Stimulation of Phosphorylation of Tyr\textsuperscript{394} by Hydrogen Peroxide Reactivates Biologically Inactive, Non-membrane-bound Forms of Lck*

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Lck, a lymphocyte-specific tyrosine protein kinase, is bound to cellular membranes as the result of myristoylation and palmitoylation of its amino terminus. Its activity is inhibited by phosphorylation of tyrosine 505 and stimulated by phosphorylation of tyrosine 394. The Tyr-505—Phe mutant of Lck (F505Lck) exhibits elevated biological activity and constitutive phosphorylation of Tyr-394 in vivo. Mutations at sites of fatty acylation that prevent F505Lck from associating with cellular membranes abrogate the biological activity of the molecule in vivo, compromise its activity as a protein kinase in vivo and in vitro, and eliminate the phosphorylation of Tyr-394. Here, we show that exposure of cells expressing cytoplasmic or nuclear forms of F505Lck to H\textsubscript{2}O\textsubscript{2}, a general inhibitor of tyrosine protein phosphatases, restores the catalytic activity of these mutant proteins through stimulation of phosphorylation of Tyr-394. H\textsubscript{2}O\textsubscript{2} treatment induced the phosphorylation of Tyr-394 on catalytically inactive forms of Lck regardless of cellular localization. Phosphorylation of Tyr-394 therefore need not occur by autophosphorylation. Thus, there appear to be two mechanisms through which the phosphorylation of Lck at Tyr-394 can occur. One is restricted to the plasma membrane and does not require the presence of oxidants. The other is operational in the nucleus as well as the cytosol and is responsive to oxidants.

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\pS6^ck, a member of the Src family of tyrosine protein kinases (1, 2), is expressed exclusively in lymphoid cells and predominantly in T cells. Lck associates with the cytoplasmic domain of the T-cell receptor-associated glycoproteins CD4 and CD8 (3, 4), membrane immunoglobulin in B cells (5), and glycosylphosphatidylinositol-anchored proteins (6, 7) through its unique amino terminus. \pS6^ck is essential for both T-cell development (8, 9) and T-cell activation (10, 11). The activity of Lck is regulated by the phosphorylation of tyrosine residues at positions 394 and 505. Phosphorylation of Tyr-505 inhibits the activity of Lck (12, 13). Mutation of Tyr-505 to phenylalanine results in a constitutively active protein that is able to transform fibroblasts (12, 13), enhance T-cell responsiveness to antigen (14), and induce antigen-independent interleukin-2 production in T cells (15). In contrast, phosphorylation of Tyr-394, the single site of autophosphorylation in vitro, enhances the catalytic activity of Lck and is required for the activation of Lck by mutation of Tyr-505 to phenylalanine (16–18).

Lck associates with the inner face of the plasma membrane through its amino terminus. This interaction is mediated by both myristic acid and palmitic acid that are bound to the amino-terminal glycine and Cys-3 and/or Cys-5, respectively (19–22). Mutations in which the amino-terminal glycine is mutated to alanine result in a protein that is neither myristoylated nor palmitoylated and is unable to associate with the plasma membrane (17). Mutation of both Cys-3 and Cys-5 to serine yields a protein that is myristoylated but not palmitoylated (21–23). The resulting Lck protein is completely cytoplasmic, indicating that myristoylation is not sufficient for anchoring Lck to the plasma membrane (22).

Hydrogen peroxide is a general inhibitor of tyrosine protein phosphatases (24). Exposure of cells to H\textsubscript{2}O\textsubscript{2} induces rapid tyrosine phosphorylation of numerous cellular proteins in vivo (24–28) and has been shown to be a potent activator of Lck (18). Activation of Lck by H\textsubscript{2}O\textsubscript{2} is due to an increase in the phosphorylation of Tyr-394 (18). In the current study, we examined the effect of H\textsubscript{2}O\textsubscript{2} on three different non-membrane-bound forms of genetically activated Lck (F505Lck). Non-myristoylated, myristoylated but non-palmitoylated, and nuclear forms of F505Lck all exhibit severely reduced activity in vivo and in vitro. H\textsubscript{2}O\textsubscript{2} is able to restore the in vitro catalytic activity of all three non-membrane-bound forms of Lck. This increase in activity correlates with, and in all probability is due to, an increase in the phosphorylation of Tyr-394. Apparently, phosphorylation of Tyr-394 occurs inefficiently when Lck is located at sites other than cellular membranes but can be induced by elevated levels of oxidants.

