MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 4996–5003 Vol. 16, No. 9
0270-7306/96/$04.00+0

Mutational Analysis of Lck in CD45-Negative T Cells: Dominant Role of Tyrosine 394 Phosphorylation in Kinase Activity

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Received 29 November 1995/Returned for modification 30 January 1996/Accepted 18 June 1996

The CD45 tyrosine phosphatase has been reported to activate the *src* family tyrosine kinases Lck and Fyn by dephosphorylating regulatory COOH-terminal tyrosine residues 505 and 528, respectively. However, recent studies with CD45$^-$ T-cell lines have found that despite the fact that Lck and Fyn were constitutively hyperphosphorylated, the tyrosine kinase activity of both enzymes was actually increased. In the present study, phosphoamino acid analysis revealed that the increased phosphorylation of Lck in CD45$^-$ YAC-1 T cells was restricted to tyrosine residues. To understand the relationship between tyrosine phosphorylation and Lck kinase activity, CD45$^-$ YAC-1 cells were transfected with forms of Lck in which tyrosines whose phosphorylation is thought to regulate enzyme activity (Tyr-192, Tyr-394, Tyr-505, or both Tyr-394 and Tyr-505) were replaced with phenylalanine. While the Y-to-F mutation at position 192 (192-Y→F) had little effect, the 505-Y→F mutation increased enzymatic activity. In contrast, the 394-Y→F mutation decreased the kinase activity to very low levels, an effect that the double mutation, 394-Y→F and 505Y→F, could not reverse. Phosphopeptide analysis of tryptic digests of Lck from CD45$^-$ YAC-1 cells revealed that it is hyperphosphorylated on two tyrosine residues, Tyr-505 and, to a lesser extent, Tyr-394. The purified and enzymatically active intracellular portion of CD45 dephosphorylated Lck Tyr-394 in vitro. These results demonstrate that in addition to Tyr-505, CD45 can dephosphorylate Tyr-394, and that in the absence of CD45 the hyperphosphorylation of Tyr-394 can cause an increase in the kinase activity of Lck despite the inhibitory hyperphosphorylation of Tyr-505. Therefore, Lck kinase activity is determined by the balance of activating and inhibitory tyrosine phosphorylations that are, in turn, regulated by CD45.

Protein tyrosine phosphorylation is a critical step in the biochemical cascade associated with T-cell activation. Activation via the T-cell antigen receptor (TCR) is rapidly followed by tyrosine phosphorylation of many substrates, and in fact this is the earliest cellular response observed (20). Inhibitors of protein tyrosine kinases (PTKs) block the TCR-induced biochemical signals that lead to T-cell activation (21). None of the chains of the TCR has intrinsic tyrosine kinase activity, suggesting that TCR engagement may recruit a membrane-associated PTK into a complex with the receptor. Different PTKs are known to be involved in T-cell activation; among these are two members of the *src* kinase family: Fyn and Lck. Fyn is constitutively associated by its N-terminal domain to the ζ chain of the TCR (39), and its activity increases with TCR stimulation (43). While overexpression of Fyn can enhance T-cell proliferation (10), its presence is not absolutely required, as Fyn knockout mice have only partial signaling defects in thymocytes and peripheral T cells (3, 41). Lck is noncovalently associated by its N-terminal domain to the intracytoplasmic tail of CD4 and CD8 (44, 46), whose binding to monomorphic regions of class II and class I molecules of the major histocompatibility complex, respectively, enhances physiological T-cell activation (16, 54). Lck kinase activity is increased by TCR engagement (12), and a mutated and hyperactive form of this kinase is able to enhance T-cell responsiveness (1). Another important observation is that T-cell lines lacking Lck have a profound defect in TCR-mediated signal transduction that is reversed by the reexpression of Lck (22, 42). Finally, Lck knockout mice have a block of thymocyte maturation resulting in fewer $CD4^+$ $CD8^+$ thymocytes and a drastic reduction in the number of mature T cells (28).

src family kinases are regulated by the phosphorylation of specific tyrosine residues. For Lck, phosphorylation of Tyr-505 inhibits, while phosphorylation of Tyr-394 (the autophosphorylation site) augments, kinase activity (2, 26, 48). Recently, in vitro phosphorylation of Tyr-192, which resides in the SH2 domain, has also been shown to increase Lck activity (11). The tyrosine phosphorylation of Lck depends upon the activity of both PTKs and protein tyrosine phosphatases (PTPs). CD45 is the major transmembrane PTP in T cells and plays a crucial role in regulating T-cell activation, as demonstrated by the profound alteration of TCR signaling that occurs in its absence, including loss of phosphoinositide hydrolysis, diminished increases in intracellular Ca^{2+} with delayed and asynchronous Ca^{2+} transients, and lack of lymphokine production or proliferation (24, 25, 50, 52). Since the enzymatic activity of *src* family PTKs is regulated, both positively and negatively, by phosphorylation of specific tyrosine residues, a role for PTPs in the control of these enzymes is generally accepted. In this regard, several studies have suggested that CD45 can dephosphorylate the negative regulatory COOH-terminal tyrosine residues of Fyn and Lck and therefore activate these PTKs (30–32, 36). Other data indicate a more complex role for CD45 in its relationship with *src* family PTKs. For example, while antibody-mediated cross-linking of CD4 increases both autophosphorylation and kinase activity of associated Lck (47), these activities are reduced by a concomitant clustering of CD4 with CD45, suggesting that this phosphatase can actually inhibit Lck activity (37). Furthermore, recent work from our

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laboratory in which the relationship between CD45 and the activity of *src* family PTKs in three different CD45$^-$ T-cell lines (YAC-1, SARKTLS, and HPB-ALL) was studied found that in the absence of this phosphatase, Fyn and Lck are hyperphosphorylated and, surprisingly, hyperactive (8). In the present study we address the mechanism by which CD45 regulates Lck activity and the regulatory tyrosine residues involved in this process.

MATERIALS AND METHODS

Cells. The YAC-1 T-lymphoma cell line and its CD45$^-$ variant N1 have been previously described (49). GA2.1 and CS269.2 cells were derived from N1 cells by transfection with cDNA encoding myr-iCD45 containing a Gly-2→Ala or a Cys-269→Ser substitution, respectively (34).

