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Activation of p56^{lck} through Mutation of a Regulatory Carboxy-Terminal Tyrosine Residue Requires Intact Sites of Autophosphorylation and Myristylation

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Mutation of the major site of in vivo tyrosine phosphorylation of p56^{lck} (tyrosine 505) to a phenylalanine constitutively enhances the p56^{lck}-associated tyrosine-specific protein kinase activity. The mutant polypeptide is extensively phosphorylated in vivo at the site of in vitro Lck autophosphorylation (tyrosine 394) and is capable of oncogenic transformation of rodent fibroblasts. These observations have suggested that phosphorylation at Tyr-505 down regulates the tyrosine protein kinase activity of p56^{lck}. Herein we have attempted to examine whether other posttranslational modifications may be involved in regulation of the enzymatic function of p56^{lck}. The results indicated that activation of p56^{lck} by mutation of Tyr-505 was prevented by a tyrosine-to-phenylalanine substitution at position 394. Furthermore, activation of p56^{lck} by mutation of the carboxy-terminal tyrosine residue was rendered less efficient by substituting an alanine residue for the amino-terminal glycine. This second mutation prevented p56^{lck} myristylation and stable membrane association and was associated with decreased in vivo phosphorylation at Tyr-394. Taken together, these findings imply that lack of phosphorylation at Tyr-505 may be insufficient for enhancement of the p56^{lck}-associated tyrosine protein kinase activity. Our data suggest that activation of p56^{lck} may be dependent on phosphorylation at Tyr-394 and that this process may be facilitated by myristylation, membrane association, or both.

The Src family of tyrosine-specific protein kinases comprises eight well-characterized members named c-Src, c-Yes, Fyn, c-Fgr, Lck, Hck, Lyn, and Blk (for a review, see reference 31) (9). Increasing evidence indicates that the tyrosine protein kinase activity of the prototype of the Src family, p60^{src}, is primarily regulated through phosphorylation. In NIH 3T3 fibroblasts, the c-*src* gene product is extensively phosphorylated in vivo at the carboxy-terminal tyrosine residue 527 (6), most likely by a cellular tyrosine protein kinase different from p60^{src} (14). Mutational substitution of Tyr-527 by other amino acid residues results in activation of p60^{src} and uncovers its oncogenic potential in rodent and avian fibroblasts (5, 12, 17, 27). Furthermore, in vitro dephosphorylation of Tyr-527 by potato acid phosphatase has been shown to significantly elevate the p60^{src}-associated tyrosine kinase activity (7). Taken together, these findings suggest that phosphorylation at Tyr-527 negatively regulates the tyrosine kinase activity of p60^{src} and that in vivo dephosphorylation of this site may result in partial p60^{src} activation.

The major site of in vitro autophosphorylation of p60^{src} (Tyr-416) is not normally phosphorylated in vivo (6). However, activated versions of p60^{src} have been found to be extensively phosphorylated at this site (5, 12, 17, 27). Occupancy of this residue may play a role in the full activation of some Src mutants, as replacement of Tyr-416 by other amino acid residues partially reduces the elevated tyrosine kinase activity and oncogenic potential of the Tyr-527-to-Phe-527 p60^{src} variant (17, 27).

Because of cotranslational myristylation of a conserved amino-terminal glycine residue, p60^{src} stably associates with plasma membranes (for a review, see reference 13). Transformation by activated versions of p60^{src} is abolished by substitution of Gly-2 by an alanine or a glutamic acid residue (15, 28). These mutations prevent p60^{src} myristylation and stable membrane association. Interestingly, it has been repeatedly demonstrated that lack of myristylation and membrane association does not alter the tyrosine kinase function of activated Src mutants (8, 15, 28). This likely indicates that the lack of oncogenic potential of myristylation-defective Src mutants relates primarily to their inability to phosphorylate critical membrane-associated substrates.

p56^{lck} is a Src-related tyrosine protein kinase found to be expressed exclusively (within normal cell populations) in cells of lymphoid lineage, most predominantly in T lymphocytes (24, 35). In T cells, part of the p56^{lck} function seems to be the transduction of intracellular tyrosine phosphorylation signals for the CD4 and CD8 surface antigens, with which p56^{lck} has been shown to be physically associated (for a review, see reference 3). Like p60^{src}, p56^{lck} is myristylated and membrane associated (21, 39), although the site of myristylation has not been directly defined. In addition, p56^{lck} is extensively phosphorylated in vivo at a carboxy-terminal tyrosine residue (Tyr-505) (2, 22, 32, 36, 37). While the exact stoichiometry of Tyr-505 occupancy remains to be established, mutation of this residue to a phenylalanine results in a constitutively activated form of p56^{lck} (2, 22). This mutant p56^{lck} is significantly phosphorylated in vivo at the site of Lck autophosphorylation (Tyr-394) and is capable of oncogenic transformation of NIH 3T3 cells (2, 22). These findings indicate that the enzymatic function of p56^{lck} is likely to be negatively regulated by Tyr-505 phosphorylation. Further evidence for such function is provided by the observation that the carboxy-terminal sequence of p56^{lck} can efficiently down regulate the activity of a juxtaposed p60^{src}

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tyrosine kinase (20). However, in contrast with p60*src*, in vitro dephosphorylation of p56*lck* by potato acid phosphatase does not result in p56*lck* activation (R. Louie and J. Cooper, unpublished results). This finding raises the possibility that the regulation of these two related products may differ. In this study, we have asked whether other known posttranslational modifications may participate in the regulation of the enzymatic activity of p56*lck*. Our data suggest that, in addition to dephosphorylation of Tyr-505, phosphorylation at Tyr-394 may be required for enhancing the p56*lck*-associated tyrosine kinase activity. Furthermore, they raise the possibility that activation of p56*lck* may be facilitated by myristylation, membrane association, or both.

MATERIALS AND METHODS

Cells. NIH 3T3 fibroblasts, Rat-1 fibroblasts, and their derivatives were grown in alpha minimal essential medium (MEM) (30) supplemented with 10% fetal calf serum (GIBCO Laboratories), penicillin, and streptomycin.

Site-directed mutagenesis. To generate mutant *lck* cDNAs, the *Eco*RI fragment of the murine *lck* cDNA NT18 (24; kindly provided by Roger Perlmutter, Seattle, Washington) was cloned in the *Eco*RI site of M13mp18. All single mutants were generated by site-directed mutagenesis according to Kunkel (19), using uracil-rich single-stranded M13 template and the following 17-mer oligonucleotides: Gly-2-to-Ala-2 mutant AGACACAGGCCATGATC, Tyr-394-to-Phe-394 mutant GGGCCGTGAACTCATTG, and Tyr-505-to-Phe-505 mutant GGGGCTGGAACTGGCCC. Positive mutants were identified by sequencing. The full-length mutant *lck* cDNAs were subsequently resequenced and found to contain no additional mutations when compared with the sequence published by Marth et al. (24) other than the changes already noted by these authors (23) and a uniform single nucleotide substitution at codon 433 (ATT replaced by ATC), which does not result in an amino acid change (data not shown). After identification of single-point mutants, the *Eco*RI *lck* fragments were isolated from double-stranded M13 DNA and cloned in the same site of pGEM3. For generation of double-point mutants, chimeric cDNAs were constructed by replacing the 5′ *Hin*dIII-*Pfl*MI fragment of the F505 *lck* cDNA with that of either the A2 or the F394 *lck* cDNA. The full-length double-mutant cDNAs were also resequenced and found to contain no modification other than the ones stated above (data not shown).

