Characterization of the in Vivo Sites of Serine Phosphorylation on Lck Identifying Serine 59 as a Site of Mitotic Phosphorylation*

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The lymphocyte-specific protein-tyrosine kinase Lck plays a critical role in T cell activation. In response to T cell antigen receptor binding Lck undergoes phosphorylation on serine residues that include serines 59 and 194. Serine 59 is phosphorylated by ERK mitogen-activated protein kinase. Recently, we showed that in mitotic T cells Lck becomes hyper-phosphorylated on serine residues. In this report, using one-dimensional phosphopeptide mapping analysis, we identify serine 59 as a site of in vivo mitotic phosphorylation in Lck. The mitotic phosphorylation of serine 59 did not require either the catalytic activity or functional SH2 or SH3 domains of Lck. In addition, the presence of ZAP-70 also was dispensable for the phosphorylation of serine 59. Although previous studies demonstrated that serine 59 is a substrate for the ERK MAPK pathway, inhibitors of this pathway did not block the mitotic phosphorylation of serine 59. These results identify serine 59 as a site of mitotic phosphorylation in Lck and suggest that a pathway distinct from that induced by antigen receptor signaling is responsible for its phosphorylation. Thus, the phosphorylation of serine 59 is the result of two distinct signaling pathways, differentially activated in response to the physiological state of the T cell.

Lck is a member of the Src family of non-receptor protein kinases. It is primarily expressed in T lymphocytes and natural killer cells and plays a critical role in T cell development and activation (1–6). The structure of Lck is typical of Src family kinases with an amino-terminal membrane-targeting Src homology (SH)1 4 domain, followed by a unique domain, an SH3 domain, an SH2 domain, and the catalytic domain (7). The extreme carboxyl terminus contains a conserved tyrosine residue that upon phosphorylation negatively regulates the catalytic activity of Lck. The SH4 domain of Lck consists of an amino-terminal myristoylation site and two sites of palmitoylation (8), and Lck is both myristoylated (9) and reversibly palmitoylated (10, 11). Recently, it was shown that the palmitoylation of Lck is essential for its signaling function in T cells (12). In addition to palmitoylation, the kinase activity and the SH2 and SH3 domains of Lck play critical roles in T cell activation.

Lck is phosphorylated on several sites in vivo, two of which, tyrosines 394 and 505, directly regulate catalytic activity. In addition to these two major tyrosine phosphorylation sites, Lck also is phosphorylated on serine residues. In response to T cell stimulation by the ligation of either the T cell antigen receptor (TCR) (13–18), the interleukin-2 receptor (19), or treatment with phorbol esters (17, 20, 21), Lck undergoes phosphorylation on amino-terminal serine residues. In all these cases, the phosphorylation on serine residues is accompanied by a decrease in the electrophoretic mobility of Lck on SDS-polyacrylamide gels. Mutational analysis revealed that the phorbol ester, PMA, induced the phosphorylation of serines 42 and 59 in the unique domain of Lck (22), and stimulation with anti-CD3 antibodies resulted in the in vivo phosphorylation of serine 59 that in turn resulted in the decreased electrophoretic mobility of Lck (18). The ERK MAP kinase was identified as the kinase responsible for the phosphorylation of serine 59 in both cells (23) and in vitro (18, 22), whereas protein kinase A and protein kinase C were both implicated in the phosphorylation of serine 42 (18, 22). Biochemical studies also revealed that serine 158 present within the SH2 domain of Lck was phosphorylated upon treatment of Jurkat T cells with PMA (17) and that anti-CD3 stimulation of Jurkat T cells resulted in the phosphorylation of tyrosine 192 (17, 24) and serine 194 (17) in cells. Several studies have reported that serine phosphorylation of Lck inhibits its enzymatic activity (13, 14, 16, 18, 25). Phosphorylation of serine 59 in particular has been reported to inhibit Lck activity and to regulate the binding specificity of the Lck SH2 domain (26, 27).

We demonstrated previously (28) that during mitosis Lck became phosphorylated on serine residues and that this phosphorylation was mimicked by the in vitro phosphorylation of Lck by Cdc2. Src itself is known to be phosphorylated at mitosis by Cdc2, and this results in the activation of Src and association with its mitotic substrate Sam68 (29, 30). Studies over the past several years have implicated Src family kinases in the progression of the cell cycle (31–37). Recently it was reported that in mature T lymphocytes, Lck was required for progression through the G2-M transition (32) and that cells treated with a Src family selective tyrosine kinase inhibitor were blocked in mitosis (35).

