The Activated Form of the Lck Tyrosine Protein Kinase in Cells Exposed to Hydrogen Peroxide Is Phosphorylated at Both Tyr-394 and Tyr-505*

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James S. Hardwick‡§ and Bartholomew M. Sefton‡
From the ‡Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies and the §Department of Biology, University of California at San Diego, La Jolla, California 92037

Members of the Src family of non-receptor tyrosine protein kinases are known to be inhibited by the intramolecular association between a phosphorylated carboxyl-terminal tyrosine residue and the SH2 domain. We have previously shown that exposure of cells to H2O2 strongly activates Lck, a lymphocyte-specific Src family kinase, by inducing phosphorylation on Tyr-394, an absolutely conserved residue within the activation loop of the catalytic domain. Here we show that Lck that has been activated by H2O2 is simultaneously phosphorylated at both the carboxyl-terminal tyrosine (Tyr-505) and Tyr-394. Thus, dephosphorylation of Tyr-505 is not a prerequisite for either phosphorylation of Lck at Tyr-394 or catalytic activation of the kinase. These results indicate that activation of Lck by phosphorylation of Tyr-394 is dominant over any inhibition induced by phosphorylation of Tyr-505. We propose that these results may be extended to all Src family members.

The Salk Institute for Biological Studies and the Department of Biology, University of California at San Diego, La Jolla, California 92037

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‡ Supported by National Institutes of Health Training Grant 2T32-GM07240. To whom correspondence should be addressed: Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, P.O. Box 58500, San Diego, CA 92186. Tel.: 619-453-4100 (Ext. 1331); Fax: 619-457-4765; E-mail: hardwick@biomail.ucsd.edu.

‡‡ The abbreviations used are: Tyr(P), phosphotyrosine; PVDF, polyvinylidene difluoride.
rabbit polyclonal antisera specific for a phosphorylated peptide identical to the sequence flanking Tyr-416 of Src (a generous gift from M. Weber) that was prebound to S. aureus cells. Immune complexes were pelleted by centrifugation and washed three times in RIPA buffer and once in TN buffer (40 mM Tris, pH 7.5, 150 mM NaCl). Remaining Lck proteins not immunoprecipitated by the α-Tyr(P)-416 sera were isolated by re-immunoprecipitation of the supernatant with the Lck-specific antisera.

**Immunoblotting**—Immunoprecipitated Lck proteins were resolved by gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF, Immobilon-P, Millipore) membrane. Western blotting was carried out with α-Tyr(P)-416, α-Tyr(P) (33), or α-Lck antibodies and 125I-protein A (ICN) (33, 34) as described previously. The α-Tyr(P)-416 antisera were diluted 1:200 before use.

**Bioassay**—105 Jurkat cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium and incubated with [32P]phosphoric acid (H3PO4, 0.5 mCi/ml; ICN) in 4 ml of medium at 37 °C for 5 h. 32P-Labeled Lck was isolated by immunoprecipitation and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin as described (35). 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 phosphochromatography buffer as described (36). Labeled peptides were visualized by autoradiography. Relative peptide phosphorylation levels were determined with a PhosphorImager (Molecular Dynamics).

**RESULTS**

A Phosphorylation State-specific Antiserum Specifically Recognizes Lck That Is Phosphorylated on Tyr-394—To examine whether Lck phosphorylated at Tyr-394 following exposure of cells to H2O2 was also phosphorylated at Tyr-505, we used an antibody raised against a phosphorylated peptide from the region in Src containing Tyr-416. This antiserum (α-Tyr(P)-416, a generous gift of M. Weber) cross-reacts with Lck phosphorylated at Tyr-394 because the amino acid sequences flanking Tyr-394 in Lck are identical to those flanking Tyr-416 in Src. To verify that this serum specifically recognized Lck that was phosphorylated at Tyr-394, we first tested it in Western blotting. We isolated Lck from Jurkat human T leukemia cells or from rat 208F fibroblast cell lines expressing Lck by immunoprecipitation with α-Lck antibodies before and after the cells had been exposed to H2O2. 208F fibroblasts express no endogenous Lck. Wild type Lck from either unstimulated T cells or fibroblasts is highly phosphorylated at Tyr-505 and poorly phosphorylated at Tyr-394 (22, 32). In contrast, a constitutively active form of Lck where Tyr-505 is mutated to phenylalanine (LckF505) is highly phosphorylated on Tyr-394 in unstimulated cells (16). A double mutant of Lck (LckA2F505), that lacks the amino-terminal myristoylation site (gly-2) and contains the carboxyl-terminal tyrosine to phenylalanine mutation completely lacks tyrosine phosphorylation in unstimulated cells (21, 37). All of these forms of Lck isolated from H2O2-stimulated cells are highly phosphorylated on Tyr-394 (22, 37).

