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Palmitoylation of Either Cys-3 or Cys-5 Is Required for the Biological Activity of the Lck Tyrosine Protein Kinase

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Palmitoylation can regulate both the affinity for membranes and the biological activity of proteins. To study the importance of the palmitoylation of the Src-like tyrosine protein kinase p56^{lck} in the function of the protein, Cys-3, Cys-5, or both were mutated to serine, and the mutant proteins were expressed stably in fibroblasts and T cells. Both Cys-3 and Cys-5 were apparent sites of palmitoylation in Lck expressed in fibroblasts, as only the simultaneous mutation of both Cys-3 and Cys-5 caused a large reduction in the incorporation of [^{3}H]palmitic acid. The double mutant S3/5Lck was no longer membrane bound when examined by either immunofluorescence or cell fractionation. This indicated that palmitoylation was required for association of Lck with the plasma membrane. Since the S3/5Lck protein was myristoylated, myristoylation of Lck is not sufficient for membrane binding. When Cys-3, Cys-5, or both Cys-3 and Cys-5 were changed to serine in activated F505Lck, palmitoylation of either Cys-3 or Cys-5 was found to be necessary and sufficient for the transformation of fibroblasts and for the induction of spontaneous, antigen-independent interleukin-2 production in the T-helper cell line DO-11.10. Nonpalmitoylated F505Lck exhibited little activity in vivo, where it did not induce elevated levels of tyrosine phosphorylation, and in vitro, where it was unable to phosphorylate angiotensin in an in vitro kinase assay. These findings suggest that F505Lck must be anchored stably to membranes to become activated. Because palmitoylation is dynamic, it may be involved in regulating the cellular localization of p56^{lck}, and consequently its activity, by altering the proximity of p56^{lck} to its activators and/or targets.

Palmitoylation of proteins is a posttranslational modification that occurs at cellular membranes, predominantly on cysteine residues (35). Palmitate is linked to cysteine through a labile thioester bond, and the modification turns over with a half-life that is often much less than that of the polypeptide to which it is attached (35). Palmitoylation differs from myristoylation in that myristoylation is a cotranslational process in which myristic acid is linked to the amino-terminal glycine through a stable amide bond (35). Therefore, the dynamic, reversible nature of palmitoylation may allow regulation of the activity and membrane affinity of proteins. Palmitoylation of the β2-adrenergic receptor is required for coupling of this receptor to G proteins (23, 27). Palmitoylation of $G\alpha_s$, the G protein regulated by the β2-adrenergic receptor, increases in response to agonist stimulation of the β2-adrenergic receptor (7, 46) and may regulate association of $G\alpha_s$ with the plasma membrane. In contrast, removal of palmitate from bovine rhodopsin by treatment with hydroxylamine in vitro enhances coupling to G_t (25). Palmitate turnover on endothelial nitric oxide synthase increases upon stimulation of aortic endothelial cells with the agonist bradykinin, and the protein translocates to the cytoplasm (30). Palmitoylation of GAP-43, a neuronal growth cone protein, reversibly blocks the ability of GAP-43 to activate $G\alpha_O$ (41).

Like GAP-43 and $G\alpha_s$, a number of Src family protein tyrosine kinases, but not Src itself, are palmitoylated at one or two cysteine residues (position 3, 5, or 6) near their myristoylated N termini (28, 31, 37). This modification has been shown to affect membrane binding and the association of the proteins with glycosylphosphatidylinositol (GPI)-linked plasma membrane proteins and caveolae (31, 36, 37). p56^{lck} (21, 45) associates with the cytoplasmic tails of T-cell receptor-associated glycoproteins CD4 and CD8 in T cells (32, 44), membrane immunoglobulin in B cells (6), and GPI-anchored proteins, such as the decay-accelerating factor and placental alkaline phosphatase, through its unique amino terminus (37, 39). p56^{lck} is essential for both T-cell development (4, 24) and T-cell activation (15, 40). Recently, several groups have shown that Lck is palmitoylated at either or both cysteine 3 and cysteine 5 in transient protein expression systems by using either HeLa or COS cells (16, 28, 31). Palmitoylation is apparently required for association of Lck with GPI-linked proteins in caveolae, as mutation of cysteines 3 and 5 abolished the ability of Lck to associate with the Triton-insoluble fraction which contained GPI-linked proteins including the decay-accelerating factor (36, 37) and placental alkaline phosphatase (31).

As the importance of palmitoylation for the biological function of Lck has yet to be analyzed, we examined the effect of mutation of Cys-3 and Cys-5 on the ability of an activated form of Lck to transform fibroblasts and induce antigen-independent interleukin-2 (IL-2) expression in T cells. Mutation of Cys-3 or Cys-5 alone had no effect on the ability of activated Lck to transform fibroblasts or to stimulate IL-2 production in T cells. Thus, palmitoylation of either site alone is sufficient to allow biological activity. In contrast, simultaneous mutation of both Cys-3 and Cys-5 reduced palmitoylation severely and rendered the protein soluble and biologically inactive. The lack of biological activity of the double mutant appears to result from an inability of the protein to interact productively with activators and polypeptide substrates. The presence of palmitate at either Cys-3 or Cys-5 in Lck may therefore be essential for specific cellular localization of the protein.

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MATERIALS AND METHODS

Construction of Lck mutants. Site-directed mutagenesis was accomplished by using PCR (33) with murine Lck cloned in the vector pcDNA I/Amp (Invitrogen). S3Lck was made by changing the codon for Ser-3 from TGT to TCT with 5′-ATCATGGGCTCTGTCTGCAGC-3′ and 5′-GCTGCAGACAGAGCCCA TGAT-3′ as the internal primers in conjunction with two external primers: a downstream primer in *lck*, 5′-TCCCTGGTTCTGGTCGAAGT-3′, and an upstream primer in pcDNA I/Amp, 5′-CCACCTGGCAGTACATCAAG-3′. S5Lck was made by changing the codon for Ser-5 from TGC to TCC with 5′-GGCTGT GTCTCCAGCTCAAAC-3′ and 5′-GTTTGAGCTGGAGACACAGCC-3′ as the internal primers and the aforementioned external primers. For the double mutant S3/5Lck, internal primers 5′-ATCATGGGCTCTGTCTCCAGCTCAAAC-3′ and 5′-GTTTGAGCTGGAGACAGAGCCCATGAT-3′ and the external primers noted above were used. In each case, the two primary PCR products were isolated and mixed, and a second round of PCR was performed with the external primers to amplify the mutated region of *lck*. *Pfu*I DNA polymerase (Stratagene) was used in all PCRs. A DNA restriction fragment from this amplified product was used to replace the equivalent DNA fragment in wild-type *lck* in pcDNA I/Amp. All mutations were confirmed by sequencing, and no additional mutations were found. Wild-type and mutant cDNAs were then inserted into the retroviral expression vector LXSN (22).

