Journal of Cellular Biochemistry 55:389–397 (1994)

Purification and Characterization of an Activated Form of the Protein Tyrosine Kinase Lck From an *Escherichia coli* Expression System

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Abstract The lymphocyte-specific, nonreceptor protein tyrosine kinase Lck has been purified from an *Escherichia coli* expression system using a monoclonal antibody column followed by dye-affinity chromatography. Polyacrylamide gel electrophoretic analysis of purified protein revealed a single 56 kDa band, indicating that recombinant Lck was purified to near-homogeneity. The purified enzyme displayed tyrosine kinase activity as measured by both autophosphorylation and phosphorylation of exogenous substrates. Biochemical properties including protein phosphorylation and kinetic characteristics of the enzyme have been assessed. Peptide map analysis revealed that bacterially expressed Lck is phosphorylated predominantly on the autophosphorylation site (tyrosine-394), which is characteristic for activated protein tyrosine kinases. Indeed, we found that the recombinant enzyme is approximately fivefold more active than Lck from resting T cells, which is extensively phosphorylated at the regulatory carboxy-terminal tyrosine residue (tyrosine-505). Thus, we have overproduced recombinant human Lck in *E. coli* and developed a simple two-step purification procedure which yields highly active enzyme. This will enable the identification and characterization of potential regulators and targets of Lck and thereby greatly facilitate studies which will clarify its role in T cell signal transduction.

Key words: signal transduction, protein tyrosine kinase, T lymphocytes, recombinant protein, $p56^{lck}$, $p60^{src}$

A number of studies have revealed the importance of the protein tyrosine kinase Lck in T lymphocyte development and activation [Glaichenhaus et al., 1991; Molina et al., 1992; Penninger et al., 1993]. This Src-family protein tyrosine kinase is associated with the cytoplasmic face of the plasma membrane and is predominantly expressed in T cells [Perlmutter et al., 1988], where it has been shown to interact with the cell surface receptors CD4 and CD8 [Rudd et al., 1988; Veillette et al., 1988a; Shaw et al., 1989; Gassmann et al., 1992] and the β subunit of the interleukin 2 receptor [Hatakeyama et al., 1991]. Like other kinases of the Src family, Lck comprises several distinct functional domains, the most highly conserved region being the kinase domain within the carboxy-terminal half of the molecule. A second region of homology comprising Src homology regions 2 and 3 (SH2 and SH3) is thought to play a role in interactions with other proteins: the SH2 region with specific phosphotyrosine residues [Koch et al., 1991] and the SH3 region with proline-rich sequences on target proteins [Cicchetti et al., 1992]. In Lck the amino-terminal myristylation site is followed by a unique sequence of about 50 residues which contains two cysteine residues (Cys-20 and Cys-23) crucial for the interaction with CD4 or CD8 [Turner et al., 1990; Shaw et al., 1990]. The interaction between Lck and CD4 is necessary for maximal antigen-dependent stimulation of certain T cell hybridomas [Glaichenhaus et al., 1991]. Mice bearing a targeted disruption of the *lck* gene [Molina et al., 1992], or overexpressing a catalytically inactive form of the enzyme [Levin et al., 1993], fail to generate mature T cells. Thus Lck is of crucial importance both in the activation of mature T cells and the

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Received December 14, 1993; revised February 2, 1994; accepted February 4, 1994.

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maturation of T cells in the thymus [Penninger et al., 1993].

The kinase activity of Lck is tightly regulated by tyrosine phosphorylation [Amrein and Sefton, 1988; Marth et al., 1988; Amrein et al., 1993]. At least two tyrosine residues, both conserved among the Src-family kinases, can undergo phosphorylation. Tyrosine-394 (Tyr-394) is the autophosphorylation site, which is thought to be involved in the full activation of Lck [Amrein and Sefton, 1988; Luo and Sefton, 1990], whereas tyrosine-505 (Tyr-505) phosphorylation is important in the suppression of kinase *activity* [Amrein and Sefton, 1988; Hurley and Sefton, 1989; Amrein et al., 1993]. The activity of Lck in vivo might ultimately depend on the relative activity of the phosphatase(s) and kinase(s) which act on this regulatory site [Mustelin and Burn, 1993]. The most likely candidates for these activities are the protein tyrosine phosphatase CD45 [Ostergaard et al., 1989] and the protein tyrosine kinase Csk [Nada et al., 1991; Partanen et al., 1991]. Indeed, we have demonstrated a functional complex between CD45 and Lck in vitro [Autero et al., 1994] and in vivo [Guttinger et al., 1992]. In addition, we have shown that Csk is capable of phosphorylating the regulatory site of Lck, thereby modulating its activity [Bergman et al., 1992].

