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Purification and initial characterization of the lymphoid-cell protein-tyrosine kinase p56^{lck} from a baculovirus expression system

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ABSTRACT **The lymphocyte-specific protein-tyrosine kinase p56^{lck} has been purified 90-fold to ≈30% purity in 30% yield from a baculovirus expression system by a two-column purification procedure. At least two forms of p56^{lck} were isolated, differing in the extent of phosphorylation and migrating as 56- and 59-kDa species on SDS/PAGE but as a single 56-kDa band after treatment with potato acid phosphatase. Autophosphorylation of purified p56^{lck} occurred at a rate of 25 fmol/min to a maximum incorporation of ≈2 mol of phosphate per mol of p56^{lck} with tyrosine-394 (but not tyrosine-505) and other, unidentified tyrosine residue(s) being the major sites of phosphorylation *in vitro*. Phosphorylation of tyrosine-containing peptides was monitored using an automated HPLC system. Although peptide substrate K_m values were in the 1–5 mM range, the V_{max} for the 13-amino acid peptide RRLIED-AEYAARG (modified p60^{src} autophosphorylation site) was 120 min^{-1} (350 min^{-1} when adjusted for p56^{lck} purity), suggesting that the enzyme purified from recombinant baculovirus-infected Sf9 cells has a high catalytic turnover compared with other tyrosine kinases.**

Considerable effort has recently been directed at understanding the mechanism of signal transduction in T-cell activation. Upon interaction of the T-cell receptor and CD4/CD8 surface proteins with an antigen-presenting cell, one of the earliest (≈30 sec) detectable changes is increased phosphorylation of cellular proteins on tyrosine residues (1–3) catalyzed by one or more protein-tyrosine kinases, including the lymphocyte-specific p56^{lck} (for reviews see refs. 4–7). p56^{lck} was first identified in the LSTRA cell line [derived from a Moloney murine leukemia virus-induced thymoma (8, 9)] where it is overexpressed ≈40 fold, resulting in increased levels of protein tyrosine phosphorylation in these cells (10, 11). After its initial identification, p56^{lck} was found to be distributed in all T cells and some B cells but only rarely in cells of nonlymphoid origin (9, 12).

p56^{lck} is the translation product of the *lck* gene and is a member of the *src* family of protein-tyrosine kinases. These proteins share a common structural organization: an N-terminal myristoyl group (13), a nonhomologous N-terminal region that may be involved in substrate and/or receptor recognition (e.g., CD4 or CD8α for p56^{lck}), a conserved kinase (catalytic) domain, and a short C-terminal regulatory sequence involved in modulating the catalytic activity (for reviews see refs. 6 and 14). The N-terminal myristoyl group is important for membrane association and may affect the association of p56^{lck} with CD4 and CD8 (15). In the C-terminal regulatory domain, phosphorylation of tyrosine-505 (527 in p60$^{c\text{-}src}$) decreases the kinase activity of p56^{lck} and is clearly important for *in vivo* regulation of p56^{lck} (16, 17). Expression of a mutant p56^{lck} in which tyrosine-505 is replaced by phenylalanine (which cannot be phosphorylated) results in transformation of NIH 3T3 fibroblasts, suggesting that the mutant kinase cannot be properly regulated (16, 17). Tyrosine-394 (416 in p60$^{c\text{-}src}$) is also conserved and is phosphorylated *in vivo* in activated forms of the kinases (such as the Y505F mutant) (16). Tyrosine-394 can be phosphorylated by p56^{lck} itself *in vitro* (autophosphorylation), a reaction that is common in protein-tyrosine kinases.

Previous characterization of p56^{lck} has been from experiments *in vivo* or with immunoprecipitated (i.e., antibody-bound) enzyme. To further understand at a molecular level this important tyrosine kinase, we report here the overexpression of p56^{lck} in a recombinant baculovirus expression system, partial purification of a highly active form of N-myristoylated p56^{lck}, and *in vitro* characterization of the detergent-solubilized protein.