A *Bam*HI restriction fragment containing the mutations to generate Phe-505 of Lck (previously described [5]) was used to replace the corresponding *Bam*HI fragments in S3Lck, S5Lck, and S3/5Lck. A2F505Lck was made from F505Lck by changing the codon for Gly-2 in Lck from GGC to GCA, with the oligonucleotide 5′-GATCATGGCATGCGTCTGC-3′ as a primer, by M13 mutagenesis (previously described [5]).

Cell lines. 208F, a rat fibroblast line, and COSm-6 (26), an African green monkey kidney cell line expressing the simian virus 40 T antigen, were maintained in Dulbecco-Vogt's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% calf serum (Gemini). Rat 208F fibroblasts expressing Lck were grown in DMEM containing 10% calf serum and 600 μg of G418 (Geneticin; Gibco/BRL) per ml. DO-11.10, a $CD4^+$ $CD8^-$ murine T-cell hybridoma (38), was grown in DMEM supplemented with 10% fetal bovine serum (Intergen), 5×10^{-5} M β-mercaptoethanol, 1× nonessential amino acids, and 2 mM sodium pyruvate (T-cell medium). NK, an IL-2- and IL-4-dependent cell line, was grown in the above-described medium supplemented with 5% conditioned medium from EL-4 cells (8).

Transfection and retroviral infection of cells. The LXSN constructs encoding S3Lck, S5Lck, S3/5Lck, F505Lck, S3F505Lck, S5F505Lck, S3/5F505Lck, and A2F505Lck were transfected individually with a viral helper plasmid, SV-ϕ$^-$-EMLV (18), into COSm-6 cells growing on 5-cm-diameter plates as previously described (42). At 24 h after transfection, 2.5×10^5 DO-11.10 cells were infected by cocultivation for 24 h with the transfected cells in 2.5 ml of T-cell medium containing 6 μg of Polybrene per ml. At 24 h postinfection, the infected DO-11.10 cells were diluted to 10^4 cells per ml, and 1-ml aliquots were seeded into 24 wells of a 24-well plate. After 24 h, G418 was added to a final concentration of 1.7 mg/ml.

For infection of fibroblasts, 1.75×10^5 208F fibroblasts were seeded onto a 5-cm-diameter tissue culture dish in medium containing 6 μg of Polybrene per ml. After 4 h, the medium was aspirated, and 200 μl of the viral supernatant collected 48 h after transfection of COSm-6 cells, supplemented with 6 μg of Polybrene per ml, was added. After incubation at 37°C for 45 min, 4 ml of medium containing 6 μg of Polybrene per ml was added. After 48 h, fresh medium containing 600 μg of G418 per ml was added. Individual colonies were picked by aspiration of the medium and the addition of 5 μl of trypsin to the colony of interest. After 1 min, the cells were transferred to a 35-mm-diameter tissue culture dish.

[^{3}H]palmitate labeling. [^{3}H]palmitic acid in ethanol (60 Ci/mmol; DuPont NEN) was dried under N_2 and solubilized in dimethyl sulfoxide to 1% of the final volume, and fetal bovine serum was added to 10% of the final volume. DMEM containing 1× nonessential amino acids and 2 mM sodium pyruvate was added to obtain a concentration of 1 mCi of [^{3}H]palmitate per ml. For labeling, 10^6 208F fibroblasts were seeded on a 35-mm-diameter tissue culture dish in 2 ml of medium. After 18 h at 37°C, cells were rinsed once with isotonic Tris-buffered saline (TBS) and incubated at 37°C for 4 h in 400 μl of medium containing 1 mCi of [^{3}H]palmitic acid per ml.

Immunoprecipitation and Western blotting (immunoblotting). For immunoprecipitation, the medium was aspirated from the dish, and the cells were rinsed once with cold TBS. Cells were lysed in RIPA buffer (10 mM sodium phosphate [pH 7.2], 150 mM sodium chloride, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] 100 kallikrein inhibitor units (KIU) of aprotinin per ml, 2 mM EDTA, 50 mM sodium fluoride, 200 μM sodium vanadate) for 20 min at 4°C at a concentration of 2×10^6 cells per ml. *Staphylococcus aureus* (30 μl; Pansorbin cells; Calbiochem) was added, and the lysates were clarified by centrifugation at $35,000 \times g$ for 45 min at 4°C. Lck was isolated from the clarified lysate with an Lck-specific polyclonal antiserum (12) prebound to *S. aureus*.

For [^{3}H]palmitate-labeled immunoprecipitates, immunoprecipitated Lck was resuspended in protein sample buffer containing 2% SDS, 5 mM sodium phosphate (pH 7.0), 10% glycerol, 0.01% bromophenol blue, and 100 mM dithiothreitol as the only reducing agent; boiled for 30 s; and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) in duplicate. The addition of β-mercaptoethanol or longer boiling times resulted in the loss of label from Lck (46a). Half of the gel was treated with Entensify (DuPont NEN) and dried, and labeled protein was visualized by autoradiography by using presensitized film and quantified by densitometry (LKB). The other half was transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) and subjected to Western blotting with anti-Lck antibodies and ^{125}I-protein A (ICN) as previously described (14, 43). Labeled proteins were visualized with a PhosphorImager (ImageQuant version 4.1 software; Molecular Dynamics).