Construction and generation of recombinant retroviruses. For expression of wild-type p56*lck* and p56*lck* variants, the *Stu*I fragments of the *lck* cDNAs were cloned in the *Hpa*I site of the retroviral vector pLXSN (24; kindly provided by D. Miller, Fred Hutchinson Cancer Research Center, Seattle, Wash.) as described previously (A. Veillette and M. Fournel, Oncogene, in press). This vector contains the neomycin resistance gene (Tn5). For production of retrovirus stocks, retroviral expression constructs were transfected by calcium phosphate precipitation (4) in ψ-2 packaging cells (provided by Philippe Gros, McGill University). Polyclonal virus-producing cell lines were established by growth in the presence of G418 (500 μg/ml; GIBCO).

Gene transfer. Retroviral infection of NIH 3T3 or Rat-1 cells was performed as described previously (1). Polyclonal as well as monoclonal cell lines were selected for growth in 250 μg of G418 per ml.

Transformation assays. To examine focus formation, either 10^3 or 10^4 Lck-expressing NIH 3T3 fibroblasts were mixed with 10^5 neomycin-resistant NIH 3T3 cells and plated in six-well Costar plates in the presence of 250 μg of G418 per ml. Foci were counted 10 days later. For growth in soft agar, 2×10^4 cells were plated in semisolid medium as described previously (10). Fresh G418-containing medium was added every 4 days, and colonies were counted after 10 and 21 days.

Antiphosphotyrosine immunoblotting. Cells in monolayers were lysed directly in boiling sample buffer. After treatment according to a previously published protocol (16), lysates corresponding to equal numbers of cells were resolved on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels and transferred onto nitrocellulose. Antiphosphotyrosine immunoblotting was performed as described previously (32, 38), using either a polyclonal rabbit antiphosphotyrosine antiserum (18; kindly provided by Tony Pawson, Toronto, Canada) or the antiphosphotyrosine monoclonal antibody PY-20 (11; purchased from ICN Immunobiologicals) with ^{125}I-labeled protein A (Amersham Corp.) and ^{125}I-labeled sheep anti-mouse immunoglobulin G (Amersham), respectively, as second-step reagents. Phosphotyrosine-containing proteins were detected by autoradiography. The presence of equivalent amounts of proteins in each lane was confirmed by amido black staining of nitrocellulose filters (data not shown). The specificity of the antiphosphotyrosine antibodies used for phosphotyrosine has previously been established (11, 18).

Metabolic labeling and peptide mapping studies. For $^{32}P_i$ labeling, cells were incubated for 4 hours in phosphate-free Dulbecco MEM supplemented with 1.0 mCi of $^{32}P_i$ (carrier free; Dupont, NEN Research Products) per ml and 2% dialyzed fetal calf serum. After a wash in phosphate-buffered saline, cells were immediately lysed in boiling 2% SDS-TNE buffer (TNE is 50 mM Tris [pH 8.0], 1% Nonidet P-40; and 2 mM EDTA [pH 8.0]). The samples were boiled for an additional 5 min, passed through no. 25 needles, and then diluted in 4 volumes of TNE buffer containing 200 μM sodium orthovanadate, 50 mM sodium fluoride, and 10 μg each of leupeptin, aprotinin, *N*-tosyl-L-phenylalanine chloromethyl ketone, *N*-*p*-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride per ml. p56*lck* was recovered by immunoprecipitation using a specific rabbit anti-Lck antiserum (33, 36) and resolved on 8% SDS-PAGE gels. The phosphorylated products were detected by autoradiography of dried gels. Cyanogen bromide cleavage of phospholabeled p56*lck* was performed as described elsewhere (32, 36).

For [^{3}H]myristic acid labeling, cells were incubated for 4 h in the presence of 0.5 mCi of [^{3}H]myristic acid (Dupont, NEN) per ml in serum-free alpha MEM. Labeling with [^{35}S]methionine and [^{35}S]cysteine was accomplished by incubating parallel cultures in methionine-cysteine-free Dulbecco MEM supplemented with 0.5 mCi of Trans-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN) per ml. After the labeling period, cells were washed with phosphate-buffered saline and lysed in TNE buffer supplemented with protease and phosphatase inhibitors as described above. p56*lck* was recovered from cell lysates by immunoprecipitation (33, 36) and resolved on 8% SDS-PAGE gels. Gels were treated with En^{3}Hance (Dupont, NEN) and dried, and the radiolabeled products were detected by fluorography.

Immune complex kinase assays and Lck immunoblot. Immune complex kinase reactions and Lck immunoblot were performed as described elsewhere (33, 34, 38; Veillette and Fournel, Oncogene, in press). Under the conditions used, the kinase reactions were linear for up to 4 min (data not shown).

Subcellular fractionation. Cells were swelled by incubation for 15 min on ice in hypotonic buffer (10 mM Tris [pH 7.4], 1 mM $MgCl_2$) supplemented with protease and phosphatase inhibitors. Membranes were then mechanically broken by 25 strokes in a tight-fitting Dounce homogenizer. Homogenates were adjusted to a final concentration of 0.15 M NaCl. Postnuclear lysates were separated by ultracentrifugation at 100,000 × *g* for 30 min into a particulate fraction (P100) and a cytosolic fraction (S100). After rinsing of the P100 pellet with phosphate-buffered saline, the fractions were adjusted to TNE buffer containing 0.1% SDS and boiled. Particulate material was removed by centrifugation for 5 min at 10,000 × *g* in an Eppendorf microfuge. p56^{lck} was recovered by immunoprecipitation from lysates corresponding to equivalent numbers of cells and quantitated by Lck immunoblot. Using this fractionation procedure, lactate dehydrogenase activity (a cytosolic marker) was detected exclusively in the S100 fraction, whereas 5′ nucleotidase activity (a plasma membrane marker) was found predominantly (over 75%) in the P100 fraction (assayed with Sigma Diagnostics kits 675 and 500; data not shown).

RESULTS

Site-directed mutagenesis. Through oligonucleotide-directed mutagenesis of the murine *lck* cDNA NT18 (24), the following p56^{lck} single-point mutants were engineered: F394, in which the autophosphorylation site Tyr-394 was substituted by a phenylalanine residue; A2, in which the putative site of myristylation of p56^{lck} (Gly-2) was replaced by an alanine residue; and F505, in which Tyr-505 was mutated to a phenylalanine. By using standard recombinant DNA technology, double mutants carrying the F505 mutation with either the F394 substitution (F394F505 Lck mutant) or the A2 mutation (A2F505 Lck mutant) were created. All cDNAs were completely resequenced and found to contain no additional alterations (see Materials and Methods; data not shown).

Expression and biological effects of mutant *lck* cDNAs in NIH 3T3 fibroblasts. Wild-type, F505, F394F505, and A2F505 *lck* cDNAs were cloned in the retroviral expression vector pLXSN, and retrovirus stocks were generated by transfection in ψ-2 packaging cells. Packaging cells expressing the neomycin resistance gene alone were also generated by transfection with pLXSN. After infection of NIH 3T3 fibroblasts with the appropriate retrovirus stocks, polyclonal cell lines were selected by growth in medium containing the aminoglycoside G418. Monoclonal cell lines were also established by clonal expansion of antibiotic-resistant cells.

To quantitate levels of p56^{lck} expression, equivalent numbers of cells from polyclonal lines were lysed in boiling sample buffer, and lysates were resolved on 8% SDS-PAGE gels. After transfer onto nitrocellulose membranes, the expression of p56^{lck} was measured in an immunoblot assay, using a high-affinity rabbit anti-Lck antiserum and ^{125}I-labeled protein A (Fig. 1A). Whereas NIH 3T3 fibroblasts expressing neomycin phosphotransferase alone did not express p56^{lck} (Fig. 1A, lane 1), all cells infected with Lck-encoding retroviruses expressed a 56-kilodalton (kDa) immunoreactive product consistent with p56^{lck} (lanes 2 to 5). The levels of the various forms of p56^{lck} did not differ by more than twofold (data not shown). The electrophoretic mobility of the A2F505 Lck protein (lane 5) was slightly slower than that of the other p56^{lck} polypeptides (lanes 2 to 4). Although the exact basis of this finding remains undefined, we have ruled out the possibility that it is consequent to additional alterations in the *lck* cDNA (data not shown).