Using a mutagenesis approach we identified serine 59 as an in vivo site of mitotic phosphorylation in Lck. Neither the kinase activity nor a functioning SH3 or SH2 domain of Lck was required for the mitotic phosphorylation of serine 59. In addition, the presence of ZAP-70 also was dispensable for the
phosphorylation of serine 59 at mitosis suggesting that a pathway distinct from that induced by antigen receptor stimulation is involved in the mitotic phosphorylation of serine 59. Indeed our results demonstrated that although two potent and specific inhibitors of the ERK MAP kinase pathway inhibited the phosphorylation of serine 59 induced by PMA, neither inhibited the mitotic phosphorylation of serine 59. These results suggest that two distinct proline-directed serine/threonine kinases are utilized by cells to phosphorylate serine 59 in a context-specific manner.

**MATERIALS AND METHODS**

**Cells and Antibodies**—The human leukemia T cell line Jurkat (clone E6-1, from American Type Culture Collection), the Lck-deficient Jurkat derivative (JCaM1.6, from American Type Culture Collection), and LSTRA cells, which naturally overexpress Lck due to retroviral promoters inserion of the Moloney murine leukemia virus (38, 39), were grown and maintained in log phase growth as described previously (28). Cells remained in log phase growth for the duration of all experiments. Polyconal antipeptide antibodies to residues 476–508 of Lck were described previously (28). Monoclonal antibodies to Lck (3A5) were purchased from Santa Cruz Biotechnology.

**Expression of Lck in JCaM1 Cells**—The murine Lck cDNA was obtained from Dr. A. Shaw, Washington University, St. Louis. The S59A, S59A/S194A, and the SH2 (R154K) and kinase-inactive (K273R) mutants were generated using the unique site elimination method (40). The S59D and the SH3 (W97A) mutants were generated using the transforme site-directed mutagenesis kit (CLONTECH). All mutations were confirmed by DNA sequencing. The wild type and mutant cDNAs were then subcloned into the EcoRI site of the pCAGGS expression vector. JCaM1 cells (1 × 10⁵ cells/ml) were transiently transfected with 15 µg of the various Lck DNA-containing plasmids by electroporation (Invitrogen, gene pulsar, pulsed at 800 microfarads, 250 V). Where specified, JCaM1 cells were transiently cotransfected with 15 µg each of Lck DNA-containing plasmid and pBbepluro empty vector to allow selection in puromycin (0.5 µg/ml) 24 h after transfection of cells expressing Lck.

**Cell Treatments and Analysis**—At 36 h post-transfection cells in log phase growth were incubated with nocodazole (Sigma, 1 µg/ml) for 12 h to obtain mitotic cells as described previously (28). For inhibitor assays, the inhibitor was added during the last 4 h of the nocodazole treatment. U0126 (Promega) and SB 203580 (Sigma) were used at 20 µM. Equivalent numbers of untreated cycling cells and mitotic cells were lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM sodium vanadate and 20 µg/ml each of leupeptin and aprotinin) for 15 min on ice. After incubation on ice, nuclei and unbroken cells were removed by centrifugation at 15,000 × g for 5 min at 4 °C. Lck was immunoprecipitated with anti-Lck polyclonal antibodies coupled to protein A-Sepharose (Sigma). For immunoblotting, the proteins were separated by SDS-PAGE of 5% acrylamide gels, transferred to Immobilon-P membranes, blocked in TBST (15 mM Tris, pH 7.2, 150 mM NaCl, 0.05% Tween) containing 5% goat serum for 1 h, and then incubated with the appropriate primary antibody. After extensive washes the blots were incubated with goat anti-mouse peroxidase-conjugated antibodies for 1 h, washed, and analyzed using the ECL detection system. Lck was detected by immunoblotting analysis with anti-Lck monoclonal antibodies.

**Metabolic Labeling and Phosphopeptide Mapping**—Untreated cycling cells and mitotic cells (treated with nocodazole, 1 µg/ml for 18 h) were incubated in 10 ml of phosphate-free RPMI 1640 for 1 h and then incubated for an additional 3–3.75 h in the presence of [32P]orthophosphate (PerkinElmer Life Sciences). In case of the mitotic cells, the incubation in phosphate-free RPMI 1640 and the subsequent labeling was done in the presence of nocodazole (1 µg/ml). Cells were collected, washed, and lysed in ice-cold RIPA buffer (10 mM Tris-Cl, pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing 5 mM EDTA, 1 mM sodium vanadate, and 40 µg/ml each of leupeptin and aprotinin. Lysates were centrifuged at 15,000 × g for 15 min at 4 °C. Lck was immunoprecipitated from precleared lysates using protein A-Sepharose previously incubated with protein A-Sepharose. The immune complexes were washed 3 times with RIPA buffer, dissociated in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Labeled proteins were detected by autoradiography.