Whole-cell lysates were prepared by adding reducing sample buffer directly to the plate of adherent cells to a final concentration of 4×10^6 cells per ml. The lysates were passed through an 18-gauge needle to shear the DNA, boiled for 5 min, spun at 13,200 rpm in a microcentrifuge (MicroMax; IEC) for 5 min, fractionated by SDS-PAGE, transferred to a PVDF membrane, and subjected to Western blotting with either anti-Lck or antiphosphotyrosine antibodies (14) and ^{125}I-protein A.

Cell fractionation. Rat 208F fibroblasts expressing wild-type or mutant Lck protein were seeded at a density of 2×10^6 cells per 10-cm-diameter dish. After 18 h, the cells were washed once with TBS, incubated with 2 ml of hypotonic lysis buffer (10 mM KCl, 1 mM EDTA, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4]) for 10 min at 4°C, scraped off the plate with a rubber policeman, and subjected to 20 strokes in a Dounce homogenizer with an A pestle. Disrupted cells were spun for 5 min at $1,000 \times g$ to pellet the nuclei and intact cells, which were discarded. The supernatant (2 ml) was collected and spun at 4°C for 30 min at $100,000 \times g$ in polyallomer centrifuge tubes (13 by 51 mm; Beckman) to pellet the membranes. The supernatant containing soluble proteins was removed and added to 0.5 ml of 5× reducing protein sample buffer. The pelleted membranes were resuspended in 0.5 ml of 5× reducing protein sample buffer and 2 ml of hypotonic lysis buffer. The samples were boiled for 5 min, proteins were analyzed by SDS-PAGE, and Lck was detected by Western blotting with antiserum against Lck followed by ^{125}I-protein A.

Immunofluorescence. Rat 208F fibroblasts expressing wild-type or mutant Lck protein were seeded on baked glass coverslips (25 by 25 mm) in 35-mm-diameter tissue culture dishes at a density of 2×10^5 cells per 35-mm-diameter dish in 2 ml of medium. After 18 h, the cells were washed once in phosphate-buffered saline (PBS) and fixed for 30 min with 3% paraformaldehyde in 1× PBS and 25 μM NaOH. The cells were then permeabilized for 10 min in rinse solution (10 mM glycine, 1% Triton X-100 in 1× PBS), incubated with antibodies against Lck (diluted 1:200 in FBS containing 1% Triton X-100) for 30 min, washed four times for 10 min in rinse solution, incubated with a goat anti-rabbit immunoglobulin G conjugated to fluorescein (Jackson Labs) (diluted 1:50 in fetal bovine serum containing 1% Triton X-100) for 30 min, and washed again. All steps were done at room temperature. Coverslips were mounted on slides with Vectashield mounting medium (Vector). Antibody staining was visualized and photographed with a fluorescence microscope.

In vitro kinase assays. For assays using [Val-5]angiotensin II (Star Biochemicals) as a substrate, Lck immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; ICN)–2 mM angiotensin in 20 μl of kinase buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 7.1], 10 mM $MnCl_2$} at room temperature. The reactions were stopped by the addition of 5% trichloroacetic acid, and the angiotensin was subsequently adsorbed onto Whatman p81 phosphocellulose paper. The paper was then washed with 0.5% phosphoric acid to remove unincorporated [γ-^{32}P]ATP. Phosphate incorporation into angiotensin was determined by scintillation counting. Lck kinase activity was normalized to the amount of Lck present in the immunoprecipitate by analyzing a fraction of the sample on an SDS-PAGE gel and Western blotting with anti-Lck antibodies as described above.

IL-2 production assays. All analyses were performed in duplicate. Infected DO-11.10 cells (10^5) were seeded in 200 μl of fresh T-cell medium in a 96-well microtiter plate and incubated for 24 h at 37°C in a CO_2 incubator. Serial dilutions of each T-cell supernatant were then incubated in duplicate in 100 μl with 5×10^3 NK cells that had last been exposed to IL-2 4 days previously and that had been washed three times to remove residual IL-2. After 24 h, 1 μCi of [^{3}H]thymidine was added. The cells were harvested after 16 h by using a PHD cell harvester (Cambridge Technology, Inc.), and [^{3}H]thymidine incorporation was quantified by scintillation counting. IL-2 activity (absolute units per milliliter) was determined by comparison with recombinant murine IL-2 (Biosource) standards.

RESULTS

Both Cys-3 and Cys-5 are palmitoylated in Lck. Mutation of Cys-3 and Cys-5 together reduce palmitoylation of Lck when the protein is overexpressed transiently in HeLa and COS cells (16, 31, 36). To examine the palmitoylation of these cysteines when the protein is expressed stably at normal levels, Lck proteins in which either or both cysteines were mutated to serine were expressed in 208F rat fibroblasts by using an LXSN-based retroviral vector. The level of Lck expressed in

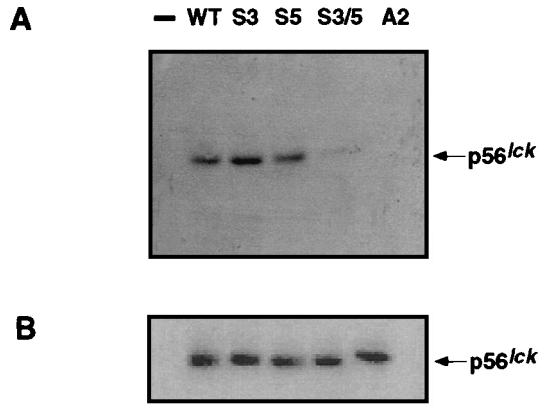

FIG. 1. Labeling of p56^{lck} mutants with [^{3}H]palmitic acid. Cells were metabolically labeled with [^{3}H]palmitic acid for 4 h. The cells were lysed, and Lck was isolated by immunoprecipitation and analyzed in duplicate on SDS-PAGE gels. (A) One gel was treated with Entensify, and the radioactive bands were detected with fluorography by using presensitized film. Autoradiograms were quantitated by densitometry. (B) The duplicate gel was transferred to PVDF and subjected to Western blotting with antibodies to Lck. ^{125}I-protein A binding to Lck was quantified on a PhosphorImager with ImageQuant software. WT, wild type.