Antibodies and reagents. The monoclonal antibody 9E10 (15) recognizes the p60^{myc} epitope EQKLISEEDL. Rabbit anti-Lck antiserum was a gift of L. Samelson (NIH). The monoclonal antibody LA074 (Quality Biotech, Camden, N.J.) recognizes amino acids 2 to 17 of Src. ^{125}I-protein A was purchased from ICN (Costa Mesa, Calif.), and streptavidin-horseradish peroxidase (HRP) and an enhanced chemiluminescence (ECL) detection system were purchased from Amersham (Arlington Heights, Ill.).

Phosphoamino acid analysis and tryptic phosphopeptide mapping. After being washed in phosphate-free RPMI 1640, cells were resuspended at 10^7 cells per ml in phosphate-free RPMI 1640 with 10% dialyzed fetal calf serum and incubated for 1 h at 37°C. At that time, 10 mCi of ^{32}Pi (specific activity, 285 Ci/mg; ICN) were added to each cell type, and the incubation was continued for an additional 4 h. The cells were washed in cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, leupeptin, aprotinin, and phosphatase inhibitors (2 mM sodium orthovanadate, 0.4 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate). The lysates were immunoprecipitated with antiserum-coated protein A-Sepharose beads. Proteins were eluted from the beads in reducing sample buffer (50 mM Tris [pH 6.9], 1% sodium dodecyl sulfate [SDS], 10% glycerol, 3% 2-mercaptoethanol, bromophenol blue), resolved by SDS–8% polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose filter. Phosphoamino acid analysis and tryptic phosphopeptide mapping were performed as previously described (7). Briefly, the Lck protein band was identified by autoradiography, excised, and digested overnight at 37°C with 100 μg of trypsin-TPCK [L-(tosylamido-2-phenyl)ethyl chloromethyl ketone)] (Worthington, Freehold, N.J.) per ml in 50 mM ammonium bicarbonate (pH 8.0), and the digested material was dried in a SpeedVac concentrator (Savant, Farmingdale, N.Y.). For phosphoamino acid analysis, the trypsin-digested material was further treated with 6 N HCl for 1 h at 110°C. After being dried, the sample was resuspended in pH 1.9 buffer with cold phosphoamino acid standards, equal amounts of radioactivity were spotted on a thin-layer chromatography (TLC) plate, and the material was resolved by two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension. For phosphopeptide mapping, all of the trypsin-digested material was resuspended in pH 8.9 buffer, spotted on TLC plates, and separated by electrophoresis at pH 8.9 in the first dimension and by ascending chromatography (*n*-butanol, acetic acid, pyridine, water [112.5:22.5:75:90, vol/vol]) in the second dimension. The peptides containing Tyr-505 and Tyr-394 were identified by their comigration with nonradiolabeled synthetic peptides corresponding to the fragments produced by trypsin digestion in which phosphate was covalently bound to the tyrosine residue. Phosphotyrosine-containing peptides were prepared by Fmoc chemistry by using Fmoc-Tyr(PO3H2) on an Applied Biosystems 430A peptide synthesizer. Deprotection and cleavage were performed with reagent K (trifluoroacetic acid, H_2O, phenol, thioanisole, ethanedithiol [82.5:5:5:5:2.5]). The crude peptides were purified by reverse-phase high-performance liquid chromatography on a Vydac C-8 column with 0.05% TFA-water acetonitrile and verified by mass spectroscopy (Finnigan SSQQ 7000). Phosphotyrosine-containing peptides in experimental samples were identified by adding control peptides corresponding to the trypsin fragment of Lck containing Tyr-192, Tyr-394, and Tyr-505 to the trypsin digests of radiolabeled material during the phosphopeptide mapping analysis. The synthetic phosphopeptides were identified by ninhydrin staining.

Construction of chimeric p57$^{lck\text{-}myc}$ and site-directed mutagenesis. pBluescript KS+/− (Stratagene, La Jolla, Calif.) containing the cDNA of murine p56^{lck} (pBS-lck) was used as the template in PCR to obtain a chimeric fragment encoding the last portion of Lck from the *Pfl*mI site to the 3′ end followed by the Myc-tag-containing sequence (amino acids in the one-letter code: SMEQKLI SEEDLN), a stop codon, and an *Eco*RI site. The *Pfl*mI-*Eco*RI chimeric PCR fragment was used to replace the *Pfl*mI-*Eco*RI fragment in pBS-lck, yielding pBS-lck-myc, containing the coding sequence for the entire Lck molecule plus a COOH-terminal Myc-tag epitope. Site-directed mutagenesis was performed with the Transformer site-directed mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, Calif.). This method works by simultaneously annealing two oligonucleotide primers to one strand of denatured double-stranded plasmid. One primer introduces the desired mutation (mutation primer), and the second mutates a restriction site unique to the plasmid (selection primer). The sequences of our mutation primers (5′→3′) were as follows: Tyr-192→Phe, AGGGGAGATGA AGAAGCCACC; Tyr-394→Phe, CCGGGCCGTGAACTCATTGTC; and Tyr5-05→Phe, CTGGGGCTGGAACTGGCCCTC. The sequence produced by PCR and the sequences surrounding the sites of mutation (100 bp) were checked with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio) and ^{35}S-dATP (Amersham). Mutated *lck-myc* fragments were excised from the pBluescript vector by *Hin*dIII-*Eco*RI digestion and cloned into the *Hin*dIII-*Eco*RI sites of the pTEJ-8 expression vector (19).

Transfection and screening of stable transfectants. Stable transfectants were obtained by electroporation of cells with 10 μg of plasmid DNA per 15×10^6 cells as previously described (49). Twenty-four hours later the cells were divided into two 96-well plates in medium consisting of RPMI 1640 plus 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, and antibiotics (complete medium) supplemented with G418 (Gibco, Grand Island, N.Y.). All G418-resistant clones were screened for protein expression by immunoprecipitation and immunoblotting with the anti-Myc monoclonal antibody 9E10. Clones expressing comparable amounts of the transfected gene product were chosen for further study.

Immunoprecipitation and immunoblotting. Cells were washed three times in cold PBS and lysed in lysis buffer. Immunoprecipitation was performed on postnuclear fractions for 3 h at 4°C with protein A-Sepharose beads (Pierce, Rockford, Ill.) precoated with the indicated antibodies. The precipitated proteins or total cell lysates were separated on reducing SDS–8% polyacrylamide gels, transferred to a nitrocellulose filter, and immunoblotted with the antibodies indicated below. The anti-Lck immunoblots were developed by ^{125}I-protein A and autoradiography. Immunoblots with biotinylated 9E10 were developed with streptavidin-HRP and an ECL detection system.

