Phosphorylation of a Src Kinase at the Autophosphorylation Site in the Absence of Src Kinase Activity*

(Received for publication, November 24, 1999, and in revised form, December 23, 1999)

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Exposure of cells to oxidants increases the phosphorylation of the Src family tyrosine protein kinase Lck at Tyr-394, a conserved residue in the activation loop of the catalytic domain. Kinase-deficient Lck expressed in fibroblasts that do not express any endogenous Lck has been shown to be phosphorylated at Tyr-394 following H2O2 treatment to an extent indistinguishable from that seen with wild type Lck. This finding indicates that a kinase other than Lck itself is capable of phosphorylating Tyr-394. Because fibroblasts express other Src family members, it remained to be determined whether the phosphorylation of Tyr-394 was carried out by another Src family kinase or by an unrelated tyrosine protein kinase. We examined here whether Tyr-394 in kinase-deficient Lck was phosphorylated following exposure of cells devoid of endogenous Src family kinase activity to H2O2. Strikingly, treatment of such cells with H2O2 led to the phosphorylation of Tyr-394 to an extent indistinguishable from that seen with wild type Lck. This finding indicates that a kinase other than Lck itself is capable of phosphorylating Tyr-394. Because fibroblasts express other Src family members, it remained to be determined whether the phosphorylation of Tyr-394 was carried out by another Src family kinase or by an unrelated tyrosine protein kinase. We examined here whether Tyr-394 in kinase-deficient Lck was phosphorylated following exposure of cells devoid of endogenous Src family kinase activity to H2O2. Strikingly, treatment of such cells with H2O2 led to the phosphorylation of Tyr-394 to an extent identical to that seen with wild type Lck, demonstrating that Src family kinases are not required for H2O2-induced phosphorylation of Lck. Furthermore, this efficient phosphorylation of Lck at Tyr-394 in non-lymphoid cells suggests the existence of an ubiquitous activator of Src family kinases.

Members of the Src family of non-receptor tyrosine protein kinases have important roles in controlling growth, proliferation, and differentiation (1). For example, the Src family kinases c-Src, Yes, and Fyn are required for integrin-mediated signaling in response to cell adhesion to the extracellular matrix (2). Additionally, Lck, a lymphoid cell-specific member of the Src family (3), is essential for both the development of T cells in the thymus and the response of mature T cells to signals arising from the T-cell antigen receptor (4, 5).

Src family kinases are 52–62-kDa cytoplasmic proteins consisting of an acylated N-terminal unique domain that mediates association with the plasma membrane, an SH3 domain, an SH2 domain, a catalytic domain, and a short C-terminal regulatory tail (6). The SH3 domain interacts with poly-proline type II helices (7), whereas the SH2 domain binds sites of tyrosine phosphorylation (8). Both the SH3 and SH2 domains play a role in intramolecular regulation of Src family kinase activity (9, 10).

The kinase activity of Src family members can be both inhibited and activated by phosphorylation. Phosphorylation of a conserved tyrosine near the C terminus (Tyr-505 in Lck, Tyr-527 in c-Src) by the ubiquitous tyrosine protein kinase Csk (11, 12) induces formation of a biologically inactive conformation by allowing intramolecular binding of the SH2 domain to the phosphorylated C terminus (9, 13, 14). This inactive “closed” conformation is further stabilized by binding of the SH3 domain to the linker between the SH2 domain and the catalytic domain (9, 10). Dephosphorylation of this site by the tyrosine phosphatase CD45, in the case of Lck, or R-PTP-α, in the case of c-Src, activates the kinase (15–18).

In contrast, phosphorylation of a conserved tyrosine in the activation loop (Tyr-394 in Lck, Tyr-416 in c-Src) enhances kinase activity (19–21). In Lck, the phosphorylation of Tyr-394 is activating because it stabilizes the catalytic active site by forming hydrogen bonds with Arg-387 and Arg-363 and hydrophobic interactions with Ile-361 and Ile-389 (22). Phosphorylation of this site can be carried out by the kinase itself, and it is the major site of “autophosphorylation” in vitro (23, 24). Studies of c-Src activity in vitro and in yeast indicate that phosphorylation of the activation loop in c-Src is an intermolecular autophosphorylation event (25, 26). The same appears to be the case with Lck and Lyn (21, 27). Dephosphorylation of Tyr-394 in Lck is mediated by the tyrosine phosphatases PEP and SHP-1 (28).