The biological effects of expression of these mutants were next examined (Fig. 1B). As previously reported by Marth et al. (22) as well as by Amrein and Sefton (2), expression of the F505 Lck mutant resulted in morphological transformation of NIH 3T3 cells. The cells acquired a typical refractile appearance with spindle-shaped morphology and developed multiple neuronal-like processes (Fig. 1B, panel 3). In contrast, cells expressing wild-type (panel 2), F394F505 (panel 4), or A2F505 (panel 5) p56^{lck} had morphologies that were indistinguishable from that of NIH 3T3 fibroblasts expressing the neomycin resistance marker alone (panel 1). Similar observations were made with multiple monoclonal cell lines expressing each of the Lck mutants (data not shown). The fibroblast lines were also tested for their ability to form foci on monolayers. F505 Lck-expressing fibroblasts developed easily identifiable foci with a swirl-like appearance at an average frequency of 9.4% (range from four separate assays, 4.7 to 20%). None of the other cell lines gave rise to identifiable foci (data not shown). Finally, cells were evaluated for their growth potential in semisolid medium. Four separate assays revealed that an average of 0.8% (range, 0.31 to 1.7%) of F505 Lck-expressing fibroblasts formed colonies in soft agar. None of the other fibroblast lines were capable of detectable growth in this medium (data not shown). Taken together, these data indicated that the oncogenic potential of the F505 p56^{lck} mutant was abolished by additional mutations of either the known site of Lck autophosphorylation (F394 mutation) or the predicted site of Lck myristylation (A2 mutation).

Analyses of the tyrosine protein kinase activity of the F394F505 Lck mutant. To confirm the mutational substitution of Tyr-394, fibroblasts expressing approximately equivalent amounts of wild-type, F505, or F394F505 p56^{lck} were metabolically labeled for 4 h in the presence of 1.0 mCi of $^{32}P_i$ per ml. Cells were then lysed in boiling SDS buffer, and the Lck polypeptides were recovered by immunoprecipitation. After separation by SDS-PAGE, peptide mapping studies were conducted by cleavage of eluted phospholabeled p56^{lck} with cyanogen bromide. The reaction products were separated on 20% SDS-PAGE gels and detected by autoradiography (Fig. 2). As previously reported (32, 36), the wild-type Lck protein (Fig. 2, lane 1) was phosphorylated on the 28-kDa C1 fragment (which contains the sites of amino-terminal serine phosphorylation) and on the 4-kDa C3 peptide (which contains the major site of in vivo tyrosine phosphorylation, Tyr-505). The F505 Lck mutant (lane 2) demonstrated significant C1 peptide phosphorylation (on both serine and tyrosine residues; data not shown) and phosphorylation of a series of fragments between 10 and 14 kDa corresponding to the Tyr-394-containing C2 peptides and, as predicted, lacked phosphorylation of the carboxy-terminal C3 fragment. The F394F505 Lck mutant (lane 3) was phosphorylated on its amino-terminal C1 fragment but had no detectable phosphorylation of the C2 and C3 peptides, consistent with the alterations of Tyr-394 and Tyr-505 in this mutant. The peptides generated by cleavage of in vitro autophosphorylated wild-type p56^{lck} are also shown as C2 peptide markers (lane 4).

To evaluate the basis for the lack of transforming potential of the F394F505 Lck mutant, the effects of expression of this product on cellular phosphotyrosine levels were examined by antiphosphotyrosine immunoblotting (Fig. 3A). Polyclonal as well as monoclonal F394F505 Lck-expressing cell lines (Fig. 3A, lanes 9 to 11) were compared with cells

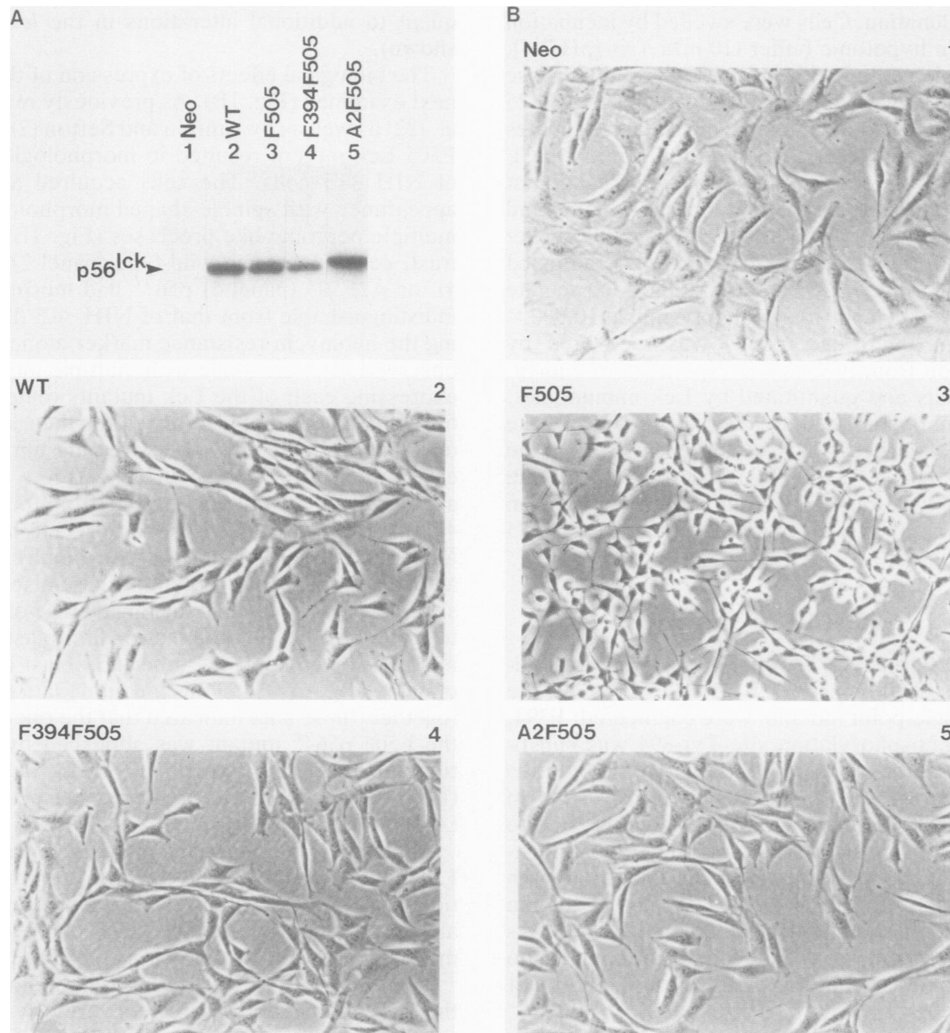

FIG. 1. Expression and effects of wild-type and variant p56^{lck} in NIH 3T3 fibroblasts. (A) Lck immunoblot. NIH 3T3 fibroblasts were infected with retroviruses encoding wild-type and variant p56^{lck} molecules as described in Materials and Methods. Polyclonal cell lines were established by growth in medium containing the aminoglycoside G418. Expression of p56^{lck} was assayed by Lck-specific immunoblot of total cell lysates. Lanes: 1, neomycin phosphotransferase (Neo) control; 2, wild-type (WT) p56^{lck}; 3, F505 p56^{lck}; 4, F394F505 p56^{lck}; 5, A2F505 p56^{lck}. The position of p56^{lck} is indicated. Exposure time, 24 h. (B) Morphology of p56^{lck}-expressing NIH 3T3 fibroblasts. Polyclonal cell lines expressing wild-type and variant p56^{lck} molecules were examined by light microscopy. Panels: 1, neomycin phosphotransferase control; 2, wild-type p56^{lck}; 3, F505 p56^{lck}; 4, F394F505 p56^{lck}; 5, A2F505 p56^{lck}. Magnification, ×112.