MATERIALS AND METHODS

Peptides were synthesized by Charles Dahl (Harvard Medical School), purified to >95% by reverse-phase HPLC, and isolated as the trifluoroacetate salts. Protein concentrations were determined using the Protein Gold reagent (Integrated Separations Systems, Hyde Park, MA) as described by the manufacturer, with bovine serum albumin as the standard. Since dithiothreitol (DTT) interferes with this reagent, samples were either diluted to a final DTT concentration of <0.05 mM or dialyzed against column buffer containing no DTT before protein determination. Polyclonal anti-phosphotyrosine antibodies were from Upstate Biotechnical (Lake Placid, NY) and polyclonal anti-p56^{lck} antibodies were a gift from Paul Burn (Hoffmann–La Roche, Basel).

Recombinant Baculovirus Construct. The recombinant baculovirus overexpression construct pAc373-lck (18) was obtained by cloning the cDNA encoding the entire human p56^{lck} into pAc373 by using the previously reported pAc373-c-src (19). The plasmid ph-lck (20) was digested with *Stu* I restriction endonuclease, ligated with *Bcl* I linkers, and digested with *Nco* I and *Bcl* I, resulting in a fragment that codes for p56^{lck}. pAc373-c-src was digested with *Bam*HI and *Nco* I, and the plasmid backbone was isolated, dephosphorylated, purified, and ligated to the *lck* fragment, generating pAc373-lck. Recombinant baculovirus was obtained by transfecting Sf9 cells with a mixture of wild-type baculoviral DNA and pAc373-lck and identifying the recombinant virus visually as occlusion-negative phenotypes (21). The recombinant virus was further purified by three rounds of plaque purification.

p56^{lck} Purification. Cultures of Sf9 cells (≈20 × 10^6 cells per 150-mm culture plate) were harvested 48 hr after infection with the recombinant baculovirus (18, 21). The infected cells were washed with phosphate-buffered saline (PBS) and lysed at 4°C with 2.5 ml of lysis buffer per plate [50 mM Hepes/5

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Abbreviation: DTT, dithiothreitol.

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mM EDTA/1% (vol/vol) Triton X-100/5 mM NaF/1 mM Na_3VO_4/2 mM phenylmethylsulfonyl fluoride, pH 7.2, containing 0.15 unit of aprotinin and 20 μg of leupeptin per ml]. The lysate was clarified by centrifugation at 1000 × *g* for 10 min and the supernatant was stored frozen at −80°C.

The purification was done at 4°C. Sf9 cell lysate (100 ml) was thawed on ice and solid $(NH_4)_2SO_4$ and glycerol were added to final concentrations of 0.8 M and 10% (vol/vol), respectively. After the $(NH_4)_2SO_4$ had dissolved (30 min), the solution was applied at 0.5 ml/min to a 75-ml column of L-tyrosine-agarose (2.5 × 15 cm, Sigma catalogue no. T0262) equilibrated in column buffer (40 mM Hepes/10% glycerol/1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.2) containing 0.8 M $(NH_4)_2SO_4$. The column was subsequently washed with 80 ml of column buffer with 0.8 M $(NH_4)_2SO_4$, followed by a linear 240-ml gradient to column buffer without salt. Fractions active in peptide phosphorylation were combined, concentrated on an Amicon PM10 membrane, and dialyzed against column buffer to remove $(NH_4)_2SO_4$.

A 10-ml Matrex gel blue A column (Amicon) was conditioned in 8 M urea/0.5 M NaOH overnight at 4°C and washed with column buffer until the eluent was colorless (>12 hr). Ten milliliters (76 units) of the dialyzed tyrosine-agarose fraction was applied at 0.1 ml/min. The column was washed at 0.5 ml/min with 10 ml of column buffer. The majority of the activity was found in the wash fractions, which were pooled and concentrated with an Amicon PM10 membrane. This was made 30% in glycerol and stored frozen at −80°C.