To obtain *in vitro* autophosphorylated Lck, lysates from equivalent numbers of untreated cycling and mitotic Jurkat cells were immunoprecipitated with anti-Lck polyclonal antibodies. The immune complexes were washed in lysis buffer followed by washes in 25 mM HEPES, pH 7.5. The immune complexes were incubated for 3 min at 30 °C in 25 mM HEPES, pH 7.4, 10 mM MnCl₂, 5 µM p-nitrophenyl phosphate, 5 µM ATP, and 20 µl of [γ-³²P]ATP, and the reactions were stopped by adding SDS sample buffer. Phosphorylated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography.

Phosphoproteins were excised from nitrocellulose membranes and digested with trypsin as described previously (41). Briefly, membranes were incubated in polyvinylpyrrolidone (PVP-10, Sigma) and 1 mM acetic acid for 30 min at 37 °C. After washing with 25 mM acetic acid, membranes were incubated for 2 h at 37 °C with 10 µg of N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin in 50 mM NH₄HCO₃ and then for an additional 2 h with 10 µg of fresh trypsin. Supernatants were lyophilized and resuspended in alkaline PAGE sample buffer containing 0.125 M Tris-HCl, pH 6.8, 6 mM urea, and bromphenol blue. The tryptic phosphopeptides were separated by 40% alkaline-acrylamide gels (42) until the blue tracking dye had migrated to R<sub>n</sub> = 0.5. The phosphopeptides were detected by autoradiography.

**Phosphoamino Acid Analysis**—Phosphopeptide bands were excised from alkaline 40% gels and extracted in 1 ml of distilled water overnight at 37 °C with constant shaking. The extract was concentrated using a Vivaspin concentrator, and the resulting pellet was resuspended in 3 µl of distilled water. Samples were spotted onto a Whatman cellulose thin layer plate. Phosphoamino acids were separated by electrophoresis at 1100 V for 45 min. Standards were visualized by ninhydrin staining, and radioactive samples were analyzed using a Storm 880 PhosphorImager.

**RESULTS**

*Lck Is Serine-phosphorylated during Mitosis in Jurkat T Cells*—Jurkat T cells and the Lck-deficient Jurkat variant JCaM1 cells were chosen to study the mitotic serine phosphorylation of Lck. As we reported previously (28), Jurkat cells arrested at mitosis by treatment with the microtubule inhibitor nocodazole contained an additional Lck band which migrated with slower electrophoretic mobility on SDS-polyacrylamide gels (Fig. 1A, lane 2). As mentioned previously, this shifted, slower mobility form of Lck is associated with increased serine phosphorylation. Although not visible in Fig. 1A, similar immunoblot analyses frequently showed the presence of a faint Lck band of decreased mobility in lysates from cycling, non-nocodazole-treated Jurkat cells (*e.g.* Fig. 1B, 1C, and 1D). The presence of the decreased mobility (shifted) form of Lck in cycling cells correlated with flow cytometric analysis, which indicated the presence of ~20% mitotic cells in the cycling population (data not shown). The slower mobility form of Lck present in the cycling cell population likely represents the small fraction of mitotic cells that are present in the dividing cultures. As expected, the lysates from Lck-deficient JCaM1 cells contained a truncated form of Lck (Δ-Lck, Fig. 1A, lanes 3 and 4) that previously was reported to be catalytically inactive (2).

As nocodazole itself recently was demonstrated to inhibit the activity of Lck (43), we sought to confirm that the shift in the electrophoretic mobility of Lck was a mitosis-specific event, rather than a nocodazole-induced effect. To determine the effect of the drug on the mobility of Lck, a time course of nocodazole treatment of Jurkat cells was performed. The results revealed that treatment of Jurkat cells with nocodazole for 15 min did indeed induce the hyper-phosphorylated, shifted form of Lck (Fig. 1B). The generation of the shifted form of Lck reached a maximum at 30 min following the addition of nocoda-
We reported previously (28) that in vitro phosphorylation of Lck by the mitotic cyclin-dependent kinase, Cdc2, induced the shifted form of Lck. This result suggested that the mitotic phosphorylation site contained either a serine or threonine residue followed by a proline residue. Four such serine or threonine residues, serines 59, 194, and 281 and threonine 330, are present in the Lck sequence. Of these four residues, only two, serine 59 and serine 194, have been reported to be phosphorylated in vivo (17, 18). Hence, our initial studies to identify the mitotic site of phosphorylation focused on these two serine residues. As illustrated in Fig. 2, the sequence surrounding serine 59 resembles the consensus sequence for phosphorylation by MAP kinases, and the sequence surrounding serine 194 is similar to the consensus phosphorylation site for Cdc2. As mentioned earlier, ERK MAP kinases phosphorylate serine 59 in vitro (18, 22) and in cells (23), and a synthetic peptide containing the serine 194 site was reported to be phosphorylated by Cdc2 (17).

