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Biochemistry

Activation of the Lck tyrosine protein kinase by hydrogen peroxide requires the phosphorylation of Tyr-394

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ABSTRACT **Exposure of cells to H_2O_2 mimics many of the effects of treatment of cells with extracellular ligands. Among these is the stimulation of tyrosine phosphorylation. In this study, we show that exposure of cells to H_2O_2 increases the catalytic activity of the lymphocyte-specific tyrosine protein kinase $p56^{lck}$ (Lck) and induces tyrosine phosphorylation of Lck at Tyr-394, the autophosphorylation site. Using mutant forms of Lck, we found that Tyr-394 is required for H_2O_2-induced activation of Lck, suggesting that phosphorylation of this site may activate Lck. In addition, H_2O_2 treatment induced phosphorylation at Tyr-394 in a catalytically inactive mutant of Lck in cells that do not express endogenous Lck. This demonstrates that a kinase other than Lck itself is capable of phosphorylating Lck at the so-called autophosphorylation site and raises the possibility that this as yet unidentified tyrosine protein kinase functions as an activator of Lck. Such an activating enzyme could play an important role in signal transduction in T cells.**

Aerobic cells are constantly exposed to reactive oxygen intermediates (ROIs). Oxidative stress induced by an excess of ROIs affects various signal transduction pathways. Exposure of cells to H_2O_2 activates the transcription factors c-*jun* (1, 2), c-*fos* (1, 2), and NF-κB (3, 4) and stimulates mitogen-activated protein kinase activity (5) and human immunodeficiency virus transcription (6). Oxidative stress may activate proteins involved in signal transduction because ROIs function as natural second messengers in some signaling pathways. *N*-Acetylcysteine, a glutathione precursor and anti-oxidant, prevents activation of NF-κB by a number of stimuli that act at the plasma membrane (1, 3, 6) and inhibits the mammalian UV response (1). The observation that H_2O_2 can induce rapid tyrosine phosphorylation of multiple cellular proteins (4, 7–10) suggests that ROIs can regulate tyrosine protein kinases and tyrosine protein phosphatases. In this study, we have examined the effect of oxidative stress on the non-receptor tyrosine protein kinase Lck.

Lck, a member of the *src* family of cytoplasmic tyrosine kinases, is expressed in T cells, natural killer cells, and some B cells. Lck binds to the cytoplasmic domain of CD4 and CD8 (11, 12) and plays an essential role in T-cell activation and development. Lck is required for T-cell receptor signaling in the human Jurkat T-cell leukemia line (13) and for antigen receptor-dependent cytolytic effector function in the CTLL-2 T-cell line (14). In addition, mice lacking expression of functional Lck or overexpressing an inactive form of Lck have severely disrupted thymocyte development (15, 16). Lck is a typical Src-like kinase in that its activity is inhibited by phosphorylation of a highly conserved tyrosine residue, Tyr-505, located near the carboxyl terminus (17, 18). The tyrosine protein kinase CSK phosphorylates Lck at this site and is a natural inhibitor of Lck kinase activity (19). Expression of the tyrosine protein phosphatase CD45 leads to dephosphorylation of Tyr-505, which suggests that CD45 is a natural activator of Lck (20–22). *In vitro*, Lck undergoes autophosphorylation at Tyr-394 (23, 24). The extent of phosphorylation of Tyr-394 *in vivo* correlates with Lck activity and appears to be required for maximum catalytic activity (25, 26).

Studies of the effects of oxidative stress on *src*-family tyrosine kinases have yielded contradictory results. Nakamura *et al.* (10) reported that treatment of human peripheral blood T lymphocytes with the oxidant diamide [1,1′-azobis(*N*,*N*-dimethylformamide)] increased $p56^{lck}$ catalytic activity and induced phosphorylation of $p56^{lck}$ at both Tyr-394 and Tyr-505. This treatment did not stimulate $p59^{fyn}$ activity in the same cells. In contrast, Schieven *et al.* (27) reported that exposure of Ramos cells to H_2O_2 alone stimulated the activity of $p72^{syk}$ but did not increase the kinase activities of $p56^{lck}$, $p53/56^{lyn}$, or $p59^{fyn}$. We found that H_2O_2 increased the catalytic activity of Lck in every cell line we examined. In addition, we found that phosphorylation of Tyr-394 is apparently required for H_2O_2-induced activation of Lck. Although Lck can phosphorylate itself at Tyr-394, we demonstrate here that Lck can be phosphorylated at Tyr-394 by a kinase other than Lck *in vivo*.