Expression of cells to oxidants such as hydrogen peroxide and pervanadate induces the rapid tyrosine phosphorylation of multiple cellular proteins (29) and mimics stimulation by growth factors such as epidermal growth factor or platelet-derived growth factor (30, 31) or antigen receptor cross-linking (32). These agents have this effect because both inhibit the activity of protein tyrosine phosphatases through the oxidation of an essential catalytic cysteine (33–36). The induced phosphorylation on tyrosine results, at least in part, from cessation of dephosphorylation in the oxidant-treated cells.

Hydrogen peroxide stimulates the activity of Src family kinases. Endogenously produced H2O2 stimulates the activity of the Src family kinases c-Fgr and Lyn in adherent neutrophils (37). Exposure of Jurkat T cells or Lck-expressing rat 208F fibroblasts to H2O2 induces the phosphorylation of Tyr-394 and enhances Lck kinase activity (21). Interestingly, this oxidant-induced phosphorylation of Tyr-394 in Lck occurs at an undiminished rate in fibroblasts expressing only kinase-deficient forms of Lck. Therefore, the kinase activity of Lck is not required for the oxidant-induced phosphorylation of Tyr-394, and...
another tyrosine protein kinase may function as an activator of Lck (21). The kinase that phosphorylates Tyr-394 in H2O2-treated fibroblasts could be either another Src kinase or an as yet unidentified kinase. To distinguish between these possibilities, we took advantage of the cell line SYF, which is derived from mutant mouse embryos lacking the Src family members c-Src, Yes, and Fyn (2). Additionally, the SYF cell line does not express Lyn (2). We expressed kinase-deficient Lck in SYF cells and analyzed the phosphorylation of Tyr-394 following cellular exposure to H2O2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293, a human embryonic kidney cell line, was grown in DMEM (Cellgro, Mediatech) supplemented with 10% calf serum (HyClone). Jurkat, a human leukemic T-cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Intergen) and 2 mM glutamine. SYF cell line was a murine embryonic fibroblast cell line derived from mutant embryos deficient for c-Src, Yes, and Fyn (a kind gift from Drs. R. Klinghoffer and P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA), was maintained in DMEM supplemented with 10% fetal calf serum.

**DNA Constructs and Retroviral Infections**—Wild type Lck (WT) and kinase-deficient Lck (R273) cDNAs have been previously described (21, 38). The lck cDNAs were subcloned into the retroviral vector MSCVhph (39) from the retroviral vector LXSN (40) to utilize hygromycin B phosphotransferase as a selectable marker. Recombinant retroviruses were produced by cotransfecting the MSCV-Lck constructs along with the viral helper plasmid SV-CAF E-MLV (41) into 293 cells using a calcium phosphate transfection system (Life Technologies, Inc.). 48 h post-transfection, supernatant containing recombinant virus particles were subjected to an immobilized protein A column (Pharmacia LKB) and used for subsequent analysis.

**Cell Lysis and Immunoprecipitations**—Cells were washed once with Tris-buffered saline and lysed in either RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 100 kallikrein-inactivating units/ml aprotinin) or Tris/Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 100 kallikrein-inactivating units/ml aprotinin) for 20 min at 4 °C. Lysates were subjected to centrifugation at 35,000 × g for 30 min. Lysates were stored at −70 °C. Lysates were used for subsequent analysis.

**Immunoprecipitates**—SYF cells or Jurkat T cells were lysed in Tris/Nonidet P-40 lysis buffer as described. Anti-Lck immunoprecipitates were reisolated by sequential precipitation using anti-Lck antibodies or were subjected to an in vitro kinase assay followed by SDS-PAGE.

**Immunoblotting**—Immunoprecipitated Lck was resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Western blotting was carried out with rabbit anti-Lck antibodies as described previously (42). Immune complexes were collected on a nitrocellulose membrane (ScilBiochem), washed three times in either RIPA or Tris/Nonidet P-40 buffer, and used for subsequent analysis.