these cells was similar to that in murine T cells (data not shown). Mutation of cysteine 3 to serine did not decrease labeling of Lck with palmitate (Fig. 1A). In fact, in all experiments it appeared to increase palmitate labeling. In contrast, mutation of cysteine 5 reduced labeling by 15 to 30%. Mutation of both cysteines to serine, however, reduced palmitate labeling by 85%. These results are similar to those seen in transiently transfected COS cells by Koegl et al. (16), except that they observed no increase in palmitoylation of S3Lck. Experiments using transient transfection of HeLa cells have given mixed results. Shenoy-Scaria et al. (36) detected palmitoylation of Cys-3 only, whereas Rodgers et al. (31) observed palmitoylation of Cys-5 but not of Cys-3. As previously reported (31), we detected no incorporation of [^{3}H]palmitate into nonmyristoylated A2Lck (data not shown). This suggests that none of the label was incorporated into Lck as a result of the metabolism of [^{3}H]palmitate to amino acids. No detectable [^{3}H]palmitate remained after treatment of immunoprecipitated Lck with NH_4OH (data not shown). This result is indicative of a thioester linkage between the [^{3}H]palmitate and Lck.

Palmitoylation of Lck is required for membrane association. The effect of mutations of Cys-3 and Cys-5 on the intracellular location of Lck was examined by indirect immunofluorescence (Fig. 2) and cellular fractionation (Fig. 3) with anti-Lck antibodies. Obvious plasma membrane staining of Lck was seen in permeabilized 208F fibroblasts expressing S3Lck, or S5Lck, or wild-type Lck (Fig. 2). Staining of Lck at the plasma membrane was actually more obvious with S5Lck than with the wild type. Additionally, perinuclear staining of Lck was less prevalent with S5Lck than with either wild-type Lck and S3Lck. This may suggest that Cys-5, or perhaps palmitate at Cys-5, provides a signal for localization of Lck to the perinuclear region. If so, mutation of Cys-5 might result in relocation of this perinuclear fraction of Lck to the plasma membrane and lead to more prominent staining of Lck at the plasma membrane. Lck staining at the membrane was slightly less striking in the case of S3Lck than with the wild type. Under conditions where 100% of wild-type Lck was found in the particulate fraction, 10% of S5Lck and 50% of S3Lck were recovered in the soluble fraction (Fig. 3). This may indicate that a lack of palmitate at Ser-5 reduces membrane binding in vivo slightly and that a lack of palmitate at Ser 3 reduces it markedly. Alternatively, S3Lck and S5Lck may associate stably with cellular membranes in vivo, but do so more tenuously than does wild-type Lck, and dissociate during the swelling and lysis of the cells. We favor the latter explanation because the subcellular localization of S3Lck and S5Lck detected by immunofluorescence differed only slightly from that of wild-type Lck in immunofluorescent studies.

Both immunofluorescence and cell fractionation showed that neither S3/5Lck nor A2Lck was associated with membranes. Only amorphous immunofluorescent staining was apparent with both S3/5Lck and A2Lck. Although both S3/5Lck and A2Lck showed clear staining of the cytoplasm, S3/5Lck appeared to be excluded from the nucleus, whereas A2Lck was not (Fig. 3). Distinctive perinuclear staining of an unknown nature was seen with wild-type Lck and S3Lck. This perinuclear staining was seen only occasionally with S5Lck.

Palmitoylation of either Cys-3 or Cys-5 is necessary for transformation of fibroblasts with activated Lck. Mutation of tyrosine 505 in Lck to phenylalanine prevents inhibitory phosphorylation of Lck and creates a constitutively activated form that will transform fibroblasts (5, 20), enhance T-cell responsiveness to antigen (1), and induce antigen-independent IL-2 production in T-helper cell hybridomas (19). We therefore asked whether mutations of Cys-3, Cys-5, or both affected the transforming ability of the activated form of Lck. 208F rat fibroblasts infected with retroviruses expressing the mutant Lck genes were selected in G418, and the morphology of drug-resistant colonies was examined microscopically. Both S3F505Lck and S5F505Lck induced morphological transformation in a manner indistinguishable from that of F505Lck (Fig. 4). Approximately 75% of all G418-resistant colonies of cells infected with retroviruses expressing F505Lck, S3F505Lck, and S5F505Lck had a rounded morphology typical of transformed cells and expressed Lck, as confirmed by Western blotting of cellular lysates with anti-Lck antibodies (data not shown). The flat, G418-resistant colonies in each case did not express Lck (data not shown). In contrast, all 98 G418-resistant colonies arising from infection with virus encoding S3/5F505Lck exhibited a morphology undistinguishable from that of uninfected cells. Three of four G418-resistant colonies examined expressed S3/5F505Lck. Therefore, palmitoylation of either cysteine 3 or cysteine 5 is necessary for activated Lck to transform fibroblasts.

Palmitoylation of either Cys-3 or Cys-5 is required for induction of spontaneous IL-2 production by activated Lck in T cells. To examine the effect of mutation of Cys-3, Cys-5, or both on the ability of activated Lck (F505Lck) to induce spontaneous IL-2 production, mouse DO-11.10 T-helper hybridoma cells were infected with retroviruses encoding F505Lck, S3F505Lck, S5F505Lck, S3/5F505Lck, or A2F505Lck. Infected cells were selected with G418 and seeded in 24-well plates. After 10 days, all wells contained G418-resistant cells, and 12 pools from each infection were screened for spontaneous, antigen-independent, production of IL-2. S3F505Lck and S5F505 Lck both induced antigen-independent IL-2 production to an extent undistinguishable from that of F505Lck (Fig. 5). Cells infected with either S3/5F505Lck or A2F505Lck produced essentially no IL-2.