In order to understand at a molecular level this important tyrosine kinase, its regulation by phosphorylation and dephosphorylation, and its intracellular targets, an abundant source of purified and active enzyme is needed. In this paper we report the overexpression and purification of an activated form of Lck from an *E. coli* expression system. In vitro characterization has been accomplished and includes biochemical and kinetic analysis of the recombinant enzyme. In addition, we have examined the phosphorylation pattern and kinase activity of the bacterially expressed enzyme and compared it with that from resting T cells.

MATERIALS AND METHODS

Reagents and Antibodies

The generation of mouse monoclonal antibodies to Lck has been described previously [Stieger et al., 1993]. Purified monoclonal anti-Lck IgG and bovine plasma gamma globulin were coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions to give a final concentration of 5.5 mg IgG per milliliter of swollen gel. Rabbit polyclonal antisera were raised to recombinant Lck expressed with a 6-histidine tail and purified by NTA chromatography [Hochuli et al., 1987] and preparative polyacrylamide gel electrophoresis.

Plasmid Constructions

The plasmid pUC12/YT16 harbouring the human *lck* gene [Koga et al., 1987] and the construction of the pDS/MRDPSlck plasmid [Stieger et al., 1993] have been described previously. pDS/lck was produced by digesting pUC12/YT16 with NcoI and HindIII and ligating the fragment encoding Lck with HindIII/PvuI and PvuI/NcoI fragments from pDS56/RBSII, NcoI [Stüber et al., 1990]. The predicted amino terminus of the protein produced from this plasmid is that of the naturally occurring Lck except for the absence of posttranslational modifications unique to eukaryotes (cleavage of the N-terminal methionine and myristylation of Gly_2). An error (A instead of C) was found at base pair 311 in the published sequence for *lck* [Perlmutter et al., 1988], corresponding to a change from proline to glutamine at amino acid 87. All expression plasmids were transformed into *E. coli* strain R3193 SE-13009 (*lonA*$^-$) harbouring the repressor plasmid pUHA1 [Stüber et al., 1990]. Expression of Lck was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the bacteria were harvested after a further 3 h of growth.

Purification of Recombinant Lck

A 5 l bacterial culture expressing plasmid pDS/lck was resuspended in 125 ml of ice-cold disruption buffer (1 mg/ml lysozyme, 10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl, 5 mM NaF, 0.2 mM Na_3VO_4, 20 μg/ml phenylmethylsulphonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin), left on ice for 30 min, and then sonicated (setting 5 on a Branson probe sonicator for 5 min on 50% time cycle). The resulting lysate was clarified by centrifugation (30 min at 44,000*g*) and passed over a 16 ml bovine gamma-globulin-sepharose column (1.5 cm × 9 cm, flow rate 15 ml/h) to remove proteins binding nonspecifically to immunoglobulins. The solution which flowed through this column was loaded onto a 5 ml anti-Lck mouse monoclonal F200-6C1 [Stieger et al., 1993] antibody column (1.5 cm × 3 cm, flow rate 15 ml/h). The column was subsequently washed with 100 ml of PBS (10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl), 50 ml of

PBHS (10 mM Na_2HPO_4, pH 7.2, 500 mM NaCl), and 50 ml of 5 mM HEPES, pH 6.8. Lck was eluted with 25 mM CAPS (3-[Cyclohexylamino]-1-propane-sulphonic acid), pH 10.0, and 1 ml fractions collected into tubes containing 25 μl 1 M HEPES, pH 6.8; the pH of the resulting solution was 7.2. After the addition of glycerol to a final concentration of 25% and dithiothreitol to 1 mM, the samples were frozen in liquid nitrogen and stored at −80°C. Aliquots of each fraction were subsequently immunoprecipitated and assayed for kinase activity, and the active fractions were pooled to give a total of 12 ml. This pooled material was further purified by dye-affinity chromatography on a 5 ml column of Reactive Green 19-agarose (1.5 cm × 3 cm, Sigma R-4004). After washing with 100 ml of column buffer (50 mM Tris-HCl, pH 7.5), 10 ml of column buffer containing 10 mM ATP and 10 mM $MgCl_2$, and 10 ml of column buffer containing 20 mM phenylphosphate, Lck was eluted on a linear 25 ml salt gradient to 1 M NaCl.

