The Protein-tyrosine Kinase Lck Associates with and Is Phosphorylated by Cdc2*

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The protein-tyrosine kinase Lck is essential for signaling through the T-cell antigen receptor. Treatment of T-cells with a variety of extracellular stimuli increases the phosphorylation of Lck on serine residues. This results in shifts in the apparent molecular weight of Lck to forms that exhibit reduced electrophoretic mobility on SDS-polyacrylamide gels. We found that as a result of arresting cells in mitosis, forms of Lck were generated that migrated with slower mobilities on SDS-polyacrylamide gels. This suggested that a serine/threonine kinase, active at mitosis, was phosphorylating Lck. Using antibodies to Lck and to the cyclin-dependent kinase affinity resin, Sucl-agarose, we detected a stable interaction between Lck and Cdc2. The interaction was mediated through the Src homology 3 domain of Lck and was selective, as only the active form of Cdc2 was found to associate with Lck. Moreover, Cdc2 was able to phosphorylate Lck in vitro and shift its electrophoretic mobility to a more slowly migrating form. An association between active Cdc2 and the Src-related kinases Lyn and Fyn was also demonstrated, although Cdc2 was not found associated with the tyrosine kinases, Csk and Syk. These results demonstrate that at mitosis, Cdc2 associates with and phosphorylates Lck.

Lck is a member of the Src family of non-receptor protein-tyrosine-kinases. It is expressed primarily in T lymphocytes, although natural killer cells and B-lymphocytes also express Lck (1). Like the other Src family members, Lck is myristoylated on its N-terminal glycine residue and contains both an SH2 and SH3 domain preceded by a unique N-terminal region (2, 3). In addition, Lck is also palmitoylated (4). In vivo studies using a variety of T-cell lines showed that Lck is phosphorylated primarily on serine and to a lesser extent on tyrosine residues (5–8). Like the other Src kinases, its phosphorylation state changes with activation. Lck activity is required for normal thymocyte development (9), and its constitutive activation results in thymic tumors in mice (10).

Lck plays a critical role in T-cell activation (1, 11–14). T-cells lacking Lck are unable to signal through their antigen receptors (15). Lck binds noncovalently to the cytoplasmic tails of the T-cell surface glycoproteins CD4 and CD8 (16, 17), which complex with the T-cell antigen receptor during signaling. Lck also is associated with the β-subunit of the IL-2 receptor (18). Stimulation of T-cells through the antigen receptor complex (19–23), with IL-2 (24, 25), or with activators of PKC (6, 22, 26, 27) results in the conversion of Lck to forms exhibiting reduced electrophoretic mobility when analyzed by SDS-PAGE. These apparent higher molecular weight forms of Lck contain increased amounts of phosphoserine (6, 19–21, 23–27) and phosphothreonine (6), which presumably account for their aberrant electrophoretic mobility.

The prototype Src family member, Src itself, exhibits a retardation in its electrophoretic mobility when phosphorylated on serine and threonine residues at mitosis (28–30). In this case, the mitotic cyclin-dependent kinase, Cdc2 (31–33), is responsible for inducing the mobility shift (28–30). It is well established that Src is activated at mitosis, and its mitosis-specific phosphorylation is thought to contribute to its activation (34, 35). The recent identification of a specific mitotic substrate for Src (36, 37) clearly implicates Src in mediating cell cycle-dependent events. In addition to Src, Src family members Fyn and Yes also have been reported to be activated during mitosis (34).

These observations prompted us to initiate studies to determine whether Lck becomes differentially phosphorylated during the cell cycle. We found that cells arrested in mitosis contained a form of Lck that exhibited a retarded mobility on SDS-polyacrylamide gels relative to Lck from cycling cells or cells arrested at the G1/S boundary of the cell cycle. This suggested that a serine kinase active at mitosis phosphorylated Lck. We found that in mitotic cells, the cyclin-dependent kinase Cdc2 associated with Lck, as well as with two other Src family members, Fyn and Lyn. Furthermore, Lck isolated from cycling cells was converted to a more slowly migrating form when phosphorylated by Cdc2.