S3/5F505Lck does not induce an increase in tyrosine-phosphorylated proteins in fibroblasts. To examine why S3/5F505Lck was not functional in vivo, lysates from 208F cell clones expressing the mutant proteins stably were analyzed for tyrosine phosphorylation of cellular proteins by Western blotting with antiphosphotyrosine antibodies. The amount of cell lysate analyzed here was adjusted so that equal amounts of

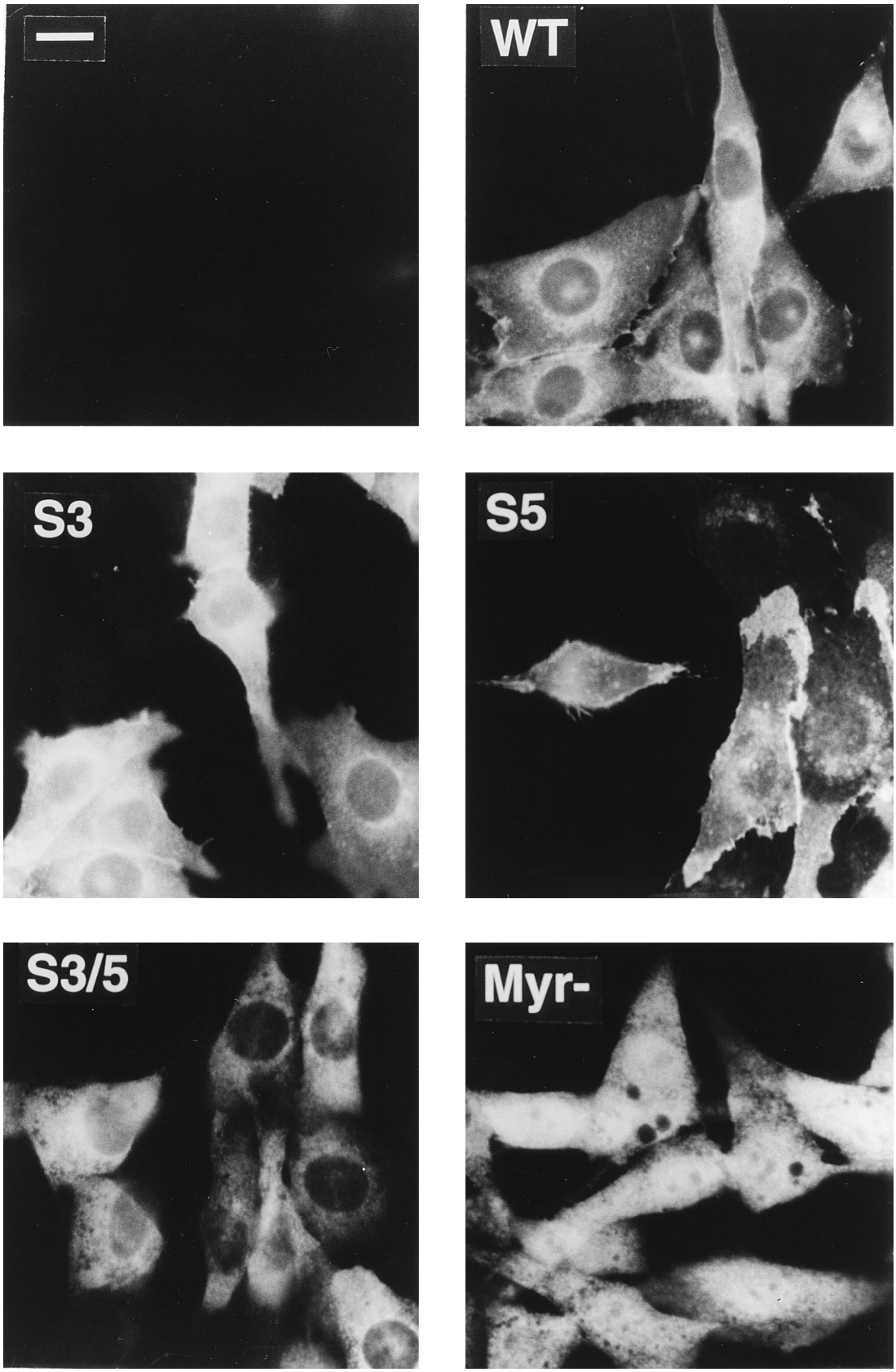

FIG. 2. Detection of Lck in 208F cells expressing wild-type (WT) Lck, S3Lck, S5Lck, S3/5Lck, and A2Lck (Myr−) by immunofluorescence. Cells were fixed, permeabilized, and incubated first with rabbit antiserum to p56^{lck} and then with a fluorescein isothiocyanate goat anti-rabbit secondary antibody. Photographs were taken with a 63× objective lens and Kodak Tri-X Pan 400 black-and-white print film.

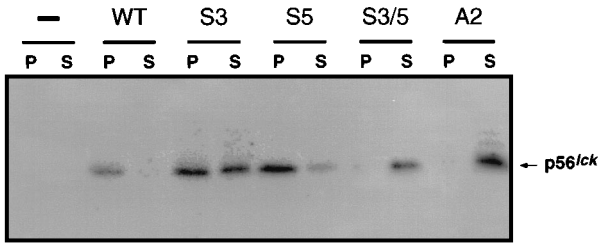

FIG. 3. Fractionation of 208F cells stably expressing wild-type (WT) Lck, S3Lck, S5Lck, S3/5Lck, and A2Lck. Unlabeled cells were subjected to hypotonic lysis, Dounce homogenization, and differential centrifugation as described in the text. The resulting P100 and S100 fractions were dissolved in SDS sample buffer and analyzed by SDS-PAGE. The gel was transferred to PVDF and subjected to Western blotting with antibodies to Lck and ^{125}I-protein A. Radioactive bands were detected with a PhosphorImager and quantified by using ImageQuant software. P, P100, or the membrane fraction; S, S100, or the cytosolic or soluble fraction.

Lck protein were present in each lane. Like F505Lck, both S3F505Lck and S5F505Lck induced an increase in tyrosine phosphorylation of at least six cellular proteins, and the pattern of phosphorylated proteins was identical to that seen with F505Lck itself (Fig. 6). In contrast, neither S3/5F505Lck nor A2F505Lck stimulated tyrosine phosphorylation of cellular proteins. Apparently, F505Lck must be membrane associated to be active.