MATERIALS AND METHODS

Construction of *lck* Mutants. Site-directed mutagenesis of the murine *lck* cDNA (28) was performed as described (17). lck^{R273} was made by changing the codon for Lys-273 from AAG to AGG using the oligonucleotide 5′-TCAGACTCCTCACCGCC-3′ as the mutagenic primer. lck^{F394} and $lck^{F394F505}$ were made from wild-type *lck* or lck^{F505}, respectively, by changing the codon for Tyr-394 from TAC to TTT using the oligonucleotide 5′-CGGGCCGTAAACTCATTGTCC-3′. lck^{F192} was made by changing the codon for Tyr-192 from TAC to TTC using PCR and the complementary oligonucleotides 5′-ACGGTGGCTTCTTCATCTC-3′ and 5′-GAGATGAAGAAGCCACCGT-3′ (29). All mutations were confirmed by sequencing. Wild-type and mutant *lck* cDNAs were inserted into the retroviral expression vector LXSN (30). Helper-free recombinant retroviruses were produced by cotransfecting COSm6 cells with retroviral vector DNA and the viral helper plasmid, SV-Ψ⁻-E-MLV (31). After 48 hr of incubation in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum, supernatants were collected and used to infect rat 208F fibroblasts (32, 33).

Cell Lines. HPB-MLT, a human T-cell leukemia line (34), was maintained in RPMI 1640 medium supplemented with 10% calf serum (HyClone). Rat 208F fibroblasts expressing wild-type or mutant Lck were grown in DMEM containing 10% calf serum and 600 μg of G418 per ml (Geneticin; GIBCO/BRL).

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; ROI, reactive oxygen intermediate.

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H_2O_2 and Phorbol 12-Myristate 13-Acetate (PMA) Treatments. Suspension cells were collected by centrifugation, resuspended at 5×10^6 cells per ml, and exposed to H_2O_2 by adding a solution of 100 mM H_2O_2 (freshly made from a 30% H_2O_2 stock solution; Mallinckrodt) to a final concentration of 5 mM. After 15 min at 37°C the cells were collected by centrifugation at $400 \times g$ for 5 min at 4°C and washed once with ice-cold, Tris-buffered saline. 208F fibroblasts were seeded at a density of 2×10^6 cells per 10-cm tissue culture dish. Freshly made 100 mM H_2O_2 solution was added to the medium to a final concentration of 5 mM 18 hr later. After incubation at 37°C for 15 min, the medium was aspirated, and the cells were washed with ice-cold, Tris-buffered saline.

Immunoprecipitation and Western Blotting. Cells were lysed in RIPA buffer (35) for 20 min at 4°C. Suspension cells were lysed at a concentration of 10^7 cells per ml, adherent cells at an approximate concentration of 2×10^6 cells per ml. Fifteen microliters of *Staphylococcus aureus* cells (Pansorbin Cells; Calbiochem) were added and the lysates were clarified by centrifugation at $35,000 \times g$ for 30 min at 4°C. Lck was isolated by immunoprecipitation with a polyclonal antiserum (36) prebound to *S. aureus* cells. Whole cell lysates were prepared by directly lysing cells in SDS/gel sample buffer.

Immunoprecipitated Lck or whole cell lysates were fractionated by SDS/polyacrylamide gel electrophoresis (SDS/PAGE), transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P; Millipore), and subjected to Western blotting with anti-phosphotyrosine (37) or anti-Lck antibodies and ^{125}I-labeled protein A (ICN) as described (37, 38). Labeled protein was visualized by autoradiography or with a PhosphorImager (Molecular Dynamics, IMAGEQUANT 4.0 software).