EXPERIMENTAL PROCEDURES

Antibodies—Antipeptide antibodies to residues 476–509 in the Lck sequence were prepared as described previously (38). Antibodies to the unique C-terminal region of Cdc2 were obtained from Upstate Biotechnology Inc. Rabbit polyclonal anti-Fyn antibodies (directed against residues 28–48) and anti-Lyn antibodies (directed against residues 44–65) were obtained from Santa Cruz Biotechnology, Inc. Antipeptide antibodies directed against residues 422–450 of the Csk sequence were prepared as described (39). Antibodies to the p40 catalytic domain of Syk were prepared as described (40).

Cells and Cell Treatments—The murine cell line LSTRA, the human leukemia T-cell line Jurkat (clone E6-1, from the American Type Culture Collection), the Lck-deficient mutant of Jurkat (J.CaM1, American Type Culture Collection), and CEM-6 were grown and maintained as described (4). For cell cycle treatment experiments, cells (4 × 10^5 cells/ml) that were maintained in the log phase of growth were either left untreated or incubated in the presence of either aphidicolin (Boeh-
ringer Manheim, 0.5 μg/ml) or nocardazole (Sigma, 1 μg/ml) for 10 h (LSTRA cells) or 14 h (Jurkat and J.CaM1 cells) at 37°C. Flow cytometric analysis of blocked cells indicated that, after nocardazole treatment, approximately 60% of the cells had undergone mitotic arrest. Cells were equalized for cell number with untreated control cells and lysed in ice-cold lysis buffer containing 0.5% Nonidet P-40, 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and 20 μg/ml each of aprotinin and leupeptin at 4°C for 15 min. Samples were centrifuged at 13,000 × g (microcentrifuge), and the supernatants were subjected to immunoblotting, immunoprecipitation, or incubation withSuc-1-agarose resin. Murine thymocytes were isolated from 6-week-old mice as described (41). Thymocytes (3 × 10^6 cells/ml) were resuspended in RPMI 1640 medium (Life Technologies, Inc.) supplemented as described (39) except that 10% inactivated fetal calf serum was used. Thymocytes were incubated in the absence or presence of ConA (5 μg/ml) for 48 h, prior to washing in phosphate-buffered saline and cell lysis. All cells were maintained at 37°C in a humidified incubator under an atmosphere of 6% CO₂.

**Immunoblotting—**Cells were equalized for cell number and lysed as described above. Samples were centrifuged at 13,000 × g (microcentrifuge) and the supernatants analyzed by SDS-PAGE followed by transfer to Immobilon membranes. The membranes were blocked in TBST (15 mM Tris/HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween 20) containing 2.5% BSA (Sigma) and 2.5% nonfat dried milk (Carnation) for 1 h followed by incubation with the rabbit antibodies described above. After extensive washings, the membranes were incubated for 1 h with goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) in TBST containing 5% goat serum, 1% BSA, and 1% nonfat dry milk. The immunoblots were analyzed using the ECL detection system (Amer sham). For immunoblotting anti-Lck immunoprecipitations, proteins were separated by SDS-PAGE, transferred to Immobilon-P, and blocked in TBST containing 0.2% methyl cellulose for 1 h, followed by incubation with anti-Cdc2 antibodies. After extensive washes, the membranes were incubated with monoclonal anti-rabbit antibodies conjugated to biotin (Sigma) for 45 min, followed by washing and incubation with the ABC peroxidase staining reagent (Pierce) for 30 min. The immunoblots were analyzed using the ECL detection system.

**Immunoprecipitation and Immune Complex Kinase Assays—**Cell lysates were incubated at 4°C with anti-Lck antibodies bound to protein A-Sepharose (Sigma). The immune complexes were washed three times with ice-cold buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, 0.5% Nonidet P-40, and 20 μg/ml each of protease inhibitors leupeptin and aprotinin. The complexes were assayed in a 50-μl volume containing 25 mM HEPES, pH 7.4, 10 mM MnCl₂, 5 mM p-nitrophenylphosphate, 5 μM sodium orthovanadate, 20 μg/ml each of aprotinin and leupeptin, 1 μM ATP (5 μM of [γ-³²P]ATP, DuPont-NEN, 600 Ci/mmol). Reactions were carried out at 30°C for 5 min. Phosphoproteins were separated by SDS-PAGE, transferred to Immobilon-P, and detected by autoradiography. Prior to autoradiography, the membranes were incubated with 1 μCi of K⁺OH at 55°C for 1 h to preferentially remove radioactive phosphate from proteins phosphorylated on serine and threonine residues.