Immunoprecipitation. To 10 μl (10–100 ng) of enzyme solution were added 40 μl of RIPA buffer (lysis buffer plus 1% sodium deoxycholate and 0.1% SDS) and 1 μl of anti-$p56^{lck}$ antibodies (1:1 in PBS). This was incubated for 2 hr at 4°C and 35 μl of 50% protein A-Sepharose in PBS was added. After another hour at 4°C, the resin was washed with ice-cold PBS (once, 500 μl), 0.5 M LiCl/20 mM Tris, pH 8.0 (twice, 500 μl), and water (once, 500 μl).

Autophosphorylation. Autophosphorylation was measured by adding 5 μl of 3× kinase buffer (1× is 50 mM Hepes/10 mM $MgCl_2$, pH 7.5) and 1 μl (10 μCi) of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) to 10 μl (10–100 ng) of purified $p56^{lck}$ and incubating at room temperature. The reaction was quenched by the addition of SDS/PAGE sample buffer and heating at 95°C for 4 min.

Alternatively, autophosphorylation of immunoprecipitated enzyme was determined by adding to the immunoprecipitate 40 μl of kinase buffer and 1 μl (10 μCi) of [γ-^{32}P]ATP (3000 Ci/mmol). After 20 min at room temperature the resin was washed with 1 ml of cold PBS, treated with SDS/PAGE sample buffer, and heated at 95°C for 4 min.

After separation in an SDS/polyacrylamide gel, the ^{32}P-containing proteins were visualized by autoradiography.

Peptide Phosphorylation. Assays were done at room temperature (23–25°C). To 5 μl (5–50 ng) of enzyme solution was added 5 μl of 0.3 mM [γ-^{32}P]ATP (0.27–1.0 Ci/mmol) in 3× kinase buffer followed by 5 μl of peptide solution (3 mM in water). Reactions were quenched at 2, 4, and 6 min by addition of 85 μl of ice-cold 1 M KP_i (pH 3.0) and 90 μl of the quenched reaction mixture was analyzed by HPLC on a Perkin–Elmer Picosphere 3-Å C_{18} column (3 mm × 3 cm) at a flow rate of 3 ml/min. A linear gradient from solvent A (25 mM KP_i, pH 3.0/1 mM EDTA) to solvent B (CH_3CN) was used and the slope was adjusted so that the phosphopeptide was eluted between 2.5 and 4 min. For example, with the RR-SRC peptide the gradient was 0 min, 100% A; 3 min, 68% A; 3.25 min, 68% A; 3.5 min, 100% A. The peptide and phosphopeptide were eluted at ≈3.3 min.

Phospho Amino Acid and Phosphopeptide Analysis. Purified $p56^{lck}$ was autophosphorylated by incubation with [γ-^{32}P]-ATP (3000 Ci/mmol) at 25°C for 30 min and, after treatment with SDS/PAGE sample buffer, separated by SDS/7.5% PAGE. The proteins were transferred by electrophoresis to poly(vinylidene difluoride) (Immobilon, Millipore) or nitrocellulose, and the location of the ^{32}P-$p56^{lck}$ was determined by autoradiography. LSTRA cells (4 × 10^6) were labeled *in vivo* in 2 ml of phosphate-free Dulbecco's modified Eagle's medium (GIBCO) with [^{32}P]P_i added at 1 mCi/ml (16). After immunoprecipitation with anti-$p56^{lck}$ antibodies the protein was separated by SDS/7.5% PAGE and transferred by electrophoresis to Immobilon or nitrocellulose.

Phospho amino acid analysis was done on protein bound to Immobilon essentially as described (22), by hydrolysis with constant-boiling HCl for 1 hr at 110°C followed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5. Two-dimensional tryptic mapping and CNBr digestion (initiated by Kurt Amrein, Hoffmann–La Roche, Basel) were done on protein bound to Immobilon or nitrocellulose, respectively, as described (23).

RESULTS

Development of a $p56^{lck}$ Assay for Peptide Phosphorylation. Protein-tyrosine kinases are often identified by their ability to self-phosphorylate on tyrosine residues (autophosphorylation). This reaction is *stoichiometric* with respect to enzyme but provides no information on kinetics and processing of external substrates, and we therefore sought to develop a *catalytic* assay for tyrosine kinase activity using short peptides as substrates.