**In Vivo Kinase Assay**—SYF cells or Jurkat cells were lysed in Tris/Nonidet P-40 lysis buffer as described. Anti-Lck immunoprecipitates were reisolated by sequential precipitation using rabbit anti-Lck antibodies or were subjected to an in vitro kinase assay followed by SDS-PAGE. Immunoaffinity-purified Lck immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblotting using anti-Lck antibodies or were subjected to an in vitro kinase assay followed by SDS-PAGE.

**Results**

**Phosphorylation of Lck at Tyr-394**—Phosphorylation at Tyr-394 in H2O2-treated fibroblasts could be another Src kinase or an as yet unidentified kinase. To distinguish between these possibilities, we took advantage of the cell line SYF, which is derived from mutant mouse embryos lacking the Src family members c-Src, Yes, and Fyn (2). Additionally, the SYF cell line does not express Lyn (2). We expressed kinase-deficient Lck in SYF cells and analyzed the phosphorylation of Tyr-394 following cellular exposure to H2O2.

**Stable Expression of WT Lck and R273 Lck in SYF Cells**—To express wild type Lck and the kinase-deficient R273 Lck in SYF cells, we infected the cells with recombinant MSCVhph retroviruses. Following antibiotic selection, pools of selected cells were tested for both Lck expression and kinase activity. Anti-Lck immunoprecipitates were analyzed by Western blotting using anti-Lck antibodies or were subjected to an in vitro kinase reaction. Uninfected SYF cells did not express Lck (Fig. IA, lane 1). SYF cells infected with WT- or R273 Lck-encoding virus expressed readily detectable levels of Lck (Fig. IA, lanes 2 and 3). Wild type Lck from the SYF cells exhibited robust kinase activity (Fig. IB, lane 2). In contrast, the R273 Lck did not exhibit any detectable kinase activity (Fig. IB, lane 3). Because we were able to detect phosphorylated bands with 0.5% of the intensity of the wild type Lck signal, we estimate that the activity of the R273 Lck is at least 200-fold lower than that of the wild type kinase. H2O2 treatment of SYF cells expressing R273 Lck had no detectable effect on the activity of the mutant kinase (data not shown).

**Kinase-deficient Lck Is Phosphorylated at Tyrosine 394 in SYF Cells following H2O2 Treatment**—It has been shown previously that exposure of both Jurkat T cells and 208F fibroblasts expressing wild type Lck to H2O2 induces the phosphorylation of Lck at Tyr-394 (21). To determine whether this is also the case in SYF cells, we biosynthetically labeled cells expressing wild type Lck with [32P]ATP and immunoprecipitated Lck both before and after exposure to 5 mM H2O2. The phosphorylation of the protein was examined by two-dimensional tryptic peptide analysis (Fig. 2). Wild type Lck was predominately phosphorylated on Tyr-505 in untreated cells (Fig. 2A). A trace of phosphorylation at Tyr-394 (2% of the level of Tyr-505) was also observed in untreated cells. Following exposure of the labeled cells to H2O2, wild type Lck became phosphorylated at Tyr-394 to an extent equal to that of Tyr-505 (Fig. 2B).

**Fig. 1. Analysis of Lck expression and kinase activity in infected SYF cells.** Lck was immunoprecipitated from SYF cells expressing WT Lck or R273 Lck and incubated with 32P-labeled Lck was visualized using a PhosphorImager.
Phosphorylation of Lck at Tyr-394

Many serine/threonine protein kinases are activated by phosphorylation in the activation loop. This activating phosphorylation is usually carried out by a specific upstream activator. For example, the cyclin-dependent kinases (Cdks) are stimulated by the Cdk-activating kinase, CAK, in this manner (45). Additionally, protein kinase B/c-Akt, cAMP-dependent protein kinase, p70 S6 kinase, and several protein kinase C isoforms are all activated by the 3-phosphoinositide-dependent kinase, PDK1 (46–50). Furthermore, mitogen-activated protein (MAP) kinases are activated by phosphorylation by MAP kinase kinases, such as MEK1 (51–54).