In vitro tyrosine kinase assay. PTK activity was assayed by using the tyrosine kinase (Src family) assay kit (UBI, Lake Placid, N.Y.) based on the phosphorylation of the peptide KVEKIGEGTYGVVKK from cdc2(6-20), as previously described (9). Briefly, washed cells were lysed in lysis buffer and postnuclear fractions were immunoprecipitated with the indicated antibodies. After extensive washing, the immunoprecipitate (10 μl) was incubated with a reaction mixture (21 μl) (125 mM Tris [pH 7], 62 mM MgCl, 12 mM MnCl, 0.5 mM Na_3VO_4) containing the cdc2(6-20) peptide substrate (0.75 mM) and 10 μCi of [γ-^{32}P]ATP (specific activity, 4,500 to 6,000 Ci/mmol; ICN) at 30°C for 10 min. Blank values were obtained for each sample in the absence of substrate. Reactions were stopped by adding 10 μl of 50% acetic acid, the samples were centrifuged in an Eppendorf (Hamburg, Germany) microcentrifuge for 30 s, and 25 μl of supernatant from each sample was spotted on a SpinZyme basic separation unit (Pierce). After two washes with 75 mM phosphoric acid, bound radioactivity was counted with a β scintillation counter. Kinase activity was calculated according to the manufacturer's instructions as follows:

$$\text{Enzyme activity} = \frac{(\text{cpm of sample} - \text{cpm of blank}) \times 41/25}{\text{specific activity of [}\gamma\text{-}^{32}\text{-P]ATP} \times \text{reaction time}}$$

Enzyme activity is expressed as pmol of PO_4 incorporated into peptide substrate per min. Phosphate incorporation into the substrate was linear during the kinase assay with the conditions described above.

In vitro dephosphorylation of recombinant autophosphorylated p57$^{lck\text{-}myc}$. Recombinant baculovirus encoding p57$^{lck\text{-}myc}$ was produced as previously described (4). Sf9 insect cells infected with baculovirus encoding p57$^{lck\text{-}myc}$ were lysed in lysis buffer without phosphatase inhibitors and immunoprecipitated with 9E10-coated protein A-Sepharose beads. After extensive washing, p57$^{lck\text{-}myc}$ on the Sepharose beads was autophosphorylated in vitro at 30°C with [γ-^{32}P]ATP, washed four times with TS buffer (50 mM Tris [pH 7.6], 150 mM NaCl) to eliminate the unincorporated radioactivity, and divided into equal parts (verified by β counting without scintillation fluid) for subsequent treatment. Untransfected N1 cells and myr-iCD45(GA2)- or myr-iCD45(CS269)-expressing cells were lysed in lysis buffer without phosphatase inhibitors and immunoprecipitated with anti-Src-protein A–Sepharose-coated beads. After extensive washing in TS buffer and a final washing in reducing buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 10 mM dithiothreitol), the anti-Src immunoprecipitated beads [containing myr-iCD45(GA2), myr-iCD45(CS269), or nonspecific immunoprecipitate] were incubated with the beads bound to autophosphorylated p57$^{lck\text{-}myc}$ in reducing buffer at 37°C with mixing by inversion every 15 min. After 4 h the beads were washed, and the proteins were eluted with sample buffer and resolved by SDS–8% PAGE transferred to a nitrocellulose filter, and analyzed by autoradiography. The same nitrocellulose filter was then immunoblotted with biotinylated anti-Myc antibody and developed with streptavidin-HRP and ECL. In vitro autophosphorylation of immunoprecipitated p57$^{lck\text{-}myc}$ was performed as previously described (8).

RESULTS

Previous work has shown that despite hyperphosphorylation of the negative regulatory tyrosine 505 (Tyr-505), Lck has higher kinase activity in CD45$^-$ YAC-1 T cells than in their CD45$^+$ counterparts (8). This increase was ascribed to hyper-

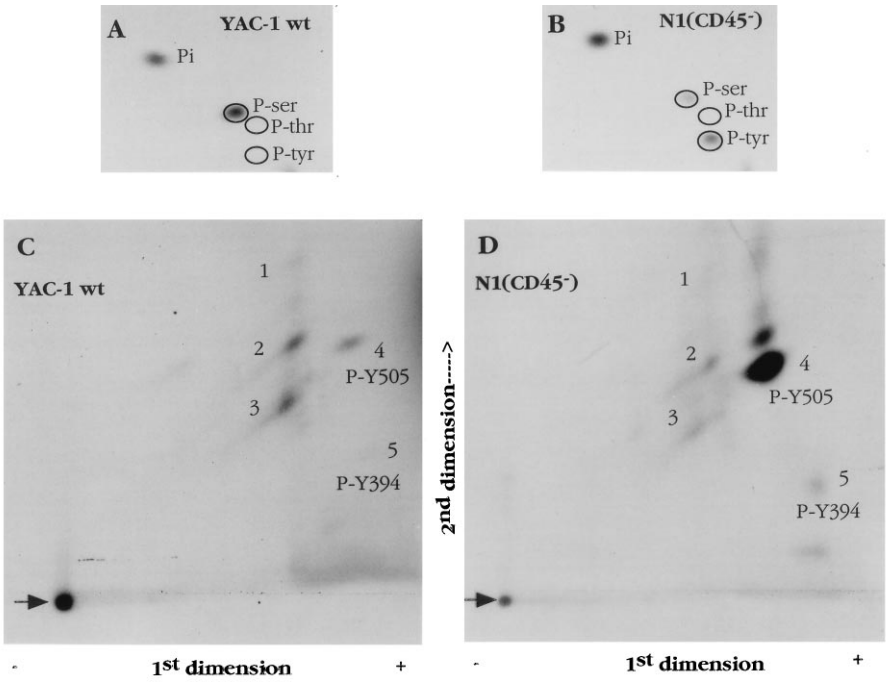

FIG. 1. Phosphoamino acid analysis (A and B) and phosphopeptide mapping (C and D) of ^{32}P-labeled Lck from YAC-1 WT (CD45$^+$) and N1 (CD45$^-$) cells. In panels A and B, equal amounts of radiolabeled material were spotted. In panels C and D, all of the trypsin-digested material was analyzed. An arrow indicates the sample origins. The phosphoamino acid analysis is representative of three independent experiments, and the phosphopeptide mapping is representative of five independent experiments.