expressing either the neomycin marker alone (lane 1), wild-type p56^{lck} (lanes 2 to 4), or the F505 Lck mutant (lanes 5 to 8). A parallel Lck immunoblot was performed to quantitate the abundance of p56^{lck} in each cell line (Fig. 3B). This assay indicated that the ranges of p56^{lck} expression in the various sets of cell lines were overlapping. As previously reported by Amrein and Sefton (2), cells expressing F505 p56^{lck} (lanes 5 to 8) contained significantly elevated levels of phosphotyrosine-containing proteins. In contrast, those expressing the F394F505 Lck mutant (Fig. 3A, lanes 9 to 11) had levels of protein tyrosine phosphorylation comparable to the low levels detected in cells expressing the neomycin resistance marker alone (lane 1) or wild-type p56^{lck} (lanes 2 to 4), although one cell line (lane 11) demonstrated increased detectability of products migrating at approximately 65 and 80 kDa. Strikingly, the prominent 36-kDa immunoreactive product detected in cells expressing the F505 Lck mutant (most likely calpactin I [2]) was not present in F394F505 Lck-expressing cells.

To further examine the effects of mutational substitution of Tyr-394 on the function of the F505 p56^{lck} mutant, the tyrosine protein kinase activities of F394F505 and F505 p56^{lck} were compared in immune complex kinase assays. Representative polyclonal and monoclonal cell populations were lysed in Nonidet P-40-containing buffer, and the Lck polypeptides were recovered by immunoprecipitation. The p56^{lck}-associated tyrosine kinase activity was measured under linear assay conditions in immune complex kinase reactions using rabbit muscle enolase as an exogenous substrate (Fig. 4A). The amount of p56^{lck} in these immunoprecipitates was quantitated by parallel Lck immunoblot (Fig. 4B). Specific tyrosine protein kinase activity was calculated as the ratio of relative enolase phosphorylation over relative p56^{lck} abundance (data not shown). A representative exper-

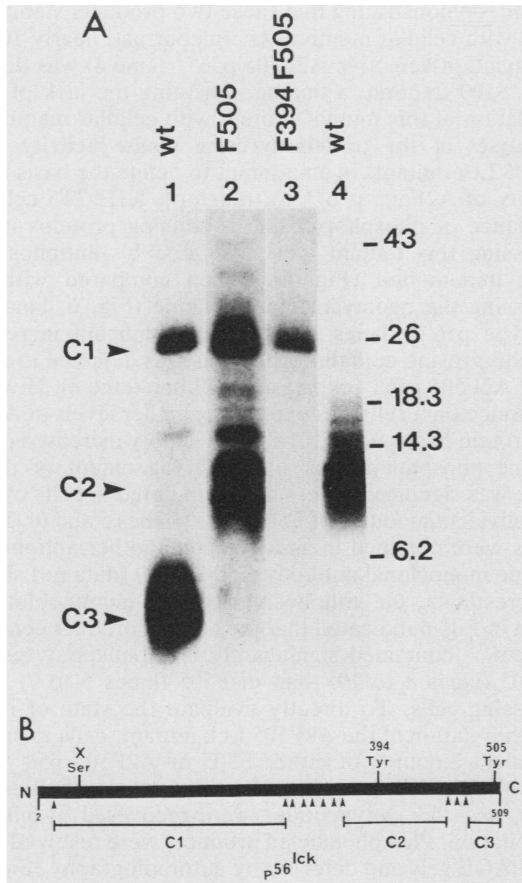

FIG. 2. (A) Cyanogen bromide cleavage analyses. In vivo phospholabeled Lck polypeptides were electroeluted from gel slices and subjected to cyanogen bromide fragmentation. The products of this reaction were resolved on 20% SDS-PAGE gels. Lanes: 1, wild-type (wt) p56^{lck}; 2, F505 p56^{lck}; 3, F394F505 p56^{lck}; 4, in vitro autophosphorylated wild-type p56^{lck}. The C1, C2, and C3 cyanogen bromide fragments are indicated. The positions of prestained molecular mass markers are shown on the right. Exposure time, 48 h. (B) Schematic diagram of the predicted cyanogen bromide cleavage sites (arrowheads). C1 corresponds to the phosphoserine-containing amino-terminal portion of the molecule; C2 and C3 contain the major sites of in vitro (Tyr-394) and in vivo (Tyr-505) tyrosine phosphorylation, respectively (32, 36). The specific serine residue(s) phosphorylated within fragment C1 has not been identified and is therefore indicated as X.

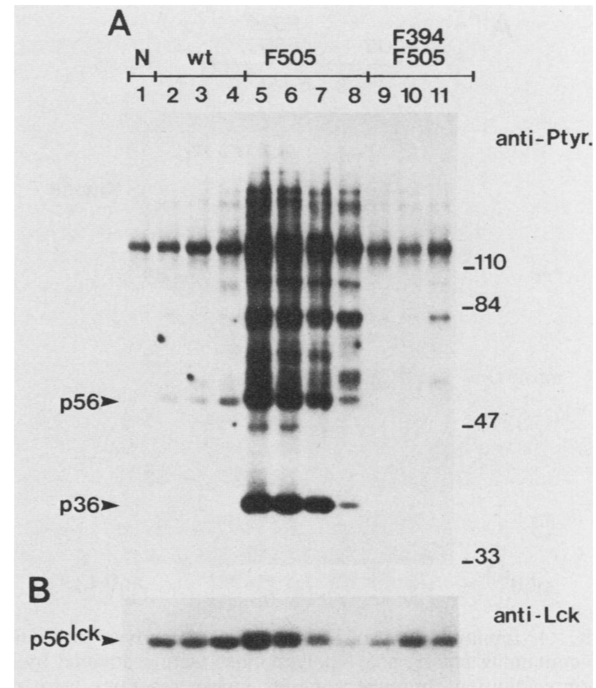

FIG. 3. Antiphosphotyrosine immunoblot. Cells were lysed directly in boiling sample buffer. Lysates from equivalent numbers of cells were analysed by antiphosphotyrosine (anti-Ptyr.) immunoblotting using a polyclonal rabbit antiphosphotyrosine antiserum and ^{125}I-labeled protein A as described in Materials and Methods (A). A parallel Lck immunoblot was performed on these lysates to determine levels of Lck expression (B). Both polyclonal and monoclonal Lck-expressing cell lines were analyzed in these assays. The first lane of each set (lanes 1, 2, 5, and 9) represents a polyclonal cell population; the remaining lanes are representative monoclonal cell lines. Lanes: 1, neomycin phosphotransferase (N) control; 2 to 4, wild-type (wt) p56^{lck}; 5 to 8, F505 p56^{lck}; 9 to 11, F394F505 p56^{lck}. The positions of p56^{lck} as well as the putative 36-kDa calpactin I (p36) are indicated. The positions of prestained molecular mass markers are shown on the right. Exposure times: (A) 6 h; (B) 7 days.

iment (Fig. 4A) revealed that the specific activity of F394F505 Lck (lanes 4 to 6) was approximately 3.5-fold less than that of F505 Lck (lanes 1 to 3). Taken together, using in vivo and in vitro assays, the data presented above indicated that the enhanced enzymatic activity of the F505 p56^{lck} mutant was significantly reduced by mutating Tyr-394 to a phenylalanine residue.