***In Vitro* Kinase Assays.** For assays using rabbit muscle enolase as a substrate, Lck immunoprecipitates were incubated with acid-denatured enolase (Boehringer Mannheim), 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; ICN), and 1 μM ATP in 20 μl of kinase buffer (40 mM Pipes, pH 7.1/10 mM $MnCl_2$) at 25°C (36). The reactions were terminated by the addition of 2× SDS/gel sample buffer. Reactions were analyzed by electrophoresis on a 15% SDS/polyacrylamide gel, and phosphate incorporation into enolase was quantified with a PhosphorImager.

For assays using [Val^5]angiotensin II (Sigma) as a substrate, Lck immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]ATP and 2 mM angiotensin in 20 μl of kinase buffer at room temperature. The reactions were stopped by the addition of 5% trichloroacetic acid, and the angiotensin was 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 measured by liquid scintillation counting.

***In Vivo* Labeling and Peptide Mapping.** Lymphoid cells (2×10^7) were washed twice with phosphate-free medium, resuspended at 5×10^6 cells/ml in the same medium supplemented with phosphate-free fetal bovine serum, and incubated with $^{32}P_i$ ($H_3{}^{32}PO_4$; 0.5 mCi/ml; ICN) at 37°C for 5 hr. Fibroblasts ($\approx 2 \times 10^6$ per 10-cm dish) were washed twice with phosphate-free medium and incubated with $^{32}P_i$ (0.5 mCi/ml) in 4 ml of medium for 5 hr.

Labeled Lck was isolated by immunoprecipitation, resolved by gel electrophoresis, and transferred to a nitrocellulose membrane (Schleicher & Schuell). It was then digested on the membrane by L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin as described (39). Lck that had been autophosphorylated *in vitro* was used as a source of the Tyr-394-containing peptide. Two-dimensional tryptic peptide mapping was carried out on cellulose thin-layer chromatography plates by electrophoresis at pH 8.9 in the first dimension, followed by ascending chromatography in phospho-chromatography buffer as described (40). Labeled peptides were visualized by autoradiography.

RESULTS

Increase in Tyrosine Phosphorylation and Activation of Lck by H_2O_2. Lck was immunoprecipitated from HPB-MLT human T leukemia cells before or after the cells were exposed to H_2O_2 and assayed for catalytic activity using enolase or [Val^5]angiotensin II as exogenous substrates. Fifteen minutes of exposure to 5 mM H_2O_2 induced a 3-fold increase in activity as measured with either angiotensin (Fig. 1*A*) or enolase as an exogenous substrate (data not shown). Three- to 8-fold increases in Lck catalytic activity were also observed in response to similar treatment in WEHI 231 murine B cells, LSTRA murine T cells that overexpress Lck, Jurkat human T leukemia cells, SAKRTLS12.1 murine T leukemia cells, murine CTLL-2 cells (data not shown), and 208F rat fibroblasts expressing Lck (see below).

To examine whether activation was a result of H_2O_2 acting directly on the kinase, Lck isolated by immunoprecipitation from untreated cells was incubated with H_2O_2 *in vitro* and then assayed for protein kinase activity using enolase as an exogenous substrate. H_2O_2 at concentrations from 1 to 100 mM induced no detectable increase in activity. At a concentration of 200 mM (50-fold higher than the concentrations used for routine stimulation), H_2O_2 inhibited Lck activity (data not shown).

To determine whether H_2O_2 stimulated the tyrosine phosphorylation of Lck, we analyzed immunoprecipitated Lck by Western blotting with anti-phosphotyrosine antibodies. In HPB-MLT cells, the binding of anti-phosphotyrosine antibodies to Lck increased 5-fold following H_2O_2 treatment (Fig. 1*B*). In other cells lines (WEHI 231, LSTRA, Jurkat, SAKR,