phosphorylation of tyrosine residues because in vitro dephosphorylation of Lck by CD45 reduced kinase activity to a level similar to that found in CD45$^+$ cells. Although phosphopeptide mapping showed that Lck Tyr-505 was the major site of phosphorylation in the absence of CD45, other sites of hyperphosphorylation were also observed. To identify which Lck residues are hyperphosphorylated in the absence of CD45, phosphoamino acid analysis and two-dimensional phosphopeptide mapping of trypsin-digested Lck from ^{32}P-labeled YAC-1 CD45$^+$ and CD45$^-$ cells were performed. The large majority of the phosphate found in Lck isolated from YAC-1 CD45$^+$ wild-type (WT) cells was covalently bound to serine (Fig. 1A). In contrast, Lck from CD45$^-$ N1 cells had abundant phosphotyrosine (Fig. 1B). Calculations based upon the total counts per minute incorporated into Lck revealed that although the ratio of phosphoserine to phosphotyrosine decreased in CD45$^-$ cells, the absolute amount of phosphoserine did not. The tryptic phosphopeptide map of Lck from CD45$^+$ cells (Fig. 1C) revealed five reproducible major radiolabeled species. The labeled material was scraped from the TLC plates, eluted from the cellulose, and analyzed for phosphoamino acid content. Spots 1, 2, and 3 contained only phosphoserine, and spots 4 and 5 contained only phosphotyrosine (data not shown). Using comigration with unradiolabeled synthetic phosphopeptides, spot 4 was identified as the tryptic fragment containing Tyr-505. The small spot above spot 4 was not seen in other experiments and probably represents incompletely digested material. Spot 5, which is barely detectable at this exposure in CD45$^+$ WT cells, was found to represent the tryptic fragment containing Tyr-394. The same analysis of Lck from CD45$^-$ cells (Fig. 1D) demonstrated a dramatic increase in phosphorylation of Tyr-505, and a lesser, although reproducible, increase in the phosphorylation of Tyr-394. No species comigrating with the synthetic peptide corresponding to the tryptic fragment containing Tyr-192 was detected in either CD45$^+$ or CD45$^-$ cells. Therefore, the loss of CD45 results in the hyperphosphorylation of two Lck residues, Tyr-505 and, to a lesser extent, Tyr-394.

Construction of p57$^{lck\text{-}myc}$ chimera and its mutagenesis. To determine the relationship between tyrosine phosphorylation and enzyme activity, site-directed mutants of Lck were generated for stable expression in YAC-1 CD45$^+$ and CD45$^-$ (clone N1) cells. Since both cell lines express endogenous Lck, the mutations were made on cDNAs encoding the entire *lck* coding region followed by a short peptide derived from c-Myc that is reactive with the monoclonal antibody 9E10 (Fig. 2). This p57$^{lck\text{-}myc}$ molecule and mutants in which Tyr-192, Tyr-394, and Tyr-505 had been changed to phenylalanine were stably expressed in N1 cells. p57$^{lck\text{-}myc}$ wild type and mutants with

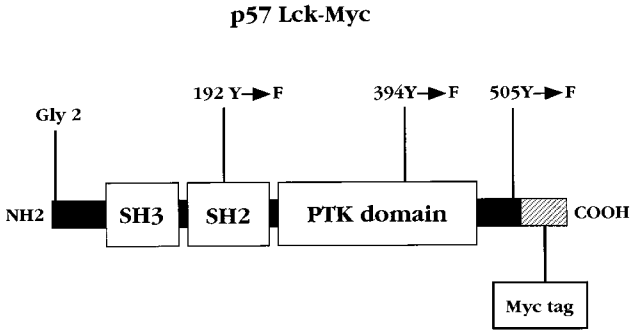

FIG. 2. Schematic representation of p57$^{lck\text{-}myc}$ and its mutations. The chimeric protein is composed of the entire sequence of Lck followed by the amino acid sequence SMEQKLISEEDLN, containing the Myc epitope recognized by 9E10.

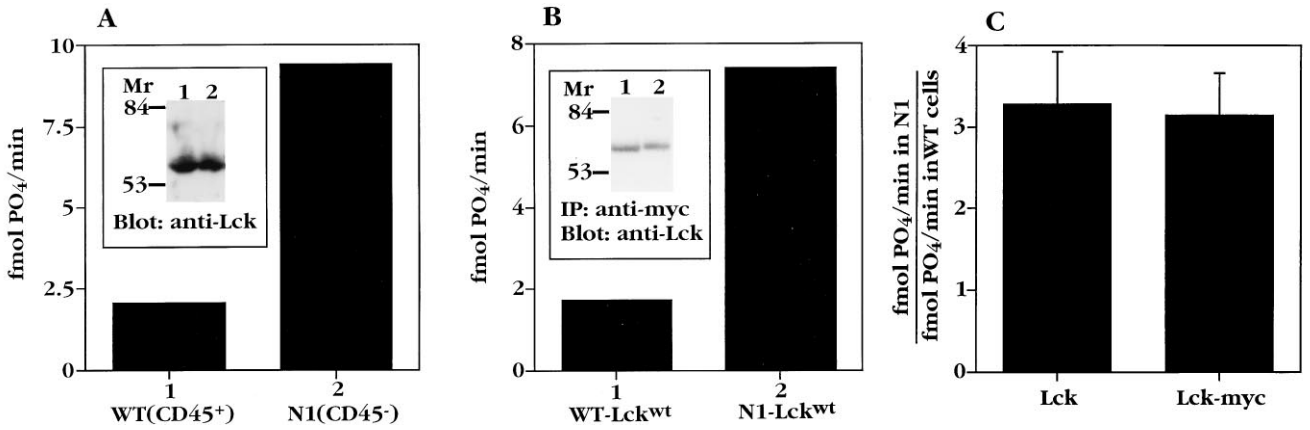

FIG. 3. Kinase activity of Lck and p57$^{lck\text{-}myc}$ from CD45$^+$ and CD45$^-$ cells. (A) YAC-1 WT and N1 cells were lysed, and 1 mg of protein cell lysate from each was incubated with anti-Lck coated beads. Kinase activity was determined by measuring the phosphorylation of cdc2(6-20) peptide. (Inset) Anti-Lck immunoblot of 100 μg of total protein lysate from YAC-1 WT (lane 1) and N1 (lane 2). (B) WT-Lck^{WT}.4 and N1-Lck^{WT}.2 clones were lysed, p57$^{lck\text{-}myc}$ was immunoprecipitated by 9E10 from 1 mg of protein cell lysate, and kinase activity was measured by phosphorylation of cdc2(6-20) peptide. (Inset) Quantitative immunoblotting of p57$^{lck\text{-}myc}$ with anti-Lck after immunoprecipitation by 9E10 from 1 mg of protein cell lysate from clones WT-Lck^{WT}.4 (lane 1) or N1-Lck^{WT}.2 (lane 2). IP, immunoprecipitation. (C) The mean activity of endogenous Lck (four independent experiments) and p57$^{lck\text{-}myc}$ (five independent experiments) in N1 cells relative to their activity in WT cells. The error bars represent the standard errors of the means. The amounts of immunoprecipitated p57$^{lck\text{-}myc}$ from the different cells were equivalent in all experiments.