S3/5F505Lck exhibits little activity in vitro. To examine whether the lack of cellular protein phosphorylation by S3/5F505Lck in fibroblasts was due to the lack of tyrosine protein kinase activity of the protein, we isolated the mutant protein by immunoprecipitation and compared its in vitro protein kinase activity with that of wild-type Lck. Autophosphorylation (data not shown) and the ability to phosphorylate two exogenous substrates, enolase (data not shown) and angiotensin (Fig. 7) were determined. The kinase activities of the proteins tested in the assay were normalized to correct for differences in the amounts of Lck in the immunoprecipitates used in the kinase assays by Western blotting with anti-Lck antibodies. S3/5F505Lck was found to have undetectable activity in vitro (Fig. 7). The activity of S5F505Lck was indistinguishable from that of F505Lck (Fig. 7). The kinase activity of S3F505Lck was reproducibly greater than that of F505Lck (Fig. 7). We do not yet understand the reasons for these variations.

DISCUSSION

Both Cys-3 and Cys-5 are apparent sites of palmitoylation of Lck in fibroblasts expressing the proteins stably because simultaneous mutation of both Cys-3 and Cys-5 was required to reduce Lck palmitoylation substantially. Evidence for the palmitoylation of Cys-3 comes only from the properties of the double mutant, as mutation of Cys-3 alone did not detectably reduce total labeling of p56^{lck} with palmitate. We observed an increase in the total amount of palmitate incorporated in S3Lck and only a 15 to 30% decrease in the labeling of S5Lck with palmitate. The limited effect of the single mutations could reflect compensatory palmitoylation of Cys-3 or Cys-5, or of other cysteines, as a result of a conformational change in the protein. It may be that there is normally, on average, only one molecule of palmitate attached to Lck, with that palmitate being present at either Cys-3 or Cys-5. Mutation of either of these cysteines could result in increased palmitoylation of the remaining amino-terminal cysteine. This increase could explain the unexpectedly small effect of mutation of Cys-3 or Cys-5 singly on palmitate incorporation. However, the observed increase in palmitoylation of S3Lck cannot be explained by this model. Mutation of Cys-3 may result in palmitoylation of Cys-5 and other cysteines in Lck, possibly cysteine 20 or cysteine 23. In contrast to our data, Rodgers et al. (31) detected a 75% reduction in palmitate labeling with S5Lck and observed no difference in palmitate labeling between S5Lck and S3/5Lck in HeLa cells transiently expressing high levels of the mutant proteins. Koegl et al. (16) found both Cys-3 and Cys-5 of Lck to be palmitoylated when the protein was expressed transiently in COS cells, although they saw no increase in palmitoylation of S3Lck as a result of the introduced mutation.

Despite the unreduced labeling with palmitate, 50% of S3Lck was recovered in the cytosolic or soluble fraction during cell fractionation under conditions with which 100% of the wild-type protein was in the particulate fraction. This recovery rate may result from the reduced ability of S3Lck to associate with GPI-linked proteins in caveolae (31, 36). Although this possibility indicates that the membrane affinity of S3Lck is reduced, it does not necessarily indicate that the binding to membranes is reduced significantly in vivo. Localization by immunofluorescence showed that the intracellular distribution of S3Lck was only slightly different from that of wild-type Lck and very different from that of S3/5Lck. It may well be that the majority of S3Lck is attached to membranes in vivo but that the lack of palmitoylation at Cys-3 prevents the tight association of S3Lck with GPI-linked proteins at the plasma membrane and causes the mutant protein to be lost partially during cell fractionation.

The undiminished biological activities of S3F505Lck and S5F505Lck show that palmitoylation of either cysteine is sufficient to allow full biological activity. However, simultaneous mutation of both Cys-3 and Cys-5 abolished membrane binding and biological activity. S3/5Lck has been shown previously to have lost its ability to associate with GPI-linked proteins that are concentrated in caveolae (31, 36). Our data, and those of Kwong and Lublin (17), suggest that S3/5Lck has lost the ability to associate with any membrane constituents, not just those found in the caveolae. The effect of mutation of both Cys-3 and Cys-5 on membrane binding of Lck was much more pronounced than was the effect of mutation of both Cys-3 and Cys-6 in Fyn (3). Although the Cys-3/6 Fyn mutant was not palmitoylated, it exhibited only a 54% reduction in membrane binding as assayed by cell fractionation (3). Because palmitoylation of Lck is required for plasma membrane association, and all wild-type Lck is normally membrane bound, as indicated by cell fractionation, essentially all Lck protein in the fibroblasts appears to be palmitoylated at at least one site.

An interesting difference was observed in the properties of S3/5Lck, which is myristoylated but not palmitoylated, and A2Lck, which is neither myristoylated nor palmitoylated. A2Lck and S3/5Lck did not exhibit the same intracellular locations. Both proteins were present in the cytosol and absent from the plasma membrane, but S3/5Lck was excluded from the nucleus, when examined by immunofluorescence, whereas A2Lck was not. The myristoyl group on S3/5Lck may prevent the protein from diffusing through the nuclear pores.

Like A2F505Lck (2), S3/5F505Lck does not phosphorylate substrates on tyrosine efficiently in vivo (Fig. 6). These cytoplasmic forms of Lck are different from the nonmyristoylated, cytoplasmic forms of v-*src* (13) and activated c-*src* (29), which are active in vivo and in vitro but are unable to transform fibroblasts. The lack of substrate phosphorylation in vivo by S3/5F505Lck appears to be due to the inability of the nonmembrane-bound F505Lck to become activated. In contrast to that which is seen with the membrane-bound F505Lck, which