Immunoprecipitation and In Vitro Kinase Assays

E. coli expressing Lck were harvested, resuspended in disruption buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mg/ml lysozyme, 5 mM NaF, 1 mM dithiothreitol, 0.2 mM Na_3VO_4, 20 μg/ml phenylmethylsulphonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin), left for 30 min on ice, and then sonicated (setting 5 on a Branson probe sonicator for 5 min on 50% time cycle). Human peripheral blood lymphocytes (treated 2 weeks previously with phytohaemagglutinin) were resuspended at 55 × 10^6 cells per milliliter in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM Na_3VO_4, 20 μg/ml phenylmethylsulphonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin), left for 15 min on ice, and DNA sheared using a 21 gauge needle. The lysates were clarified by centrifugation (30 min at 26,000*g*), protein concentrations were determined (bacterial lysate 12 mg/ml, T cell lysate 13 mg/ml), and aliquots were frozen in liquid nitrogen and stored at −80°C. Portions of each cleared lysate were added to equal volumes of 2 × RIPA buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% NP40, 2% sodium deoxycholate, 0.2% sodium dodecyl sulfate), made to a final volume of 100 μl with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate), and rabbit polyclonal antiserum specific for Lck (1 μl) was added. After incubation for 10 min on ice, 30 μl of a 1:1 suspension of *Staphylococcus aureus* (Pansorbin cells, Calbiochem 507861) in RIPA buffer were added, and the immunoprecipitation continued for a further 60 min on ice. Acid-denatured enolase was prepared by adding 100 μl of 50 mM acetic acid to 100 μl of enolase solution (10 mg/ml), incubating at 30°C for 10 min, neutralizing with 50 μl of 1 M HEPES, pH 7.5, and then diluting with 1.75 ml of kinase buffer (10 mM HEPES, pH 7.0, 5 mM $MnCl_2$). *Staphylococcus aureus*–bound immunoprecipitates were centrifuged (20 seconds on a microcentrifuge at 4°C), washed twice by resuspension in kinase buffer, and then individually resuspended in 20 μl of acid-denatured enolase. The kinase reaction was started by adding 5 μl [γ-^{32}P]ATP (5,000 Ci/mmol, 0.1 mCi/ml in kinase buffer) and stopped after incubation at 30°C for 5 min by the addition of EDTA to a concentration of 20 mM. Proteins were separated by reducing SDS gel electrophoresis on 10% polyacrylamide gels, and the gel was fixed, dried, and autoradiographed.

SDS/PAGE and Western Blotting

Proteins were separated by reducing SDS gel electrophoresis on 10% polyacrylamide gels. The gels were either stained with Coomassie blue or electrophoretically transferred onto polyvinyldifluoride membranes (Immobilon P; Millipore Corporation, Beford, MA). The membranes were then probed with rabbit polyclonal antiserum specific for Lck. After washing, the presence of antibody bound to Lck was detected using ^{125}I-protein-A and autoradiography.

ELISA-Based Lck Assay

Immunoaffinity-purified Lck (10 ng) in 25 μl of dephospho-α-casein solution (Sigma C-8032, 0.1 mg/ml in 30 mM HEPES, pH 6.6) was added to a microtitre plate well containing 25 μl of 200 μM ATP in 2 × kinase buffer (60 mM HEPES, pH 6.6, 20 mM $MnCl_2$). After incubation at 30°C for 1 h the reaction was stopped by adding EDTA to a final concentration of 30 mM, and proteins were allowed to adsorb to the microtitre plate overnight at 4°C. Tyrosine phosphorylation was then quantified using an enzyme-linked immunoassay with peroxidase-linked monoclonal antiphosphotyrosine antibody.

Phosphopeptide Maps

LSTRA cells [Marth et al., 1985] were grown for 4 h in phosphate-free DMEM containing 10% fetal bovine serum (dialyzed against phosphate-free saline) to which 1 mCi/ml [^{32}P]orthophosphate had been added. Bacteria harbouring the Lck expression plasmid pDS/MRDPSlck were grown in DMEM containing 10% glucose and induced (by the addition of 1 mM IPTG in phosphate-free DMEM containing 10% glucose to which 1 mCi/ml [^{32}P]orthophosphate had been added. Immunoprecipitated Lck was purified by SDS polyacrylamide gel electrophoresis, electrophoretically transferred onto nitrocellulose, and digested with trypsin [Beemon and Hunter, 1978]. Peptides were separated in two dimensions on 100 μm cellulose thin-layer plates. The first dimension was electrophoresis in 0.1% ammonium carbonate (pH 8.9) for 30 min at 1.0 kV, and the second dimension was ascending chromatography in 1-butanol/pyridine/acetic acid/water (75/50/15/60, vol/vol) [Hunter and Sefton, 1980].