Low reported turnover numbers for protein-tyrosine kinases (24) and particularly for $p60^{src}$ [1.5 min^{-1} for $p60^{c\text{-}src}$ (25) and 14 min^{-1} for $p60^{v\text{-}src}$ (26)] necessitate a radioisotope-based assay, and the transfer of the γ phosphate from [γ-^{32}P]ATP to a tyrosine-containing peptide is used routinely (27–29). Product phosphopeptide is separated from unreacted [γ-^{32}P]ATP by electrophoresis (27, 29) or by preferential binding of the phosphorylated peptide to phosphocellulose paper (30, 31). To develop an assay that could be applied to a wide variety of substrates and easily quantitated, HPLC was used to separate phosphopeptide from [γ-^{32}P]ATP (8). A 3-Å C_{18} column (3 mm × 3 cm) was used, which allowed high flow rates (3 ml/min) and short equilibration times (1–2 min) while still retaining good resolution. It proved necessary to include 1 mM EDTA in the aqueous solvent to prevent the [γ-^{32}P]ATP from being eluted as a broad peak. An autoinjector and a radioisotope detector allowed multiple sample processing with minimal operator interaction.

The HPLC-based assay is rapid (6 min between injections), sensitive (1000–2000 dpm of product representing <0.1% conversion), and versatile. All the peptides described can be assayed rapidly with baseline separation of the phosphopeptide from [γ-^{32}P]ATP by adjusting the slope of the CH_3CN gradient.

For a standard peptide substrate the modified $p60^{c\text{-}src}$-(412–424) autophosphorylation peptide RRLIEDAEYAARG (RR-SRC) was used (8, 32). This peptide had been shown to be a substrate for $p56^{lck}$ (8, 33) in lysate from LSTRA cells. To confirm that this peptide was phosphorylated by $p56^{lck}$ from recombinant baculovirus, lysate from infected Sf9 cells expressing $p56^{lck}$ was treated with RR-SRC, Mg^{2+}, and [γ-^{32}P]ATP and the products were analyzed by HPLC. We concluded that baculovirus-infected Sf9 cells did not contain endogenous protein-tyrosine kinase activity not ascribable to $p56^{lck}$, because the lysate from cells infected with wild-type baculovirus did not phosphorylate RR-SRC (data not shown).

Purification of $p56^{lck}$ from Recombinant Baculovirus-Infected Sf9 Cells. Eukaryotic proteins produced from baculovirus expression systems have been reported to be post-translationally modified efficiently and correctly (for reviews see refs. 34–36), including N-terminally myristoylated $p60^{src}$ (19, 37). $p56^{lck}$ from T cells is myristoylated on the N-terminal

glycine (13) as well as phosphorylated on serine, threonine, and tyrosine residues (38–40). To obtain active $p56^{lck}$ that was likely to be functionally posttranslationally modified, Sf9 cells infected with recombinant baculovirus containing the gene for human $p56^{lck}$ (18) were harvested 48 hr after infection and lysed in a buffer containing the nonionic detergent Triton X-100 and aprotinin, leupeptin, and phenylmethylsulfonyl fluoride as protease inhibitors to prevent the significant proteolysis that has often been observed and been problematic in $p60^{src}$ purifications (25, 41–44).

Our initial attempts to isolate $p56^{lck}$ based on reported procedures for purifying $p60^{src}$ showed that $p56^{lck}$ exhibits very different chromatographic properties from $p60^{src}$ (43–47). An L-tyrosine-agarose column run at high ionic strength as a hydrophobic interaction column provided up to 15-fold purification of $p56^{lck}$ in 30% yield. $p56^{lck}$ activity (measured by peptide phosphorylation) was found in the last half of the gradient as a broad peak of activity. The high concentrations of $(NH_4)_2SO_4$ inhibited the ability of $p56^{lck}$ to phosphorylate peptide (data not shown), but this inhibition was reversed by dialysis. The L-tyrosine-agarose column repeatedly gave 10- to 15-fold purification with 30–50% recovery of activity.

