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Neoplastic Transformation Induced by an Activated Lymphocyte-Specific Protein Tyrosine Kinase (pp56*lck*)

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The *lck* proto-oncogene encodes a lymphocyte-specific member of the *src* family of protein tyrosine kinases. Here we demonstrate that pp56^{lck} is phosphorylated in vivo at a carboxy-terminal tyrosine residue (Tyr-505) analogous to Tyr-527 of pp60$^{c\text{-}src}$. Substitution of phenylalanine for tyrosine at this position resulted in increased phosphorylation of a second tyrosine residue (Tyr-394) and was associated with an increase in apparent kinase activity. In addition, this single point mutation unmasked the oncogenic potential of pp56^{lck} in NIH 3T3 cell transformation assays. Viewed in the context of similar results obtained with pp60$^{c\text{-}src}$, it is likely that the enzymatic activity and transforming ability of all *src*-family protein tyrosine kinases can be regulated by carboxy-terminal tyrosine phosphorylation. We further demonstrate that overexpression of pp56^{lck} in the murine T-cell lymphoma LSTRA as a result of a retroviral insertion event produces a kinase protein that despite wild-type primary structure is nevertheless hypophosphorylated at Tyr-505. Thus, control of normal growth in this lymphoid cell line may have been abrogated through acquistion of a posttranslationally activated version of pp56^{lck}.

The *src* gene family includes seven closely related sequences (*fgr, fyn, hck, lck, lyn, src,* and *yes*), all of which diverged from a common evolutionary precursor before the mammalian radiation (25, 26, 32, 34, 38, 41, 43, 46, 48, 49). Each of these genes has the potential to encode a membrane-associated protein tyrosine kinase composed of a specialized amino-terminal domain of about 70 residues that is unique to each *src* family member joined to a common region containing a prototypical kinase domain. Unlike the related growth factor receptor kinases, which demonstrate ligand-induced increases in kinase activity, physiologic activators of the *src*-like kinases have not been identified. Thus, the normal function of each of these molecules is obscure. Nevertheless, considerable evidence suggests that the *src* family kinases can participate in the regulation of cell growth. Three of these kinases (*fgr, src,* and *yes*) were originally identified in mutant forms as retroviral oncogenes capable of inducing cell transformation (reviewed in reference 2). Similarly, the *lck* gene is rearranged and overexpressed in some murine lymphomas (32, 46).

Comparison of the deduced structures of the *src*-like protein tyrosine kinases permits some inferences regarding their physiology. Each of the *src* family kinases contains an analogous tyrosine residue in a conserved carboxy-terminal sequence context. In pp60$^{c\text{-}src}$ this tyrosine (Tyr-527) is phosphorylated in vivo at high stoichiometry (10). Activated forms of pp60^{src} are either hypophosphorylated at Tyr-527, for example, when pp60$^{c\text{-}src}$ associates with polyomavirus middle T antigen, or lack Tyr-527 altogether, as for pp60$^{v\text{-}src}$ (6; reviewed in reference 19). In addition, removal of the phosphate from Tyr-527 in pp60$^{c\text{-}src}$ increases its kinase activity in vitro (12, 13), and mutation of Tyr-527 to phenylalanine increases the kinase activity and activates the transforming potential of pp60$^{c\text{-}src}$ (5, 27, 37, 39).

In contrast to other members of the *src* gene family, transcripts encoded by the *lck* gene accumulate only in lymphocytes (among normal cells) and particularly in T lymphocytes (32). Thus, pp56^{lck} probably subserves a specialized function in lymphoid cells. Nevertheless, pp56^{lck} contains a typical *src*-like protein tyrosine kinase catalytic domain including a carboxy-terminal tyrosine (Tyr-505) analogous to Tyr-527 of pp60$^{c\text{-}src}$. We used site-directed mutagenesis to substitute a phenylalanine residue for Tyr-505 of pp56^{lck} and here report that *lck* expression constructs bearing this point mutation transform NIH 3T3 fibroblasts. In addition, the enzymatic activity of the mutant kinase, judged by measuring phosphotyrosine levels in intact cells in vivo, was increased as much as 10-fold over that of the wild-type enzyme. We also learned that high-level expression of the *lck* kinase in the murine lymphoma LSTRA is associated with hypophosphorylation at Tyr-505. These results further implicate pp56^{lck} in lymphomagenesis and strongly support the view that all *src* family members will be regulated by carboxy-terminal tyrosine phosphorylation.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis and expression vector construction. A 20-base oligomer complementary to *lck* mRNA at nucleotide positions 1664 to 1683 (32) was synthesized which contained a base pair mismatch in Tyr-505 of pp56^{lck} (5'-GGCTGGAACTGGCCCTCTGT-3'). Site-directed mutagenesis (51) was performed with a 520-base-pair *Hin*cII-*Stu*I *lck* cDNA fragment cloned into M13mp18 with this oligomer and was confirmed by complete DNA sequence analysis (40). Reconstructed full-length F505 and wild-type *lck* cDNAs (*Stu*I fragments) were then inserted into the blunt-ended *Eco*RI site of the pNUT expression vector containing the mouse metallothionein-I promoter and human growth hormone 3' untranslated sequences and polyadenylation site (35). The retroviral vector pLSHL was constructed by inserting the hygromycin phosphotransferase (*hph*) gene from pLG90 (a gift from Linda Gritz [17])

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downstream of the internal simian virus 40 early region promoter of pLSDL (33). pLSDL was digested with *Hin*dIII and ligated with a *Bam*HI fragment containing the *hph* gene after both fragment and vector had been blunt ended with T4 DNA polymerase. The *Bam*HI fragment from LG90 contains the entire coding sequence of the *hph* gene (L. Gritz, personal communication). pLSHL*lck* was constructed by inserting the *Stu*I fragment of the *lck* cDNA (32) at the *Xho*I site of pLSHL with *Xho*I linkers.

Cell culture and calcium phosphate-mediated transfection of NIH 3T3 fibroblasts. NIH 3T3 cells were cultured in Dulbecco modified Eagle medium supplemented with glucose (1 mg/ml), glutamine (60 mg/ml), and 5% fetal calf serum at 37°C in 7% CO_2. Cells (5×10^5/100-mm culture dish) were transfected with calcium phosphate (47) with 5 μg of *lck* expression plasmid in the presence of 5 μg of salmon sperm DNA as the carrier and 1 μg of a plasmid encoding the hygromycin resistance gene driven by the simian virus 40 early promoter (pSV2-Hygro; kindly provided by Paul Berg). Forty-eight hours after transfection, 500 μg of hygromycin B (Calbiochem-Behring, La Jolla, Calif.) per ml was added to the cultures. After 2 weeks, hygromycin-resistant colonies from the Y505 and F505 transfections were isolated with cloning cylinders and propagated. A polyclonal cell line (pNUT; containing over 500 clones) was obtained by transfection with pNUT (without an *lck* cDNA insert) and with pSV2-Hygro and was also maintained in hygromycin-containing medium.

Retrovirus production and cell infections. Clones of hygromycin-resistant *psi*-2 cells producing recombinant retrovirus were isolated after transfection with the pLSHL or pLSHL*lck* retroviral constructs by standard techniques (29). The viral titers were 5×10^4 and 1.5×10^4 hygromycin-resistant CFU/ml for the LSHL and LSHL*lck* retroviruses, respectively, measured on BALB 3T3 cells. Exponentially growing BALB 3T3 cells (2×10^5/100-mm dish) or NIH 3T3 cells (5×10^5/100-mm dish) were infected with 6 ml (BALB 3T3) or 2 ml (NIH 3T3) of the retroviral stocks for 20 h in the presence of Polybrene (4 μg/ml). Hygromycin-resistant clones were selected in bulk, and these polyclonal lines were used in assays of transformation and kinase activity.