Lck Lacking Serine 59 Fails to Shift Its Electrophoretic Mobility on SDS-Polyacrylamide Gels—To determine whether serines 59 or 194 were phosphorylated at mitosis, alanine substitution mutants were made to generate the S59A, S194A single mutants and the S59A/S194A double mutant. The wild type and mutant forms of Lck transiently were expressed in the Lck-deficient JCaM1 cells, and Lck was immunoprecipitated from cycling and mitotic cells. Lysates from both cycling and mitotic Jurkat cells demonstrated a decreased electrophoretic mobility of serine 194 similar to wild type Lck, indicating that the phosphorylation of serine 194 was not necessary for the mitotic induced shift in the mobility of Lck. However, the additional mutation of serine 59 to alanine blocked the mitotic shift (Fig. 3B, lane 4). This result suggested that serine 59 was phosphorylated at mitosis either causing or contributing to the formation of the decreased mobility form of Lck. The mobility of the S59A mutant could not be determined, as its expression level was too low to permit analysis.

To investigate whether phosphorylation of serine 59 is sufficient to change the electrophoretic mobility of Lck, an aspartic acid residue was substituted for serine 59 to position a negatively charged residue at this site. Wild type and S59D Lck were expressed in JCaM1 cells, and the electrophoretic mobilities of Lck from cycling and mitotic cells were compared. As shown in Fig. 3C, the S59D mutant from both cycling and mitotic cells demonstrated a decreased electrophoretic mobility. This result indicated that positioning a negatively charged residue at position 59 in the Lck sequence was sufficient to generate the shifted mobility form of Lck and supported the...
Serine 59 is specifically phosphorylated at mitosis.

Phosphorylation of Serine 59 Is Independent of the Catalytic Activity and Intact SH3 or SH2 Domains of Lck and the Presence of ZAP-70—Results presented above suggested that serine 59 was a site of mitotic phosphorylation in Lck. In the case of antigen receptor signaling, it was suggested that phosphorylation of serine 59 by ERK MAP kinase is the result of a negative feedback pathway designed to inhibit activated Lck (18, 25). To assess the requirement for the catalytic activity of Lck for the mitotic phosphorylation of serine 59, a point mutant (K273R) that disrupts the ATP binding capacity and hence the catalytic activity of Lck was generated and expressed in JCaM1 cells. Western blotting analysis of cycling and mitotic cells expressing wild-type Lck (lanes 2 and 3), S99D Lck (lanes 4 and 5), or pCAGGS vector (lanes 6 and 7) were immunoblotted with anti-Lck monoclonal antibodies.

Fig. 3. Serine 59 is phosphorylated at mitosis. A, detergent lysates (lanes 1 and 2) and Lck immunoprecipitates (lanes 3–9) from mitotic (M) and cycling (U) Jurkat (lanes 1 and 2 and 8 and 9) and JCaM1 cells expressing wild-type Lck (lanes 3 and 4), S194A Lck (lanes 5 and 6), or pCAGGS vector only (lane 7) were immunoblotted with anti-Lck monoclonal antibodies. B, detergent lysates (lane 1) and Lck immunoprecipitates (lanes 2–6) from mitotic (M) and cycling (U) Jurkat (lane 1) and JCaM1 cells expressing wild-type Lck (lanes 2 and 3), S99A/S194A double mutant Lck (lanes 4 and 5), or pCAGGS vector (lane 6) were immunoblotted with anti-Lck monoclonal antibodies. C, detergent lysates (lane 1) and Lck immunoprecipitates (lanes 2–7) from mitotic (M) and cycling (U) Jurkat (lane 1) and JCaM1 cells expressing wild-type Lck (lanes 2 and 3), S99D Lck (lanes 4 and 5), or pCAGGS vector (lanes 6 and 7) were immunoblotted with anti-Lck monoclonal antibodies.

Fig. 4. Mitotic phosphorylation of Ser59 is independent of the early components of the TCR signaling pathway. A, detergent lysates (lanes 1 and 2) and Lck immunoprecipitates (lanes 3–7) from mitotic (M) and cycling (U) Jurkat (lanes 1 and 2) and JCaM1 cells expressing wild-type Lck (lanes 3 and 4), K273R Lck (lanes 5 and 6), or pCAGGS vector (lane 7) were immunoblotted with anti-Lck monoclonal antibodies. B, detergent lysates from mitotic (M) and cycling (U) Jurkat (lanes 1 and 2), JCaM1 (lanes 3 and 4), and ZAP-70 deficient P116 (lanes 5 and 6) cells were immunoblotted with anti-Lck antibodies. C, detergent lysates (lanes 1 and 13) and Lck immunoprecipitates (lanes 2–12) from mitotic (M) and cycling (U) Jurkat (lanes 1 and 13) and JCaM1 cells expressing wild-type Lck (lanes 2 and 3 and 6 and 7), R154K Lck (lanes 4 and 5), W97A Lck (lanes 8 and 9), W97A/R154K Lck (lanes 10 and 11), or pCAGGS vector (lanes 12) were immunoblotted with anti-Lck monoclonal antibodies.