The A2F505 Lck mutant is not myristylated and lacks detectable association with cellular membranes. Previous studies have shown that p56^{lck} is membrane associated and contains covalently bound myristic acid (21, 39). On the basis of sequence homology with p60^{src}, it appeared likely that the amino-terminal glycine (Gly-2) represented the site of p56^{lck} myristylation. To evaluate the effects of mutating Gly-2 to an alanine (A2 mutation) on myristylation, A2F505 and F505 Lck-expressing fibroblasts were metabolically labeled with either [^{3}H]myristic acid or a mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans-label). After lysing in detergent-containing buffer, p56^{lck} was recovered by immunoprecipitation and resolved on 8% SDS-PAGE gels. The labeled p56^{lck} polypeptides were detected by fluorography (Fig. 5A and C). Serial dilutions of F505 Lck-expressing fibroblast lysates were assayed to facilitate our analysis (lanes 2 to 5). By comparing dilutions containing equivalent amounts of newly synthesized ^{35}S-labeled p56^{lck} quantitated by densitometry (Fig. 5A; lane 1 for A2F505 Lck and lane 4 for F505 Lck), we determined that only F505 p56^{lck} detectably incorporated [^{3}H]myristic acid (Fig. 5C, lane 4). This result indicated that A2F505 p56^{lck} was not myristylated.

To evaluate whether lack of myristylation resulted in altered p56^{lck} cellular localization, cell fractionation experiments were conducted. Cells expressing wild-type, F505, and A2F505 p56^{lck} were mechanically lysed in hypotonic buffer, and postnuclear lysates were separated into particulate (P100) and cytosolic (S100) fractions by high-speed centrifugation. The abundance of p56^{lck} in each fraction was measured by immunoblot of anti-Lck immunoprecipitates (Fig. 5B). In several experiments including the one depicted in Fig. 5B, we found that between 60 and 70% of wild-type (lane 1) and F505 (lane 3) p56^{lck} was present in the P100

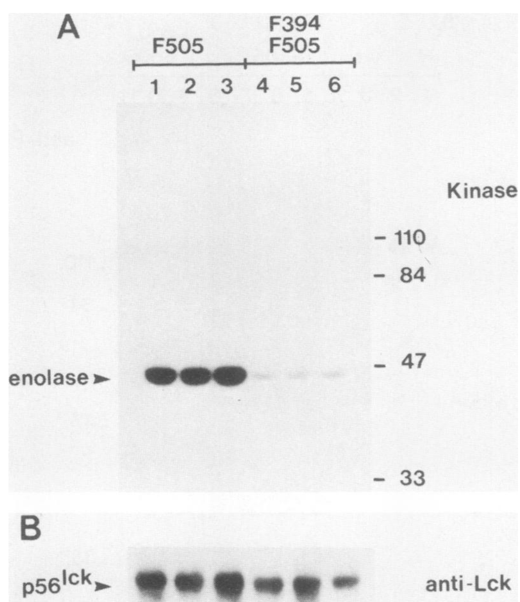

FIG. 4. Immune complex kinase assay. After lysis in Nonidet P-40-containing buffer, p56^{lck} polypeptides were recovered by immunoprecipitation. Immune complex kinase reactions were performed in the presence of the exogenous substrate rabbit muscle enolase. The reactions were terminated after 2 min by the addition of sample buffer, and the products were resolved on 8% SDS-PAGE gels (A). A p56^{lck} immunoblot was performed on parallel immunoprecipitates to determine levels of Lck expression (B). The first lane of each set of cell lines (lanes 1 and 4) represents a polyclonal cell population; the remaining lanes are representative monoclonal cell lines. Lanes: 1 to 3, F505 p56^{lck}; 4 to 6, F394F505 p56^{lck}. The positions of p56^{lck} and enolase are shown. The positions of prestained molecular mass markers are indicated on the right. Exposure times: (A) 12 h; (B) 48 h.

fraction, demonstrating that these two products stably associated with cellular membranes. In contrast, nearly 100% of myristylation-defective A2F505 p56^{lck} (lane 6) was detected in the S100 fraction, a finding indicating the lack of stable association of this mutant protein with cellular membranes.

Analyses of the protein tyrosine kinase activity of the A2F505 Lck mutant. In an attempt to define the basis for the inability of A2F505 p56^{lck} to transform NIH 3T3 cells, the abundance of phosphotyrosine-containing proteins in cells expressing this mutant was evaluated by antiphosphotyrosine immunoblot (Fig. 6). When compared with cells expressing the neomycin marker alone (Fig. 6, lane 1) or wild-type p56^{lck} (lanes 2 to 4), no significant increase in phosphotyrosine-containing proteins was detected in a polyclonal A2F505 p56^{lck}-expressing cell line (lane 8). However, two monoclonal cell lines expressing higher levels of A2F505 Lck protein (lanes 9 and 10) had detectably increased protein tyrosine phosphorylation, although the extent of this increase was significantly less than that noted in cells expressing equivalent amounts of F505 p56^{lck} (lanes 5 and 6). Similar results were obtained in assays using another antiphosphotyrosine monoclonal antibody (PY-20; 11) (data not shown).

Interestingly, the antiphosphotyrosine immunoblot assay shown in Fig. 6 indicated that the 56-kDa product consistent with p56^{lck} contained significantly less phosphotyrosine in A2F505 (lanes 8 to 10) than in F505 (lanes 5 to 7) p56^{lck}-expressing cells. To directly evaluate the state of in vivo phosphorylation of the A2F505 Lck mutant, cells expressing equivalent amounts of either F505 or A2F505 p56^{lck} were metabolically labeled with $^{32}P_i$ and lysed in boiling SDS buffer, and Lck polypeptides were recovered by immunoprecipitation. Phospholabeled products were resolved on 8% SDS-PAGE gels and detected by autoradiography (Fig. 7A). The results of this experiment indicated that the overall phosphorylation of the A2F505 Lck protein (Fig. 7A, lane 2) was significantly less than that of the F505 Lck mutant (lane