RNA solution hybridization. In a modification of the method devised by Durnam and Palmiter (15), *lck* mRNA levels were assayed in total RNA samples by solution hybridization with *lck*-complementary RNA probes synthesized in vitro with radiolabeled [^{35}S]UTP (New England Nuclear Corp., Boston, Mass.) as previously described (31). Samples were analyzed at least three times with less than 5% variation between reactions.

Antibody production and characterization. Antisera specific for residues 476 to 505 (CWKERPEDRPTFDYLR SVLDDFFTATEGQY) of murine pp56^{lck} (32) were obtained through immunization of New Zealand White rabbits as previously described (31). A second antiserum directed against an amino-terminal peptide corresponding to residues 71 to 96 (SYEPSHDGDLGFEKGEQLRILEQSGE) of murine pp56^{lck} was generated by similar procedures. These antisera specifically immunoprecipitate pp56^{lck} but at relatively low efficiency and hence have been used principally in immunoblot analyses (31).

RNA blot analyses and membrane isolation. RNA was isolated and analyzed by Northern (RNA) blotting as previously described (32). For protein blot analyses, cells were scraped from culture dishes and harvested by low-speed centrifugation to remove culture medium. Cells were then washed once in phosphate-buffered saline (pH 7.4) and suspended in 5 ml of hypotonic lysis buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), 10 mM EGTA, 2 mM EDTA, and 2 mM dithiothreitol. Homogenization was achieved with 20 strokes in a Dounce homogenizer, and the homogenate was then centrifuged at 40,000 × *g* at 4°C for 30 min to separate cellular membranes from cytosolic components. Membranes were resuspended in hypotonic lysis buffer with 1.0% Triton X-100 before centrifugation and supernatant isolation. Triton-soluble membrane protein was quantitated by the method of Bradford (4).

Cell transformation and tumorigencity assays. Stably transfected or retrovirally infected cell lines expressing wild-type or F505 *lck* cDNAs, as well as polyclonal control cell lines, were assayed for transformation by several criteria. Focus formation was tested in mixing experiments when 10^3 cells from F505 lines F1, F4, and F5 were seeded with 10^5 wild-type NIH 3T3 cells and cultured for 2 to 3 days. Cells were also tested for growth in bacterial petri plates, a characteristic of some transformed cells (50). Tumorigenicity was assessed 30 days after subcutaneous injection of 10^7 cells from either wild-type or mutant *lck* lines into BALB *nu*/*nu* mice (3).

pp56^{lck} protein abundance and tyrosine kinase activity assays. Triton-soluble membrane proteins (1 mg/ml) were incubated at 0°C in 20 mM HEPES (pH 7.5)–10 mM $MnCl_2$–0.1% Triton X-100–5 mM dithiothreitol–1 mM Na_3VO_4, and the reaction was started by the addition of [^{32}P]ATP at 3,000 Ci/mmol (New England Nuclear Corp.) to a concentration of 0.3 μM. After 30 s the reaction was stopped by the addition of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer and boiled for 5 min before SDS-polyacrylamide gel electrophoretic analysis and immunoblotting procedures (31). The amount of protein loaded was adjusted to reflect equivalent cell number, equal levels of *lck* mRNA (determined by solution hybridization), or equivalent levels of immunoreactive pp56^{lck}, quantitated by densitometric analyses with an LKB laser densitometer. The phosphorylated pp56^{lck}-containing bands were extirpated, and ^{32}P disintegrations were measured by scintillation counting. Incorporation of ^{32}P in these samples was linear over the protein concentrations used.

Phosphoprotein analysis. LSTRA cells (4×10^6) or confluent 50-mm dishes of Y2 or F5 cells were labeled by incubation for 4 h with 5 mCi of $^{32}P_i$ (carrier-free) in 2 ml of phosphate-free Dulbecco modified Eagle medium with 4% fetal calf serum (final phosphate concentration about 50 μM). Cells were lysed and pp56^{lck} was immunoprecipitated with antiserum to residues 476 to 505 by the method of Cooper and King (12). Radioactive pp56^{lck} bands were excised from dried SDS-polyacrylamide gels, eluted, and digested with trypsin as described previously (1). Phosphopeptides were separated on cellulose thin-layer plates by electrophoresis at pH 8.9 (anode at left) and perpendicularly by chromatography (21).

For immunoprecipitation of tryptic phosphopeptides, about 70 cpm of ^{32}P-labeled phosphopeptides were incubated for 60 min at 0°C with fixed *Staphylococcus aureus* that had been precoated with antibody to residues 476 to 505. This was done in 10 μl of 50 mM NH_4HCO_3, pH 7.6. After centrifugation, the supernatant unbound fraction was lyophilized. The pellet was washed twice in 50 mM NH_4HCO_3, and then bound peptides were eluted at 70°C with two applications of 20 μl of 2.25% formic acid–7.75% acetic acid. Unbound peptides, bound peptides, and 50 cpm of total peptides were separated by thin-layer electrophoresis and

chromatography before autoradiography for 10 days at −70°C with an intensifying screen.

The synthetic peptide SVLDDFFTATEGQYQPQP was phosphorylated in vitro by incubation for 60 min at 30°C with [^{32}P]ATP, and an immunoprecipitate containing pp60$^{c\text{-}src}$ and ^{32}P-peptide was separated from free ATP by electrophoresis at pH 3.5 (10). ^{32}P-peptide (250 cpm) was analyzed alone or after mixing with 250 cpm of ^{32}P-pp56^{LSTRA} tryptic phosphopeptides by thin-layer electrophoresis and chromatography. Thin-layer plates were exposed for 2 days at −70°C with an intensifying screen.

Phosphoamino acid analysis. Phosphoamino acid analysis of cells labeled as described above was performed after acid hydrolysis at 110°C for 2 h in 5.7 M HCl (42).

RESULTS

Oligonucleotide-directed mutagenesis and *lck* expression vector construction. Because of the close structural relationship between pp56^{lck} and pp60$^{c\text{-}src}$, we reasoned that mutation of the carboxy-terminal tyrosine (Tyr-505) of pp56^{lck} would produce a constitutively active kinase molecule. Oligonucleotide-directed mutagenesis (see Materials and Methods) was therefore used to substitute a phenylalanine codon (TTC) for the wild-type tyrosine codon (TAC) at position 505 in the *lck* coding region (Fig. 1A) (32, 46). Full-length wild-type (Y505) and mutant (F505) *lck* cDNA expression vectors were assembled in pNUT driven by the mouse metallothionein I promoter (Fig. 1B; see Materials and Methods). Transcription from this promoter can be induced 5- to 20-fold by exposure to 50 to 100 μM zinc (14, 35). A second type of expression construct was generated by incorporating the Y505 wild-type *lck* cDNA into a retroviral vector (see Materials and Methods) under the control of regulatory sequences in the Moloney murine leukemia virus long terminal repeat (Fig. 1C).

Derivation of fibroblast cell lines expressing pp56^{lck}. To analyze the effects of the F505 mutation on pp56^{lck} activity, we introduced Y505 and F505 *lck* expression constructs into NIH 3T3 cells by calcium phosphate transfection (see Materials and Methods). Simultaneously, these cells were cotransfected with pSV2-Hygro (kindly provided by Paul Berg). Transfectants were selected for growth in hygromycin and subjected to one round of isolation with cloning cylinders. Polyclonal *lck*-expressing fibroblast cell lines were derived by infecting NIH 3T3 cells or BALB/c 3T3 cells with an *lck* retrovirus (see Materials and Methods) and again selecting for growth in hygromycin.