RESULTS

Purification of Recombinant Human Lck From *E. coli*

Recombinant human Lck was overexpressed in *E. coli* and subsequently purified as described in Materials and Methods. Our initial attempts to purify Lck were based on an immunoaffinity purification scheme using monoclonal antibodies that specifically recognize an amino-terminal epitope of Lck [Stieger et al., 1993]. First, several different methods, including low pH (100 mM glycine, pH 2.5), high pH (50 mM diethylamine, pH 11.5), and high salt (5 M $MgCl_2$), were screened for their ability to elute ^{32}P-autophosphorylated Lck from each of four different immobilized monoclonal antibodies. The most efficient elution (74% recovery) was obtained by using 50 mM diethylamine pH 11.5 to elute the enzyme from monoclonal antibody F200-6C1, but the eluate from the column had no measurable enzyme activity. We therefore eluted from the immunoaffinity column with a pH gradient from 9.5–11.8 and checked each fraction for Lck by western blotting (Fig. 1A) and by kinase activity (Fig. 1B). Both immunoreactive Lck and kinase activity were first detected in the fraction eluted above pH 9.5 (Fig. 1A,B, lane 5), but as the pH was increased above 10.3 the kinase activity began to decrease, whereas

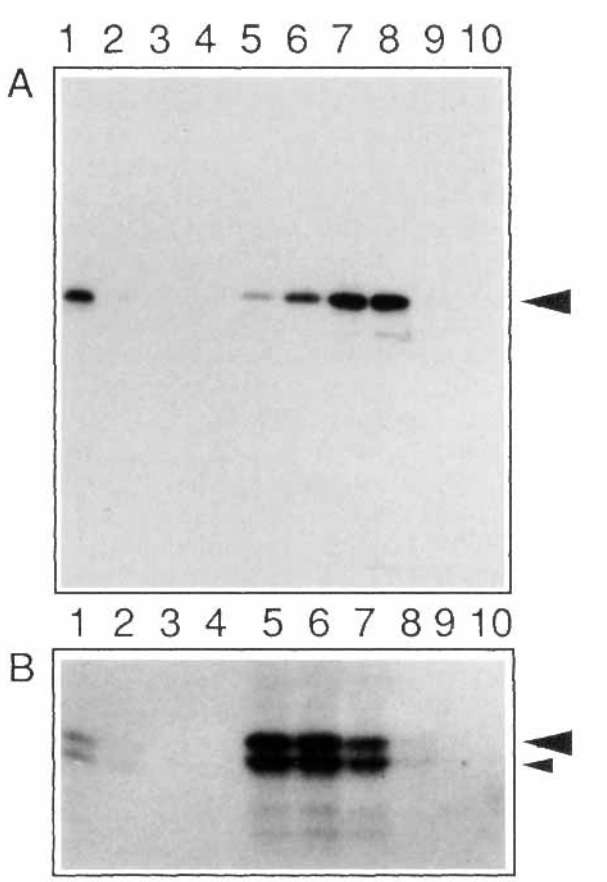

Fig. 1. Elution of Lck from an immunoaffinity column by increasing pH. Clarified lysate from *E. coli* expressing plasmid pDS/lck **(lane 1)** was passed through an anti-Lck monoclonal antibody column as described in Materials and Methods. After collecting the flow-through **(lane 2)** and washing the column **(lanes 3, 4),** Lck was eluted in steps of 50 mM CAPS buffer at pH 9.5 (lane 5), pH 10.0 (lane 6), pH 10.3 (lane 7), pH 11.0 (lane 8), pH 11.5 (lane 9), and pH 11.8 (lane 10). Collection was made into a neutralizing buffer (0.08 volumes of 1 M HEPES, pH 6.8). The positions of Lck (large arrow) and enolase (small arrow) are indicated. Each fraction was analyzed by **(A)** immunoblotting with antiserum specific for Lck and **(B)** immunoprecipitation of Lck followed by in vitro kinase reaction with acid-denatured enolase as a substrate.