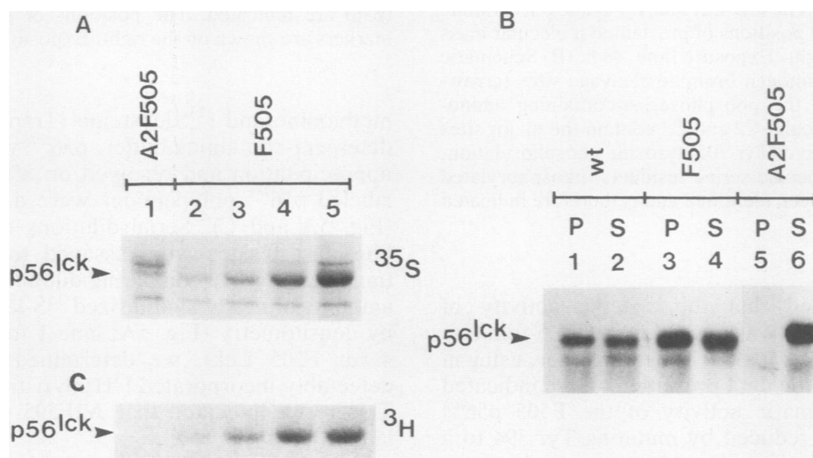

FIG. 5. Myristylation and subcellular localization studies. (A and C) Metabolic labeling studies with [^{3}H]myristic acid and [^{35}S]methionine-[^{35}S]cysteine. Parallel cultures were incubated for 4 h in 0.5 mCi of [^{35}S]methionine-[^{35}S]cysteine (A) or [^{3}H]myristic acid (C) per ml as described in Materials and Methods. After lysis in TNE buffer, A2F505 p56^{lck} was recovered from 500 μg of cellular proteins, whereas F505 p56^{lck} was recovered from serial dilutions of lysate. Labeled polypeptides were resolved on 8% SDS-PAGE gels. Lanes: 1, A2F505 p56^{lck} (500 μg); 2 to 5, F505 p56^{lck} (2, 31 μg; 3, 62.5 μg; 4, 125 μg; 5, 250 μg). The position of p56^{lck} is shown. Exposure times: (A) 3 days; (C) 7.5 weeks. (B) Subcellular localization studies. Cells were mechanically lysed after swelling in hypotonic buffer, and S100 and P100 fractions were separated by centrifugation as described in Materials and Methods. The particulate (P100) and cytosolic (S100) fractions were adjusted to 0.1% SDS-TNE buffer and boiled. p56^{lck} was recovered from each fraction by immunoprecipitation, resolved on 8% SDS-PAGE gels, and visualized by Lck immunoblot. Lanes: 1, 3, and 5, P100 (particulate fraction); 2, 4, and 6, S100 (cytosolic fraction); 1 and 2, wild-type (wt) p56^{lck}; 3 and 4, F505 p56^{lck}; 5 and 6, A2F505 p56^{lck}. The position of p56^{lck} is indicated. Exposure time, 3 days.

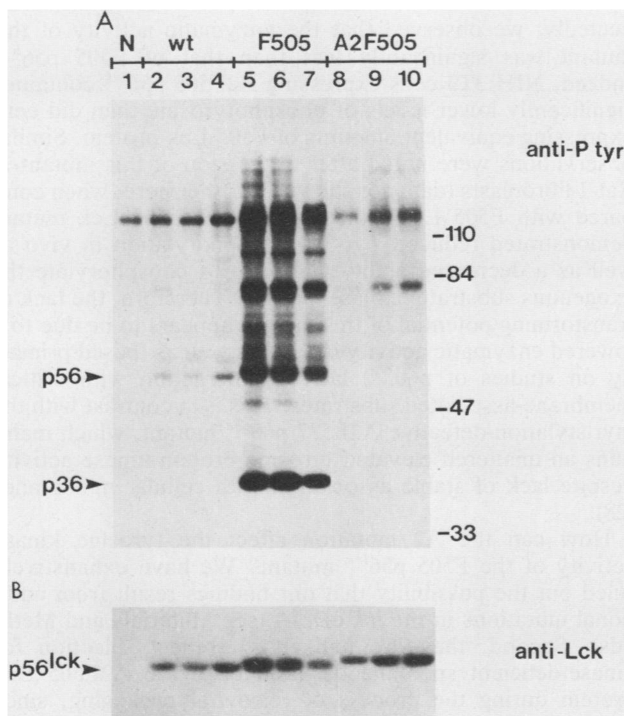

FIG. 6. Antiphosphotyrosine immunoblot. Antiphosphotyrosine immunoblot analysis was performed as described for Fig. 3A. A parallel Lck immunoblot was also done to determine levels of Lck expression (B). Both polyclonal and monoclonal Lck-expressing cell lines were analyzed. The first lane of each set (lanes 1, 2, 5, and 8) represents a polyclonal cell population; the remaining lanes are representative monoclonal cell lines. Lanes: 1, neomycin phosphotransferase (N) control; 2 to 4: wild-type (wt) $p56^{lck}$; 5 to 7: F505 $p56^{lck}$; 8 to 10, A2F505 $p56^{lck}$. The positions of $p56^{lck}$ and of the putative 36-kDa calpactin I (p36) are shown. The positions of prestained molecular mass markers are shown on the right. Exposure times: (A) 8 h; (B) 7 days.

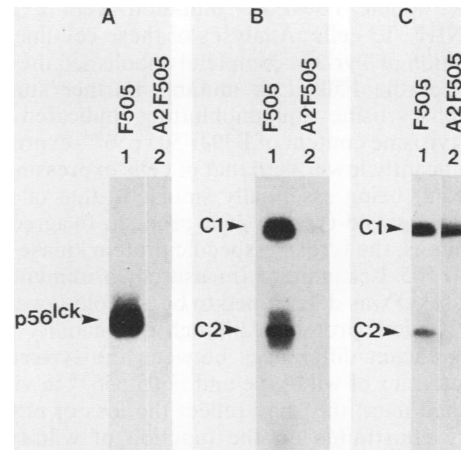

FIG. 7. A2F505 $p56^{lck}$. (A) Metabolic labeling studies with $^{32}P_i$. Cells were labeled with $^{32}P_i$ as described in Materials and Methods. $p56^{lck}$ polypeptides were recovered by immunoprecipitation from cell lysates. Lanes: 1, F505 $p56^{lck}$; 2, A2F505 $p56^{lck}$. The position of $p56^{lck}$ is indicated. Exposure time: 12 h. (B) Cyanogen bromide cleavage analyses. Conditions were as for Fig. 2A. Lanes: 1, F505 $p56^{lck}$; 2, A2F505 $p56^{lck}$. The C1 and C2 cyanogen bromide fragments (as depicted in Fig. 2B) are indicated. Exposure time, 12 h. (C) Cyanogen bromide cleavage analyses. Relative phosphorylation of C2 was examined by different autoradiographic exposures of the experiment shown in panel B. Lanes: 1, F505 $p56^{lck}$ (7-h exposure); 2, A2F505 $p56^{lck}$ (3-day exposure). The positions of fragments C1 and C2 are shown.

1). Mapping studies using cyanogen bromide (Fig. 7B) revealed that whereas the F505 Lck protein (lane 1) was extensively phosphorylated on the amino-terminal C1 and Tyr-394-containing C2 peptides, the A2F505 $p56^{lck}$ mutant was noticeably phosphorylated only on the amino-terminal C1 fragment (lane 2). Different autoradiographic exposures of this gel (Fig. 7C) indicated the relative lack of C2 peptide phosphorylation in digests of A2F505 $p56^{lck}$ (lane 2). Although not apparent from this experiment, we have occasionally observed small degrees of in vivo C2 phosphorylation in A2F505 $p56^{lck}$ molecules (data not shown).

To further evaluate the enzymatic function of A2F505 $p56^{lck}$, the catalytic activity of this product was measured by immune complex kinase assay (Fig. 8). Under linear assay conditions, this analysis demonstrated that the specific activity of A2F505 $p56^{lck}$ (Fig. 8, lanes 4 to 6) was approximately 2.5-fold less than that of F505 $p56^{lck}$ (lanes 1 to 3). Taken together, these findings showed that mutation of the site of $p56^{lck}$ myristylation significantly decreased the tyrosine-specific protein kinase activity of the F505 Lck mutant.