Phenotypes of 3T3 fibroblasts expressing wild-type and mutant *lck* mRNA and pp56^{lck}. Table 1 summarizes the results obtained when *lck*-expressing 3T3 cells were examined for *lck* mRNA and protein, morphology, growth characteristics, and tumorigenicity. Cells expressing either Y505 or F505 *lck* constructs contained similar levels of *lck* mRNA; however, the pp56$^{lck\text{-}F505}$ protein levels judged by immunoblotting were comparatively low on a per molecule of *lck* mRNA basis (Table 1). These protein assays utilize triton-soluble membrane components; thus, it is possible that the lower levels of pp56$^{lck\text{-}F505}$ observed after *lck* mRNA standardization reflect differences in detergent solubility as shown previously for transformation-competent pp60^{src} molecules (18). Nevertheless, all cells expressing pp56$^{lck\text{-}F505}$ showed substantial morphologic alterations when compared with cells expressing the wild-type protein. None of the Y505 transfectants showed any morphologic abnormalities even though expression of *lck* mRNA and of pp56^{lck} protein was detected. Although the extent of morphologic alteration varied in F505-transfected cells, it clearly correlated with the level of *lck*-F505 mRNA detected by solution hybridization and RNA blot analyses (Table 1; data not shown). Zinc treatment provoked a three- to fivefold increase in mRNA abundance in the Y2 and F5 cell lines.

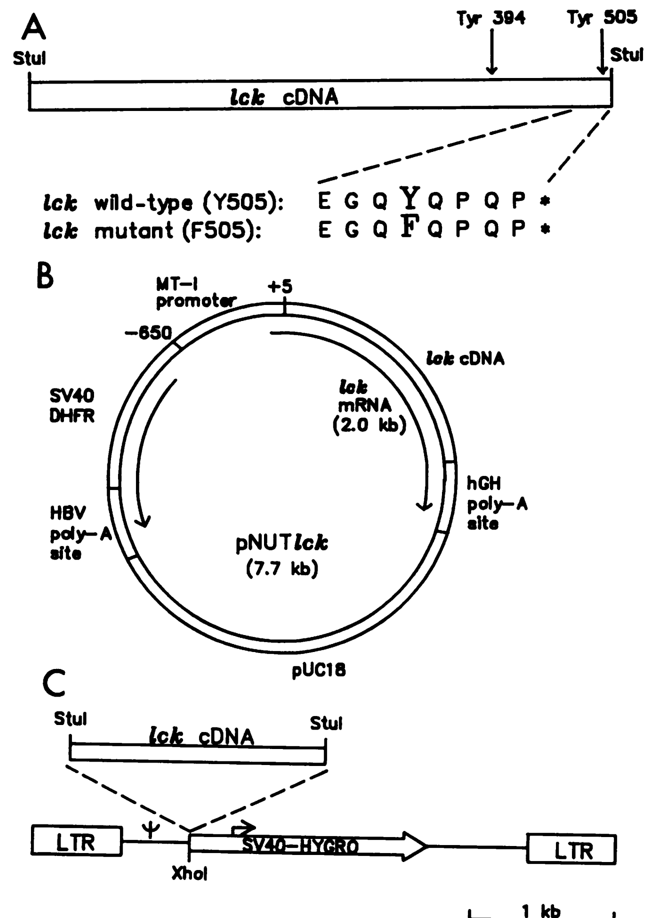

FIG. 1. Structure of constructs used to direct expression of pp56^{lck} in fibroblasts. Tyrosine 505 of pp56^{lck} was changed to phenylalanine by oligonucleotide-mediated mutagenesis. (A) Position of the substitution and the *Stu*I fragment containing the complete *lck* coding region that was used to generate expression constructs. (B) pNUT*lck* expression vector. Arrows indicate the directions of transcription from the metallothionein and simian virus 40 (SV40) promoters; numbers indicate positions relative to the start of transcription. (C) Provirus structure of the retroviral expression vector (LSHL*lck*) with the *lck* cDNA insert site diagrammed. The position of the viral packaging sequence (psi) is noted. See Materials and Methods for details. kb, Kilobases; DHFR, dihydrofolate reductase; LTR, long terminal repeat; HYGRO, *hph* gene.

Figure 2A compares the morphology of a representative cell line expressing the wild-type *lck* construct (Y2) with the morphologies of two cell lines expressing the F505 mutation (F1 and F4) and a control line containing the pNUT vector and the hygromycin resistance plasmid but lacking an *lck* cDNA insert. Each clone of F505-transfected cells was morphologically distinguishable and appeared transformed as compared with the Y505-transfected cells (Table 1; Fig. 2). That these morphologic disturbances reflect the activity of the *lck* expression construct is most dramatically shown by cell line F5 which displays a reversible zinc-induced

TABLE 1. Characteristics of fibroblast lines expressing pp56^{lck}

Cell line	*lck* RNA[a]	pp56^{lck} protein[b]	Morphology[c]	Zinc sensitivity[d]	Focus formation	Growth properties[e]	Tumorigenicity[f]
pNUT	UD[g]	0	−	−	0	−	ND
Y1	UD	ND[h]	−	−	ND	−	0/5
Y2	54.8	3.3	−	−	0	−	1/10
Y3	17.5	ND	−	−	ND	ND	0/5
Y4	35.0	ND	−	−	0	−	ND
F1	112.0	>1	+	+/−	>50	+	ND
F2	15.3	ND	±	+/−	ND	ND	1/5
F3	8.0	ND	±	+/−	ND	ND	ND
F4	88.8	1.0	+	+/−	>50	+	5/5
F5	120.0	1.8	+	+	>50	+	5/5
LSHL-*lck*1[i]	226.0	1.2	−	NA[k]	0	−	0/5
LSHL-*lck*2[j]	>200	5.6	±	NA	0	ND	0/5

[a] Picograms of *lck* mRNA per microgram of total cellular RNA by solution hybridization (31).
[b] Relative levels of pp56^{lck} protein per cell as judged by immunoblotting and densitometry, normalized to the level in F4 cells.
[c] Morphology on near-confluent plates scored as normal (−), mildly aberrant (±), or aberrant (+). See text for description.
[d] Zinc-induced (60 μm) changes in cell morphology assayed from 24 to 48 h. −, No change; ±, some alterations; +, clear morphologic change.
[e] Growth in bacterial culture plates. −, No growth, +, growth.
[f] Tested in BALB *nu/nu* mice. Number of mice yielding tumors/number of mice injected. See Materials and Methods for details.
[g] UD, Undetectable above background.
[h] ND, Not determined.
[i] LSHL*lck*-infected NIH 3T3 cells.
[j] LSHL*lck*-infected BALB 3T3 cells.
[k] NA, Not applicable.

morphologic alteration (Fig. 3) that correlates with an increase in the levels of mutant *lck* mRNA and of pp56$^{lck\text{-}F505}$ (Table 1, Fig. 4A). Although zinc treatment induced *lck* mRNA levels three- to fivefold in the Y2 cell line, no morphologic abnormality resulted (Table 1; data not shown). While wild-type *lck* sequences do not appear to produce profound morphologic changes when expressed in NIH 3T3 cells, infection of BALB/c 3T3 fibroblasts with a retroviral vector containing wild-type *lck* sequences (Fig. 1B) resulted in a subtle yet distinct rounding of cell shape. By RNA and immunoblotting analyses, these retro*lck*$^+$ BALB/c 3T3 cells expressed the highest levels of *lck* mRNA and of pp56^{lck} of any of our cell lines (Table 1; data not shown). It is therefore likely that high levels of wild-type *lck*-encoded protein can induce morphologic changes in BALB/c 3T3 cells.