EXPERIMENTAL PROCEDURES

Construction of lck Mutants—All mutations were introduced into murine lck cDNA by oligonucleotide-directed mutagenesis using the M13 bacteriophage except for lck\textsuperscript{S5F505}, which was constructed by polymerase chain reaction mutagenesis. The construction of lck\textsuperscript{S5F505} and lck\textsuperscript{S5F505} has been described (12, 22). lck\textsuperscript{A2F505} was made from lck\textsuperscript{S5F505} by site-directed mutagenesis of glycine at position 2 to alanine using the oligonucleotide 5'–GATCGGTCAGTCGTCGTCG–3'. The Lck\textsuperscript{S5F505} and Lck\textsuperscript{A2F505} mutants were assembled by cloning a 33-base pair synthetic Sphl fragment (5'-TCCATAGAAGAAGCTGAGTCGAGATCCAG3') encoding the nuclear localization signal (PKKKRKVE)PC from SV40 large T antigen.
(29) into a SpI site in genes encoding lckA2 or lckA2,F505 that was generated by changing the codon for Cys-3 (TGT) to (TGC). All mutations were confirmed by sequencing. A BamHI- EcoRI restriction fragment of the murine lck cDNA containing the Lys-273 → Arg mutation (18) was used to replace the corresponding BamHI-EcoRI fragment in genes encoding lckA2,F505 to lckA2,F505 and lckA2,R273, respectively. All mutant lck cDNAs were subcloned into the retroviral expression vector LXSN (30). Recombinant retroviruses were produced by cotransfecting the LXSN constructs and the viral helper plasmid SV-ϕ -E-MLV (31) into COSm-6 cells (32). 48 h after cotransfection, supernatant containing recombinant virus particles was collected and used to infect 208F cells as described previously (18).

**Cell Lines—** 208F rat fibroblasts expressing Lck were maintained in Dulbecco-Vogt modified Eagle’s medium (DMEM) (Mediatech, Wash-

**Preparation of Cell Lysates and Immunoprecipitation—** Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 1% (w/v) sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 200 μM Na3VO4, 50 mM NaF, 2 mM EDTA, 100 kallikrein inhibitor units/ml aprotinin) for 20 min at 4°C at a concentration of 2 × 106 cells/ml. 30

**In Vivo Labeling and Peptide Mapping—** 208F cells expressing Lck (approximately 2 × 106 per 10-cm dish) were washed twice with phosphate-free DMEM and incubated with 0.5 μCi/ml [32P]ATP (3000 Ci/mmol; Amersham Corp.) and 2 mM Val3-angiotensin II (Star Bioc
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**In Vitro Kinase Assays—** Lck immunoprecipitates were incubated with 5 μCi of (γ-32P)ATP (3000 Ci/mmol; Amersham Corp.) and 2 mM Val3-angiotensin II (Star Biochemicals, Torrance, CA) in 20 μl of kinase buffer (40 mM PIPES, pH 7.0, 10 mM MnCl2) at room temperature. The reactions were stopped by the addition of 5% trichloroacetic acid, and the angiotensin was adsorbed onto Whatman p81 phosphocellulose paper (Whatman, Fairfield, NJ). The paper was then washed with 0.5% phosphoric acid to remove unincorporated (γ-32P)ATP. Phosphopeptide incorporation into angiotensin was measured by liquid scintillation counting of the p81 paper.