the amount of immunoreactive Lck was still increasing (Fig. 1A,B, lane 7). We concluded that the enzyme was irreversibly denatured above pH 10.3 and chose to elute at pH 10.0 (see Materials and Methods, Purification of Recombinant Lck) in subsequent experiments. Three major polypeptides were revealed in the material eluted from the immunoaffinity column by 25 mM CAPS, pH 10.0 (Fig. 2, lane 2). Western blotting with anti-Lck antiserum identified a band migrating, as expected, at a position corresponding to a molecular weight of ~56 kDa. Approximately 10% of the total protein in the

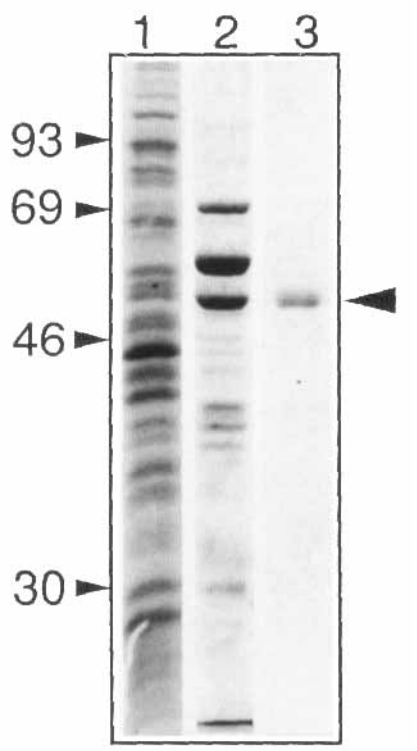

Fig. 2. SDS-PAGE analysis of Lck-containing fractions from each purification step. **Lane 1:** Bacterial lysate. **Lane 2:** Immunoaffinity-purified Lck. **Lane 3:** Reactive Green 19 peak. Molecular size markers and the 56 kDa band of Lck are indicated.

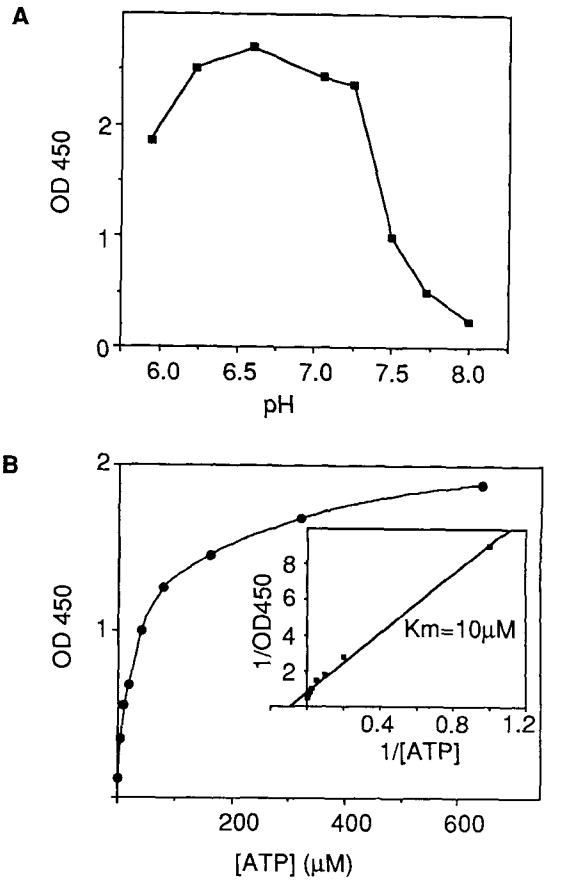

Fig. 3. Characterization of immunopurified recombinant Lck. Dependence of Lck activity on (**A**) pH and (**B**) ATP concentration; the insert in B shows a double-reciprocal plot of the same data. Note that the Km for ATP is 10 μM.

fraction was in this band (Fig. 2, large arrow). Lck could also be eluted from the affinity column using a synthetic peptide corresponding to the epitope recognized by the monoclonal antibody (data not shown). The contaminating *E. coli* polypeptides could not be removed by more stringent washing of the column. Immunoaffinity-purified Lck was further purified by dye-affinity chromatography on Reactive Green 19-agarose. Most of the contaminating proteins flowed through the column under the conditions used, and when a salt gradient was applied Lck eluted as the major protein peak at 0.35 M NaCl concentration (Fig. 2, lane 3).