The alteration in fibroblast morphology induced by expression of pp56$^{lck\text{-}F505}$ is associated with acquisition of other properties of transformed cells. For example, F1, F4, and F5 cells formed foci when mixed with normal 3T3 cells in culture and grew without substrate adherence in bacterial culture plates, and F4 and F5 cells formed tumors in nude mice at high frequency (Table 1). In contrast, the 3T3 lines expressing the wild-type pp56^{lck} protein did not exhibit these transformed properties. Thus, by the criteria of morphologic alteration, growth properties, and tumorigenicity, a single codon substitution converts the *lck* gene into an oncogene.

In vivo tyrosine kinase activity of fibroblasts expressing pp56^{lck}. Expression of pp60$^{c\text{-}src}$ at high levels in fibroblasts has only minor effects on cellular protein phosphorylation (reviewed in reference 20). In contrast, expression of pp60$^{v\text{-}src}$ or of pp60$^{c\text{-}src}$ with the phenylalanine 527 mutation stimulates phosphorylation of cell proteins on tyrosine (11, 37). We tested whether a similar situation applied with fibroblasts expressing pp56^{lck}.

Y2 and F5 cells were labeled with $^{32}P_i$ for 4 or 17 h, and phosphoproteins were isolated. Phosphoamino acids were quantified after partial acid hydrolysis (Table 2). F5 cells contained 10-fold more phosphotyrosine than Y2 cells. The percentage of phosphotyrosine depended on the labeling time (24), but the ratio of phosphotyrosine in F5 cells to that in Y2 cells was constant. Incubating the cells in the presence of Zn^{2+} to induce increased expression of pp56^{lck} had little effect on the phosphotyrosine level in Y2 cells (1.4-fold increase), but increased the level in F5 cells by a factor of 2.8 (Table 2). The increased phosphotyrosine content of F5 cells resulted from phosphorylation of a number of different cellular proteins (data not shown).

Abundance of pp56^{lck} and in vitro kinase activity of membranes containing wild-type and mutant pp56^{lck} molecules. As the F505 substitution in pp56^{lck} results in cellular transformation and increased phosphotyrosine content in vivo, we tested whether these cell lines were expressing similar amounts of intact pp56^{lck} protein and whether an increase in the autophosphorylating activity of pp56$^{lck\text{-}F505}$ could be measured in vitro. Immunoblot analyses of membranes from cell lines containing the *lck*-F505 mutant construct (F4 and F5) compared with cell lines expressing wild-type *lck* expression constructs (Y2 or LSHL-*lck*1) are shown in Fig. 4. By loading a constant amount of Triton-soluble membrane protein, the per cell protein abundance of pp56$^{lck\text{-}Y505}$ was equal to or greater than that of pp56$^{lck\text{-}F505}$ (Fig. 4A). In Fig. 4B, the levels of immunoreactive pp56^{lck} protein were normalized and quantitated by densitometry (given in relative values below the figure). For each sample, the Triton-soluble membrane preparations were first incubated with ^{32}P-labeled ATP in an in vitro autophosphorylation reaction before SDS-polyacrylamide gel electrophoresis and immunoblotting. Figure 4C shows an autoradiogram of the immunoblot presented in Fig. 4B. The ^{32}P measurements were determined directly by scintillation counting (see Materials and Methods). Values below Fig. 4C were adjusted in pairs to represent equivalent levels of pp56^{lck} as determined by immunoblotting and densitometry in Fig. 4B. Although we cannot be certain that all the ^{32}P detected in the 56-kilodalton

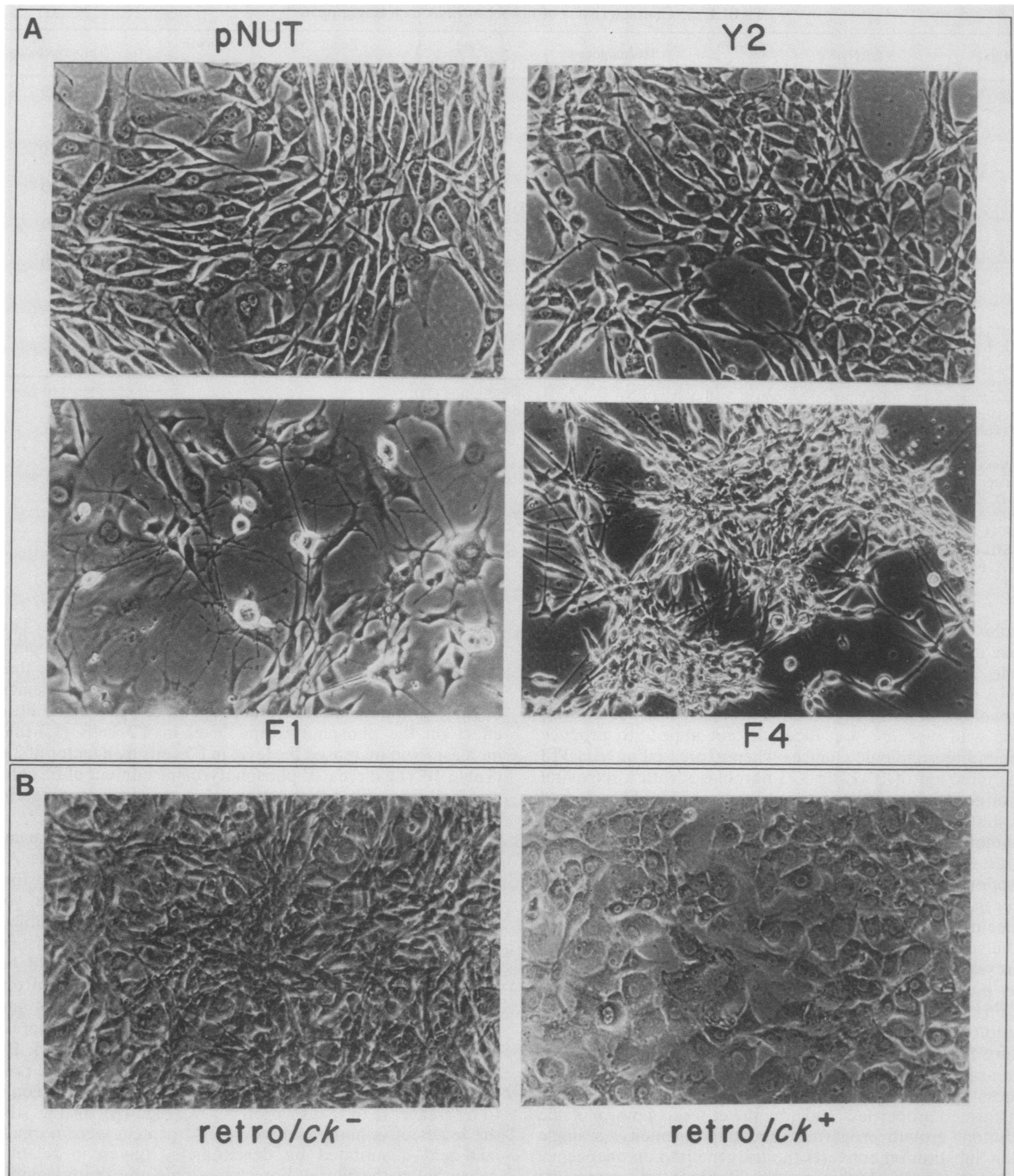

FIG. 2. Morphologic alterations induced by expression of pp56^{lck} in 3T3 fibroblasts. (A) Clones of NIH 3T3 cells transfected with the pNUT vector alone (pNUT), with pNUT containing wild-type *lck*-Y505 (Y2), or with the mutant *lck*-F505 construct (F1, F4) were grown under hygromycin selection, and the morphologies of the cells were compared. (B) BALB 3T3 cells were infected with a retroviral vector carrying only the hygromycin resistance gene (retro*lck*$^{-}$) or with the same virus containing a wild-type *lck* sequence (retro*lck*$^{+}$). Polyclonal cell lines were selected for hygromycin resistance, and the morphologies were compared at cell confluence.