Precipitations with Suc-1-Agarose—Suc-1-agarose (Oncogene Science) was incubated in blocking buffer containing 5% Ig-free BSA and 1% ovalbumin at 4°C for 1 h. The resin was washed in lysis buffer and incubated at 4°C for 1 h with lysates from cells that had undergone the various treatments indicated. Resins were analyzed by immunoblot analysis (Figs. 2, 3, and 7) or in vitro kinase assays (Figs. 2 and 7). For immunoblot analysis, the resins were washed twice with Suc-1-bound proteins were released by boiling in SDS-sample buffer for 5 min, followed by SDS-PAGE and transfer to Immobilon-P before incubation with the antibodies indicated. For in vitro kinase assays, the Suc-1 resins were phosphorylated as described above for immune complex kinase assays. For the phosphorylation of Lck by Cdc2 (Fig. 2), Suc-1 resin containing both Cdc2 and Lck was resuspended in a final volume of 50 μl containing 25 mM HEPES, pH 7.4, 10 mM MgCl₂, 20 μg/ml each of aprotinin and leupeptin, and 100 μM ATP. The kinase assays were performed at 24°C for 10 min. The phosphorylated Lck was released from the resin by boiling in SDS-sample buffer and resolved by SDS-PAGE. Lck was visualized by anti-Lck immunoblot analysis after the gel had been transferred to Immobilon-P.

**RESULTS**

**Cdc2 Phosphorylates Lck—**

In order to detect cell cycle-dependent covalent modifications of Lck, detergent-solubilized extracts were prepared from LSTRA or Jurkat T-cells that were left untreated or treated with the cell cycle blockers aphidicolin and nocardazole. The extracts were separated by SDS-PAGE and analyzed by immunoblot analysis with antibodies to Lck. As shown in Fig. 1A, anti-Lck antibodies detected a protein of M₀ = 56,000 in lysates from cycling cells or from cells blocked in S phase with aphidicolin. However, lysates from cells blocked in mitosis by treatment with nocardazole contained an additional immunoreactive protein that exhibited a retarded electrophoretic mobility relative to the 56-kDa form of Lck. This slower migrating form of Lck was also observed in lysates of cells treated with colcemid, but not in lysates of cells treated with hydroxyurea (data not shown). As illustrated in Fig. 1B, similar results were obtained when Lck was isolated in anti-Lck immune complexes and detected by an in vitro kinase assay. Anti-Lck immunoprecipitates were formed from lysates of nocardazole-treated LSTRA cells (Fig. 1B) or Jurkat or CEM-6 T-cells (data not shown) and incubated in the presence of [γ-³²P]ATP to generate autophosphorylated Lck. Again, only a single band of autophosphorylated Lck appeared in anti-Lck immune complexes formed from cycling cells or cells treated with aphidicolin, while Lck obtained from nocardazole-treated cells showed an additional, more slowly migrating band. Densitometric analysis revealed that the more slowly migrating band represented approximately 30% of the total Lck. Because approximately 60% of nocardazole-treated cells underwent mitotic arrest (see “Experimental Proce-
from LSTRA cells were incubated with agarose beads coupled to Cdc2.

Gated, therefore, whether that enzyme might be the cyclin-dependent kinase, Cdc2. We investigated the possibility that Lck was a substrate for a serine/threonine kinase. Electrophoretic mobility shift of Lck, coupled with our observations, suggested that Lck was a substrate for a serine/threonine kinase that was specifically activated at mitosis. We investigated whether Lck was a substrate for a serine/threonine kinase that was specifically activated at mitosis.