Kinetic Properties of Bacterially Expressed Lck

Immunoaffinity-purified Lck was used in an ELISA-based kinase assay for an initial characterization of the enzyme. The principal advantage of this nonradioactive assay is that saturating concentrations of ATP can be used without the need for impractically high amounts of radioisotopes. A variety of proteins and tyrosine-containing peptides were shown to be substrates for Lck, and a time course of the reaction at various enzyme concentrations revealed that phosphorylation of 2.5 μg of α-casein by 10 ng of Lck in a 50 μl reaction volume was linear for up to 60 min (data not shown). $MnCl_2$ (optimally 10 mM) was found to be a better source of divalent cations than $MgCl_2$ (data not shown). Investigation of the effect of varying the pH from 6.0 to 8.0 showed that the pH optimum for the kinase reaction was ~6.7 (Fig. 3A). A wide range of ATP concentrations (1–640 μM) was used, and saturation kinetics were observed (Fig. 3B). Lineweaver-Burk analysis of the results gave a value of 10 μM for the Km for ATP.

Phosphorylation Status of Bacterially Expressed Lck

In unstimulated T cells Lck is extensively phosphorylated at Tyr-505 [Hurley and Sefton, 1989], a modification not expected to occur in

bacteria (see Discussion). We therefore analyzed the phosphorylation pattern of human Lck expressed in *E. coli* and compared it to the phosphorylation pattern of Lck isolated from the murine thymoma cell line LSTRA. Two-dimensional phosphopeptide maps of Lck immunoprecipitated from LSTRA cells, which overexpress it fiftyfold [Marth et al., 1988], revealed the presence of at least three major phosphorylation sites (Fig. 4A). One tryptic fragment contains the autophosphorylation site (Tyr-394; Fig. 4A, lower arrow), and one the regulatory phosphorylation site (Tyr-505; Fig. 4A, upper arrow). The third major spot has not yet been clearly assigned but may correspond to a third tyrosine phosphorylation site, Tyr-192 within the SH2 domain, which has recently been shown to increase in phosphorylation in response to anti-CD3 treatment [Soula et al., 1993]. When the same analysis is performed on Lck expressed in *E. coli* one major tryptic fragment is labeled (lower arrow Figure 4B). This fragment is indistinguishable from that which becomes autophosphorylated in vitro (verified by mixing the two samples together before separation; data not shown). Thus, Lck expressed in *E. coli* is phosphorylated predominantly on Tyr-394.

Comparison of the Kinase Activity of Bacterially Expressed and T Cell Lck

Since Lck expressed in *E. coli* is predominantly phosphorylated on Tyr-394, whereas in resting T cells it is extensively phosphorylated on Tyr-505, we compared the relative activity of the two forms (Fig. 5). Immunoprecipitates were made from serial fivefold dilutions of lysates from either human peripheral T lymphocytes or Lck-expressing *E. coli*. The immunoprecipitates were divided into two samples; in one sample the amount of Lck was estimated by Western blotting (Fig. 5A), and in the other kinase activity was determined using acid-denatured enolase as a substrate (Fig. 5B). When samples containing the same amount of Lck are compared (Fig. 5A, lanes 3, 6) bacterially expressed Lck is more active than T cell Lck (Fig. 5B, lanes 3, 6). Indeed, the same kinase activity is present in immunoprecipitates containing one-fifth the amount of bacterially expressed Lck indicating that the bacterially expressed enzyme is approximately fivefold more active than Lck from resting T cells.