When Lck from untreated cells was examined, α-Tyr(P)-416 reacted strongly with LckF505 but poorly or not at all with wild type Lck and LckA2F505 (Fig. 1A). In contrast, α-Tyr(P)-416 reacted with all forms of Lck isolated from H2O2-treated cells. H2O2 did not alter the level of Lck in either T cells or fibroblasts (Fig. 1B). These results rule out the possibility that significant amounts of contaminating anti-peptide reactivity or α-Tyr(P) reactivity that recognizes Tyr(P)-505 are present in the α-Tyr(P)-416 sera. If present, such antibodies would have recognized Lck from unstimulated cells because the majority of Lck in these cells is not phosphorylated on Tyr-394, but highly phosphorylated on Tyr-505.

**Tyr(P)-416-specific Antisera Only Immunoprecipitates Lck from H2O2-stimulated Cells**—We also tested whether the α-Tyr(P)-416 sera exhibited specificity for Tyr(P)-394 in immunoprecipitation. Total Lck was isolated by immunoprecipitation from Jurkat cells with α-Lck antibodies before and after H2O2 stimulation, and the Lck immunoprecipitates were subsequently boiled to both disassociate the complex and inactivate the α-Lck immunoglobulin. Lck molecules containing Tyr(P)-394 were then isolated by immunoprecipitation with α-Tyr(P)-416 antisera. We analyzed the resulting immune complexes by Western blot with α-Lck antibodies (Fig. 2A) and α-Tyr(P) antibodies (Fig. 2B).

Lck from unstimulated cells was immunoprecipitated with α-Tyr(P)-416 very inefficiently, consistent with the low level of Tyr(P)-416 phosphorylation. In contrast, the α-Tyr(P)-416 serum readily immunoprecipitated Lck from Jurkat cells that had been exposed to H2O2. Equal amounts of Lck were present in both stimulated and unstimulated samples as determined by α-Lck Western blot (Fig. 2A, lanes 3 and 4). To exclude the possibility that any α-Lck antibodies from the initial immunoprecipitation renatured following the boiling step, we immunoprecipitated with S. aureus cells alone. No Lck was immunoprecipitated indicating that no functional α-Lck antibodies remained after boiling (data not shown). Thus, the α-Tyr(P)-416 serum exhibited good specificity for Lck phosphorylated at Tyr-394 in both Western blotting and immunoprecipitation.

The shift in electrophoretic mobility seen in Lck following cellular exposure to H2O2 (Figs. 1 and 2) appears to be a result of protein phosphorylation because bacterial alkaline phosphatase is able to collapse the multiple Lck bands to a single 56-kDa band. In addition, it is likely that serine phosphorylation is more important than tyrosine phosphorylation for H2O2-induced Lck mobility shifts because stimulation of cells with 12-O-tetradecanoylphorbol-13-acetate induces identical shifts without inducing tyrosine phosphorylation of Lck or activating Lck.2

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2 J. S. Hardwick and B. M. Sefton, unpublished results.
**H$_2$O$_2$-activated Lck Is Phosphorylated at Tyr-394 and Tyr-505**

**FIG. 2. Analysis of the specificity of α-Tyr(P)-416 antiserum by immunoprecipitation.** Total Lck from Jurkat T cells was isolated by immunoprecipitation with α-Lck antibodies. Following dissociation of the initial immune complexes, Lck proteins were reprecipitated with α-Tyr(P)-416 antibodies. Remaining Lck proteins not immunoprecipitated by the α-Tyr(P)-416 sera were isolated by re-immunoprecipitation of the supernatant with α-Lck antibodies. Each sample of isolated Lck was divided into two fractions, resolved electrophoretically in a 15% gel and transferred to PVDF membranes. The two fractions were detected with either α-Lck antibodies (A) or α-Tyr(P) antibodies (B) combined with $^{32}$P-protein A. Lane 1, α-Tyr(P)-416 re-precipitation from unstimulated Jurkat cells; lane 2, α-Tyr(P)-416 re-precipitation from from Jurkat cells stimulated with 5 mM H$_2$O$_2$; lane 3, α-Lck reprecipitation from unstimulated Jurkat cells; lane 4, α-Lck re-precipitation from H$_2$O$_2$-stimulated Jurkat cells.