The presence of Cdc2 on the Suc1-agarose was verified by both immunoblotting analyses with anti-Cdc2 antibodies and histone H1 kinase assays (data not shown). In addition to the phosphorylation of histone H1 during these assays, proteins bound to Suc1-agarose also catalyzed the phosphorylation of a 56-kDa protein that co-migrated on SDS-PAGE with Lck (data not shown).

To investigate the possibility that Lck was bound to Suc1-agarose, cell lysates from unsynchronized or nocodazole-treated LSTRA cells were adsorbed to Suc1-agarose and the resin was analyzed for the presence of Lck. As illustrated in Fig. 2, incubation of the Suc1-resins with \[^{32}P\]ATP led to the phosphorylation on tyrosine of proteins in the molecular mass range of 56–60 kDa. Multiple bands were observed in lysates from nocodazole-treated cells, while only a single protein species migrating at 56 kDa was detected in lysates from unsynchronized cells (Fig. 2A). To verify that this phosphoprotein was Lck, proteins bound to the Suc1-resins were eluted and immunoblotted with anti-Lck antibodies. The results, shown in Fig. 2B, confirmed that the radiolabeled bands seen in Fig. 2A represented Lck that was bound to the Suc1-agarose and became autophosphorylated during the kinase reaction. As shown in Fig. 2 (C and D), neither the protein-tyrosine kinase Syk nor the protein-tyrosine kinase Csk bound to Suc1, although both were present in LSTRA cell lysates.

To ensure that the binding of Lck to the Suc1-resin was specific, lysates were prepared from resting mouse thymocytes, which do not express Cdc2 (44), or from ConA-activated thymocytes, where Cdc2 expression has been induced (44). The relative difference in the level of Cdc2 between resting and activated thymocytes was verified by the immunoblotting studies shown in Fig. 3 (lanes 1 and 2), which revealed a much higher level of Cdc2 in the ConA-treated cells. In contrast, the relative abundance of Lck was unchanged in activated cells compared with resting cells (Fig. 3, lanes 5 and 6). Lysates prepared from resting and activated thymocytes were adsorbed to Suc1-agarose and examined for the presence of Lck by immunoblot analysis. Despite its equal abundance in both cell types, only Lck from cells expressing Cdc2 bound to the Suc1 resin, as shown in Fig. 3 (lanes 3 and 4). These results indicated that Lck did not bind directly to the Suc1 protein, or nonspecifically to the Suc1 resin, but was likely associating either directly with Cdc2 or indirectly through another Cdc2-associated protein.

**Fig. 2.** Lck binds selectively to Suc1-agarose. Detergent-solubilized lysates were prepared from LSTRA cells that were untreated (U) or treated with aphidicolin (A) or nocodazole (N). A, lysates (2 × 10^6 cells/sample) were adsorbed on Suc1-agarose and the washed resin was phosphorylated in the presence of \[^{32}P\]ATP. Proteins were separated on an 8% SDS-polyacrylamide gel and transferred to Immobilon-P. The membrane was treated with KOH, and the phosphorylated proteins were detected by autoradiography. B, lysates (2 × 10^6 LSTRA cells/sample) were adsorbed to Suc1-agarose. The bound proteins were eluted in SDS-sample buffer, separated on an 8% SDS-polyacrylamide gel, transferred to Immobilon-P, probed with anti-Lck antibodies, and detected by ECL. C, detergent-solubilized lysates (2 × 10^6 cells/lane) from untreated (U) or treated (A and N) LSTRA cells were loaded directly onto an 8% gel or adsorbed to Suc1-agarose as indicated. Suc1-bound proteins were loaded in SDS-sample buffer. The gel was transferred to Immobilon-P and probed with anti-Csk antibodies and analyzed using the ECL detection system. D, same as C, except the membranes were probed with antibodies to Syk.
We reasoned that if Lck was present on the Suc-1-resin because it associated with Cdc2, then Cdc2 should be present in anti-Lck immune complexes. To test this, LSTRA cells, which express high amounts of Lck (45), were compared with J.CaM1 cells, which are deficient in Lck (46), and Jurkat cells, which express intermediate levels of Lck. Cells were untreated or treated with either aphidicolin (N) or nocodazole (L). Samples were separated on a 12% SDS-polyacrylamide gel, transferred to Immobilon-P, and probed with anti-Lck immune complexes. To test this, LSTRA cells, which express high amounts of Lck (15), showed much lower levels of Cdc2 in anti-Lck immunoprecipitations upon prolonged exposure of the autoradiographic film. Analysis of identical immune complexes with antibodies to Cdk2 indicated that no Cdk2 was found associated with Lck (data not shown). Analysis with antibodies to Lyn, however, revealed that anti-Lck immune complexes formed from mitotic lysates did contain Lyn (B). These results are consistent with Lck binding only the active form of Cdc2, which would be expected to also complex with Lyn.