region represents autophosphorylation of pp56^{lck} nor whether we selected for a particular subpopulation of pp56^{lck} molecules in our membrane preparation procedure (18), the increase in autophosphorylation observed here most simply reflects enzymatic activation of pp56$^{lck\text{-}F505}$ molecules compared with wild-type pp56^{lck}.

Sites of phosphorylation in pp56^{lck}. To examine whether pp56^{lck} is phosphorylated at Tyr-505 in normal lymphocytes,

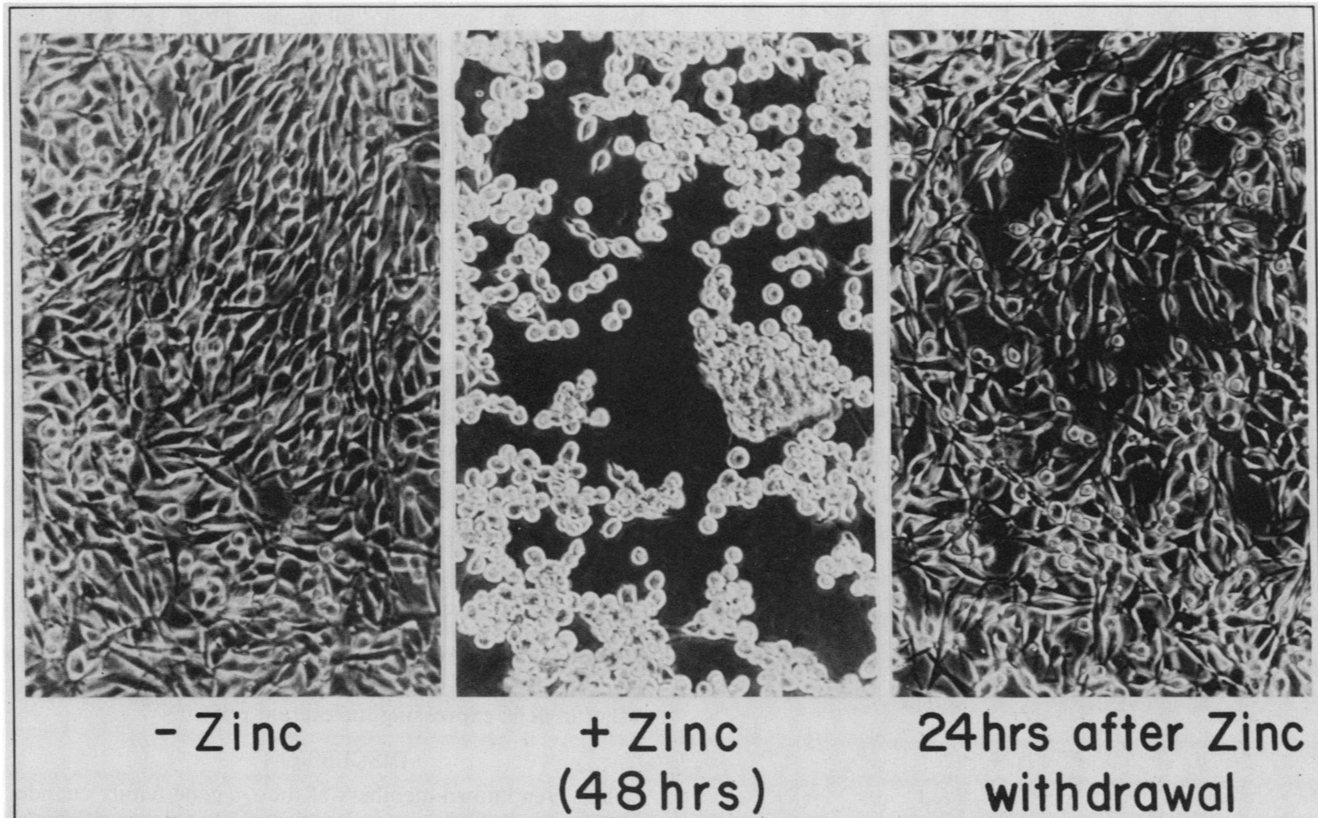

FIG. 3. Reversible alteration of fibroblast morphology induced by high-level expression of pp56$^{lck\text{-}F505}$. Shown are photographs of a single plate of F5 transfectants expressing the *lck*-F505 construct under the control of the zinc-inducible metallothionein promoter. Zinc addition stimulates *lck* expression (Table 1; Fig. 4) and results in reduced cell adherence and a marked rounding complete within 48 h (compare left and center panel), an effect that is fully reversed after removal of zinc (right panel).

we attempted directly to label and immunoprecipitate the protein from normal lymphoid cells. Unfortunately, the level of pp56^{lck} in these cells was not sufficient for analysis. Therefore, we studied pp56^{lck} phosphorylation in the murine lymphoma LSTRA. We and others have previously shown that the *lck* gene is overexpressed in LSTRA cells as a result of insertion of Moloney murine leukemia virus immediately upstream of the *lck* transcriptional unit (32, 46; unpublished data). Nevertheless, the primary sequence of pp56^{LSTRA} deduced from a full-length cDNA clone does not differ from that encoded by the germ line gene (30, 32, 46).

LSTRA cells were labeled for 4 h with $^{32}P_i$, and pp56^{lck} was isolated by immunoprecipitation. Acid hydrolysis released phosphotyrosine and phosphoserine in a ratio of aproximately 3 to 1 (Fig. 5A, inset). After trypsin digestion, peptides were separated in two dimensions in cellulose thin layers by electrophoresis and chromatography (Fig. 5). pp56^{lck} from LSTRA cells was phosphorylated at three major and three minor sites (Fig. 5A). Each of the three major peptides (peptides 1 to 3) contained phosphotyrosine, peptide 4 contained both phosphoserine and phosphotyrosine, and peptides 5 and 6 contained phosphoserine. Of the major phosphotyrosine-containing peptides, peptide 1 was most heavily labeled (42 to 48% of the sum of radioactivity in peptides 1 to 3), peptide 2 slightly less so (36 to 40%), and peptide 3 least (13 to 20%). Peptides 1 and 2 were found in the same 26-kDa fragment produced by partial cleavage with *S. aureus* V8 protease (9; data not shown). This fragment was immunoprecipitated by antiserum against a synthetic peptide containing residues 476 to 505 of pp56^{lck}, indicating that two tyrosine phosphorylation sites lie within 26 kilodaltons of the carboxy terminus.