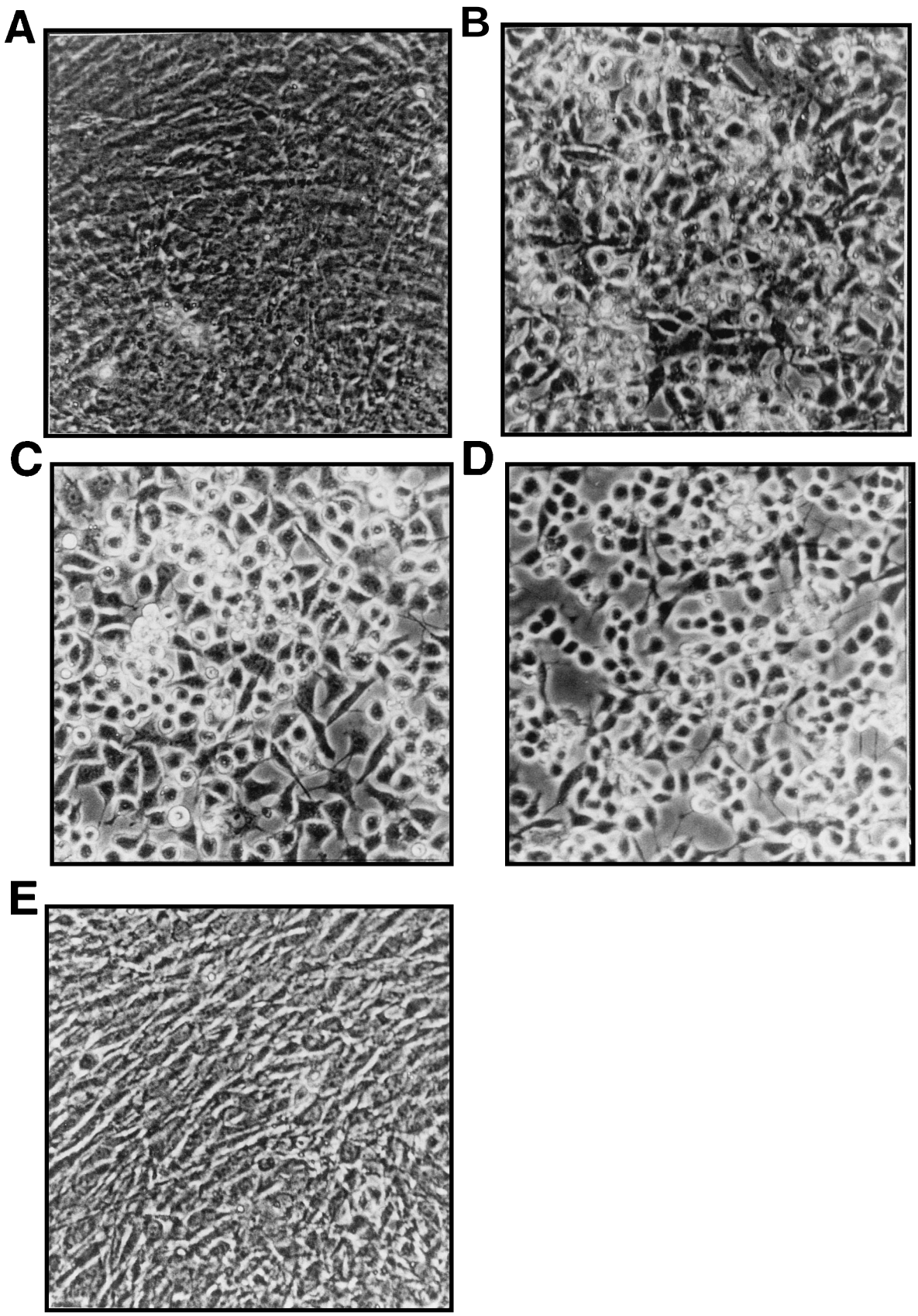

FIG. 4. Morphology of 208F fibroblasts that were uninfected (A) or infected with F505Lck (B), S3F505Lck (C), S5F505Lck (D), or S3/5F505Lck (E). Cells were infected with retroviruses expressing p56^{lck} mutants. G418-resistant colonies expressing Lck were isolated. For photography, 4×10^6 cells were seeded per 5-cm-diameter dish and photographed 18 h later with Kodak Plus-X Pan 125 black-and-white print film.

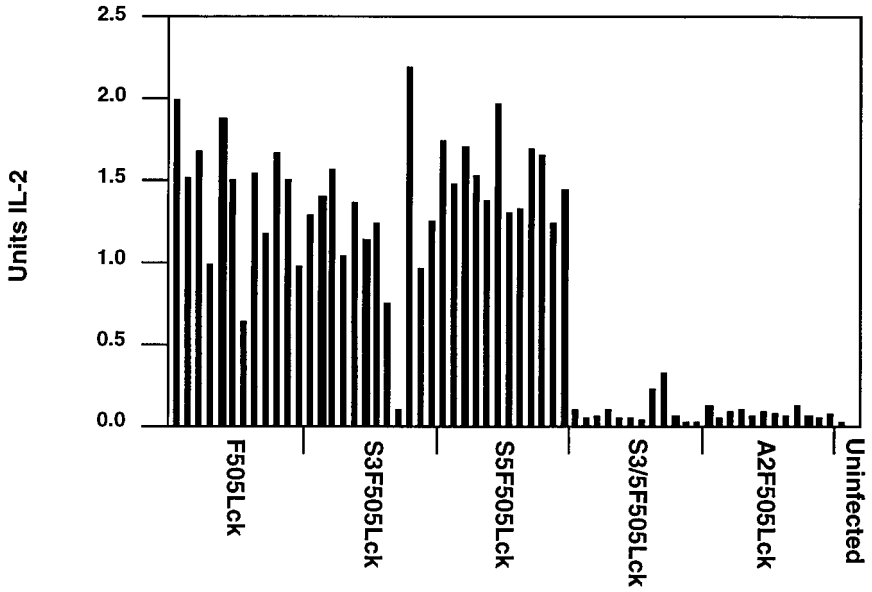

FIG. 5. Antigen-independent production of IL-2 in DO-11.10 cells infected with F505Lck, S3F505Lck, S5F505Lck, S3/5F505Lck, and A2F505Lck. DO-11.10 cells were infected with retroviruses encoding p56^{lck} mutants and selected in G418. Twelve G418-resistant pools for each mutant were screened for spontaneous IL-2 production. IL-2 in the culture supernatants was determined by its ability to stimulate the incorporation of [^{3}H]thymidine by IL-2- or IL-4-dependent NK cells. The activity of IL-2 (in absolute units of IL-2 per milliliter of culture) was determined by comparison with recombinant murine IL-2 standards. Each bar represents a single pool.

is phosphorylated heavily at tyrosine 394 (5, 20) and is active as a protein tyrosine kinase in vitro, phosphorylation of S3/5F505Lck at tyrosine 394 is barely detectable (46a), and the protein shows little activity in in vitro kinase assays. Phosphorylation of Lck at tyrosine 394 can activate the protein (11) and may be carried out by an as-yet-unidentified protein kinase, rather than occurring by autophosphorylation (11). The phosphorylation of Lck at tyrosine 394, and hence activation of Lck, may normally occur only at cell membranes. This process is apparently different from that which occurs with the cytoplasmic forms of both v-*src* and activated c-*src*, which are fully active.