**Hydrogen Peroxide Treatment of Cells—** 208F cells expressing Lck were seeded in 10 ml of DMEM supplemented with 10% calf serum onto a 10-cm plate and allowed to recover for 18 h. H2O2 was added directly to the cell medium to a final concentration of 5 mM. The cells were incubated at 37°C for 15 min, washed once with iced-cold isotonic Tris-buffered saline, and lysed directly on the tissue culture dish in 1 ml of RIPA buffer. For experiments in which cells were biosynthetically labeled with 32P, the 5-h labeling period (see above) preceded the addition of H2O2 to the media.

**Lck (33) antibodies and 32P-protein A (ICN, Costa Mesa, CA) as described previously (34, 35).**

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Immunofluorescence— Approximately 2 × 10^5 208F cells expressing Lck were seeded on 25 × 25-mm glass coverslips placed in 35-mm Petri dishes. After 18 h of recovery at 37 °C, the cells were washed once with phosphate-buffered saline (PBS) and fixed for 30 min in PBS containing 3% paraformaldehyde and 25 mM NaOH. Fixing and all subsequent manipulations were performed at room temperature. The cells were then permeabilized with rinse solution (PBS containing 10 mM glycine and 1% Triton X-100) for 10 min, incubated with rabbit anti-Lck antibodies (diluted 1:200 in fetal bovine serum (Intergen, Purchase, NY) containing 1% Triton X-100) for 30 min, washed four times with rinse solution, incubated with goat anti-rabbit IgG conjugated to fluorescein (Jackson Laboratories) (diluted 1:50 in fetal bovine serum), and washed an additional four times in rinse solution. The coverslips were mounted on slides with Vectashield mounting medium (Vector, Burlingame, CA). Antibody staining was visualized by fluorescence microscopy.

RESULTS

A Form of Activated Lck Localized to the Nucleus Does Not Induce an Increase in Tyrosine Phosphorylation of Cellular Substrate—To determine if Lck, which had been both activated by mutation (F505) and retargeted to the nucleus, was able to transform fibroblasts, we constructed a form of Lck in which the nuclear localization signal from SV40 large T antigen was attached to the amino terminus, Gly-2 was mutated to alanine, and Tyr-505 was mutated to phenylalanine. This protein (nucF505Lck) was concentrated in the nucleus when expressed in fibroblasts that express no endogenous Lck (Fig. 1E). In contrast to cells expressing F505Lck, which were highly transformed, cells expressing nucF505Lck were not transformed and were indistinguishable from uninfected fibroblasts (data not shown). To determine why nucF505Lck had no transforming activity, we examined the tyrosine phosphorylation of proteins in cells that expressed the mutant protein. Tyrosine phosphorylation in cells expressing nucF505Lck was unchanged from the level observed in uninfected cells (Fig. 2A). This is similar to what is observed in cells expressing A2F505Lck, a non-myristoylated form of activated Lck (17) or S3/5F505Lck, a form of activated Lck which is myristoylated but not palmitoylated (22), two other non-membrane-bound forms of Lck, and contrasted strikingly with the elevated tyrosine phosphorylation levels observed in cells expressing F505Lck, which is associated with the cell membrane (Fig. 2A).

Non-membrane-associated Forms of Activated Lck Have Reduced Catalytic Activity in Vitro—To determine whether the inability of nucF505Lck to phosphorylate cellular proteins was due to reduced enzymatic activity or due to a lack of access to substrates, nucF505Lck was isolated by immunoprecipitation and assayed for its ability to phosphorylate an exogenous substrate, Val5-angiotensin II. Assay results are expressed as the rate of labeled phosphate incorporation by the substrate per arbitrary unit of Lck. For normalization, Lck protein levels in the samples were measured by Western blotting of a fraction of the immunoprecipitates with antibodies to Lck and 125I-protein A. A, relative activities of wild-type (WT) Lck, F505Lck, nucF505Lck, S3/5Lck, and A2F505Lck from unstimulated cells. B, activities of non-membrane-bound forms of F505Lck before and after exposure to H2O2.