Incubation of solubilized LSTRA cell membranes with radioactive ATP in vitro results in labeling of pp56^{lck} at a tyrosine embedded in the same sequence as that surrounding Tyr-416 of pp60^{src} (8, 45). The *lck* cDNA sequence shows that this tyrosine is Tyr-394 (32, 46). Comparison of tryptic phosphopeptides of pp56^{lck} labeled in vivo and in vitro showed that peptide 1 contains Tyr-394 (Fig. 5). The identity of peptide 1 and the in vitro phosphorylation site was confirmed by secondary cleavage with V8 protease (data not shown). Peptide 2 is a minor in vitro phosphorylation site (Fig. 5B).

We tested whether any of the phosphotyrosine-containing peptides of pp56^{lck} from LSTRA cells came from the carboxy terminus by immunoprecipitating a trypsin digest of ^{32}P-labeled pp56^{lck} with antiserum against *lck* peptide 476 to 505 (Fig. 6, upper). The sequence of pp56^{lck} predicts that two tryptic peptides may be recognized by this antiserum, peptides 459 to 491 and 492 to 509. These peptides contain tyrosines at positions 470, 489, and 505. Phosphopeptide 2, but not phosphopeptide 1 (Tyr-394) or 3, bound to the antibody (Fig. 6, upper). To test which of the three candidate tyrosines is contained in phosphopeptide 2, we synthesized the 492 to 509 peptide. The single tyrosine in this peptide could be phosphorylated in vitro by an immunoprecipitate containing pp60$^{c\text{-}src}$ (data not shown). Phosphorylated peptide was separated from ATP by thin-layer electrophoresis and analyzed in two dimensions by electrophoresis and chromatography (Fig. 6, bottom, panel B). One major phos-

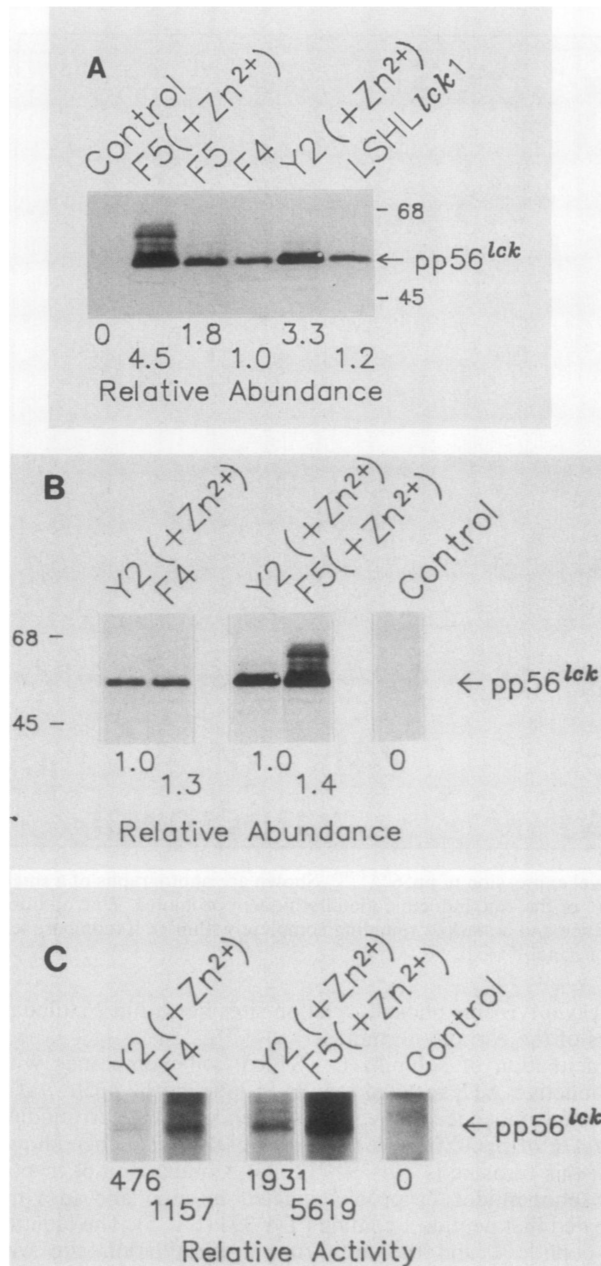

FIG. 4. pp56^{lck} abundance and autophosphorylation activity in membranes containing either wild-type or mutant pp56^{lck}. Membrane samples from cells expressing wild-type pp56^{lck} (Y2 and LSHL-*lck*1) or pp56$^{lck\text{-F505}}$ (F4 and F5) were labeled with [^{32}P]ATP in an in vitro autophosphorylation reaction, resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotted. The figure shows the results of immunoblotting with *lck* antiserum (A and B) or autoradiography (C). Similar results were obtained in separate experiments with an N-terminal antiserum (data not shown). (A) Equal amounts of membrane protein were loaded to demonstrate that wild-type and mutant versions of pp56^{lck} are present in similar amounts on a per cell basis. (B) The amount of membrane protein loaded was adjusted to give approximately equal levels of immunoreactive pp56^{lck}. The actual amounts of pp56^{lck} detected by immunoblotting were quantitated by densitometry, and relative values, determined pairwise, are given below the figure. (C) An autoradiogram of the immunoblot presented in panel B was obtained, and the apparent kinase activity of pp56^{lck} molecules was compared by measuring ^{32}P incorporation into immunoreactive pp56^{lck} quantitated by scintillation counting (see Materials and Methods). The relative activity was calculated by subtracting the background ^{32}P incorporation from each experimental determination and dividing the remainder by the relative pp56^{lck} abundance (B).

phopeptide and one minor phosphopeptide (×) were detected. The latter is assumed to result from phosphorylation of a trace contaminant from the peptide synthesis. The major peptide comigrated with peptide 2 (Fig. 6, bottom, panels A and C). This shows that pp56^{lck} from LSTRA cells contains phosphotyrosine at residue 505.

In vivo phosphorylation of pp56^{lck} in 3T3 fibroblasts. The nontransformed phenotypes of Y2 cells and of 3T3 cells bearing the LSHL*lck* retrovirus suggest that pp56^{lck} in these cells is comparatively inactive. In this case, we predicted that pp56^{lck} would lack phosphate at the major autophosphorylation site (Tyr-394) but contain phosphate at the C terminus (Tyr-505). To test this hypothesis, Y2 cells and F5 cells were labeled with $^{32}P_i$, pp56^{lck} was isolated by immunoprecipitation, and tryptic phosphopeptides were analyzed. The phosphorylation pattern of pp56^{lck} isolated from these cells closely resembled that previously observed with LSTRA cells, except that peptide 1 (Tyr-394) contained less than one-fifth the radioactivity found in peptide 2 (Tyr-505) (Fig. 7A; data not shown). In addition, there was no evidence of phosphorylation at a third site (peptide 3) found in pp56^{lck} from LSTRA cells (compare Fig. 5 and 7).

Separation of ^{32}P-labeled tryptic phosphopeptides of pp56^{lck} from the F5 cell line showed phosphorylation of peptide 1 (Tyr-394), consistent with autophosphorylation (Fig. 5B). As expected, phosphopeptide 2 (Tyr-505) was absent in cells expressing the mutant pp56$^{lck\text{-F505}}$.