Further analysis of the interaction between Lck and Cdc2 revealed that Cdc2 interacted specifically with the SH3 domain of Lck (Fig. 4C). In these experiments, lysates from untreated or cell cycle-arrested Jurkat cells were incubated with various GST/Lck domain fusion proteins and immunoblotted for the presence of Cdc2. As can be seen, only the construct containing the SH3 domain of Lck bound Cdc2. Moreover, this SH3 domain-containing construct bound only a single form of Cdc2 that co-migrated with the faster migrating active form of Cdc2. Consistent with this result, it was found that only Cdc2 from nocodazole-treated cells bound to the GST/SH3 fusion protein. These results corroborate our previous findings (Fig. 4B) that Lck binds only the active form of Cdc2.

Similar results were obtained when immunoprecipitates were formed with antibodies to the Src-related kinases Lyn and Lyn. As shown in Fig. 5, immune complexes containing Lyn (lane 9) or Lyn (lane 12) obtained from nocodazole-treated cells also contained Cdc2. Again, only the active, more rapidly migrating form of Cdc2 was found in these immune complexes. Neither anti-Csk nor anti-Syk immune complexes formed from nocodazole-treated LSTRA contained Cdc2 (data not shown).

**The Interaction between Lck and Cdc2 Is Not Mediated by Lyn**—Because association with Lyn B is a prerequisite for activation of Cdc2, it was of interest to determine whether
The Lck/Cdc2 association is not mediated through cyclin B. Detergent lysates from S9 cells expressing GST-cyclin B (A, lane 2; B and C, lanes 1) or expressing GST-cyclin B in the presence of co-expressed Cdc2 (A, lane 3; B and C, lanes 2) were adsorbed to GST-agarose. A, washed GST-agarose resins were immunoblotted for the presence of Cdc2 with antibodies to Cdc2. Lane 1 represents whole cell lysates from LSTRA cells and serves as a marker for Cdc2. B, washed resins were assayed for histone H1 kinase activity and the presence of phosphorylated histone H1 was detected by autoradiography. C, washed resins were incubated with detergent lysates of LSTRA cells, washed, and subjected to in vitro kinase assays. The presence of Lck was detected by autoradiography. Samples were analyzed on a 12% gel by SDS-PAGE. The immunoblot in panel A was detected by ECL.

In the absence or presence of 100 μM Mg-ATP for 10 min and analyzed by immunoblot analysis using antibodies to Lck. The results showed that, in the absence of Mg-ATP, only a single, 56-kDa form of Lck was present on the glutathione resin. This protein co-migrated with Lck from whole cell lysates. However, resins incubated in the presence of Mg-ATP, contained two forms of Lck, one co-migrating with the 56-kDa species, and an additional slower migrating form. These results, together with those described in Fig. 7, indicated that Cdc2 phosphorylated Lck in vitro and that this Cdc2-dependent phosphorylation resulted in a form of Lck that migrated with a slower electrophoretic mobility.

**DISCUSSION**

We observed that cells arrested during mitosis exhibited forms of Lck that migrated with reduced electrophoretic mobilities on SDS-polyacrylamide gels. Numerous studies have demonstrated that these altered mobility forms of Lck are hyperphosphorylated on serine residues in the region N-terminal to the kinase domain (6, 19–22, 26, 27). A recent report also has revealed the cell cycle-dependent serine phosphorylation of Lck, but in this IL-2-induced system, the increase was detected at the G1/S interface (25). Our results suggested that a serine/threonine kinase, active at mitosis, was responsible for the mitosis-specific serine phosphorylation of Lck.