Although $p56^{lck}$ has only a weak affinity for blue A resin, in contrast to other kinases (48), a substantial purification was achieved by applying the enzyme to Matrex blue A in a low-ionic-strength buffer in 0.05% Triton X-100 so that $p56^{lck}$ was eluted during the wash. Consistently a 6- to 8-fold increase in specific activity was obtained, with >80% of the activity recovered. A net 88-fold purification to a final specific activity of 880 nmol of RR-SRC peptide phosphorylated per min per mg of protein was achieved with a 25% recovery of activity to a final yield of 73 μg of protein of ≈30% purity.

Properties of Purified $p56^{lck}$. When $p56^{lck}$ was stored at 4°C it showed a half-life of ≈1 week. However, enzyme stored in buffer containing 30% glycerol at −80°C retained activity indefinitely, even with repeated thawing and refreezing. Fig. 1*A* shows a Coomassie-stained SDS/polyacrylamide gel of tyrosine-agarose and blue A fractions, indicating a purity of 30% for the blue A fraction. Immunoblot analysis with anti-$p56^{lck}$ antibodies revealed that two forms of $p56^{lck}$, with apparent molecular masses of 59 and 56 kDa, were present in the blue A fraction and a third form, 54 kDa, was detected in the tyrosine-agarose fraction (Fig. 1*B*) and in baculovirus lysate (18). It is uncertain at this time whether the 54-kDa form is a truncated $p56^{lck}$ (calculated molecular mass of 58.2 kDa) or a fully dephosphorylated form (see below).

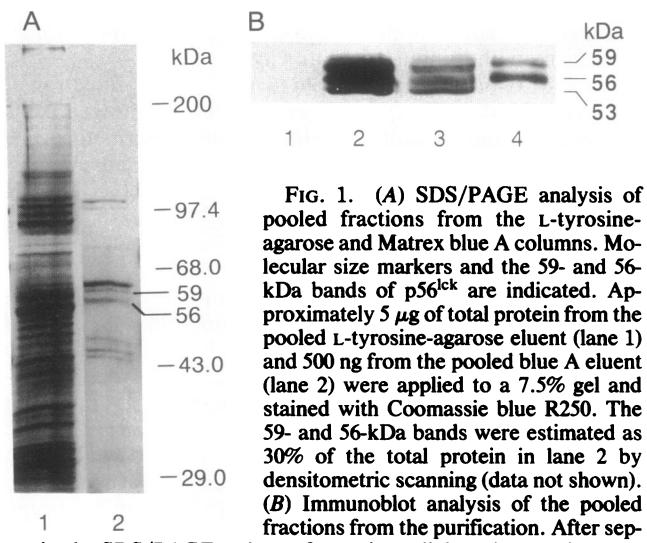

FIG. 1. (*A*) SDS/PAGE analysis of pooled fractions from the L-tyrosine-agarose and Matrex blue A columns. Molecular size markers and the 59- and 56-kDa bands of $p56^{lck}$ are indicated. Approximately 5 μg of total protein from the pooled L-tyrosine-agarose eluent (lane 1) and 500 ng from the pooled blue A eluent (lane 2) were applied to a 7.5% gel and stained with Coomassie blue R250. The 59- and 56-kDa bands were estimated as 30% of the total protein in lane 2 by densitometric scanning (data not shown). (*B*) Immunoblot analysis of the pooled fractions from the purification. After separation by SDS/PAGE and transfer to nitrocellulose the proteins were visualized by immunoblot staining with anti-$p56^{lck}$ antibodies and alkaline phosphatase-linked goat anti-rabbit antibodies. Lysate (8 μg of total protein) from Sf9 cells infected with wild-type baculovirus (lane 1) or recombinant baculovirus expressing $p56^{lck}$ (lane 2), 2.5 μg of protein from the pooled L-tyrosine-agarose fraction (lane 3), and 0.3 μg of protein from the blue A fraction (lane 4) were analyzed.