Phosphorylation of Serine 59 at Mitosis Is Independent of ERK1/2 MAP Kinase Pathway and p38—The ERK MAP kinase was reported to phosphorylate serine 59 in Lck in vitro (18, 22) and in cells in response to TCR ligation (23) and to associate with the SH3 domain of Lck (45). However, the data from the catalytically inactive Lck mutant and the ZAP70-deficient P116 cells presented above suggested that a pathway distinct from that induced by antigen receptor stimulation resulted in the mitotic phosphorylation of serine 59. To test whether ERK MAP kinase was responsible for the mitotic phosphorylation of serine 59, cycling and nocodazole-arrested mitotic Jurkat cells were treated with the MEK1/2 inhibitor (U0126) and immunoblotted with anti-Lck antibodies. As a positive control, Jurkat cells that were either untreated or were pre-treated with U0126 were stimulated with PMA, and the lysates were included on the same gel. The phorbol ester PMA is a potent activator of the serine/threonine kinase protein kinase C, and treatment of T cells with PMA both activates the MEK1/2 inhibitor (PD98059) yielded identical results (data not shown). Consistent with these results, we find that ERK MAP
kinase is not active in nocodazole-arrested Jurkat cells. Another member of the MAP kinase superfamily, p38, has been reported to be activated in nocodazole-arrested mitotic cells (46). To determine whether p38 phosphorylated serine 59 at mitosis, the p38 inhibitor SB 203580 was used to treat cycling and mitotic Jurkat cells. Similar to the results seen with the ERK MAP kinase pathway-specific inhibitors, the p38 inhibitor had no effect on the mitotic phosphorylation of serine 59 (Fig. 5B).

**Lck Phosphorylation Sites Determined by One-dimensional Phosphopeptide Mapping Analysis—**Although the above results strongly suggested that serine 59 was a mitotic site of phosphorylation in Lck, they did not directly demonstrate phosphorylation at this site, nor did they address whether additional mitotic sites existed. To accomplish this, a one-dimensional phosphopeptide mapping strategy using 40% acrylamide allows peptides as small as 4 amino acids to be resolved (41) and permits the direct comparison of different samples on the same gel. Lck from cycling and mitotic cells labeled in vivo with [32P]orthophosphate was immunoprecipitated with anti-Lck antibodies, and the lower (unshifted) and upper (shifted) mobilities were separated by SDS-PAGE (Fig. 6A). Both [32P]-Lck bands were excised from the gels and thoroughly digested with trypsin. Tryptic phosphopeptides were resolved on 40% alkaline-polyacrylamide gels. This phosphopeptide mapping technique recently was used successfully for mapping the sites of phosphorylation in the unshifted form of Lck (41). This technique preferentially allows negatively charged peptides to enter the gel and separates peptides on the basis of their charge to mass ratio. The high percentage of acrylamide allows peptides as small as 4 amino acids to be resolved (41) and permits the direct comparison of different samples on the same gel.

Lck from cycling and mitotic cells labeled in vivo with [32P]orthophosphate was immunoprecipitated with anti-Lck antibodies and the lower (unshifted) and upper (shifted) mobility forms were separated by SDS-PAGE (Fig. 6A). Both [32P]-Lck bands were excised from the gels and thoroughly digested with trypsin. Tryptic phosphopeptides were resolved on 40% alkaline-polyacrylamide gels. Fig. 6 shows the results of such an analysis of Lck labeled in vivo with [32P]orthophosphate (Fig. 6B) and in vitro in an autokinase assay utilizing [γ-32P]ATP (Fig. 6C). It is immediately obvious that the lower (L) and upper (U) mobility forms of Lck displayed distinct phosphopeptide mapping patterns consistent with their differential phosphorylation. The lower mobility forms of in vivo labeled Lck from cycling and mitotic cells generated identical phosphopeptide maps (Fig. 6B, left panel, lanes 1 and 2) indicating that the in vivo sites of phosphorylation in the unshifted form of Lck are unchanged at mitosis, at least within the limits of the resolution of this analysis. As indicated previously and illustrated in Fig. 6A (lane 1), a faint but distinct upper mobility form of Lck (U1) is present in unsynchronized cycling Jurkat cells, which represents the small percentage of cells in this population that are undergoing mitosis. Importantly, both the upper mobility, shifted forms of Lck, one generated from a cycling, non-nocodazole-treated cell population (Fig. 6B, left panel, lane 3 (U1)), and the other generated from a nocodazole-arrested mitotic population (Fig. 6B, right panel, lane 5 (U2)) generated identical phosphopeptide maps. This indicated that the increased in vivo phosphorylation of Lck seen at mitosis is a mitosis-specific event rather than a direct effect of nocodazole on Lck. The two panels in Fig. 6B are from two separate experiments. The bands shown in Fig. 6A were used to generate the data in Fig. 6B, right panel, whereas the bands used to generate the data in Fig. 6B, left panel, are not shown. In addition to the two experiments illustrated in Fig. 6B, two other experiments yielded identical results when the phosphopeptide maps of the upper forms of Lck from cycling and nocodazole-arrested cells were compared (data not shown).