![Fig. 2. Tyrosine protein phosphorylation in cells expressing non-membrane-associated F505Lck](image)

![Fig. 3. In vitro protein kinase activity of non-membrane-bound forms of F505Lck](image)
H2O2 is a potent activator of Lck that reduces phosphorylation at Tyr-394 (8, 18). Thus, differences in Lck catalytic activity and phosphorylation of Tyr-394 may result from reduced phosphorylation of Tyr-394 in non-membrane-bound Lck.

**Fig. 4.** Analysis of Lck phosphorylation by two-dimensional tryptic peptide mapping. Lck was isolated by immunoprecipitation from cells that had been labeled biosynthetically with 32P. Lck was purified by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and digested with trypsin. The resulting peptides were separated horizontally by electrophoresis at pH 8.9 and then vertically by ascending chromatography. The peptides containing Tyr-394 are marked with open arrowheads. A, nucF505Lck; B, S3/S5F505Lck; C, A2F505Lck; D, nucF505Lck + H2O2; E, S3/S5F505Lck + H2O2; F, A2F505Lck + H2O2.

**Fig. 5.** The effect of H2O2 treatment on the phosphorylation of catalytically inactive, non-membrane-associated Lck. Cells were labeled biosynthetically with 32P, and then exposed to H2O2 as described. Two-dimensional tryptic peptide mapping was performed as in Fig. 4. Sample origins are indicated by arrowheads. A, nucR273Lck from unstimulated fibroblasts; B, nucR273Lck from fibroblasts stimulated with H2O2; C, A2R273Lck from unstimulated fibroblasts; D, A2R273Lck from fibroblasts stimulated with H2O2.

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**Non-membrane-associated Forms of Activated Lck Are Not Phosphorylated on Tyr-394—Phosphorylation of Tyr-394 in Lck stimulates the activity of Lck considerably (18). In contrast to membrane-bound F505Lck, which is highly phosphorylated at this residue, A2F505Lck is poorly phosphorylated (18). To determine whether the phosphorylation of nucF505Lck and S3/S5F505Lck at Tyr-394 was similarly impaired, cells expressing the mutant Lck proteins were labeled biosynthetically with 32P, and Lck was immunoprecipitated and analyzed by two-dimensional tryptic peptide mapping. The total labeling of S3/S5F505Lck and nucF505Lck with 32P was much less than labeling seen in F505Lck, and phosphorylation of Tyr-394 was barely detectable (Fig. 4). nucF505Lck contained several labeled peptides not seen in A2F505Lck or S3/S5F505Lck that reflect an increased level of serine and threonine in phosphorylation of Lck that presumably occurs in the nucleus (data not shown). The low activity of non-membrane-bound forms of activated Lck may result from reduced phosphorylation at Tyr-394.

**Exposure of Cells to H2O2 Activates Non-membrane-associated Forms of Lck—**H2O2 is a potent activator of Lck that stimulates the catalytic activity of Lck by inducing the phosphorylation of Tyr-394 (18). To examine whether H2O2 could increase the phosphorylation of Tyr-394 and possibly restore activity to non-membrane-bound forms of Lck, nucF505Lck, S3/S5F505Lck, and A2F505Lck were immunoprecipitated from cells before and after exposure to H2O2, and assayed for catalytic activity in vitro. In addition, they were labeled biosynthetically with 32P, and subjected to two-dimensional tryptic peptide mapping. H2O2 exposure resulted in a dramatic increase in the catalytic activity of each mutant (Fig. 3). Thus, nucF505Lck, S3/S5F505Lck, and A2F505Lck have neither intrinsic defects that occurred during mutagenesis nor an inability to be activated. In each case, biosynthetic labeling showed that H2O2 induced the appearance of an additional labeled peptide that comigrated precisely with the peptide containing phosphorylated Tyr-394 (Fig. 4). Therefore, as is the case with membrane-bound Lck, H2O2 appears to activate non-membrane-bound forms by stimulating phosphorylation of Tyr-394.