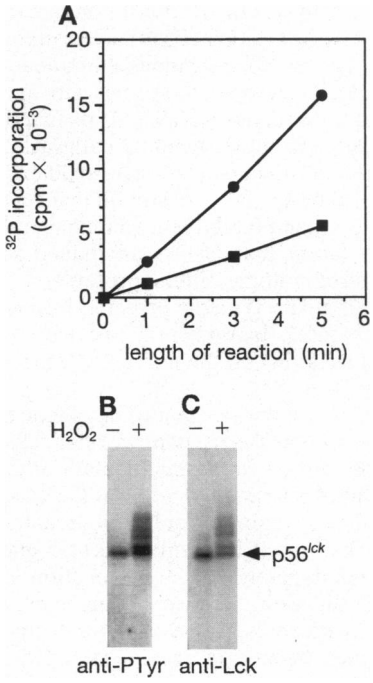

FIG. 1. Effect of H_2O_2 on *in vitro* kinase activity and tyrosine phosphorylation of p56^{lck} in HPB-MLT cells. p56^{lck} immunoprecipitates were prepared from HPB-MLT cells before and after exposing the cells to 5 mM H_2O_2 (see text). (*A*) *In vitro* kinase assay using 2 mM [Val^5]angiotensin II as an exogenous substrate. ■, Untreated; ●, H_2O_2 treated. (*B*) Anti-phosphotyrosine immunoblot of p56^{lck} immunoprecipitated from untreated (−) or treated (+) cells. (*C*) Anti-p56^{lck} immunoblot of p56^{lck} immunoprecipitated from untreated (−) or treated (+) cells. The arrow indicates the position of p56^{lck}.

CTLL-2, and 208F fibroblasts expressing Lck), increases between 3- and 5-fold were observed (data not shown). Treatment of cells with H_2O_2 also induced a shift in the electrophoretic mobility of the protein (Fig. 1*C*). In unstimulated HPB-MLT cells, Lck migrates as a homogeneous 56-kDa species. Multiple forms of Lck were observed in H_2O_2-treated HPB-MLT cells and exhibited apparent molecular masses between 56 and 65 kDa. A similar shift in the mobility of Lck is seen in Lck isolated from T cells stimulated with PMA (41–43).

The modification inducing this shift does not, however, appear to be involved in H_2O_2-induced activation. Even though treatment of cells with 100 ng of PMA per ml for 30 min induced a similar shift in the mobility of Lck in HPB-MLT cells, this treatment did not increase the catalytic activity of Lck (data not shown). In addition, neither H_2O_2 nor PMA induced a shift in the mobility of Lck expressed in 208F cells, even though H_2O_2 activated Lck enzymatically (data not shown). Thus, the modification of Lck responsible for the shift in mobility following exposure to H_2O_2, which may be the same as the modification induced by PMA, is apparently not responsible for enzymatic activation.

H_2O_2 Treatment Results in Increased Phosphorylation of Tyrosine Residues 394 and 505. Lck contains two major sites of tyrosine phosphorylation, Tyr-394 and Tyr-505. In addition, the phosphorylation of Tyr-192 in phenylarsine oxide-treated LSTRA cells, possibly mediated by the tyrosine protein kinase Syk, was reported recently (44). To identify residues phosphorylated to an increased extent in cells exposed to H_2O_2, HPB-MLT cells were labeled biosynthetically with $^{32}P_i$, and the phosphorylation of Lck was examined by two-dimensional tryptic peptide mapping. The heterogeneously migrating forms of Lck from cells treated with H_2O_2 were combined for analysis.

Lck from untreated HPB-MLT cells yields only one major phosphorylated tryptic peptide that contains Tyr-505 (Fig. 2, panel 1). H_2O_2 treatment increased the phosphorylation of an additional peptide that migrated precisely with the peptide containing phosphorylated Tyr-394 4-fold (Fig. 2, panel 2). Similar results were obtained with Lck isolated from LSTRA and WEHI 231 cells as well as from 208F fibroblasts expressing Lck (data not shown). The level of phosphorylation of the Tyr-505-containing peptide also appeared to increase slightly. No significant increase in phosphorylation of other peptides resolved by this technique was observed following H_2O_2 treatment.

