 mg of complex/liter of induced *E. coli*.

**Gel Filtration**—Samples (0.5 ml) of CD4L-LckN32 complex were applied to a Superdex 200 sizing column (Amersham Pharmacia Biotech) that had been equilibrated in at least 1.5 column volumes of standard buffer. Gel filtration experiments were performed using the Pharmacia FPLC system. Elution profiles were monitored by A280 and plotted; 0.5-m1 or 1.0-m1 fractions were collected.

**Native PAGE Experiments**—Samples of purified CD4L-LckN32 complex were incubated in buffers containing the desired reagents (EDTA, phenanthroline, dithiothreitol, etc.). All solutions were buffered at pH 7.5, and contained 200 mM NaCl, at least 0.2% β-ME and 20% glycerol. Samples were incubated at room temperature for at least 1 h, separated by electrophoresis on a 6% native gel at pH 8.8, and visualized by Coomassie staining.

**Inducively Coupled Plasma (ICP) Spectroscopy and Zinc Quantitation**—ICP spectroscopic analysis was performed by Robert Foster (Thermo Jarrell Ash Corp.). Five samples were analyzed. Two contained CD4L-LckN32 complex from two separate bacterial preparations. Two contained blanks taken from the same preparations (buffer that eluted from the Superdex 200 column just before the complex), and the last contained ~0.5 mg/ml MBP. The concentration (in parts per billion) of 25 different metal atoms was determined for each sample. The samples containing CD4L-LckN32 complex were then subjected to amino acid analysis to determine their exact protein concentration. The analysis was performed at the HHMI Biopolymers facility at Harvard Medical School.

**RESULTS AND DISCUSSION**

Various portions of CD4 and Lck were coexpressed in bacteria. Fragments encompassing the cytoplasmic tail of CD4 were expressed as fusion proteins with MBP, chosen because it is monomeric. Five CD4 fusion proteins were expressed, two with wild type CD4 sequences and three with mutations (Fig. 1A). The larger of the wild type constructs (CD4L) include almost the entire C-terminal tail (residues 396–433); the smaller (CD4S) was seven residues shorter, beginning after the N-terminal stretch of arginines. In the three mutant constructs, alanines were substituted for Cys-420 and Cys-422, the cysteines shown previously to be critical for association (20, 22). Alanines were substituted for Cys-420 and Cys-422, the cysteines shown previously to be critical for association (20, 22). Coexpression of CD4 and Lck—CD4m1, CD4m2, and CD4m1+2 are indicated bands), but the presence of fragments shorter than the full-length induced band suggested some degree of proteolytic degradation or premature termination. Coexpression of the MBP CD4 fusion proteins with Lck had no effect on this distribution of bands.

Two Lck fragments were coexpressed with the MBP-CD4 fusion proteins. LckN32 and Lck32 (Fig. 1B) LckN32 (residues 7–226) includes the unique region, the SH3 domain, and the
SH2 domain of Lck, while Lck32 (residues 53–226) bears the SH3 and SH2 domains, but lacks the unique region (Fig. 2, indicated bands). The presence of an SH2 domain in both fragments provides for simple, one step purification by phosphotyrosine affinity chromatography. The Lck32 fragment, like the CD4m fusions, was used in coexpression experiments as a control for any nonspecific interaction.

The expressed CD4 fusion proteins and Lck fragments are all soluble, fractionating in the supernatant portion of the cell lysate after ultracentrifugation. As an assay for CD4-Lck association, soluble fractions from all coexpression strains (CD4L-LckN32, CD4S-LckN32, CD4m1-LckN32, CD4m2-LckN32, CD4m1+2-LckN32, CD4L-Lck32) were subjected to phosphotyrosine affinity chromatography. If the two proteins are associated, they will both appear to bind to the column and will elute together. Conversely, if they are not associated, the CD4 fusion protein will not be retained and will not coelute with Lck. The results from these experiments are shown in Fig. 2. Significant coelution was observed only when CD4L was coexpressed with LckN32 (Fig. 2A). We concluded that this was the result of protein-protein association, as CD4L was not retained by phosphotyrosine columns when expressed by itself (data not shown). Only full-length CD4L (the highest induced band) associates with LckN32, consistent with the assumption that the other induced bands are CD4 tail truncations of some sort. When LckN32 and MBP-CD4L are separately expressed and purified, they co-purify on phosphotyrosine-Sepharose when mixed in the presence, but not absence of zinc (data not shown).