As an initial assessment of the integrity of the 90-fold purified protein, autophosphorylation capacity was tested by treatment of $p56^{lck}$ with [γ-^{32}P]ATP. Both the 59- and 56-kDa forms of $p56^{lck}$ from the blue A column incorporated [^{32}P]P_i. The incorporation of ^{32}P into $p56^{lck}$ and two unidentified Sf9 proteins (103 and 43 kDa) was time-dependent but was 10-fold faster for intramolecular phosphorylation (26) of $p56^{lck}$ (25 fmol/min) than for the intermolecular phosphorylation of the Sf9 proteins (2.5 fmol/min). If $p56^{lck}$ constituted 30% of the protein in the blue A fractions, the maximal incorporation of 1000 fmol of ^{32}P after 60 min represents 2 mol of ^{32}P per mol of $p56^{lck}$. Phospho amino acid analysis demonstrated that only tyrosines (and not serines or threonines) were phosphorylated (data not shown). Tryptic digestion and two-dimensional phosphopeptide analysis (23) showed that tyrosine-394 (but not tyrosine-505) was phosphorylated as determined by comparison with *in vivo* ^{32}P labeling of LSTRA cells and with published phosphopeptide analyses of $p56^{lck}$ (11, 16, 49) (data not shown).

The stoichiometry of the autophosphorylation (≈2 mol of ^{32}P per mol of $p56^{lck}$), however, indicates that tyrosines other than residue 394 are autophosphorylated. Since it has been suggested that tryptic peptides from the N-terminal region of $p56^{lck}$ may be lost during two-dimensional tryptic mapping (40), CNBr cleavage (23) of purified $p56^{lck}$ autophosphorylated in solution (Fig. 2, lane A) or bound to antibody (lane B) was used to show incorporation of ^{32}P into the 10-kDa band (containing tyrosine-394) as well as the 32-kDa peptide that derives from the N-terminal region of $p56^{lck}$ (12, 23). The precise tyrosine(s) phosphorylated in the 32-kDa peptide has not been established and is a previously unreported N-terminal autophosphorylation.

To determine whether the gel mobility difference between the observed isoforms was due to phosphorylation (38), $p56^{lck}$ was treated with potato acid phosphatase, which dephosphorylates protein serine and tyrosine phosphates (50). Subsequent immunoblot analysis with anti-$p56^{lck}$ antibodies showed collapse of the two bands at 59 and 56 kDa to the 54-kDa form (data not shown), demonstrating that the 59- and 56-kDa bands were differentially phosphorylated forms

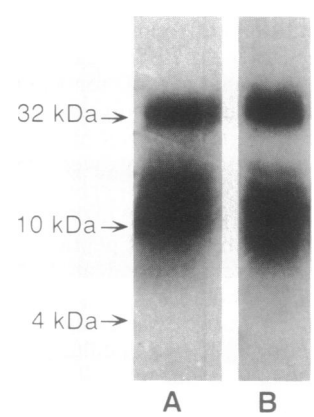

FIG. 2. CNBr cleavage of autophosphorylated $p56^{lck}$ shows that a tyrosine residue in the N-terminal region of $p56^{lck}$ is phosphorylated. $p56^{lck}$ phosphorylated in solution (lane A) or after immunoprecipitation (lane B) was cleaved with CNBr, and the fragments were separated by SDS/24% PAGE and visualized by autoradiography. Arrows indicate the calculated locations of the 32-, 10-, and 4-kDa peptides. The 10-kDa fragment contains tyrosine-394 and the 32-kDa fragment contains unidentified N-terminal phosphorylated tyrosine(s) (40).