Phosphorylation of Tyr-394 Is Required for H_2O_2-Induced Activation of Lck. To ascertain the role of phosphorylation of Tyr-394 and Tyr-505 in the activation of Lck by H_2O_2, we asked whether mutant forms of Lck in which either Tyr-394, Tyr-505, or both residues had been converted to phenylalanine (Lck^{F394}, Lck^{F505}, and $Lck^{F394F505}$) were activated following exposure of cells to H_2O_2. The mutant Lck proteins were expressed in 208F rat fibroblasts that express no endogenous Lck (32, 33). Under conditions where H_2O_2 stimulated the activity of the wild-type protein 3-fold and stimulated the activity of Lck^{F505} 2-fold, the catalytic activities of Lck^{F394} and $Lck^{F394F505}$ were unchanged (Fig. 3*A*). It appears, therefore, that Tyr-394 and, presumably,

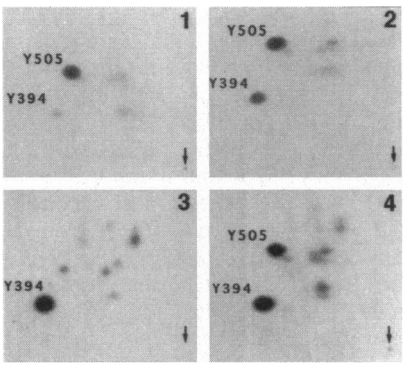

FIG. 2. Analysis of $p56^{lck}$ phosphorylation following treatment of cells with H_2O_2. Immunoprecipitates were prepared from cells that had been labeled biosynthetically with $^{32}P_i$ and exposed to 5 mM H_2O_2. Lck was purified by SDS/PAGE, transferred to nitrocellulose, and digested with trypsin. The resulting peptides were separated horizontally by electrophoresis at pH 8.9 and by ascending chromatography. Panels: 1, $p56^{lck}$ from unstimulated HPB-MLT cells; 2, $p56^{lck}$ from H_2O_2-treated HPB-MLT cells; 3, $p56^{lck}$ autophosphorylated *in vitro*; 4, mixture of panels 2 and 3. The Tyr-containing tryptic peptides are labeled. The sample origins are indicated by arrows. The autoradiograms were exposed at −70°C for 80 hr.