Examination of the phosphopeptides generated from the lower, unshifted form of Lck labeled in vivo revealed the presence of seven phosphopeptides (phosphopeptides 1–3 and 6–9; Fig. 6B, lanes 1, 2, and 6). This pattern was consistent throughout this study where 10 separate in vivo labeling experiments and subsequent phosphopeptide mapping analyses were performed. The pattern was altered, however, in the upper, shifted, mitotic form of Lck, where phosphopeptides 1 and 2 showed a faster mobility, resulting in a shift in their migration distance (phosphopeptides 1a and 2a, Fig. 6B, lanes 3–5). The increased mobility of phosphopeptides 1a and 2a is consistent with increased phosphorylation of these peptides, as the addi-

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tion of a phosphate with its two negative charges would increase peptide mobility in this alkaline gel system. In addition, a new phosphopeptide, phosphopeptide 4, was evident in the mitotic form of Lck (Fig. 6B, lanes 3–5).

Phosphopeptide mapping analysis of both the lower and upper mobility forms of Lck labeled in an in vitro autokinase assay showed a similar but not identical pattern (Fig. 6C). Phosphopeptide 9 in particular showed a dramatic increase in phosphorylation suggesting that this band represented the tryptic peptide containing tyrosine 394, the autophosphorylation site. By using phosphopeptides with known molecular weights and charge, it is possible to construct a standard curve and predict where various Lck phosphopeptides will migrate (47). Such an analysis using phosphopeptides generated from the tyrosine kinase Syk as standards revealed that phosphopeptide 9 migrates at a position that is predicted for the tryptic peptide containing phosphorylated tyrosine 394 (data not shown). Additionally, phosphoamino acid analysis of peptide 9 revealed that the only phosphorylated amino acid in this peptide was phosphotyrosine (Fig. 8B). Therefore, phosphopeptide 9 is the fragment, LIEDNEnPTAR, (amino acids 388–397; where pY is phosphotyrosine) containing the auto-phosphorylation site at tyrosine 394. As was seen in Lck labeled in vivo, the pattern of phosphopeptides generated from the lower and upper mobility forms of Lck differed (Fig. 6C) consistent with the differential phosphorylation of these two forms of Lck. In particular, phosphopeptides with mobilities similar to the serine 59-containing peptide (see next section) were present in in vitro phosphorylated Lck. Also, a mitotic specific phosphopeptide similar to band 4 (Fig. 6B) appeared directly above band 5 in the upper mobility form of Lck (Fig. 6C, lane 2).

The phosphopeptides of immediate interest in Fig. 6 were those migrating near the top of the gel, where phosphopeptides derived from the upper mobility mitotic form of Lck (phosphopeptides 1a and 2a) were altered in their migration relative to those derived from the lower mobility, non-mitotic form of Lck (phosphopeptides 1 and 2). Based on the results in Fig. 3, which suggested that serine 59 was a target for the mitotic phosphorylation of Lck, it seemed likely that these phosphopeptides contained serine 59.

The nonphosphorylated form of the tryptic peptide containing serine 59 has a molecular mass of 4166 Da and contains one threonine, four serine, and two tyrosine residues. By using a standard curve constructed from the migration of known phosphopeptides derived from the tyrosine kinase Syk, phosphopeptides 1 and 2 migrate at positions predicted for the serine 59 tryptic peptide containing one (phosphopeptide 1) or two (phosphopeptide 2) phosphates. We therefore suspected that phosphopeptides 1 and 2 represented singly and doubly phosphorylated versions of the serine 59 tryptic peptide. If this were the case, then the additional phosphorylation of serine 59 induced at mitosis would be predicted to increase the mobility of the more acidic phosphoserine 59-containing peptides. Assuming that phosphopeptide 1 (Figs. 6B and 7) represents a singly phosphorylated form of the serine 59-containing peptide, then phosphopeptide 1a (Fig. 6B and 7) would represent this same peptide additionally phosphorylated on serine 59 due to the activation of a serine 59 kinase at mitosis. Therefore, phosphopeptide 2 (Figs. 6B and 7) is a doubly phosphorylated version of the same peptide, where the additional phosphorylation of serine 59 generates phosphopeptide 2a.