To exclude the trivial possibility that our S3/5F505Lck carried a nonfunctional catalytic domain, disabling the kinase activity of the protein, cells expressing S3/5F505Lck were treated with hydrogen peroxide (H_2O_2), a potent activator of Lck activity (11). S3/5F505Lck isolated from cells treated with H_2O_2 was found to be phosphorylated at tyrosine 394 and able to phosphorylate angiotensin in an in vitro kinase assay at a rate similar to that of F505Lck (11a). Thus, there is nothing intrinsically wrong with the S3/5F505Lck protein characterized here; it just exhibits dramatically reduced activity when expressed in cells.

Cys-3, and presumably palmitoylation of Cys-3, appears to be necessary for the localization of Lck to the caveolae and/or Triton X-100-insoluble fraction of cells (31, 36). The importance of this association in the function of Lck is unclear since T cells are thought to lack caveolae and caveolin (9). Interestingly, a recent study by Gorodinsky and Harris (10) demonstrates that Triton-insoluble complexes containing all major constituents of caveolae except caveolin can form in cells lacking both caveolae and caveolin. These detergent-resistant complexes could correspond to microdomains of the plasma membrane of T cells, containing both GPI-linked proteins and kinases. Localization of Lck to either the caveolae or detergent-resistant complexes does not, however, appear to be required for transformation of fibroblasts by F505Lck, as mutation of Cys-3 to serine has no effect on the transforming ability of F505Lck. Additionally, localization to caveolae or detergent-resistant complexes is apparently not essential for the propagation of signals that induce transcription of the IL-2 gene in T cells because S3F505Lck induces antigen-independent production of IL-2 at an undiminished rate in T cells. Therefore, the functional importance of the interaction of Lck with caveolae or any potentially existing detergent-resistant

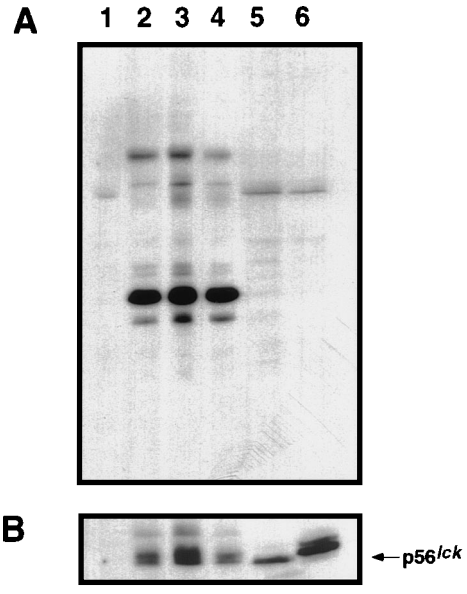

FIG. 6. Tyrosine phosphorylation of cellular proteins in 208F cells expressing F505Lck, S3F505Lck, S5F505Lck, and S3/5F505Lck (lanes 2 to 5, respectively). Cells expressing p56^{lck} mutant proteins were lysed directly in sample buffer, resolved by SDS-PAGE, transferred to PVDF, and subjected to Western blotting with either antiphosphotyrosine (A) or anti-Lck (B) antibodies and ^{125}I-protein A. Radioactive bands were visualized with a PhosphorImager.

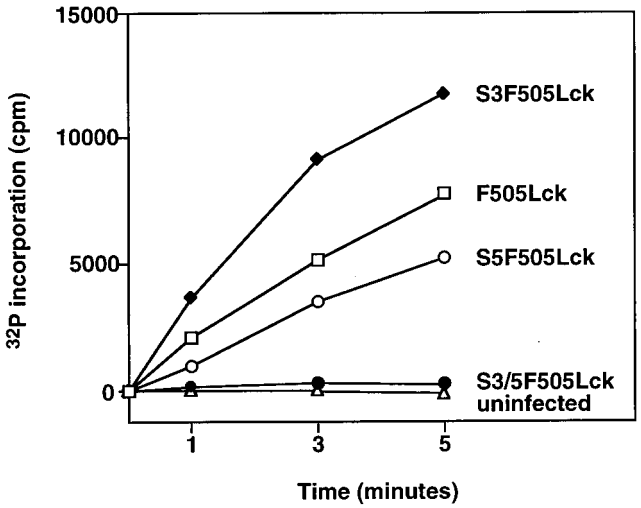

FIG. 7. Protein kinase activities of F505Lck mutants in vitro. Cells expressing F505Lck, S3F505Lck, S5F505Lck, and S3/5F505Lck were lysed, and Lck was isolated by immunoprecipitation. Immunoprecipitates were incubated with angiotensin in the presence of [γ-^{32}P]ATP in vitro for the indicated times, and [γ-^{32}P]ATP incorporation into angiotensin was determined by scintillation counting. Protein levels were normalized by Western blotting of a fraction of the immunoprecipitates with antibodies to Lck and ^{125}I-protein A.

microdomains in T cells remains unclear. Reversible palmitoylation of Lck could play a role in regulating the cellular localization of p56^{lck} and thus the accessibility of p56^{lck} to its intracellular substrates and/or activators. It will be interesting to see whether T-cell activation or exposure of T cells to cytokines modulates the turnover rate of palmitate on Lck.

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