Src family kinases are also activated by phosphorylation of a conserved tyrosine in the activation loop. This phosphorylation traditionally has been thought to occur as an intermolecular event carried out by the kinase itself (25, 26). Our data, however, suggest that phosphorylation of the activation loop tyrosine need not be performed by Src family kinases. Using SYF cells, which are devoid of all known Src family kinase activity, we have demonstrated that H$_2$O$_2$ and pervanadate induce phosphorylation of kinase-deficient Lck at Tyr-394 to an extent equal to that of wild type Lck. Therefore, our results argue strongly that the oxidant-induced phosphorylation of Lck at Tyr-394 is not catalyzed exclusively by Src family members and may be carried out by another tyrosine protein kinase(s) that is functionally analogous to activators of serine/threonine protein kinases.

In the absence of a completely sequenced mammalian genome, the formal possibility that the tyrosine protein kinase responsible for the phosphorylation of Lck at Tyr-394 is a Src family kinase that has escaped detection to date cannot be excluded. However, the defective integrin-mediated signaling phenotype of the SYF cells (2) argues against the expression of a heretofore unidentified Src family member that can functionally complement for the c-Src, Yes, and Fyn deficiency in these cells. Therefore, the simplest interpretation of our results is that a non-Src family kinase is responsible for the oxidant-induced phosphorylation of Lck. We have not yet determined whether the kinase(s) responsible for phosphorylation of Lck at Tyr-394 is itself stimulated by hydrogen peroxide or whether its activity is constitutive and the increased phosphorylation of Tyr-394 is due simply to inhibition of a tyrosine phosphatase. Because Lck is normally expressed only in lymphoid cells, the fact that kinase-deficient Lck is phosphorylated in non-lymphoid cells following exposure to oxidants suggests that a general activator of Src family kinases may exist. Characterization of this kinase(s) in SYF cells may provide insight into this alternative mechanism of Src family kinase activation. We suspect that phosphorylation of the activation loop tyrosine in Src kinases will be shown to be carried out by Src family kinases in some circumstances and by one or more non-Src kinases in others.

Acknowledgments—We are indebted to Richard Klinghoffer and Philippe Soriano for the very generous gift of the SYF cells, and we thank Kambiz Amdjadi and Roberta Schulte for critical reading of the manuscript.