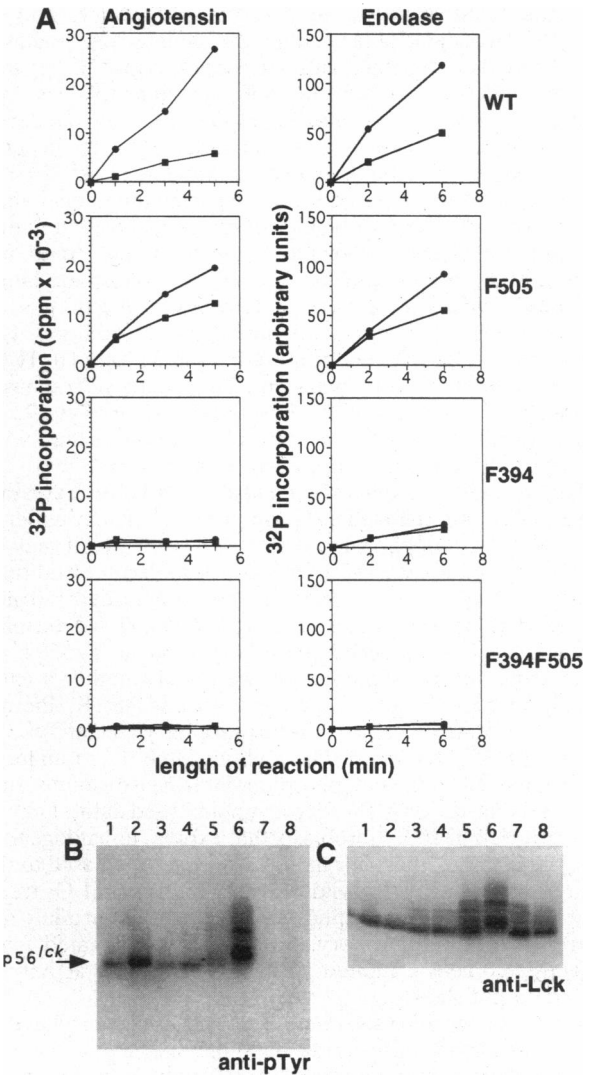

FIG. 3. Effect of H_2O_2 on the activity of Lck mutants lacking sites of tyrosine phosphorylation. Mutant and wild-type (WT) forms of $p56^{lck}$ were expressed in 208F fibroblasts and isolated by immunoprecipitation before or after exposing the cells to 5 mM H_2O_2. (*A*) *In vitro* kinase assays of wild-type or mutant $p56^{lck}$ using [Val^5]angiotensin II (left) or enolase (right) as an exogenous substrate. ■, Untreated; ●, treated. (*B*) Anti-phosphotyrosine immunoblot. $p56^{lck}$ isolated from untreated (lanes 1, 3, 5, and 7) and H_2O_2-treated (lanes 2, 4, 6, and 8) fibroblasts was resolved on a 15% polyacrylamide gel, transferred to PVDF membrane, and detected with anti-phosphotyrosine antibodies and ^{125}I-labeled protein A. Lanes 1 and 2, wild-type Lck; lanes 3 and 4, Lck^{F394}; lanes 5 and 6, Lck^{F505}; lanes 7 and 8, $Lck^{F394/F505}$. (*C*) Corresponding anti-$p56^{lck}$ immunoblot of the $p56^{lck}$ immunoprecipitates from *B*.

its phosphorylation are required for H_2O_2-induced activation of Lck. Although the *in vitro* kinase activities of Lck^{F394} and $Lck^{F394F505}$ were lower than the activity of wild-type Lck, both proteins could phosphorylate enolase *in vitro* (Fig. 3*A*). The basal kinase activities of Lck^{F394} and $Lck^{F394F505}$ were 50% and 10–20% of that in wild-type Lck, respectively (Fig. 3*A*). Although a direct comparison between the activity of Lck^{F394} and $Lck^{F394F505}$ has not been made in previous studies, Lck^{F394} (45) and $Lck^{F394F505}$ (26, 46, 47) have been studied independently by other researchers who have observed levels of catalytic activity similar to those of our mutants.

We also examined the tyrosine phosphorylation of Lck^{F394}, Lck^{F505}, and $Lck^{F394F505}$ following treatment of cells with H_2O_2 by Western blotting with anti-phosphotyrosine antibodies (Fig. 3*B*). Tyrosine phosphorylation of Lck^{F505} increased strongly in H_2O_2-treated cells. The tyrosine phosphorylation on Lck^{F394} was stimulated slightly. No tyrosine phosphorylation of $Lck^{F394F505}$ was detectable in either unstimulated or stimulated cells. These data, together with our peptide maps, suggest that Tyr-394 and Tyr-505 are the only tyrosine residues that become phosphorylated following H_2O_2 treatment. To determine more rigorously if any other tyrosine residues become phosphorylated, we labeled 208F cells expressing $Lck^{F394F505}$ *in vivo* with $^{32}P_i$ and subjected Lck to phosphoamino acid analysis. We observed a very low level of tyrosine phosphorylation that did not change following H_2O_2 treatment (data not shown). Therefore, it appears that extensive phosphorylation of Tyr-192, which can be carried out by Syk *in vitro* (44), is not induced by H_2O_2 treatment in fibroblasts. In addition, Lck containing the Tyr-192 → Phe mutation was activated by H_2O_2 to the same degree as wild-type Lck (data not shown). Although Lck may become phosphorylated on Tyr-192 in lymphoid cells expressing Syk, phosphorylation of this site is not required for H_2O_2-induced activation of Lck.

H_2O_2-Induced Phosphorylation of Tyr-394 Is Not Necessarily a Result of Autophosphorylation. *In vivo*, Lck may undergo intramolecular autophosphorylation at Tyr-394, or it may be phosphorylated in *trans* by a second Lck molecule. Additionally, there may be one or more as yet unidentified tyrosine kinases that phosphorylate Lck at Tyr-394. To determine whether the H_2O_2-induced phosphorylation at Tyr-394 resulted from autophosphorylation, we asked whether a catalytically inactive form of Lck (refs. 16 and 48; K. Pierno, unpublished data) became tyrosine phosphorylated at Tyr-394 following H_2O_2 treatment. The ability of Lck^{R273} to undergo autophosphorylation and phosphorylate an exogenous substrate is reduced >98% (K. Pierno, unpublished data). Lck^{R273} was expressed in 208F fibroblasts that express no endogenous Lck. The tyrosine phosphorylation of Lck^{R273} increased to the same extent as seen with wild-type Lck following H_2O_2 treatment (Fig. 4*A*). This phosphorylation is almost certainly not the result of autophosphorylation as Lck^{R273} isolated from H_2O_2-treated cells exhibited no detectable kinase activity *in vitro* (data not shown).