**Phosphorylation of Non-membrane-associated Forms of Lck at Tyr-394 Need Not Occur through Autophosphorylation—**To determine whether phosphorylation of non-membrane-associated Lck at Tyr-394 following exposure to H2O2 was due only to increased autophosphorylation, we asked whether catalytically inactive forms of Lck that were localized to the cytoplasm or nucleus became phosphorylated at Tyr-394 following exposure to H2O2. Mutation of a conserved lysine residue in the ATP phosphate anchor at position 273 to arginine results in a greater than 95% reduction of Lck catalytic activity in vitro (8, 18). Fibroblasts expressing introduced nucR273Lck or A2R273Lck, but no endogenous Lck, were labeled biosynthetically with 32P, and then exposed to H2O2. Lck was isolated and subjected to two-dimensional tryptic peptide mapping. Both forms of catalytically inactive Lck were phosphorylated on Tyr-394 in untreated cells, but phosphorylation of Tyr-394 was not detectable (Fig. 5). A2R273Lck treatment as indicated by the appearance of a labeled peptide that comigrated with a marker peptide containing labeled Tyr-394 (Fig. 5). In contrast, A2R273Lck from unstimulated fibroblasts did not show detectable labeling. This suggests that H2O2 does not activate Lck in the absence of autophosphorylation at Tyr-394.

**H3P5 Does Not Induce Reorganization of the Cellular Location of Non-myristoylated F505Lck—**The data presented above are consistent with the phosphorylation of non-membrane-bound Lck at Tyr-394 being mediated by an as yet unidentified kinase in the cytosol and nucleus. However, it was possible that...
H2O2-induced non-membrane-associated Lck to relocalize to the plasma membrane where it was phosphorylated on Tyr-394 and activated. To determine if such a relocalization occurred, we examined the cellular location of F505Lck and A2F505Lck by immunofluorescence microscopy before and after H2O2 treatment (Fig. 1, panels F–I). The transformed cells expressing F505Lck were rounded up and displayed a much more three-dimensional morphology than the cells expressing A2F505Lck, which were flattened and had a normal fibroblastic morphology. Unlike F505Lck, which showed a pattern of staining typical of a membrane-associated protein in untreated cells, A2F505Lck was present throughout the cell, including the nucleus (Fig. 1, panels F and H). Following H2O2 treatment, the locations of F505Lck and A2F505Lck in the cell were not altered detectably (Fig. 1, panels G and I).

**DISCUSSION**

The lack of in vivo catalytic and biological activity of A2F505Lck, S3/S5F05Lck, and nucleF505Lck, none of which can bind stably to cellular membranes, shows that F505Lck must be able to associate with membranes to be active. All of these non-membrane-associated forms of F505Lck differ from F505Lck in that they lack detectable phosphorylation on Tyr-394, a residue at which phosphorylation positively regulates Lck and that is highly phosphorylated in membrane-bound F505Lck. It is likely that this lack of phosphorylation in vivo and in vitro is responsible for their low activity (17, 18, 39). This implies that there is some protein or cofactor localized to membranes that is normally required for the activation of F505Lck through phosphorylation of Tyr-394. Phosphorylation of Tyr-394, however, can occur at intracellular sites other than the cellular membranes. When cells expressing cytoplasmic or nuclear F505Lck are exposed to H2O2, both the phosphorylation of Tyr-394 and the in vitro catalytic activity of the mutant Lcks are stimulated markedly. This rules out the possibility that intrinsic defects in the mutated genes encoding nucleF505Lck, S3/S5F505Lck, and A2F505Lck are responsible for the lack of activity in these proteins in unstimulated cells. However, the mechanism by which phosphorylation of Tyr-394 in the cytosol and nucleus occurs is unclear. It is not a result of relocalization to the plasma membrane. Immunofluorescence microscopy (Fig. 1, panels H and I) as well as cell fractionation experiments (data not shown) show clearly that the bulk of A2F505Lck remains cytosolic in H2O2-treated cells. While a small population of A2F505Lck may relocalize to the plasma membrane in the presence of H2O2, the extensive phosphorylation of A2F505Lck on Tyr-394 indicates that reorganization of cellular location is not the mechanism by which the kinase is activated.