To verify the identity of phosphopeptides 1, 1a, 2, and 2a, we took a mutational approach where serine 59 was mutated to aspartic acid (S59D) to partially mimic the negative charge generated by the mitotic phosphorylation of serine 59. We chose to analyze these mutants in the Lck-deficient JCaM1 cells. We initially determined that the phosphorylation patterns of both the upper and lower mobility forms of wild type Lck transiently expressed in JCaM1 cells were similar to those seen in endogenous Lck from Jurkat cells (data not shown). By using this expression system the S59D mutant of Lck was transiently expressed in JCaM1 cells that were either allowed to cycle or were arrested in mitosis. Cycling and mitotic Jurkat cells and S59D-expressing JCaM1 cells were metabolically labeled with [32P]orthophosphate. Lck was immunoprecipitated, and the two mobility forms were resolved by SDS-PAGE. As was seen previously in Fig. 3C, the S59D Lck mutant migrated with a single mobility from both cycling and mitotic cells, and this mobility corresponded to the mobility of the upper mitotic form of Lck (data not shown). The upper and lower mobility forms of endogenous Lck from mitotic Jurkat cells and the single S59D mobility form from cycling and mitotic JCaM1 cells were excised and subjected to phosphopeptide mapping analysis. As seen in Fig. 7, the phosphorylation pattern of Lck that contained an acidic residue at amino acid 59 (lanes 3 and 4) was similar to the phosphorylation pattern of the mitotic, upper mobility form of Lck. In particular, the aspartic acid containing versions of the serine 59 tryptic peptides displayed migrations that were identical to the migration of phosphopeptides 1a and 2a in the upper mobility form of Lck. We interpret these data as follows: phosphopeptides 1a and 2a from the S59D mutant are singly (phosphopeptide 1a) and doubly (phosphopeptide 2a) phosphorylated versions of the serine 59-containing phosphopeptide. Neither can be phosphorylated on serine 59 because aspartic acid was substituted for serine. The additional charge introduced by the aspartic acid residue caused the phosphopeptides to migrate with mobilities similar to the singly (phosphopeptide 1) and doubly (phosphopeptide 2) phosphorylated, serine 59-containing phosphopeptides additionally phosphorylated on serine 59 in the mitotic form of Lck (phosphopeptides 1a and 2a, lane 1, Fig. 7). These data confirm that phosphopeptides 1 and 2 represent different phosphorylation states of the serine 59-containing peptide and that this residue is a site of mitotic phosphorylation of Lck.
The fact that phosphopeptides 1a and 2a containing a non-phosphorylatable residue at serine 59 (Fig. 7, lanes 3 and 4) were labeled in vivo with $^{32}$P indicates the presence of additional phosphorylation sites in the serine 59-containing tryptic peptide. Phosphoamino acid analysis of in vivo labeled phosphopeptides 1 and 2 revealed the presence of only phosphoserine (Fig. 8B, samples 1 and 2). This indicated that the novel phosphorylation sites in the serine 59-containing tryptic peptide are additional serine residues.

In addition to containing increased phosphorylated peptide 9, in vitro phosphorylated Lck contained a new phosphorylated peptide, phosphopeptide 5 (Fig. 6C and Fig. 8C). This phosphopeptide migrates at a position predicted for the tryptic peptide-containing phosphorylated tyrosine 505 using a standard curve constructed from the migration of known phosphopeptides derived from the tyrosine kinase Syk. Although phosphopeptide 5 is only faintly visible in in vivo labeled Lck from Jurkat cells (Fig. 6B), it is prominent in in vivo labeled Lck from LSTRA cells (Fig. 8A), which naturally overexpress Lck due to insertional mutagenesis of the Moloney leukemia virus upstream of the Lck promoter (38, 39). Phosphoamino acid analysis of phosphopeptide 5 from in vivo labeled LSTRA cells showed that phosphopeptide 5 contained only phosphotyrosine (Fig. 8B, sample 5). Moreover, when a mixture of the labeled tryptic phosphopeptides from Lck labeled in vitro was immunoprecipitated with an antibody raised against a synthetic Lck peptide containing amino acids 476–509, the antibody specifically recognized phosphopeptide 5 (Fig. 8C, lane 3). Therefore, phosphopeptide 5 is the tryptic fragment, SVLEDFTTATEGQpYQPQP, containing phosphorylated tyrosine 505.