All three of the cysteine mutant fusions failed to coelute with LckN32 (Fig. 2, D–F), and CD4L did not coelute with Lck32 (Fig. 2C). These results show that the interaction observed in the CD4L-LckN32 coexpression experiments depend upon the N-terminal unique region of Lck and upon cysteines 420 and
The ICP-determined zinc concentration (in ppb) is displayed along with the background zinc level for that sample. Background levels of zinc were determined using sample buffer blanks taken from the same purifications. Zinc was the only metal to appear at levels above background in the CD4L·LckN32 samples (Table I). The protein concentration in both samples was determined by absolute amino acid analysis, and the number of zinc atoms per complex was calculated to be 0.90 for sample 1 and 1.05 for sample 2. As a control, a sample of MBP lacking a CD4 from p56

The CD4S also copurified with LckN32 (Fig. 2B), but not as strongly. This observation suggests that the N-terminal arginine-rich segment lacking in CD4S but present in CD4L (residues 396–402) may be necessary for proper association. Indeed, a somewhat larger deletion, encompassing residues 395–412 in murine CD4 (397–414 in humans) abolishes interaction with Lck in cell culture (20), consistent with the importance of the membrane-proximal region for stable interaction.

Large quantities of CD4L·LckN32 complex were purified using phosphotyrosine affinity chromatography (see also “Experimental Procedures”) and concentrated to between 20 and 30 mg/ml. When CD4L·LckN32 complex prepared in this manner was subjected to gel filtration on a Superdex 200 sizing column (Amersham Pharmacia Biotech), the sample eluted in two primary peaks (Fig. 3A); a large peak at 13.8 ml, followed by a smaller peak at 16 ml (the peak observed at 8.5 ml is aggregated protein and contains neither CD4L nor LckN32). SDS-PAGE of this elution pattern (Fig. 3A) revealed that the first peak contained CD4L and LckN32 in roughly 1:1 stoichiometry. The smaller second peak contained unbound, monomeric LckN32. When the gel filtered complex was diluted and reapplied to the sizing column, it eluted in a single peak at 14.1 ml, indicating that the association is relatively stable (Fig. 3B).

Comparison of elution volumes of the complex with those of known standards sets its molecular mass at ~90 kDa, ~17 kDa higher than the expected mass of a 1:1 CD4L·LckN32 complex.

This effective mass could reflect either an extended structure or some tendency to form higher aggregates.

CD4L·LckN32 complex purified by phosphotyrosine affinity and gel filtration yielded a single major band when subjected to native PAGE (Fig. 4, lane 2), the band was identified as a 1:1 CD4L·LckN32 complex by experiments in which it was excised from the gel and analyzed by SDS-PAGE (data not shown). Preincubation with 0.5 mM EDTA led to complete dissociation of the complex and appearance of two new bands (Fig. 4, lane 3). SDS-PAGE analysis identifies the higher mobility band as unbound LckN32 and the lower as CD4L (data not shown). By contrast, neither 1% β-ME nor 25 mM dithiothreitol affects the complex (lanes 4 and 5, respectively). The zinc chelator 1,10-ortho-phenanthroline also disrupts the CD4L·LckN32 association, but much less efficiently than does EDTA (approximately 50% dissociation at 14 mM 1,10-ortho-phenanthroline, data not shown). These variations in chelator effectiveness probably reflect the structural intricacies of the interaction itself.

We used ICP atomic absorption spectroscopy to detect the presence of metal ions in the CD4L·LckN32 complex. Two samples of complex from separate bacterial preparations were purified by phosphotyrosine affinity chromatography and gel filtration. ICP spectroscopy was then used to determine the concentration of 25 separate metal ions relative to blanks taken from the same purifications. Zinc was the only metal to appear at levels above background in the CD4L·LckN32 samples (Table I). The protein concentration in both samples was determined by absolute amino acid analysis, and the number of zinc atoms per complex was calculated to be 0.90 for sample 1 and 1.05 for sample 2. As a control, a sample of MBP lacking a CD4 cytoplasmic tail was also subjected to ICP analysis, which gave only background levels of zinc (data not shown). Taken together with the published mutational studies and with our native gel experiments, these compositional data strongly support a model in which CD4 and Lck associate by cysteine-mediated co-coordination of a zinc ion (Fig. 5). Zinc is a common structural element in small domains of intracellular proteins.
Reconstitution of a Zinc-mediated CD4 and Lck Complex

(471, 601, 599, 722) and their interactions with Lck. A deletion of 18 residues in the arginine-rich region of the CD4 tail disrupts its interaction with Lck (20), and a VSV-G fusion protein with the seven-residue CD4 sequence TC-QCPHR does not interact with Lck (22). In contrast, a VSV-G fusion protein with corresponding region of CD8α does bind Lck (22). See "Results and Discussion" for further discussion.

![Image](image.jpg)

**FIG. 6.** Comparison of the cytoplasmic regions of CD4 and CD8α, and their interactions with Lck. A deletion of 18 residues in the arginine-rich region of the CD4 tail disrupts its interaction with Lck (20), and a VSV-G fusion protein with the seven-residue CD4 sequence TC-QCPHR does not interact with Lck (22). In another experiment, a CD8α-fusion protein with the seven-residue CD4 sequence TC-QCPHR does not interact with Lck (22). In contrast, a VSV-G fusion protein with corresponding region of CD8α does bind Lck (22).

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