Tyr-394→Phe and/or Tyr-505→Phe substitutions were also expressed in the CD45$^+$ parental cells.

Effect of amino acid substitution on tyrosine kinase activity of Lck. Kinase activity of the different chimeric proteins was assessed with an assay previously described for *src* family enzymes by using the cdc2(6-20) peptide as a substrate (9). As previously shown, while the amounts of endogenous Lck protein expressed by the two cell lines were equal (Fig. 3A [inset]), the activity of endogenous Lck was substantially higher in CD45$^-$ than in CD45$^+$ cells (Fig. 3A). Similarly, when 9E10 was used to immunoprecipitate equal amounts of Myc-tagged Lck (Fig. 3B [inset]) from CD45$^+$ (clone WR-Lck^{WT}.4) and CD45$^-$ (clone N1-Lck^{WT}.2) transfectants, the enzyme was more active in the CD45$^-$ cells than in the wild type (Fig. 3B). When multiple independent experiments were averaged, there was no difference between the degrees to which endogenous Lck and p57$^{lck\text{-}myc}$ activities were enhanced in CD45$^-$ cells (Fig. 3C). Although we cannot completely exclude the possibility that the addition of the Myc tag has an unanticipated effect on enzymatic activity, these experiments indicate that the p57$^{lck\text{-}myc}$ chimeric protein behaves similarly to wild-type Lck with regard to the effect that loss of CD45 has on kinase activity.

Kinase assays were performed with various p57$^{lck\text{-}myc}$ mutant molecules immunoprecipitated from stably transfected CD45$^-$ N1 cells. The results of two independent experiments with multiple transfectants are shown in Fig. 4A and B. Anti-Lck immunoblotting of 9E10 immunoprecipitates shows the amount of chimeric protein immunoprecipitated from the different transfectants (Fig. 4 [insets]). Note that p57$^{lck\text{-}myc}$ appears as a doublet in SDS-PAGE gels, with the minor band migrating with a M_r of approximately 61,000. This minor band was typically observed with p57$^{lck\text{-}myc}$ expressed in either WT or N1 cells, and it was also observed with endogenous Lck immunoprecipitated from WT and N1 cells labeled with ^{32}P (data not shown). It has been reported that Lck phosphorylated on serine residues migrates with an abnormally high apparent molecular mass (51, 53), and indeed phosphoamino acid analysis of the upper band confirmed this to be the case in WT and N1 cells (data not shown). 9E10 specifically precipitated kinase activity from N1 cells expressing wild-type p57$^{lck\text{-}myc}$ and not untransfected cells. The 394-Y→F mutation (two independent clones shown) had little enzymatic activity, even when the protein was overexpressed (Fig. 4A, lane 4), while the 505-Y→F mutation (three independent clones shown) had substantially higher activity than the wild-type p57$^{lck\text{-}myc}$. p57$^{lck\text{-}myc}$ with phenylalanine substitutions at both Tyr-394 and Tyr-505 (N1-Lck$^{394F+505F}$) also had very little activity (two independent clones shown). Substitution of Tyr-192 with phenylalanine (N1-Lck^{192F}) had little if any effect on kinase activity, and the Tyr-192→F–Tyr-505→F double mutation (N1-Lck$^{192F+505F}$) had the same activity as the Tyr-505→F mutation alone, suggesting that in YAC-1 cells Tyr-192 does not play a major role in the regulation of Lck activity. Identical results were obtained with five independent clones expressing Lck^{394F}, four independent clones expressing Lck^{505F}, and two independent clones for each of the other mutations. The average enzymatic activity for each mutation relative to wild-type p57$^{lck\text{-}myc}$ from multiple independent experiments was determined and is shown in Fig. 4C.

To determine the effect of mutations at Lck Tyr 394 and/or Tyr 505 in the presence of CD45, mutagenized chimeric p57$^{lck\text{-}myc}$ molecules were expressed in wild-type YAC-1 cells and their kinase activity was measured (Fig. 5). As in CD45$^-$ cells, the 394-Y→F mutation resulted in decreased kinase activity, while the 505-Y→F substitution caused an increase in kinase activity. p57$^{lck\text{-}myc}$ with phenylalanine substitutions at both Tyr-394 and Tyr-505 had a level of activity similar to that of the single 394-Y→F mutation. These results could not be accounted for by the levels of protein expression, since if anything, the level of Lck$^{394F+505F}$ chimeric protein was slightly higher than those of the others (Fig. 5A). The statistical analysis of multiple independent experiments is shown in Fig. 5B. Together, these results indicate that phosphorylation of Tyr-394 is necessary for Lck activity in CD45$^+$ cells, for the enhancement of kinase activity in CD45$^-$ cells, and for the further increase in activity caused by the mutation of Tyr-505. Since the degree of inhibitory phosphorylation of Tyr-505 is greater in CD45$^-$ cells, it would be predicted that in these cells removal of Tyr-505 (Lck^{505F}) would result in even greater kinase activity than it would in CD45$^+$ cells, and, in fact, the relative activity of this mutated protein was substantially higher