In order to test whether this kinase was the cyclin-dependent serine/threonine kinase Cdc2, we utilized a cyclin-dependent kinase affinity resin, Suc1. When cell lysates were incubated with the Suc1-resin, we noticed that, in addition to Cdc2, this resin also bound a 56-kDa protein. We suspected that this protein was Lck, and immunoblot analysis with antibodies to Lck confirmed this. We hypothesized that the binding of Lck to Suc1 was mediated by Cdc2. We were concerned, however, that the presence of Lck on the Suc1-agarose reflected a nonspecific interaction between Lck and the resin. Therefore, the dependence of the Lck/Suc1 interaction on the presence of Cdc2 was tested. Resting thymocytes were used in these studies because in the absence of activation they do not express Cdc2. We utilized this activation-dependent expression of Cdc2 to test whether Lck bound Suc1 in the absence of Cdc2. We found that only Lck from activated thymocytes was able to bind Suc1, despite the equivalent expression of Lck in unactivated and activated cells. This finding ruled out the possibility that Lck was binding nonspecifically to the resin and suggested that Lck...
was interacting with Cdc2. We confirmed the Lck-Cdc2 association by demonstrating the presence of Cdc2 in Lck immunoprecipitates. These studies also confirmed that Lck bound only the active, mitotic form of Cdc2. Finally, we were able to show that in vitro, Cdc2 could phosphorylate the 56-kDa form of Lck and convert it to a form that migrated with a reduced electrophoretic mobility on SDS-PAGE.

We found that two other Src family members, Lyn and Fyn, also bound to active Cdc2 during mitosis. An association between Lyn and Cdc2 has been reported in irradiated cells (49). In this system, Lyn phosphorylated and inactivated Cdc2 (50, 51). Our demonstration that Cdc2 associates with Fyn is consistent with previous observations that Fyn localizes with the mitotic spindle (52) and is active at mitosis (34). Several Src family members are known to be activated at mitosis, including Src itself (34). At mitosis, Src becomes phosphorylated on one serine and two threonine residues within its unique N-terminal region (28, 30). The kinase responsible for these phosphorylations is thought to be Cdc2 (35). However, in vitro phosphorylation of Src by Cdc2 does not result in its activation, suggesting a more complex pathway in vivo (34). Quite recently, a substrate for mitotically activated Src was identified as a 68-kDa heterogeneous nuclear ribonucleoprotein (36, 37).

The finding that Lck specifically interacts with and can be phosphorylated by Cdc2 implicates Cdc2 in the regulation of Lck function. Many studies have demonstrated that T-cell signaling is directly dependent on Lck (53, 54). For example, mutant T-cell lines that lack Lck activity fail to respond to stimulation through the T-cell receptor (15, 55). This signaling defect was restored by transfection with kinase-active Lck (15), but not with kinase-inactive Lck (56). However, a kinase-active Lck lacking the unique N-terminal region (amino acids 17–57) also failed to restore signaling in this system (57). This is in agreement with findings indicating that aspects of Lck function appear to be mediated by domains other than the kinase domain (58). Thus, in addition to its enzymatic activity, Lck also functions by coupling with other cellular signaling molecules through its protein binding domains.

Metabolic labeling studies using a variety of unstimulated T-cell lines indicate that in vivo Lck is phosphorylated primarily on serine residues and to a significantly lesser extent on tyrosine residues (5–8, 59). In fact, studies utilizing mutant T-cell lines that fail to express the tyrosine phosphatase CD45 have revealed that much of the Lck in normal T-cell lines is hypophosphorylated on tyrosine (5, 59–61). However, the phosphotyrosine that is present is located on residue 505, the negative regulatory site (5, 7, 8, 19, 27, 59, 61–63).