DISCUSSION

The seven known members of the *src* gene family encode closely related protein tyrosine kinases that may participate in the control of cell growth (reviewed by J. A. Cooper *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). Differences among these proteins are concentrated in an amino-terminal domain of about 70 residues that may assist in the selection of substrates and in the regulation of kinase activity (49). In other regions, all *src*-like kinases are extremely similar, especially within the carboxy-terminal kinase domain. This close structural conservation suggests that all the *src* family kinases share similar functional properties. In accord with this prediction, we found that the biologic behavior and apparent enzymatic activity of pp56^{lck} are both regulated by phosphorylation of a carboxy-terminal tyrosine residue (Tyr-505), resembling in many respects a similar control mechanism modulating pp60$^{c\text{-}src}$ activity. Hence, it is likely that carboxy-terminal tyrosine phosphorylation not only regulates the activities of both pp56^{lck} and pp60$^{c\text{-}src}$, but probably of all *src*-like protein tyrosine kinases.

Cell transformation and reversible morphologic alterations induced by pp56$^{lck\text{-F505}}$. The *lck* gene is implicated in oncogenesis by virtue of its similarity to *src*, its overexpression in some murine lymphomas (32, 46), and its localization at a site of frequent chromosomal abnormalities in human malignancies (30). However, overexpression of wild-type pp56^{lck} in 3T3 fibroblasts does not result in transformation. Only a subtle morphologic change was detected (Fig. 2B) and that only when very high levels of pp56^{lck} were expressed in BALB/c 3T3 cells, a cell type relatively sensitive to the transforming effects of some protein tyrosine kinases (50). In contrast, pp56$^{lck\text{-F505}}$ is a transforming protein. Comparable results have been obtained with pp60$^{c\text{-}src}$ expression constructs: the wild-type protein cannot transform cells but influences morphology when overexpressed (reviewed in reference 20), while mutant constructs substituting phenylalanine for Tyr-527 transform cells (5, 27, 37, 39).

TABLE 2. Phosphoamino acid content of fibroblasts expressing pp56^{lck} [a]

Expt	Zn^{2+} [b]	Labeling time (h)	Cells	Phosphotyrosine	Phosphothreonine	Phosphoserine
1	−	4	Swiss	0.07	9.2	90.7
			Y2	0.07 (1.0) [c]	9.89	90.0
			F5	0.75 (10.1)	8.9	90.4
	+	4	Y2	0.11 (1.4)	8.13	91.8
			F5	2.11 (28.5)	7.67	90.2
2	−	17	Y2	0.02 (1.0)	5.67	94.3
			F5	0.18 (8.8)	6.27	93.6

[a] The amount of each phosphoamino acid is tabulated as a percentage of total phosphoamino acids. Numbers in parentheses are ratios of the percentage of phosphotyrosine in the indicated cell type as compared with that in a Y2 cell control for each experiment.
[b] Zn^{2+} (60 μM) was added to cultures for 48 h before labeling with $^{32}P_i$ in the absence of Zn^{2+}.
[c] Phosphotyrosine content relative to that in Y2 cells (−zinc).

The extent of morphologic alteration of the 3T3 transfectants correlated directly with the level of pp56$^{lck\text{-F505}}$ protein produced (Table 1: Fig. 2; data not shown). This was especially clear in the F5 cells in which reversible morphologic alteration could be achieved by inducing transcription from the metallothionein-I promoter driving *lck* expression (Fig. 3). Morphologic alteration to rounded, less adherent cells was the most dramatic effect of pp56$^{lck\text{-F505}}$ expression in 3T3 fibroblasts.

Expression of *lck* is restricted predominantly to T cells and modulated by signals that induce lymphokine production, suggesting the pp56^{lck} participates in a lymphocyte-specific activation pathway (31, 32). Thus, it is interesting that pp56$^{lck\text{-F505}}$ transforms 3T3 cells since it is unlikely that the normal physiologic substrate for pp56^{lck} is present in fibroblasts. Transformation of 3T3 cells by pp56$^{lck\text{-F505}}$ implies either that legitimate *lck* substrates are widely expressed or that the mutant kinase, perhaps by virtue of its apparently increased activity, is able to phosphorylate proteins that may not be physiologic substrates for the wild-type kinase.

Control of pp56^{lck} kinase activity. We showed that pp56^{lck}, like pp60$^{c\text{-}src}$, is phosphorylated at a carboxy-terminal tyrosine in vivo. By substituting phenylalanine for tyrosine at this phosphorylation site, the in vivo phosphotyrosine content of cells expressing pp56$^{lck\text{-F505}}$ increased by 10-fold and the apparent autophosphorylating activity of pp56$^{lck\text{-F505}}$ was also increased (Table 2; Fig. 4). Although we cannot be certain that this substitution does not substantially alter the normal conformation of the pp56^{lck} protein irrespective of the change in phosphorylation state, removal of phosphate from Tyr-527 of pp60$^{c\text{-}src}$ has similar activating effects (12, 13).

Whether the carboxy-terminal phosphorylation is self-catalyzed or mediated by another tyrosine kinase remains undetermined. Both pp56^{lck} (Fig. 4) and pp60$^{c\text{-}src}$ (12) are capable of phosphorylating their carboxy-terminal tyrosines in vitro. However, experiments involving pp60$^{c\text{-}src}$ expressed in *Saccharomyces cerevisiae* and expression of mutant forms of pp60$^{c\text{-}src}$ that lack kinase activity in chicken cells suggest that Tyr-527 is phosphorylated by heterologous cellular kinases (12a, 24a). Since pp56^{lck} is not normally present in fibroblasts, it is provocative that fibroblasts are capable of supporting phosphorylation of Tyr-505. These observations suggest that fibroblasts express a kinase for which Tyr-505 of pp56^{lck} is an acceptable substrate, possibly the same kinase that catalyzes phosphorylation of Tyr-527 of pp60$^{c\text{-}src}$ and, more generally, the carboxy-terminal tyrosines of other *src* family members normally expressed in fibroblasts, e.g., pp62$^{c\text{-}yes}$. Interestingly, polyomavirus middle T antigen can associate with and activate pp62$^{c\text{-}yes}$ as well as pp60$^{c\text{-}src}$ (28).

Molecular basis of transformation in LSTRA cells. Overexpression of pp60$^{c\text{-}src}$ does not lead to frank transformation, nor does it lead to a large increase in tyrosine phosphorylation (23, 24, 36, 44). Why then should overexpression of structurally normal pp56^{lck} in LSTRA cells be associated with an increase in tyrosine phosphorylation (8, 16)? The phosphorylation pattern of LSTRA cell pp56^{lck} may be pertinent. LSTRA cell pp56^{lck} contains phosphate at Tyr-394 and Tyr-505 (Fig. 5). Similar results were obtained by Casnellie (7). In contrast, wild-type pp56^{lck} from 3T3 transfectants has phosphate at Tyr-505 but very little at Tyr-394 (Fig. 7). Two-dimensional gel electrophoresis of samples of immunoprecipitated LSTRA cell pp56^{lck} that have been treated with phosphatase and carbamylated, or not treated,

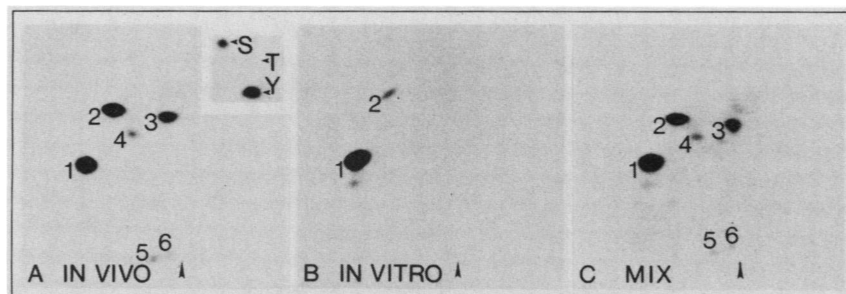

FIG. 5. Phosphopeptide maps of pp56^{lck} from LSTRA cells. (A) LSTRA cells were labeled for 4 h with $^{32}P_i$, pp56^{lck} was isolated by immunoprecipitation and digested with trypsin, and the resulting peptides were separated in two dimensions in cellulose thin layers. The major phosphopeptides visualized by autoradiography are numbered. The inset at top right presents the results of phosphoamino acid analysis of in vivo ^{32}P-labeled pp56^{lck}. (B) Similar analysis of pp56^{lck} labeled by autophosphorylation in vitro. Peptide 1 contains Tyr-394 of pp56^{lck} and comigrates with the dominant peptide in the in vivo-labeled material as shown by a mixing experiment (C).