DISCUSSION

Upon activation by mutation of Tyr-505, $p56^{lck}$ becomes extensively phosphorylated in vivo at Tyr-394, the site of in vitro autophosphorylation (2, 22). To evaluate whether Tyr-394 phosphorylation may participate in regulation of the tyrosine-specific protein kinase activity of $p56^{lck}$, this residue was replaced by a phenylalanine (thereby preventing phosphorylation at this site), and mutant polypeptides car-

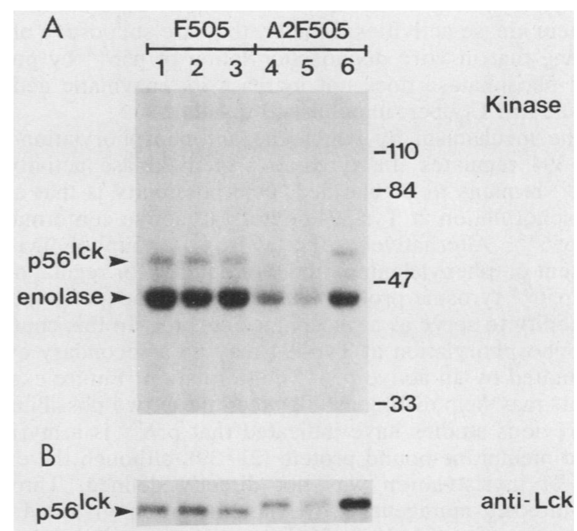

FIG. 8. Immune complex kinase assay. Conditions were as for Fig. 4. The first lane of each set of cell lines (lanes 1 and 4) represents a polyclonal cell population; the remaining lanes are representative monoclonal cell lines. Lanes: 1 to 3, F505 $p56^{lck}$; 4 to 6, A2F505 $p56^{lck}$. The positions of enolase and $p56^{lck}$ are shown. The positions of prestained molecular mass markers are indicated on the right. Exposure times: (A) 12 h; (B) 4 days.

rying the double F394F505 mutation were expressed in murine NIH 3T3 cells. Analyses of these cell lines revealed that mutation of Tyr-394 completely abolished the oncogenic potential of the F505 Lck mutant. Further studies using antiphosphotyrosine immunoblotting indicated that the phosphotyrosine content of F394F505 $p56^{lck}$-expressing cells was significantly lower than that of cells expressing the F505 Lck mutant, being essentially similar to that of fibroblasts expressing the wild-type *lck* gene product. In agreement with these findings, the tyrosine-specific protein kinase activity of the F394F505 Lck mutant (measured in immune complex kinase assays) was determined to be 3.5-fold lower than that of the F505 Lck protein. Although the inability to demonstrate significant differences between the tyrosine protein kinase activities of wild-type and F505 $p56^{lck}$ in vitro (2; our unpublished data; this may reflect the loss of physiological inhibitory constraints on the function of wild-type $p56^{lck}$ after detergent extraction) limits further assessments of the function of F394F505 $p56^{lck}$ relative to that of the wild-type polypeptide, our data nevertheless indicate that the tyrosine protein kinase activity of the F394F505 Lck mutant is either similar or marginally elevated compared with that of the wild-type protein. Although similar to the effect of mutation of Tyr-416 on the F527 Src mutant, the impact of alteration of Tyr-394 on the activity of F505 $p56^{lck}$ appears to be more dramatic. Indeed, mutation of Tyr-416 of $p60^{src}$ results in partial suppression of the oncogenic potential of the F527 Src mutant (17, 27).

These data suggest that lack of phosphorylation at Tyr-505 may facilitate but is not sufficient for $p56^{lck}$ activation. Additional Tyr-394-dependent $p56^{lck}$ modifications appear to be required for the enzyme to become activated. This may explain why, despite a low apparent stoichiometry of Tyr-505 phosphorylation before CD4 cross-linking (32), $p56^{lck}$ molecules associated with CD4 do not have elevated enzymatic activity in vitro (34). These findings may also provide an explanation for the observation of Ostergaard and colleagues (26) that $p56^{lck}$ isolated from $CD45^+$ and $CD45^-$ T-cell hybridomas (which have different extents of Tyr-505 phosphorylation) do not have significantly different in vitro protein kinase activities. Finally, they are supportive of the finding that in vitro dephosphorylation of $p56^{lck}$ by potato acid phosphatase does not increase its enzymatic activity (Louie and Cooper, unpublished results).

The mechanism by which the autophosphorylation site Tyr-394 regulates the tyrosine protein kinase activity of $p56^{lck}$ remains to be clarified. One possibility is that autophosphorylation at Tyr-394 confers an active conformation to $p56^{lck}$. Alternatively, the hydroxyl group of Tyr-394 (absent on phenylalanine) may be required for regulation of the $p56^{lck}$ tyrosine protein kinase activity independently of its ability to serve as a phosphate acceptor. In this context, autophosphorylation at Tyr-394 may be a secondary event facilitated by an active $p56^{lck}$ conformation. Future experiments may help distinguish between these two possibilities.

Previous studies have indicated that $p56^{lck}$ is a myristylated membrane-bound protein (21, 39), although the exact site of myristylation was not directly defined. Through site-directed mutagenesis, we have now demonstrated that replacement of Gly-2 of $p56^{lck}$ by an alanine abolishes Lck myristylation, suggesting that Gly-2 may be the residue targeted by this modification. Furthermore, we have shown that Gly-2 is required for stable association of $p56^{lck}$ with fibroblast cellular membranes.

As one would predict from studies of $p60^{src}$ (8, 15, 28), a myristylation-defective F505 Lck mutant (A2F505 mutant) was incapable of transforming NIH 3T3 cells. Quite unexpectedly, we observed that the enzymatic activity of this mutant was significantly less than that of F505 $p56^{lck}$. Indeed, NIH 3T3 cells expressing A2F505 $p56^{lck}$ contained significantly lower levels of phosphotyrosine than did cells expressing equivalent amounts of F505 Lck protein. Similar observations were noted after expression of this mutant in Rat-1 fibroblasts (data not shown). Furthermore, when compared with F505 Lck molecules, the A2F505 Lck mutant demonstrated reduced Tyr-394 phosphorylation in vivo as well as a decreased ability (2.5-fold) to phosphorylate the exogenous substrate enolase in vitro. Therefore, the lack of transforming potential of this mutant appears to be due to a lowered enzymatic activity in vivo as well as (based primarily on studies of $p60^{src}$) lack of interaction with critical membrane-associated substrates. This is in contrast with the myristylation-defective A2F527 $p60^{src}$ mutant, which maintains an unaltered elevated tyrosine protein kinase activity despite lack of stable association with cellular membranes (28).

How can the A2 mutation affect the tyrosine kinase activity of the F505 $p56^{lck}$ mutant? We have exhaustively ruled out the possibility that our findings result from additional mutations in the *lck* cDNA (see Materials and Methods). Second, they are unlikely to reflect selection for kinase-deficient spontaneous variants of the A2F505 Lck protein during the process of retroviral packaging, since similar results were obtained after transfection by calcium phosphate precipitation (data not shown). Third, since mutation of a glycine to an alanine represents a relatively conserved amino acid substitution, it appears doubtful that the A2 mutation drastically alters the conformation of $p56^{lck}$. Indirect support for this view is provided by the observations that the A2F505 Lck mutant is capable of physically associating with the CD4 T-cell surface antigen and that an A2 Lck mutant with a tyrosine residue at position 505 remains extensively phosphorylated at Tyr-505 in vivo (our unpublished data). One possible explanation is that myristylation or interaction with the plasma membrane is needed for efficient activation of $p56^{lck}$. Since the A2F505 Lck mutant shows a low extent of Tyr-394 phosphorylation in vivo, one plausible scenario is that myristylation, membrane association, or both are important for Tyr-394-dependent activation of $p56^{lck}$. Although less likely, it is also conceivable that the cytoplasmic localization of this mutant interferes with its activation as a result of, for example, rapid dephosphorylation of Tyr-394 by cytosolic tyrosine phosphatases.