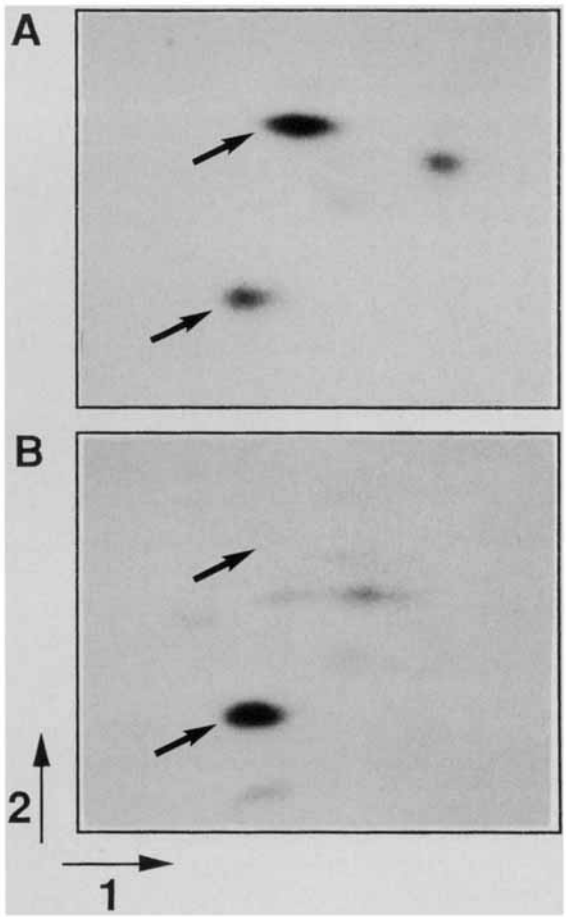

Fig. 4. Phosphopeptide analysis of Lck. 2-D tryptic phosphopeptide maps of immunoprecipitated Lck from (**A**) biosynthetically labeled LSTRA cells and (**B**) *E. coli* expressing plasmid pDS/MRDPSlck. Tyr-394– (lower arrow) and Tyr-505–containing peptides (upper arrow) are indicated. 1, electrophoresis; 2, ascending chromatography.

DISCUSSION

In this study we have described the overexpression in *E. coli,* purification, and initial characterization of human Lck, a member of the Src family of protein tyrosine kinases. Considerable effort has recently been directed towards elucidating the role of this lymphoid cell kinase in T-cell activation and development. To assess the role of Lck in vivo and to unravel the mechanisms and the elements involved in Lck-mediated T-cell signal transduction, it is crucial to identify the substrates and regulators of Lck. For this purpose an abundant source of the purified enzyme is needed. To attain this goal we have used overexpression in *E. coli* of human Lck, followed by immunoaffinity purification using immobilized monoclonal antibodies. We chose a bacterial expression system, rather than eu-

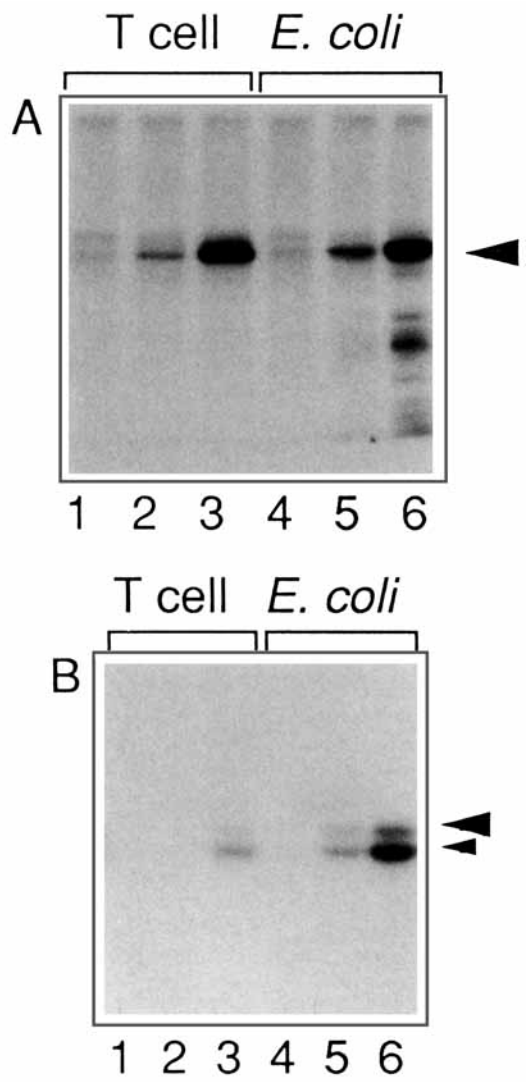

Fig. 5. Comparison between T-cell– and *E. coli*–expressed Lck. Lck immunoprecipitates from either human T cells or *E. coli* expressing plasmid pDS/lck were each divided into two portions and processed for **(A)** Western blotting and **(B)** kinase activity with enolase as a substrate. The positions of Lck (large arrow) and enolase (small arrow) are indicated.