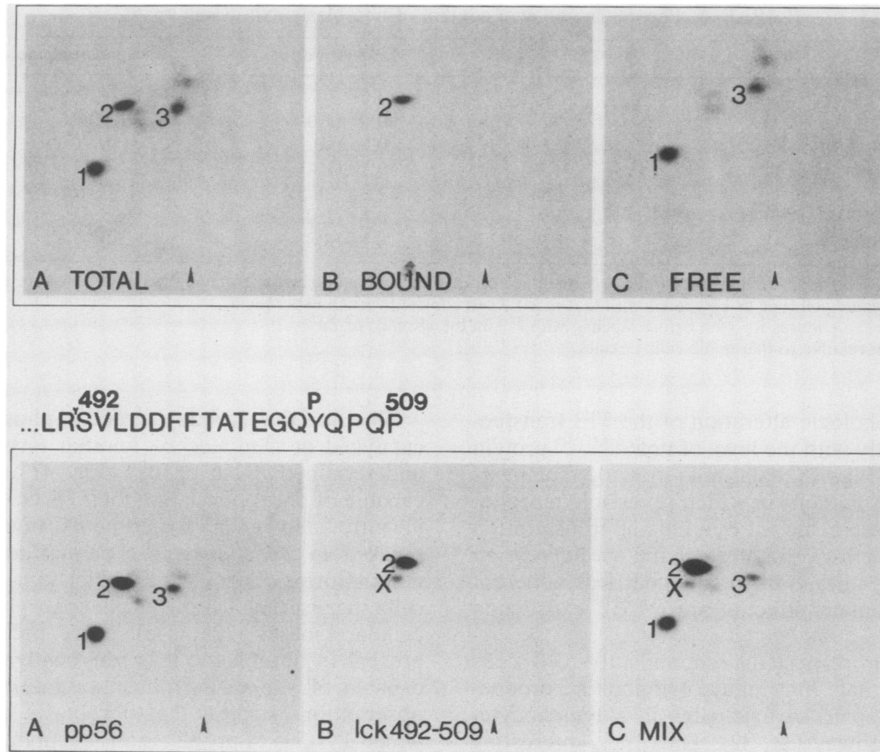

FIG. 6. Tyrosine 505 of pp56^{lck} is phosphorylated in vivo in LSTRA cells. Top: ^{32}P-labled pp56^{lck} described in the legend to prepared as Fig. 5A was digested with trypsin and analyzed in two dimensions to yield the phosphopeptide pattern shown in panel A. Using an antiserum directed against residues 476 to 505, peptide 2 was immunoprecipitated (B), while peptides 1 and 3 did not react with the antiserum (C). Bottom: Shown is a comparison of the migration in two dimensions of tryptic phosphopeptides from in vivo-labeled pp56^{lck} (A) with the migration of a synthetic peptide corresponding to residues 492 to 509 of pp56^{lck} that was phosphorylated in vitro with [^{32}P]ATP by pp60$^{c\text{-}src}$ (B). (C) The two samples are mixed, and peptide 2 of pp56^{lck} comigrates with the synthetic peptide.

shows that most of the pp56^{lck} molecules have a single phosphate (unpublished data). Therefore, LSTRA cell pp56^{lck} probably comprises two classes of molecules. Molecules phosphorylated at Tyr-505 but not Tyr-394 would resemble the pp56^{lck} expressed in fibroblasts which appears to be less active (Table 2; Fig. 4 and 7); molecules phosphorylated at Tyr-394 but not Tyr-505 would resemble the *lck*-F505 mutant in phosphorylation state (Fig. 7) and be activated (Table 2; Fig. 4). The second class of pp56^{lck} molecules is presumably responsible for the 10-fold-increased tyrosine phosphorylation in LSTRA cells (8), since 3T3 cells expressing the *lck*-F505 mutant had a similar increase in phosphotyrosine (Table 2).

Since the primary structure of pp56^{lck} in LSTRA cells is normal, why then is Tyr-505 hypophosphorylated? Three explanations may be invoked. First, Tyr-505 of pp56^{lck} may be hypophosphorylated in all lymphoid cells but fail to transform unless expressed at a sufficient level. This possibility cannot be tested until the sensitivity of peptide mapping is increased to the degree required to determine the phosphorylation state of pp56^{lck} in normal lymphoid cells. Alternatively, LSTRA cells may have sustained a mutation at a second locus that results in aberrant tyrosine phosphorylation of pp56^{lck}. This same mutation might be responsible for the utilization of a third tyrosine phosphorylation site in pp56^{LSTRA} (Fig. 5). Last, lymphocytes may contain a limited

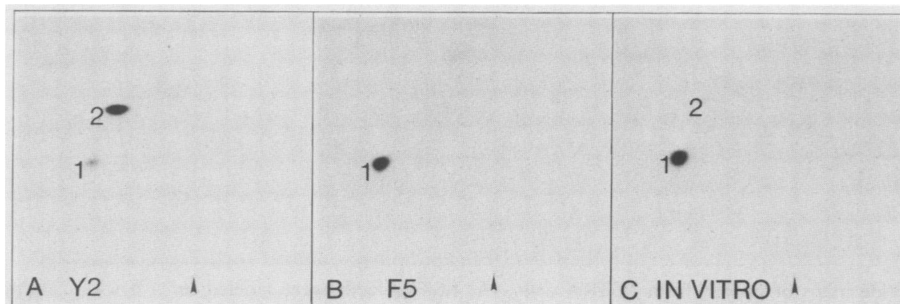

FIG. 7. Phosphorylation of pp56^{lck} in 3T3 fibroblasts. Y2 (A) or F5 (B) cells were labeled in vivo with ^{32}P, and tryptic peptides from pp56^{lck} immunoprecipitated from each cell line were separated in two dimensions as described in the legend to Fig. 5. Peptide 1 contains Tyr-394 as shown by the results of an in vitro autophosphorylation experiment (C).

amount of the factor responsible for directing phosphorylation to Tyr-505. Under these circumstances, simple overexpression of wild-type pp56^{lck} might be sufficient to transform lymphoid cells by titrating out this regulatory factor. Importantly, 3T3 cells are clearly able to suppress pp56^{lck} activity by promoting Tyr-505 phosphorylation even when the kinase is expressed at high levels. Additional studies will be required to determine whether this is a cell-type-specific effect.

For the reasons outlined above, we think it likely that normal lymphoid cells regulate pp56^{lck} activity by controlling the phosphorylation state of Tyr-505. In the absence of an established physiologic means of regulating the pp56^{lck} kinase, pp56$^{lck\text{-}F505}$ should serve as a useful preactivated surrogate and hence aid in the elucidation of the function of the *lck* gene product in lymphocytes.

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