Previous studies have indicated that phosphorylation at Tyr-505 of $p56^{lck}$ down regulates its tyrosine kinase activity (2, 22). However, several lines of evidence indicate that lack of phosphate on Tyr-505 is not sufficient for elevation of the $p56^{lck}$ tyrosine kinase activity (26, 32, 34; R. Louis and J. Cooper, unpublished data). The data presented in this report suggest that activation of $p56^{lck}$ requires additional posttranslational modifications dependent on Tyr-394, the site of Lck autophosphorylation (most likely phosphorylation at this site). Our findings also indicate that these alterations may be facilitated by $p56^{lck}$ myristylation, membrane association, or both. Because of the differences noted between the regulation of $p56^{lck}$ and $p60^{src}$, analyses of the functions of carboxy-terminal tyrosine phosphorylation, autophosphorylation, and myristylation in the regulation of other members of the Src family appear to be warranted. Such studies may help identify the central events involved in the posttranslational regulation of this family of closely related gene products.

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LITERATURE CITED

1. **Albritton, L. M., L. Tseng, D. Scadden, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57:**659–666.
2. **Amrein, K., and B. M. Sefton.** 1988. Mutation of a site of tyrosine phosphorylation in the lymphocyte-specific tyrosine protein kinase, p56^{lck}, reveals its oncogenic potential in fibroblasts. Proc. Natl. Acad. Sci. USA **85:**4247–4251.
3. **Bolen, J. B., and A. Veillette.** 1989. A function for the *lck* proto-oncogene. Trends Biochem. Sci. **14:**404–407.
4. **Cartier, M., M. W. M. Chang, and C. P. Stanners.** 1987. The use of the *Escherichia coli* gene for asparagine synthetase as a selectable marker in a shuttle vector capable of dominant transfection and amplification in animal cells. Mol. Cell. Biol. **7:**1623–1628.
5. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60^{c-src} mutated in the carboxy-terminal regulatory domain. Cell **49:**83–91.
6. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr^{527} is phosphorylated in pp60^{c-src}: implications for regulation. Science **231:**1431–1434.
7. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60^{c-src}. Mol. Cell. Biol. **6:**4467–4477.
8. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the p60^{src} terminus is required for p60^{src} myristylation and membrane association and for cell transformation. Mol. Cell. Biol. **4:**1834–1842.
9. **Dymecki, S. M., J. E. Niederhuber, and S. V. Desiderio.** 1990. Specific expression of a tyrosine kinase gene, *blk*, in B lymphoid cells. Science **247:**332–336.
10. **Freedman, V. H., and S. Shin.** 1974. Cellular tumorigenicity in *nude* mice: correlation with cell growth in semi-solid medium. Cell **3:**355–359.
11. **Glenney, J. R., Jr., L. Zokas, and M. P. Kamps.** 1988. Monoclonal antibodies to phosphotyrosine. Immunol. Methods **109:** 277–285.
12. **Harvey, R., K. M. Hehir, A. E. Smith, and S. H. Cheng.** 1989. pp60^{c-src} variants containing lesions that affect phosphorylation at tyrosines 416 and 527. Mol. Cell. Biol. **9:**3647–3656.
13. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54:**897–930.
14. **Jove, R., S. Kornbluth, and H. Hanafusa.** 1987. Enzymatically inactive p60^{c-src} mutant with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory region. Cell **50:**937–943.
15. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1985. Mutation of NH_2-terminal glycine of p60^{src} prevents both myristoylation and morphological transformation. Proc. Natl. Acad. Sci. USA **82:**4625–4628.
16. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ros*, and v-*erb*-B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2:**305–315.
17. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60^{c-src} transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.
18. **Koch, C. A., M. Moran, I. Sadowski, and T. Pawson.** 1989. The common *src* homology region 2 domain of cytoplasmic signaling proteins is a positive effector of v-*fps* tyrosine kinase function. Mol. Cell. Biol. **9:**4131–4140.
19. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
20. **MacAuley, A., and J. A. Cooper.** 1988. The carboxy-terminal sequence of p56^{lck} can regulate p60^{src}. Mol. Cell. Biol. **8:**3560–3564.
21. **Marchildon, G. A., J. E. Casnellie, K. A. Walsh, and E. G. Krebs.** 1984. Covalently bound myristate in a lymphoma tyrosine protein kinase. Proc. Natl. Acad. Sci. USA **81:**7679–7682.
22. **Marth, J. D., J. A. Cooper, C. S. King, S. F. Ziegler, D. A. Tinker, R. W. Overell, E. G. Krebs, and R. M. Perlmutter.** 1988. Neoplastic transformation induced by an activated lymphocyte-specific protein tyrosine kinase (pp56^{lck}). Mol. Cell. Biol. **8:**540–550.
23. **Marth, J. D., C. Disteche, D. Pravtcheva, F. Ruddle, E. G. Krebs, and R. M. Perlmutter.** 1986. Localization of a lymphocyte-specific protein tyrosine kinase gene (*lck*) at a site of frequent chromosomal abnormalities in human lymphomas. Proc. Natl. Acad. Sci. USA **83:**7400–7404.
24. **Marth, J. D., R. Peet, E. G. Krebs, and R. M. Perlmutter.** 1985. A lymphocyte-specific protein tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA. Cell **43:**393–404.
25. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
26. **Ostergaard, H. L., D. A. Shackelford, T. R. Hurley, P. Johnson, R. Hyman, B. Sefton, and I. S. Trowbridge.** 1989. Expression of CD45 alters phosphorylation of the *lck* encoded tyrosine protein kinase in murine lymphoma T-cell lines. Proc. Natl. Acad. Sci. USA **86:**8959–8963.
27. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60^{c-src}. Cell **49:**75–82.
28. **Reynolds, A. B., D. J. Roesel, S. B. Kanner, and J. T. Parsons.** 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. Mol. Cell. Biol. **9:**629–638.
29. **Shaw, A. S., J. Chalupny, J. A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56^{lck} tyrosine protein kinase. Mol. Cell. Biol. **10:**1853–1862.
30. **Stanners, C. P., G. L. Eliceiri, and H. Green.** 1971. Two types of ribosomes in mouse-hamster hybrids. Nature (London) **230:**52–54.
31. **Veillette, A., and J. B. Bolen.** 1989. *Src*-related protein tyrosine kinases, p. 121–142. *In* C. Benz and E. Liu (ed.), Oncogenes. Kluwer Academic Publishers, Norwell, Mass.
32. **Veillette, A., J. B. Bolen, and M. A. Bookman.** 1989. Alterations in tyrosine protein phosphorylation induced by antibody-mediated cross-linking of the CD4 receptor of T lymphocytes. Mol. Cell. Biol. **9:**4441–4446.
33. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T-cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56^{lck}. Cell **55:**301–308.
34. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}. Nature (London) **338:**257–259.
35. **Veillette, A., F. M. Foss, E. A. Sausville, J. B. Bolen, and N. Rosen.** 1987. Expression of the *lck* tyrosine kinase gene in human colon carcinoma and other non-lymphoid human tumour cell lines. Oncogene Res. **1:**357–374.
36. **Veillette, A., I. D. Horak, and J. B. Bolen.** 1988. Post-translational alterations of the tyrosine kinase p56^{lck} in response to activators of protein kinase C. Oncogene Res. **2:**385–401.
37. **Veillette, A., I. D. Horak, E. M. Horak, M. A. Bookman, and**

J. B. Bolen. 1988. Alterations of the lymphocyte-specific protein tyrosine kinase ($p56^{lck}$) during T-cell activation. Mol. Cell. Biol. **8:**4353–4361.

38. **Veillette, A., J. C. Zuniga-Pflucker, J. B. Bolen, and A. M. Kruisbeek.** 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. J. Exp. Med. **170:**1671–1680.
39. **Voronova, A. F., J. E. Buss, T. Patchinsky, T. Hunter, and B. M. Sefton.** 1984. Characterization of the protein apparently responsible for the elevated tyrosine protein kinase activity in LSTRA cells. Mol. Cell. Biol. **4:**2705–2713.