karyotic expression [Ramer et al., 1991; Watts et al., 1992; Carrera et al., 1991], as a homogenous source of fully active Lck for the following reasons: (1) the activity of Src-family kinases is downregulated in most eukaryotic cells by the action of a carboxy-terminal Src kinase, Csk [Nada et al., 1991; Partanen et al., 1991; Bergman et al., 1992]; (2) detergents are needed to solubilize and purify the enzyme from eukaryotic sources [Ramer et al., 1991; Watts et al., 1992]; (3) related protein tyrosine kinases present in eukaryotes but absent from *E. coli* [Wang et al., 1982] may copurify with the recombinant protein; and (4) in eukaryotes posttranslational modification leads to heterogeneity of the recombinant protein [Carrera et al., 1991]. Immunoblotting analysis of Lck expressed in *E. coli* showed that it did indeed migrate as a single 56 kDa band on SDS/polyacrylamide gels (Fig. 1, lane 1).

Our two-step purification scheme involved an immunoaffinity column using immobilized anti-Lck monoclonal antibodies followed by dye affinity chromatography on Reactive Green 19-agarose. Applying these procedures resulted in the purification of recombinant human Lck to near homogeneity (Fig. 2, lane 3). Using bacterially expressed, immunopurified, recombinant human Lck in an ELISA-based kinase assay, we determined the pH optimum for the reaction to be ~6.7 and the Km value for ATP to be 10 μM, in agreement with previously reported values determined using immune-complex kinase assays [Hurley and Sefton, 1989].

We have used 2-D tryptic phosphopeptide map analysis of biosynthetically labeled bacteria expressing Lck to examine its phosphorylation state. Results demonstrated that it is phosphorylated predominantly on Tyr-394, the autophosphorylation site. Many other studies have indicated that in unstimulated T cells Lck is phosphorylated mainly on the regulatory carboxy-terminal tyrosine residue, Tyr-505 [Hurley and Sefton, 1989; Veillette et al., 1988b], and that triggering with anti-CD4 antibodies induces phosphorylation at Tyr-394 [Luo and Sefton, 1990]. Indeed, phosphorylation of Tyr-505 plays a major role in downregulation of the activity of Src-family kinases [Mustelin and Burn, 1993]. The mechanism for this regulation is thought to involve an intramolecular binding of the phosphorylated carboxy-terminal, regulatory tyrosine residue to the SH2 domain [Matsuda et al., 1990; Amrein et al., 1993]. This leads to inhibition of both kinase and SH2 activities and thereby affects both the activity of the enzyme and its interaction with other proteins [Amrein et al., 1993]. Changes in the level of phosphorylation at Tyr-505 can be brought about by the actions of the protein tyrosine phosphatase CD45 [Ostergaard et al., 1989; Mustelin et al., 1992] and the protein tyrosine kinase Csk [Nada et al., 1991; Partanen et al., 1991; Bergman et al., 1992]. In particular, we have shown that a functional complex exists between CD45 and Lck in human T lymphocytes [Guttinger et al., 1992] and that Csk can modulate the activity

of Lck by phosphorylating its regulatory site [Bergman et al., 1992]. Here, we have demonstrated that bacterially expressed Lck is approximately fivefold more active than Lck from unstimulated T cells. This evaluation was made at an ATP concentration of 4 nM, which is below the Km value (10 μM) but still permits a qualitative comparison of the enzyme from the two sources. The difference in activity correlates with a difference in the phosphorylation pattern of Lck (i.e., phosphorylation on Tyr-394 in bacterially expressed Lck and phosphorylation on Tyr-505 in Lck from unstimulated T cells). Thus, the recombinant bacterially expressed Lck may be viewed as an activated form of the enzyme.

In summary, we have reported a fast two-step procedure for the purification of an activated form of recombinant human Lck from an *E. coli* expression system. Purified Lck may now be used in studies with potential regulators in order to determine apparent regulatory sites on Lck, as well as with potential substrates to determine their sites of phosphorylation. Moreover, the availability of the purified enzyme should help to identify new substrates and regulators of Lck and thereby clarify its role in T-cell signal transduction and development. Indeed, in several different studies we have already reported on the identification of candidate substrates [Amrein et al., 1992; Gulbins et al., 1993; von Willebrand et al., 1994] and regulators [Autero et al., 1994] of Lck and examined the effect of phosphorylation by purified recombinant Lck on their biological activity [Gulbins et al., 1993, *1994*; Amrein et al., in press; von Willebrand et al., 1994].

ACKNOWLEDGMENTS

The authors are grateful to Dr. M. Gassmann for many stimulating discussions and to B. Panholzer and J. Molnos for technical assistance.

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