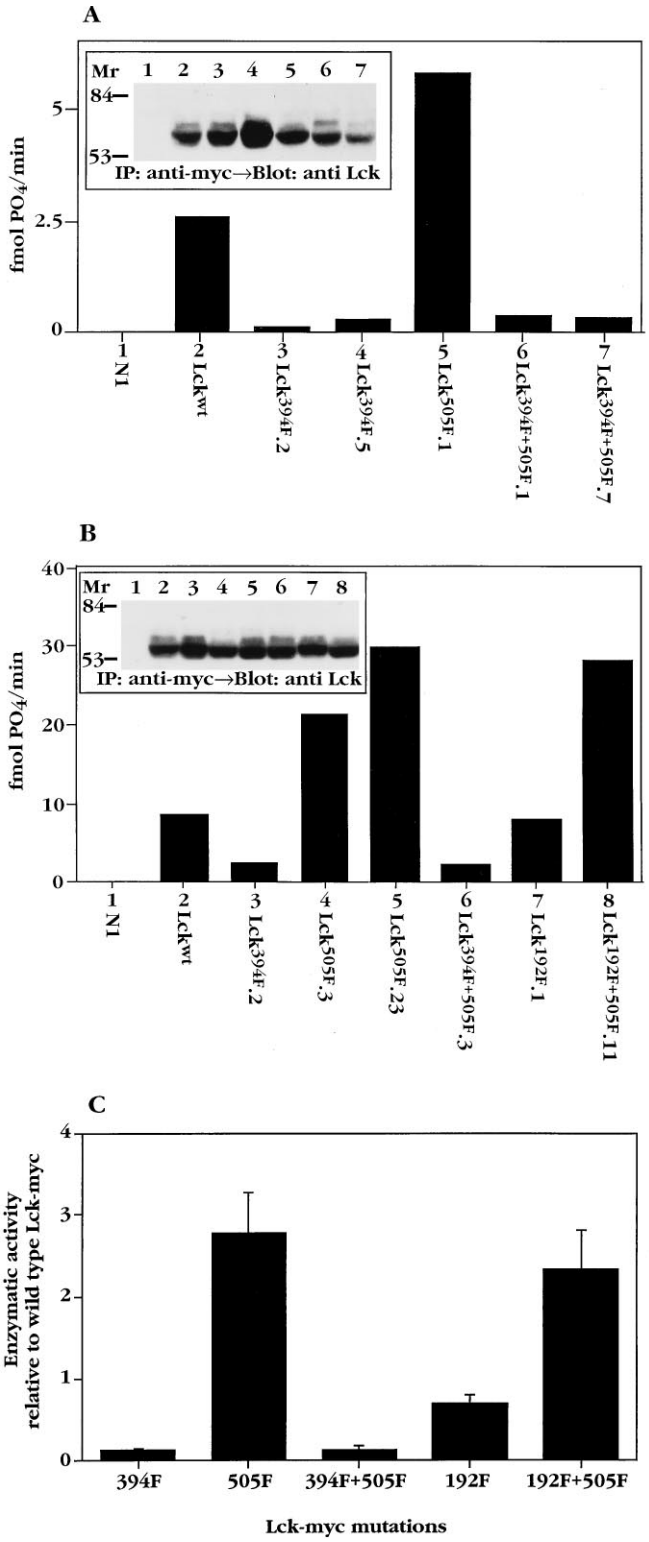

FIG. 4. Effect of amino acid substitutions on tyrosine kinase activity of $p57^{lck\text{-}myc}$ in $CD45^-$ cells. (A and B) N1 untransfected cells and the indicated transfected clones were lysed, $p57^{lck\text{-}myc}$ was immunoprecipitated by 9E10 from 1 mg of protein lysate, and kinase activity was determined by measuring the phosphorylation of cdc2(6-20) peptide. (Insets) Quantitative immunoblotting of $p57^{lck\text{-}myc}$ with anti-Lck after immunoprecipitation by 9E10 from 1 mg of protein lysate of the same cells used in the kinase assay. The lane numbers correspond to the numbers shown for the kinase assay. IP, immunoprecipitation. (C) The relative enzymatic activity of mutated $p57^{lck\text{-}myc}$ compared with that of wild-type $p57^{lck\text{-}myc}$ in N1 cells. 394F ($n = 10$), 505F ($n = 6$), 394F+505F ($n = 8$), 192F ($n = 4$), 192F+505F ($n = 4$). The error bars represent the standard errors of the means. The amounts of immunoprecipitated $p57^{lck\text{-}myc}$ from the different cells were equivalent in all experiments.

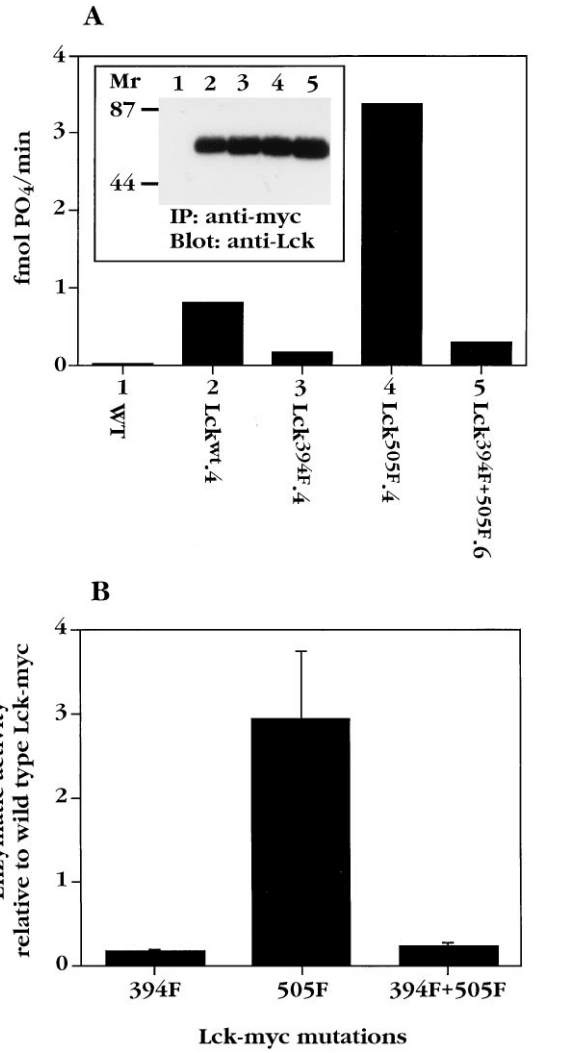

FIG. 5. Effect of amino acid substitutions on tyrosine kinase activity of $p57^{lck\text{-}myc}$ in $CD45^+$ cells. (A) YAC-1 wild-type (WT) untransfected cells and the indicated transfected WT $CD45^+$ clones were lysed, $p57^{lck\text{-}myc}$ was immunoprecipitated by 9E10 from 1 mg of protein lysate, and kinase activity was determined by measuring the phosphorylation of cdc2(6-20) peptide. (Inset) Quantitative immunoblotting of $p57^{lck\text{-}myc}$ with anti-Lck after immunoprecipitation by 9E10 from 1 mg of protein lysate of the same cells used in the kinase assay. The lane numbers correspond to the numbers shown for the kinase assay. IP, immunoprecipitation. (B) The relative enzymatic activity of mutated $p57^{lck\text{-}myc}$ compared with that of wild-type $p57^{lck\text{-}myc}$ in WT cells; the results represent the means and standard errors of the means of four independent experiments. The amounts of immunoprecipitated $p57^{lck\text{-}myc}$ from the different cells were equivalent in all experiments.

in $CD45^-$ than in $CD45^+$ cells (Fig. 6). Given that there is a relatively small amount of Tyr-394 phosphorylation in $CD45^-$ N1 cells, and even less in wild-type $CD45^+$ cells, the data indicate that only a small amount of Tyr-394-phosphorylated Lck is enough to substantially increase enzymatic activity.