We were unable to identify unequivocally phosphopeptides 4 and 6–8, although phosphoamino acid analysis revealed the presence of only phosphoserine in phosphopeptides 6 and 7 derived from the unshifted, lower form of Lck labeled in vivo in LSTRA cells (Fig. 8B, samples 6 and 7). Phosphopeptide 4 is especially intriguing because it appears to be a site of mitotic phosphorylation in Lck. Phosphopeptide 4 was intensely labeled with $^{32}$P in vivo in the shifted form of Lck that resulted from treatment of Jurkat cells with the phorbol ester, PMA (data not shown). Additionally, phosphopeptide 4 migrates with a mobility predicted for the serine 158-containing tryptic peptide (data not shown).

DISCUSSION

Stimulation of T cells through antigen receptor cross-linking or by treatment with phorbol esters is associated with an increase in the serine phosphorylation of Lck and a concomitant decrease in its electrophoretic mobility on SDS-polyacrylamide gels (13–18, 20, 21). A similar increase in the serine phosphorylation of Lck is seen at mitosis (28). Here we identify serine 59 in the unique region of Lck as a site of mitotic phosphorylation. Previously, serine 59 was shown to be a site of TCR-induced phosphorylation, and the kinase responsible for its phosphorylation was identified as ERK MAP kinase (18, 23). However, we conclude that ERK MAP kinase is not responsible for the phosphorylation of serine 59 at mitosis based on the following observations: (i) tyrosine kinase activities and binding domains that are necessary for the activation of the ERK MAP kinase pathway (e.g. Lck, ZAP-70, SH2 and SH3 domains of Lck) were not required for the mitotic phosphorylation of serine 59; and (ii) inhibitors of the ERK MAP kinase pathway failed to inhibit the mitotic phosphorylation of serine 59. Thus a proline-directed serine/threonine kinase distinct from ERK MAP kinase phosphorylates Lck on serine 59 at mitosis.

The mitotic serine phosphorylation of Lck is reminiscent of the mitotic phosphorylation of Src by Cdc2 (48). The phosphorylation of Lck and Src at mitosis occurs in the unique domains of both kinases, but at non-conserved residues (Ref. 48 and this report). Purified Cdc2 can phosphorylate Lck in vitro, and this phosphorylation results in a shift in the electrophoretic mobility of Lck (28). Two other proteins are phosphorylated at mitosis at sites identical to (49) or nearly identical to (50) serine 59 in Lck. BCL-2 is phosphorylated on an identical site by ASK1/Jun amino-terminal protein kinase (49), and the protein-tyrosine phosphatase PTP-1B (where the glutamine in Lck is an asparagine in PTP-1B) is phosphorylated by an unknown mitotic kinase that is not Cdc2 (50). However, purified JNK1 fails to phosphorylate Lck in vitro making it an unlikely candidate for the mitotic serine 59 kinase. Regardless of which serine/threonine kinase is responsible for the mitotic phosphorylation of Lck, the fact that it is not ERK MAP kinase reveals that two distinct signaling pathways phosphorylate serine 59 depending on the physiological state of the cell.

Like Src, the function of Lck at mitosis is not known, although both kinases associate with and phosphorylate Sam68 (10, 42). However, unlike Src, it isn’t clear that the activity of Lck is activated at mitosis. In fact, it seems likely that just the opposite is true. Several studies (13, 14, 16, 18, 25) have reported that the activity of the slower mobility form of Lck is decreased, and we have observed a decrease in the phosphorylation of tyrosine 394 (the autophosphorylation site) in the mitotic, slower mobility form of Lck (Figs. 6 and 7). Phosphorylation of tyrosine 394 is known to activate Lck (51). However, some basal activity of Lck is present in mitotic cells based on the finding that in vitro kinase assays of Lck from mitotic cells result in both Lck autophosphorylation and the phosphorylation of co-immunoprecipitating Sam68 and Raf-1 (52). It is...
also possible that the phosphorylation of serine 59 alters the binding of Lck to cellular partners or its submembrane localization.

The peptide mapping studies revealed the existence of several additional phosphorylation sites on Lck. The in vivo labeling of the S59D mutant with 32P indicated that at least two serine residues in the serine 59-containing tryptic peptide were basally phosphorylated in cycling cells. Although we identified the serine 59-containing peptide as well as the tyrosine 394- and tyrosine 505-containing peptides, the identities of three additional in vivo labeled phosphoserine-containing peptides (4, 6, 7) are still under investigation. The identity of phosphopeptide 4 is especially important because it appears specifically in the shifted form of Lck, generated both at mitosis and by treatment of cells with the phorbol ester, PMA. Phosphopeptide 4 migrates with a mobility that is predicted for the serine 158-containing tryptic phosphopeptide, and it was reported that serine 158 is a major site of in vivo phosphorylation following PMA treatment of Jurkat T cells (17). Therefore we think it likely that serine 158 is a second site of mitotic phosphorylation in Lck.

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Characterization of the in Vivo Sites of Serine Phosphorylation on Lck Identifying Serine 59 as a Site of Mitotic Phosphorylation
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