Table 1. Apparent kinetic constants for peptide substrates of $p56^{lck}$

Sequence	Identity	K_m, mM	V_{max}, nmol·min^{-1}·μg^{-1}
EDNEYT	$p56^{lck}$-(390–395)	4.1 ± 0.7	2.0 ± 0.2
EDNEYTAR	$p56^{lck}$-(390–397)	4.3 ± 0.8	3.0 ± 0.3
IEDNEYTAREG	$p56^{lck}$-(389–399)	1.2 ± 0.8	0.4 ± 0.09
RLIEDNEYTAREG	$p56^{lck}$-(386–399)	1.8 ± 0.6	0.5 ± 0.05
RRLIEDAEYAARG	RR-$p60^{c\text{-}src}$-(414–424)*	1.4 ± 0.4	2.1 ± 0.3
EGQYQPQPG	$p56^{lck}$-(502–509)G	—	<0.02
RREEYDVLEKK	ζ-(79–89)	2.3 ± 0.7	0.7 ± 0.09
QEGVYNALQKD	ζ-(107–117)	4.3 ± 0.8	2.8 ± 0.3

*Called RR-SRC (8).

of $p56^{lck}$. The bands in the tyrosine-agarose fraction did not collapse to the 54-kDa band, suggesting that this form of $p56^{lck}$ is perhaps truncated or otherwise modified (18).

Quantitative Evaluation of $p56^{lck}$ Activity with Tyrosyl Peptide Substrates. Using the blue A-purified $p56^{lck}$ and the [γ-^{32}P]ATP/HPLC assay, we investigated the ability of $p56^{lck}$ to phosphorylate a variety of small peptides. Kinetic constants (K_m and V_{max}, summarized in Table 1) were determined for peptides of 6–13 residues derived from the sequence flanking the known autophosphorylation (tyrosine-394) and regulatory (tyrosine-505) sites of $p56^{lck}$ and two peptides derived from the cytoplasmic region of the ζ chain of the T-cell receptor.

The kinetic constants in Table 1 were determined by varying the peptide concentration from 0.5 to 2 K_m at a fixed concentration of ATP (100 μM) and analyzing the data by the method of Cleland (51). The K_m and V_{max} values thus obtained are apparent values but are useful for comparing the relative affinities of $p56^{lck}$ for these related peptides. RR-SRC was the best of the substrates tested but still had only a 1.4 mM K_m and a high V_{max}. If one assumes that $p56^{lck}$ comprised ≈30% of the protein in the blue A fraction, the catalytic turnover number with RR-SRC peptide would extrapolate to ≈350 min^{-1}. The K_m values of peptides from the $p56^{lck}$ autophosphorylation site are almost invariant. However, $p56^{lck}$-(390–397) had the highest V_{max} (an estimated k_{cat} of 500 min^{-1}), which may reflect decreased substrate inhibition from this lower-affinity peptide as compared with RR-SRC. Even the short, 6-amino acid peptide $p56^{lck}$-(390–395) was a good substrate with a V_{max} comparable to that of RR-SRC and a K_m only 3 times greater. However, the 9-amino acid peptide derived from the regulatory site of phosphorylation was a very poor substrate, in accord with the observed lack of autophosphorylation at this site. A separate tyrosine-505 kinase must exist in LSTRA cells.

To evaluate the ability of $p56^{lck}$ to recognize tyrosine residues in the ζ subunit of the T-cell receptor, two 11-amino acid ζ-chain peptides chosen from the six tyrosines in the intracellular domain of the ζ chain, were assayed, and both were substrates for $p56^{lck}$. The K_m values for these two peptides were of a similar low affinity (2–4 mM) but ζ-(107–117) had a 4-fold higher V_{max}.

DISCUSSION

Here we describe the solubilization and substantial purification of human $p56^{lck}$, a member of the *src* tyrosine kinase family. Much effort has been expended recently to assess the role of this lymphoid cell kinase *in vivo*, in terms of its postulated function in T-cell signal transduction, its specific association with the cytoplasmic region of CD4 or CD8α, its intermolecular phosphorylation of the ζ chain of the T-cell receptor complex, and the regulation of $p56^{lck}$ activity by T-cell membrane protein-tyrosine phosphatases. To assess the molecular basis of $p56^{lck}$ catalytic efficiency and specificity, effector roles of CD4 or CD8α, and interaction with protein substrates as well as kinases and phosphatases specific for tyrosine or serine/threonine residues requires the availability of purified $p56^{lck}$. This work documents the initial steps toward these goals.