CD45 directly dephosphorylates Lck Tyr-394. The mutational analysis of Lck in the present study suggests that CD45 is required to maintain not only Tyr-505 but also Tyr-394 in a dephosphorylated state, an effect that could be direct or indirect. It has previously been shown that in vitro dephosphorylation of Lck isolated from $CD45^-$ cells by CD45 reduced kinase activity to a level similar to that found in $CD45^+$ cells (8). By using peptides derived from Lck as a substrate, CD45

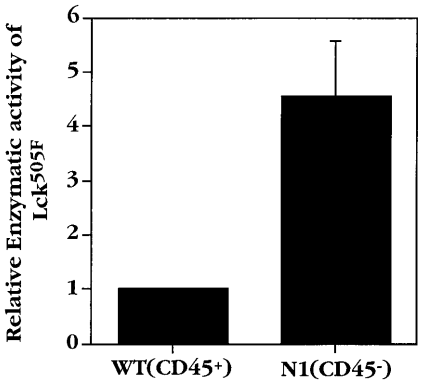

FIG. 6. Lck^{505F} has higher kinase activity in $CD45^-$ than in $CD45^+$ cells. The relative activity of Lck-myc^{505F} expressed in $CD45^+$ (value of 1) or $CD45^-$ N1 cells (± standard error of the mean) is shown ($n = 6$). The amounts of immunoprecipitated $p57^{lck\text{-}myc}$ from the different cells were comparable in all experiments.

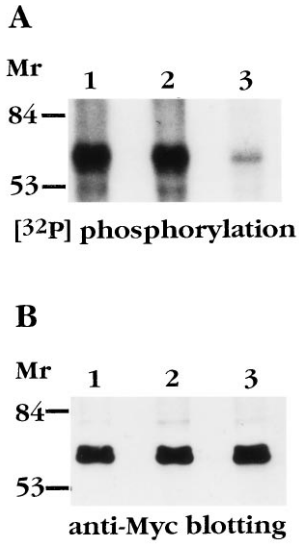

FIG. 7. CD45 directly dephosphorylates Lck Tyr-394. (A) Recombinant $p57^{lck\text{-}myc}$ was immunoprecipitated with 9E10-coated protein A-Sepharose beads from Sf9 insect cells expressing a baculovirus-encoded $p57^{lck\text{-}myc}$, autophosphorylated in vitro with [γ-^{32}P]ATP, and incubated with protein A-Sepharose beads from anti-Src immunoprecipitates of untransfected N1 cells (lane 1), N1 cells transfected with myr-iCD45(CS269) (lane 2), or N1 cells transfected with myr-iCD45(GA2) (lane 3) at 37°C in reducing buffer with periodic mixing. After 4 h the beads were washed, eluted in sample buffer, resolved by SDS–8% PAGE, transferred to a nitrocellulose filter, and analyzed by autoradiography. (B) The nitrocellulose filter described above was immunoblotted with biotinylated 9E10 and developed with streptavidin-HRP and ECL. The data are representative of three independent experiments.

has been shown to dephosphorylate the Tyr-394-containing phosphopeptide as efficiently as the Tyr-505-containing phosphopeptide (14). We therefore determined if CD45 directly dephosphorylates Lck Tyr-394 when the kinase is in its native conformation and enzymatically active. Baculovirus-encoded recombinant $p57^{lck\text{-}myc}$ was immunoprecipitated from SF9 insect cells and autophosphorylated in vitro with [γ-^{32}P]ATP. Labeling in this manner results in phosphorylation of Tyr-394 (26). We confirmed this by phosphopeptide mapping of tryptic digests of the autophosphorylated recombinant $p57^{lck\text{-}myc}$, which revealed that only Tyr-394 was labeled under these conditions (data not shown). As the source of CD45 we used a chimeric molecule consisting of the entire intracellular (and enzymatically active) domain of CD45 attached to an amino-terminal Src epitope tag, myr-iCD45 (49). As a control, an enzymatically inactive form of this molecule, myr-iCD45(CS269), was used (34). N1 YAC-1 cells stably expressing these chimeric molecules were lysed, and enzymatically active or inactive intracellular CD45 was immunoprecipitated with an anti-N-terminal Src monoclonal antibody. Incubation of P-Tyr-394 $p57^{lck\text{-}myc}$ with the enzymatically active form resulted in almost complete dephosphorylation of the recombinant protein (Fig. 7A, lane 3). In contrast, the enzymatically inactive myr-iCD45(CS269) did not affect the phosphorylation state of P-Tyr-394 $p57^{lck\text{-}myc}$ (lane 2) compared with a sham anti-Src immunoprecipitate from untransfected N1 cells (lane 1). Equal amounts of $p57^{lck\text{-}myc}$ protein were present in the three samples, as evidenced by a control anti-Myc immunoblot (Fig. 7B) of the same filter shown in Fig. 7A. This demonstrates that CD45 tyrosine phosphatase can directly dephosphorylate Tyr 394 in conformationally native Lck, an event previously shown to inhibit the activity of Lck isolated from $CD45^-$ cells (8), and is consistent with the hypothesis that the autophosphorylation site of the kinase is a target of negative regulation by the CD45 phosphatase.