We have demonstrated that the phosphorylation state of Lck changes in mitosis. Although our findings and the report by Churcher et al. (25) are the first indications that at least some serine phosphorylation of Lck is cell cycle dependent, changes in the serine phosphorylation state of Lck, in response to a variety of extracellular stimuli, are well documented. Increases in Lck serine phosphorylation have been shown to occur after: 1) the engagement of the CD3 component of the T-cell antigen receptor (22), 2) the binding of IL-2 to its receptor (24), 3) cross-linking CD4 and the T-cell receptor (64), 4) the treatment of cells with the co-mitogenic phorbol ester, PMA (6, 26, 27), and 5) the cross-linking of the antigen receptor on B-cells (65), among others. In all cases, reduced electrophoretic mobility forms of Lck were generated that contained increased amounts of phosphoserine (6, 19–21, 23–27). In several cases, the serine phosphorylation sites were localized to within an N-terminal stretch comprising amino acids 14–261 (6, 19).

The electrophoretic mobility changes in Lck that result from signaling-induced serine phosphorylations have been most thoroughly documented in the case of PMA. Treatment of a variety of T-cell lines with the phorbol ester PMA results in the generation of more slowly migrating forms of Lck that contain increased amounts of phosphoserine relative to the 56-kDa form of Lck from untreated cells (6, 19, 26). However, not all serine phosphorylation results in shifted mobility forms of Lck, although all altered mobility forms of Lck have been shown to contain elevated levels of phosphoserine. In addition to the generation of more slowly migrating forms of Lck, PMA treatment also results in a form of Lck that migrates with the 56-kDa band seen in untreated cells (26). Despite its unaltered mobility, this form also showed increased serine phosphorylation on several of the same peptides as those found in the higher migrating forms (27). In addition, treatment of cells with calcium ionophores also raises the phosphoserine content of a form of Lck that doesn't change its mobility on SDS-polyacrylamide gels (19). As has been suggested (19), this raises the possibility that at least two separate serine phosphorylation pathways contribute to the signaling-dependent increases in the phosphoserine content of Lck.

Several of these signaling-induced serine phosphorylation sites have been identified. Treatment of cells with antibodies to the CD3 component of the T-cell antigen receptor has been reported to induce the serine phosphorylation of serine 59 (23), and serine 194 (22), and well as tyrosine 192 (22). Treatment of cells with the phorbol ester PMA has been reported to result in the phosphorylation of serines 42, 59, and 158 (22, 23). In addition, serine 59 has been reported to become phosphorylated after cross-linking the antigen receptor on B-cells (65). Examination of the amino acid sequence surrounding the known signaling-induced serine phosphorylation sites suggested various kinases that might be responsible for these phosphorylations. Using in vitro systems, kinases capable of phosphorylating these sites have been identified. Thus, mitogen-activated protein kinase has been shown to phosphorylate serine residue 59 (23) and protein kinase A and protein kinase C have been reported to phosphorylate serine 42 (66). However, it is unclear which if any of these kinases phosphorylate Lck in vivo.

Based upon our demonstration of the mitotic phosphorylation of Lck, it is likely that Cdc2 phosphorylates Lck in vivo. Although we have not formally demonstrated that the slower mobility, mitotic form of Lck contains elevated levels of phosphoserine, we think that this is likely for the following reasons. During mitosis, Lck and Cdc2 were found to associate. Moreover, isolated Cdc2 was shown to phosphorylate Lck and shift its migration to that observed during mitosis. Taken together, these results suggest that during mitosis activated Cdc2 phosphorylates Lck. Consistent with this conclusion is the report that Cdc2 can phosphorylate a synthetic peptide containing amino acids 185–196 in the Lck sequence (22). This sequence is found in the SH2 domain of Lck and contains only two potential phosphorylation sites, tyrosine 192 and serine 194 (22). Like serine 59, serine 194 is followed by a proline residue, which makes either of these serines candidates for phosphorylation by a proline-directed serine/threonine kinase such as Cdc2 or mitogen-activated protein kinase. Thus it is likely that more than one serine phosphorylation pathways exists and that several kinases contribute to the overall serine phosphorylation of Lck.

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