Lck Proteins from H$_2$O$_2$-stimulated Cells Are Simultaneously Phosphorylated on Tyr-394 and Tyr-505—To determine whether Lck proteins that are phosphorylated on Tyr-394 following H$_2$O$_2$ stimulation are also phosphorylated on Tyr-505, we labeled Jurkat cells biosynthetically with $^{32}$P and then exposed the cells to H$_2$O$_2$. Total Lck was isolated first by immunoprecipitation with α-Lck antibodies, and the immunoprecipitates were then boiled. Lck proteins phosphorylated on Tyr-394 were then reimmunoprecipitated with α-Tyr(P)-416 antisera. The isolated Lck was subjected to two-dimensional trypsin peptide analysis (Fig. 3).

Analysis of the total population of Lck from unstimulated T cells or rat fibroblasts, immunoprecipitated with α-Lck antibodies, showed that a very small fraction of Lck molecules was phosphorylated on Tyr-394; the Tyr(P)-505:Tyr(P)-394 ratio was 15:1 in T cells and 34:1 in fibroblasts (Fig. 3, A and E). In contrast, the ratio of Tyr(P)-505 to Tyr(P)-394 in Lck immunoprecipitated with α-Tyr(P)-416 from unstimulated T cells was 0.35:1 (Fig. 3C). This showed that approximately one-third of the small percentage of Lck molecules from unstimulated T cells that were phosphorylated on Tyr-394 were also phosphorylated on Tyr-505. No Lck molecules phosphorylated on Tyr-394 could be detected by immunoprecipitation with α-Tyr(P)-416 from unstimulated fibroblasts (Fig. 3G). Following H$_2$O$_2$ stimulation of T cells and rat fibroblasts, both the phosphorylation of Tyr-394 and the amount of Lck precipitable by α-Tyr(P)-416 increased dramatically (Fig. 2A, compare lanes 1 and 2; Fig. 3, compare B and D to A and compare F and E to F). We found that the ratio of Tyr(P)-505 to Tyr(P)-394 in Lck isolated with α-Tyr(P)-416 from H$_2$O$_2$-stimulated T cells and fibroblasts ranged from 0.940:1 to 0.955:1 in three independent experiments. (Fig. 3, D and H). This result indicates that approximately 95% of the population of Lck molecules phosphorylated on Tyr-394 following H$_2$O$_2$ stimulation was also phosphorylated on Tyr-505.

**FIG. 3. Analysis of Lck phosphorylation following stimulation by H$_2$O$_2$.** Lck was isolated by immunoprecipitation from cells labeled with $^{32}$P, before or after the cells were exposed to 5 mM H$_2$O$_2$, and tryptic peptide mapping was carried out as described under “Experimental Procedures.” Origins are indicated by arrowheads. A, Lck proteins isolated with α-Lck antibodies from unstimulated Jurkat cells; B, Lck proteins isolated with α-Lck antibodies from Jurkat cells stimulated with 5 mM H$_2$O$_2$; C, Lck proteins isolated from unstimulated Jurkat cells and reprecipitated with α-Tyr(P)-416 antibodies; D, Lck proteins isolated from H$_2$O$_2$-stimulated Jurkat cells and reprecipitated with α-Tyr(P)-416 antibodies; E, Lck proteins isolated with α-Lck antibodies from unstimulated rat 208F fibroblasts expressing murine Lck; F, Lck proteins isolated with α-Lck antibodies from H$_2$O$_2$-stimulated 208F fibroblasts; G, Lck proteins isolated from unstimulated 208F fibroblasts and reprecipitated with α-Tyr(P)-416 antibodies; H, Lck proteins isolated from H$_2$O$_2$-stimulated 208F fibroblasts and reprecipitated with α-Tyr(P)-416 antibodies.