DISCUSSION

One way by which the activity of *src* family kinases is controlled is by phosphorylation of specific tyrosine residues. All the members of this family have at least two sites of tyrosine phosphorylation with opposite effects on kinase activity. A tyrosine in the kinase domain is the site of autophosphorylation and functions as a positive regulator, increasing the activity of the kinase and making the cellular oncogenic forms of the *src* family kinases more efficient at cell transformation (48). In contrast, phosphorylation of the carboxy-terminal tyrosine inhibits kinase activity. It is thought that upon phosphorylation this tyrosine binds to the SH2 domain of the same molecule, or of another identical molecule, forcing the kinase domain into an enzymatically inactive conformation (38, 40). For Lck, the carboxy-terminal tyrosine is at position 505, and its substitution with phenylalanine activates the kinase, resulting in oncogenicity (2) and enhanced T-cell responsiveness to antigen (1). The ubiquitous cytosolic kinase Csk is responsible for phosphorylation of the negative regulatory tyrosine of *src* family members, and it is able to phosphorylate and inhibit Src in yeast cells (29, 33) and Fyn (35) and Lck (6) in vitro. Disruption of the Csk locus in mice results in embryonic lethality, and cell lines derived from these embryos have increased Src and Fyn kinase activity (17). A number of *csk*-related PTKs, such as Ctk (23) and Matk (5), have also been identified, although their role in vivo is not clear.

Another enzyme that participates in the regulation of lymphocyte *src* family kinases is the protein tyrosine phosphatase CD45, whose presence is essential for normal T-cell activation (24, 25, 50, 52). Early reports on the relationship between *src* family kinases and CD45 demonstrated that this phosphatase can dephosphorylate the COOH-terminal tyrosine of Lck and Fyn in vitro and increase their activity (31, 32). Several studies have analyzed Lck or Fyn in T cells lacking CD45. One of the first found that in the $CD45^-$ mutant of a murine lymphoma T-cell line, Lck has increased levels of phosphorylation of tyrosine 505 (36). The COOH-terminal region of Lck is also hyperphosphorylated in a $CD45^-$ variant of the human leukemic T-cell line Jurkat (40). In a CD45-deficient murine T-cell clone there was an increase in COOH-terminal phosphorylation of Lck as well as Fyn, and there was a decrease in the

kinase activity of both enzymes (27). The conclusion of these studies was that since the negative regulatory COOH-terminal tyrosines of Lck and Fyn are substrates for CD45, this phosphatase must play a role in T-cell activation by keeping these kinases in an active state. There have been other reports, however, suggesting a different view of the relationship between CD45 and *src* family kinases. In a $CD45^-$ variant of the human T-cell line HPB-ALL, the kinase activity of Lck, and in particular the CD4-associated fraction, was higher than that in parental cells in spite of its hyperphosphorylation, and the cross-linking of CD3 and CD4 was able to restore activation signals of even higher intensity than in cells with CD45 (13). In another study of independently derived $CD45^-$ Jurkat T cells, Lck activity was higher than in wild-type cells (18). A negative regulatory role for CD45 in Lck activity is also suggested by the observation that while the cross-linking of CD4 increases both phosphorylation and kinase activity of the associated Lck, clustering CD45 with CD4 inhibits these responses (37).

Recent work from our group has provided further evidence to support a more complex role for CD45 in the regulation of the *src* family kinases (8). It was found that Lck and Fyn had increased activity in three independent $CD45^-$ T-cell lines compared with the corresponding $CD45^+$ controls. This was despite hyperphosphorylation of Lck in different parts of the molecule, particularly Tyr-505. The enhancement of CD45 activity appeared to be directly related to its tyrosine phosphorylation status, since CD45-mediated in vitro dephosphorylation of Lck from $CD45^-$ YAC-1 cells reduced kinase activity almost to the level of Lck taken from $CD45^+$ YAC-1 cells. In the present study we have extended these observations with phosphopeptide mapping and mutational analysis to investigate in detail the correlation between Lck phosphorylation and kinase activity. There were low levels of constitutive serine phosphorylation of Lck from $CD45^+$ and $CD45^-$ cells; loss of CD45 resulted in a selective increase in tyrosine phosphorylation. Two sites of tyrosine phosphorylation were identified, Tyr-505 and Tyr-394, the latter being phosphorylated to a much lesser extent than Tyr-505. Despite this, the biological importance of Tyr-394 phosphorylation was shown by the effect of 394-Y→F substitutions in epitope-tagged Lck molecules. Lck^{394F} had a very low level of activity in $CD45^+$ cells which, importantly, did not increase in cells lacking CD45. Furthermore, the 505-Y→F substitution, which normally results in a substantial increase in kinase activity, failed to do so in Lck bearing the 394-Y→F mutation. Although it is possible that the tyrosine-to-phenylalanine mutation at residue 394 has some unanticipated effect on Lck, the simplest interpretation of the data is that the biochemical consequences of this conservative substitution are due to prevention of phosphorylation at this site. The finding that CD45 can dephosphorylate Tyr-394 in vitro is consistent with, although it does not prove, the possibility that CD45 dephosphorylates both Tyr-394 and Tyr-505 in vivo, and it explains the previous finding that treatment of hyperphosphorylated Lck isolated from $CD45^-$ decreases kinase activity despite dephosphorylation of the negative regulatory residue Tyr-505. It is worth noting that the relationship between Lck phosphorylation status and kinase activity in $CD45^-$ cells is similar to that observed in normal lymphocytes following CD4 cross-linking: a large increase in Tyr-505 phosphorylation was accompanied by a much less pronounced increase in Tyr-394 phosphorylation but was nonetheless associated with a dramatic increase in Lck kinase activity (45).

These results demonstrate that the kinase activity of Lck depends on a delicate balance between phosphorylation of tyrosine residues at positions 394 and 505. Although phosphorylation of Tyr-192 has a positive effect on Lck activity (11), it appears that this event does not occur in $CD45^-$ YAC-1 T cells. CD45 directly dephosphorylates both Tyr-394 (this report) and Tyr-505 (30), and its absence results in increased phosphorylation of each residue. The net biological result, increased or decreased kinase activity, may depend upon cell type, activation state, and the complement of other tyrosine phosphatases and kinases expressed. Indeed, this may account for some of the variability in the results obtained by different laboratories using different cell lines. It is clear that Lck activity in vivo is regulated by CD45. What these findings underscore is that a relatively small number of molecules phosphorylated at Tyr-394 has a dominant effect on net Lck activity. Given the results previously obtained with Fyn, it is likely that a similar mechanism accounts for its hyperactivity in $CD45^-$ cells, and indeed such a mechanism may be a critical determinant in the kinase activity of all *src* family kinases.

ACKNOWLEDGMENTS

We are grateful to Stephen Ullrich for advice on phosphopeptide mapping and Astrid Eder and Allan Weissman for critical review of the manuscript.

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