To determine which sites were becoming phosphorylated in Lck^{R273}, we carried out peptide mapping experiments. Lck^{R273} was phosphorylated predominantly at Tyr-505 in unstimulated cells (Fig. 4*B*, panel 1). H_2O_2 treatment induced the appearance of an additional phosphorylated peptide that comigrated precisely with a marker peptide containing phosphorylated Tyr-394 (Fig. 4*B*, panel 2). This indicates that one or more kinases expressed in fibroblasts have the ability to phosphorylate Lck at Tyr-394 and that the phosphorylation of Tyr-394 need not occur intramolecularly.

DISCUSSION

Treatment of cells with H_2O_2 increases the catalytic activity of Lck through a mechanism other than direct oxidation of the protein. Lck is known to be activated by dephosphorylation of

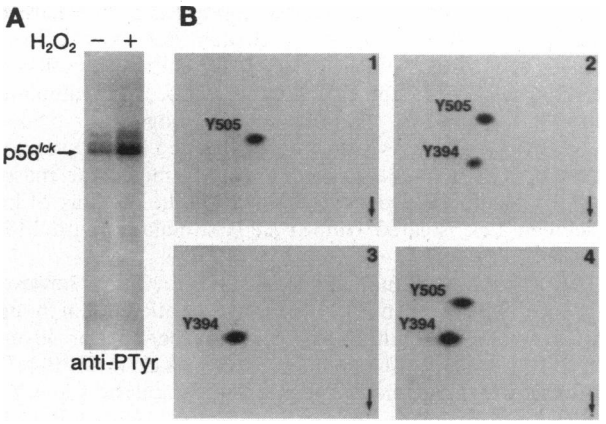

FIG. 4. Effect of H_2O_2 on catalytically inactive Lck. (*A*) Anti-phosphotyrosine immunoblot of Lck^{R273} isolated from fibroblasts before (−) or after (+) exposure to H_2O_2. The arrow indicates the position at which wild-type Lck from unstimulated cells migrated on the same gel. [Val^5]angiotensin II was used as an exogenous substrate (see text). (*B*) Two-dimensional tryptic peptide maps of Lck^{R273}. Panels: 1, Lck^{R273} from unstimulated fibroblasts labeled biosynthetically with $^{32}P_i$; 2, Lck^{R273} from cells labeled biosynthetically with $^{32}P_i$ and treated with H_2O_2; 3, Lck autophosphorylated *in vitro*; 4, mixture of autophosphorylated Lck and Lck^{R273} from H_2O_2-treated fibroblasts. The Tyr-505- and Tyr-394-containing tryptic peptides of Lck are labeled. Sample origins are indicated by arrows.

Tyr-505 by CD45 (20). Phosphorylated Tyr-505 can apparently bind the SH2 domain of Lck, thereby inducing a molecular conformation that inhibits catalytic activity (49, 50). Dephosphorylation of Tyr-505 relieves this repression. H_2O_2 does not, however, appear to increase Lck activity through induced dephosphorylation of Tyr-505. We observed no decrease in Tyr-505 phosphorylation following H_2O_2 treatment (Figs. 2 and 3*B*). Additionally, Lck^{F505}, which is not phosphorylated at position 505, could still be activated by H_2O_2 (Fig. 3*A*). If dephosphorylation of Tyr-505 were the sole mechanism of Lck activation, Lck^{F505} activity should not be stimulated by H_2O_2. Furthermore, the finding that H_2O_2 can stimulate Lck activity in a T-cell line lacking CD45 (data not shown) argues against induced dephosphorylation of Tyr-505 playing a primary role in H_2O_2-mediated activation.