by mutation of Tyr-505 to phenylalanine loses its transforming ability when Tyr-394 is also mutated to phenylalanine (21, 40). We previously showed that the extent of Tyr-505 phosphorylation in Lck from H$_2$O$_2$-stimulated cells was at least as great as that of Tyr-394. Thus, H$_2$O$_2$ activation appeared not to require Tyr-505 dephosphorylation. These observations suggested that Tyr-394 phosphorylation could override any negative regulation of Lck due to Tyr-505 phosphorylation. However, it was impossible to rule out the possibility that two differentially phosphorylated subpopulations of Lck existed in H$_2$O$_2$-stimulated cells, a relatively inactive population phosphorylated only on Tyr-505 and an activated population phosphorylated only on Tyr-394. Thus, it was formally possible that activation of Lck by Tyr-394 phosphorylation occurred only in the absence of phosphorylation of Tyr-505.

Through the use of a phosphorylation state-specific antibody, we have now shown formally that Lck that is phosphorylated on Tyr-505 may be additionally phosphorylated on Tyr-394. If dephosphorylation of Tyr-505 were required for phosphorylation of Tyr-394, we would expect that Lck immunoprecipitated

**DISCUSSION**

The catalytic activity of Lck is greatly influenced by the phosphorylation state of Tyr-394 (22, 38, 39). Mutation of Tyr-394 to phenylalanine not only decreases Lck activity in unstimulated cells, but also prevents activation of Lck by oxidative stress. In addition, Lck that has been genetically activated...
by α-Tyr(P)-416 sera would only be phosphorylated on Tyr-394. This was not seen. The observation that the Tyr(P)-505:Tyr(P)-394 ratio is approximately 1:1 suggests that the molecules phosphorylated on Tyr-394 are also phosphorylated on Tyr-505. Thus, the activating effects of Tyr-394 phosphorylation are dominant over the inhibitory effects of Tyr-505 phosphorylation. Apparently, even when Lck is in a “closed” conformation with Tyr(P)-505 bound to the SH2 domain and the SH3 domain bound to the polyproline type II helix in the linker region between the SH2 and catalytic domains (19, 20), Tyr-394 is still accessible as a substrate for phosphorylation. Comparison of the crystal structure of Lck phosphorylated at Tyr-394 (24) with that of Src and Hck lacking phosphorylation at this site (19, 20) suggests that phosphorylation of Tyr-394 allows the formation of hydrogen bonds between Tyr(P)-394 and Arg-387 (19, 20) suggests that phosphorylation of Tyr-394 allows the repositioning of Glu-288, Leu-385, and Arg-387 (24), and this in turn allows Lys-273, Glu-288, Asp-364, Asn-369, and Asp-382, residues critical to ATP binding and phosphate transfer, to assume positions characteristic of an active catalytic site (24–26). Our data suggest that such repositioning of residues in the catalytic site of Lck can occur while the SH3 domain is still intramolecularly bound to the SH2-kinase linker region.

Our results agree with data presented by others who showed that the Src tyrosine kinase retains activity when phosphorylated on Tyr-416 and Tyr-527 (41). Previous work in our laboratory as well as kinetic data by other groups suggest that the activating phosphorylation of Tyr-394 in Src family members is an intermolecular event rather than intramolecular reaction (22, 42–44). Intermolecular phosphorylation of Tyr-394 may be carried out by Lck in vivo, but it is quite possible that other Src family members, or non-Src tyrosine kinases, may also act to phosphorylate Tyr-394 and activate Lck. Consistent with this second possibility is our finding that H2O2 stimulates the activation loop and the C-terminal tyrosine simultaneously. Apparently, even when Lck is in a “closed” conformation in the absence of dephosphorylation of the conserved Tyr505 is catalytically activated. Thus, activation of Lck can occur in the absence of Tyr505 dephosphorylation or SH3 domain dissociation from the SH2-kinase linker. It is reasonable to predict that phosphorylation of the absolutely conserved tyrosine in the activation loop of other Src kinases will also activate them in the absence of dephosphorylation of the conserved carboxyl-terminal tyrosine.

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3 J. D. Bjorge and D. J. Fujita, personal communication.
4 K. Pierro and B. M. Sefton, unpublished results.