REFERENCES
1. Thomas, S. M., and Brugge, J. S. (1997) Annu. Rev. Cell Dev. Biol. 13, 513–609
2. Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A., and Soriano, P. (1999) EMBO J. 18, 2449–2471
3. Sefton, B. M., and Campbell, M. A. (1991) Annu. Rev. Cell Biol. 7, 257–274
4. Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Veillette, A., Davidson, D., and Mak, T. W. (1992) Nature 357, 161–164
5. Straus, D. B., and Weiss, A. (1992) Cell 70, 585–593
6. Brown, M. T., and Cooper, J. A. (1996) Biochem. Biophys. Acta 1287, 121–149
7. Yu, H., Chen, J. K., Peng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) Cell 76, 933–945
8. Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1983) Nature 362, 87–91
9. Sicheri, F., Morrell, I., and Kuriyan, J. (1997) Nature 385, 603–609
10. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Nature 385, 595–602
11. Thomas, J. E., Soriano, P., and Brugge, J. S. (1991) Science 254, 568–571
12. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) Nature 351, 69–72
13. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P., and Alitalo, K. (1992) *EMBO J.* **11**, 2919–2924
14. Sáez, M., Bolon, J. B., and Weiss, A. (1999) *EMBO J.* **18**, 351–352
15. Oestergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 257–262
16. Oestergaard, H. L., and Trowbridge, I. S. (1990) *J. Exp. Med.* **172**, 347–350
17. Mustelin, T., Coggeshall, K. M., and Altman, A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6302–6306
18. Su, J., Muranjan, M., and Sap, J. (1999) *Curr. Biol.* **9**, 505–511
19. Boulet, I., Fagard, R., and Fischer, S. (1987) *Biochem. Biophys. Res. Comm. 149*, 56–64
20. Abraham, N., and Veillette, A. (1990) *Mol. Cell. Biol.* **10**, 5197–5206
21. Hardwick, J. M., and Sefton, B. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4527–4531
22. Yamaguchi, H., and Hendrickson, W. A. (1996) *Nature* **384**, 48–49
23. Cassel, J. E., Harrison, M. L., Hellingh, K. E., and Krebs, E. G. (1982) *J. Biol. Chem.* **257**, 13877–13879
24. Voronova, A. F., Buss, J. E., Patschinsky, T., Hunter, T., and Sefton, B. M. (1984) *Mol. Cell. Biol.* **4**, 2705–2713
25. Barker, S. C., Kassel, D. B., Weigl, D., Huang, X., Luther, M. A., and Knight, W. B. (2000) *Biochemistry 34*, 14843–14851
26. Cooper, J. A., and MacAuley, A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4232–4236
27. Sotirellis, N., Johnson, T. M., Hibbs, M. L., Stanley, I. J., Stanley, E., Dunn, A. R., and Cheng, H. C. (1995) *J. Biol. Chem.* **270**, 29773–29780
28. Cloutier, J. F., and Veillette, A. (1999) *Curr. Biol.* **9**, 505–511
29. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) *Free Radical Biol. Med.* **22**, 269–285
30. Saj, G. N. (1996) *Oncogene 13*, 713–719
31. Sundaesren, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science 270*, 296–299
32. Sechrist, J. P., Burns, L. A., Karnitz, L., Kozelty, G. A., and Abraham, R. T. (1993) *J. Biol. Chem. 268*, 5886–5893
33. Hecht, D., and Zick, Y. (1992) *Biochem. Biophys. Res. Commun. 188*, 773–779
34. Sullivan, S. G., Chiu, D. T., Errada, M., Wang, J. M., Qi, J. S., and Stern, A. (1994) *Free Radical Biol. Med. 16*, 369–403
35. Denk, J. M., and Tanner, K. G. (1998) *Biochemistry 37*, 5633–5642
36. Huyer, G., Liu, S., Kelly, J., Moffat, J., Payette, P., Kennedy, B., Tsapralis, G., Greger, M. J., and Ramachandran, C. (1997) *J. Biol. Chem. 272*, 843–851
37. Yan, S. R., and Bertin, G. (1996) *J. Biol. Chem. 271*, 23464–23471
38. Vornov, A. F., and Sefton, B. M. (1986) *Nature 319*, 682–685
39. Hawley, R. G., Lien, P. H., Pong, A. Z., and Hawley, T. S. (1994) *Gene Ther.* **1**, 136–138
40. Miller, A. D., and Rosman, G. J. (1989) *Biotechniques 7*, 880–990
41. Muller, A. J., Young, J. C., Pendergast, A. M., Pondel, M., Landau, N. R., Littman, D. R., and Witte, O. N. (1991) *Mol. Cell. Biol. 11*, 1785–1792
42. Hurley, T. R., and Sefton, B. M. (1989) *Oncogene 4*, 265–272
43. Luo, K., Hurley, T. R., and Sefton, B. M. (1990) *Oncogene 5*, 921–925
44. Hunter, T., and Sefton, B. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1311–1315
45. kaldis, P. (1999) *Cell. Mol. Life Sci. 55*, 284–296
46. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol. 7*, 261–269
47. Cheng, X., Ma, Y., Moore, M., Hemmings, B. A., and Taylor, S. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9849–9854
48. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr. Biol. 8*, 69–81
49. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) *Science 281*, 2042–2045
50. Dutil, E. M., Toker, A., and Newton, A. C. (1998) *Curr. Biol. 8*, 1366–1375
51. Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem. 270*, 14843–14848
52. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) *Cell 90*, 859–869
53. Su, B., and Karin, M. (1996) *Curr. Opin. Immunol. 8*, 402–411
54. Garrington, T. P., and Johnson, G. L. (1999) *Curr. Opin. Cell Biol. 11*, 211–218
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J. Biol. Chem. 2000, 275:6055-6058.
doi: 10.1074/jbc.275.9.6055

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