Because $p56^{lck}$ is likely to be posttranslationally phosphorylated *in vivo* with different regio- as well as chemoselectivities (tyrosine, serine, and threonine residues) (38, 40, 52) and in particular because of N-terminal myristoylation (13), which is thought to be of major consequence in plasma membrane localization (37, 53, 54), we turned to a recombinant baculovirus vector (18) as the initial source for the enzyme purification. While amounts of protein may eventually be limiting, the baculovirus-produced $p56^{lck}$ should serve as one of the benchmarks for $p56^{lck}$ catalytic properties and provide a system for mutant $p56^{lck}$ expression and purification. In this work we report an ≈90-fold purification of active $p56^{lck}$ (in Triton micelles) to ≈30–50% purity.

To identify and characterize $p56^{lck}$ we have used the tyrosine-specific kinase activity with defined peptide substrates, anti-$p56^{lck}$ antibodies, and stoichiometric phosphorylation capacity with [γ-^{32}P]ATP. Each of these probes revealed the not-unanticipated microheterogeneity one has come to expect for *src*-type tyrosine kinases. Possible sources of $p56^{lck}$ isoforms are the posttranslational modifications, which can proceed to different fractional stoichiometries at different sites. It was anticipated that the baculovirus system would yield N-myristoylated $p56^{lck}$, and this was validated by [^{3}H]myristic acid incorporation (18). However, the $p56^{lck}$ produced in the late stages of baculovirus infection is not uniformly myristoylated; the 59-kDa isoform incorporates less [^{3}H]myristate than the 56- and 53-kDa forms, suggesting fractional or substoichiometric myristoylation (18). This $p56^{lck}$ preparation carries out autophosphorylation on tyrosine-394 and other, unidentified tyrosines, but does not modify tyrosine-505. To what extent tyrosine, serine, and threonine residues are endogenously phosphorylated during $p56^{lck}$ production in baculovirus remains to be tested. In the end one wants a single, well-defined molecular entity to assess quantitatively the behavior of $p56^{lck}$ in, for example, autophosphorylation, catalytic efficiency and regioselectivity, and interaction with CD4 or CD8α. This is an issue not only for $p56^{lck}$ but also for the other members of the *src* kinase family, and only recently has $p60^{c\text{-}src}$ been purified to homogeneity (25) to permit such characterization to begin with that best-studied tyrosine kinase.

The use of an automated HPLC assay to separate [γ-^{32}P]ATP and ^{32}P-labeled peptide permitted the acquisition of full steady-state kinetic data for an initial series of tyrosyl peptides with the purified $p56^{lck}$ and the estimates of k_{cat}. The baculovirus produces a rather active catalyst in both an absolute sense, at 350–500 catalytic events per minute per kinase molecule, and in a relative sense compared with other members of the *src* family of tyrosine kinases. $p60^{c\text{-}src}$ (95% pure) has a turnover number of only 1.5 min^{-1} for enolase (25) and $p60^{v\text{-}src}$ of 14 min^{-1} for casein (26). It would be useful to test such peptides as RR-SRC with these purified kinases,

since the peptide resembles a highly conserved *src*-family autophosphorylation peptide sequence.

While $p56^{lck}$ turnover numbers are usefully high, permitting a sensitivity of ≈1 pmol of phosphorylated peptide per minute and consuming <1 ng of enzyme, the affinity for both autophosphorylation-site peptides and external tyrosyl peptides was only modest, in the 1–5 mM range. It remains to be seen whether $p56^{lck}$ will be relatively promiscuous, with low affinity in tyrosine residue regioselectivity despite fastidious tyrosyl chemospecificity. Physiologic protein substrates in the T cell, such as full-length ζ chain, may be high-affinity substrates or become so in the presence of a CD4 or CD8 complex, but this remains to be demonstrated. Such studies could guide inhibitor design.

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