The phosphorylation of Tyr-394 increases following H_2O_2 treatment (Figs. 2, 3*B*, and 4*B*). Because treatment of cells with H_2O_2 did not activate Lck mutants (Lck^{F394} and $Lck^{F394F505}$) in which Tyr-394 had been converted to phenylalanine (Fig. 3*A*), the phosphorylation of Tyr-394 appears to be required for H_2O_2-mediated activation of Lck. In addition, the low catalytic activities of Lck^{F394} and $Lck^{F394/F505}$ from untreated cells (50% and 10% of wild type, respectively) suggest that phosphorylation of Tyr-394 may be of central importance to Lck kinase activity. Supporting this assertion are the observations that mutation of Tyr-394 interferes with the activation of Lck following cross-linking of CD4 (45), renders LckF505 incapable of transforming fibroblasts (26) and stimulating interleukin 2 production in BI-141 cells (46), and abolishes the ability to stimulate the expression of a c-*fos* reporter gene (47). Because the extent of phosphorylation of Tyr-505 is at least as great as that of Tyr-394 in cells treated with H_2O_2, it appears that stimulation of activity resulting from the induced phosphorylation of Tyr-394 may override inhibition of activity resulting from phosphorylation of Tyr-505. We cannot, however, rule out the possibility that two differentially modified subpopulations of Lck are present following H_2O_2 treatment, an activated population phosphorylated on Tyr-394, but not on Tyr-505, and a less active population phosphorylated only on Tyr-505. Determining the stoichiometry of phosphorylation of Lck at Tyr-394 and Tyr-505 is difficult because the protein is

phosphorylated at additional sites and because it is hard to resolve the multiply phosphorylated forms of Lck by isoelectric focusing (51). Phosphorylation-state-specific antibodies should allow an estimation of the extent of phosphorylation of Tyr-394 and Tyr-505, but these reagents are not currently available.

It is unclear whether phosphorylation of Tyr-394 induced by H_2O_2 treatment is due to activation of a kinase, inhibition of a phosphatase, or both. Because Secrist *et al.* (9) have shown that H_2O_2 treatment of cells inactivates CD45, perhaps by oxidizing the critical cysteine residue in the active site, it seems very likely that the effect of H_2O_2 is mediated in part by inhibition of one or more tyrosine phosphatases. If Tyr-394 were subject to constitutive phosphorylation, H_2O_2 treatment could induce an increase in phosphorylation simply by reducing the rate of dephosphorylation. One model for the increase in catalytic activity we have seen is that H_2O_2 inhibits dephosphorylation of Tyr-394 and allows Lck to activate itself by autophosphorylation. However, because catalytically inactive Lck is phosphorylated at Tyr-394 following exposure of cells that express no active Lck to H_2O_2, it is clear that Lck is subject to phosphorylation at Tyr-394 by at least one as yet unidentified tyrosine protein kinase. This unidentified kinase(s) may therefore be a natural activator of Lck and could well play a role in T-cell activation, which is known to require active Lck. It is possible that the induced phosphorylation of Lck at Tyr-394 results from H_2O_2-mediated activation of this activating kinase. Because it has been shown that treatment of cells with pervanadate (9) or diamide (10) also activates Lck and induces phosphorylation of Tyr-394, it is possible that these agents act through a similar mechanism.

The activation of Lck by H_2O_2 that we have observed is an extreme example of regulation by oxidants. However, it raises the possibility that Lck may be subject to regulation during oxidative stress and that ROIs may be involved in normal lymphocyte signaling pathways. An important question that remains is whether other *src* family members are subject to a similar form of regulation. In $pp60^{c\text{-}Src}$, phosphorylation of Tyr-416, the site analogous to Tyr-394 in Lck, has also been shown to affect the activity of the kinase in some situations. Overexpressed $pp60^{c\text{-}Src}$ that carries the Tyr → Phe-416 mutation has decreased transforming ability and reduced *in vitro* kinase activity compared to the wild-type protein (52–54). The possibility exists, therefore, that there is an activator of $pp60^{c\text{-}Src}$ that acts by phosphorylating Tyr-416. If our results with Lck extend to $pp60^{c\text{-}Src}$, this regulation by phosphorylation of Tyr-416 may not be due simply to intermolecular autophosphorylation (53).

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