The phosphorylation of A2V505Lck and nucleF505Lck is also not necessarily the result of autophosphorylation since catalytically inactive A2Lck and nucleLck become phosphorylated at Tyr-394 when cells lacking active Lck are treated with H2O2. There must, therefore, be at least one tyrosine kinase in the cytoplasm and nucleus that has the ability to phosphorylate Lck at Tyr-394. This unidentified kinase may normally be subject to regulation by oxidant levels within the cell. In addition, apparently undiminished phosphorylation of Tyr-505 was observed in cytosolic and nuclear-targeted forms of kinase-inactive Lck. The tyrosine protein kinase CSK phosphorylates the carboxyl-terminal tyrosine of Src family members, including Tyr-505 of Lck, and negatively regulates the activities of these proteins (40). Our results demonstrate that Csk is able to phosphorylate Tyr-505 of non-membrane-bound, kinase-inactive Lck. The fact that Tyr-505 was phosphorylated in non-membrane-bound forms of kinase-inactive Lck suggests that either CSK itself or a CSK-like activity is present in the nucleus as well as the cytosol.

S3/S5F505Lck and A2F505Lck, which cannot be phosphorylated at position 505, are less active as tyrosine kinases in vitro than S35Lck or A2Lck, which contain phosphorylated Tyr-505 (data not shown). This difference may result from the fact that the SH2 domain of A2F505Lck and S3/S5F505Lck does not have a phosphorylated Tyr-505 with which to form an intramolecular complex. In wild-type Lck, the SH2 domain binds to phosphorylated Tyr-505 (41) and apparently yields a conformation that exhibits in vitro activity in the absence of extensive phosphorylation at Tyr-394. In contrast, the untreated SH2 domain of F505Lck may induce a conformation that requires phosphorylation of Tyr-394 for catalytic activity.

The properties of nucleF505Lck, S3/S5F505Lck, and A2F505Lck differ from those of analogous cytosolic forms of the activated Src kinase. A2v-Src and A2F527c-Src, although non-transforming when expressed in fibroblasts, both retain the ability to phosphorylate most cellular substrates in vivo, and A2v-Src exhibits undiminished activity to phosphorylate exogenous substrates in vitro (42, 43). The reason for the differences between the cytosolic, non-membrane-bound forms of activated Lck and Src is not clear.

It appears that there are at least two activities that can phosphorylate Tyr-394. One is restricted to the plasma membrane and does not require the presence of oxidants for activity. Most likely, this is the mechanism that is responsible for the high level of Tyr-394 phosphorylation and increased activity of F505Lck at the plasma membrane. This membrane-restricted activity could be due to Lck itself or another Src family member. Additionally, there are one or more activities in the cytosol and nucleus that can phosphorylate Tyr-394, but these kinases require activation by oxidants. It is unlikely that a Src family kinase is responsible for this phosphorylation since Src family proteins are generally associated with cellular membranes. In the absence of oxidants, the lack of phosphorylation at Tyr-394 in forms of F505Lck not tethered to membranes could result from simple lack of phosphorylation of this site or a more rapid rate of dephosphorylation than phosphorylation of the site. Since H2O2 will almost certainly inhibit the phosphatase responsible for dephosphorylating Tyr-394 in the cytosol, we cannot determine whether the phosphorylation of Tyr-394 induced by H2O2 results from activation of the kinase phosphorylating the site or simply from inhibition of the phosphatase dephosphorylating the site. Nevertheless, it is clear that the steady-state phosphorylation of Tyr-394 in F505Lck is greatly reduced when the protein is removed from membranes and that there must exist one or more enzymes or functions restricted to membranes that facilitate constitutive phosphorylation of Tyr-394. An important question is the identity of the normal substrates of the oxidant-regulated tyrosine protein kinases in the cytosol and nucleus and the role of these tyrosine protein kinases in normal signal transduction.

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