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Modification of Ser^{59} in the unique N-terminal region of tyrosine kinase p56^{lck} regulates specificity of its Src homology 2 domain

(tyrosine phosphorylation/glutathione *S*-transferase fusion protein/surface plasmon resonance)

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ABSTRACT **During T-cell activation, Ser^{59} in the unique N-terminal region of p56^{lck} is phosphorylated. Mutation of Ser^{59} to Glu^{59} mimics Ser^{59} phosphorylation, and upon CD4 crosslinking, this mutant p56^{lck} induces tyrosine phosphorylation of intracellular proteins distinct from those induced by wild-type p56^{lck}. Mutant and wild-type p56^{lck} have similar affinities for CD4 and similar kinase activities. In glutathione *S*-transferase fusion proteins, the p56^{lck} Src homology 2 (SH2) domain with the SH3 domain and the unique N-terminal region (including Ser^{59}) has a different binding specificity for phosphotyrosyl proteins than the SH2 domain alone. Either deletion of the unique N-terminal region or mutation of Ser^{59} to Glu^{59} in the fusion protein reverts the phosphotyrosyl protein binding specificity back to that of the SH2 domain alone. These results suggest that phosphorylation of Ser^{59} regulates the function of p56^{lck} by controlling binding specificity of its SH2 domain.**

The association of p56^{lck}, a lymphoid cell-specific Src-family protein-tyrosine kinase, with the intracellular domains of the CD4 and CD8 molecules (1) and the direct involvement of p56^{lck} in T-cell activation (2), cytolytic activity of cytotoxic T lymphocytes (3), and thymic differentiation (4) have all been described (reviewed in ref. 5). Members of the Src family of cytoplasmic tyrosine kinases, including p56^{lck}, have an N-terminal myristoylation site, a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, and a C-terminal catalytic domain with conserved regulatory tyrosine phosphorylation sites (6). SH2 and SH3 domains mediate protein–protein interactions by binding to proteins containing phosphotyrosine and a proline-rich region, respectively (7). The first 66 aa of p56^{lck} are unrelated in amino acid sequence to similarly placed unique regions in other members of the Src family (6).

Tyrosine kinase activity of p56^{lck} is suppressed by phosphorylation of the C-terminal inhibitory tyrosine residue (Tyr^{505}) that is catalyzed by another tyrosine kinase, Csk (8). The catalytic activity of p56^{lck} is upregulated by a CD45-mediated dephosphorylation of Tyr^{505} (9) and an increase in the autophosphorylation of Tyr^{394} (10). It has been proposed that phosphorylated C-terminal Tyr^{505} binds intramolecularly to the SH2 domain, blocking the kinase domain from its substrate (5, 11). In this model, dephosphorylated Tyr^{505} is released from the SH2 domain, and the SH2 domain becomes free for binding to other signaling proteins.

Although SH2 domains are structurally homologous, those from different proteins have distinct binding specificities for particular phosphotyrosyl proteins, determined by the amino acid sequence surrounding the phosphotyrosine residue (12). Many of the complexes formed by binding of SH2 domains to phosphotyrosine-containing proteins have been described as having critical roles in signal transduction (7), including the Ras signal pathway (13).

As the N and C termini of the SH2 domain are in close proximity on opposite sides of the phosphotyrosine-binding region in the three-dimensional structure, it has been suggested that other regions of these proteins may not significantly affect function and folding of the SH2 domain (14, 15). However, some binding studies suggest that SH2 domains can be intramolecularly modified by other regions of these SH2 domain-containing proteins (16, 17). Regulatory events for the p56^{lck} SH2 domain could also take place in the context of the full-length protein.

Transient phosphorylation of Ser^{59} in the unique N-terminal region of p56^{lck} by a proline-directed kinase [most likely by mitogen-activated protein kinase (MAP kinase)] during T-cell activation has been observed (refs. 18 and 19; unpublished work). Phosphorylation at this site induces a shift in apparent molecular mass of p56^{lck} from 56 kDa to 61 kDa in SDS/polyacrylamide gels. We demonstrate that the Ser^{59} phosphorylation site regulates p56^{lck} by modifying the binding affinity and specificity of the p56^{lck} SH2 domain for phosphotyrosine-containing proteins.

MATERIALS AND METHODS

Site-Directed Mutagenesis, Expression, and Activation of Cells. Site-directed mutagenesis was performed on uracil-containing phage DNA (20). HeLa and CD4$^+$ HeLa cells (21) and Jurkat T cells were maintained in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium and RPMI 1640, respectively. HeLa cells were transiently transfected with 20 μg of cDNA in the pCDNA-1 expression vector (Invitrogen) per 10-cm plate by the calcium phosphate precipitation method (22) and used for assays 48–72 hr later. v-*src* transformation was induced by transient expression of v-*src*. For antibody crosslinking, OKT4 and T3b monoclonal antibodies were used to activate cells through CD4 and CD3, respectively. Cells were incubated at 4°C for 30 min with primary antibody and washed twice at 4°C. Crosslinking was initiated by the addition of goat anti-mouse IgG antibody at 37°C and terminated by washing with ice-cold phosphate-buffered saline or by boiling in SDS/PAGE loading buffer.

Production of Glutathione *S*-Transferase (GST) Fusion Proteins. GST fusion proteins were produced as described (23). Portions of the *lck* gene were isolated by PCR and were subcloned into the *Nde* I and *Eco*RI sites of pGEX-3Xb vector. When the bacteria bearing the vector reached an OD_{555} of 0.6, protein expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside for 3 hr. Bacterial pellets were lysed by sonication in 10 mM Hepes, pH 7.5/150 mM NaCl/0.5% (vol/vol) Triton X-100/10 mM dithiothreitol. The lysate was cleared by centrifugation at 15,000 rpm in a Sorvall SS34 rotor for 30 min. GST fusion proteins were purified on glutathione-

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Abbreviations: SH, Src homology; GST, glutathione *S*-transferase; MAP kinase, mitogen-activated protein kinase.

Sepharose, dialyzed against 10 mM Hepes, pH 7.5/150 mM NaCl/10 mM dithiothreitol, aliquoted, and stored frozen at −80°C.

Protein Precipitation. Cells were lysed for 10 min at 10^7 cells per ml of lysis buffer [10 mM Tris, pH 7.4/150 mM NaCl/1% Triton X-100 supplemented with phenylmethanesulfonyl fluoride (1 mM), leupeptin (1 mg/ml), Na_3VO_4 (1 mM), and NaF (1 mM)]. Insoluble material was removed by centrifugation at 16,000 × *g* for 30 min at 4°C. Lysates were precleared for 1 hr with glutathione-Sepharose (Pharmacia). For each 150 μl of precleared cell lysate, 5 μg of GST fusion protein and 10 μl of glutathione-Sepharose were used for precipitation. For phosphotyrosyl peptide competition, the stated amounts of phosphotyrosyl peptides were added to the incubation mixture. After 1–2 hr at 4°C, precipitates were washed three times with lysis buffer. Bound proteins were eluted from the glutathione-Sepharose beads by boiling in SDS/PAGE loading buffer. Four phosphotyrosyl peptides, pY324 [EPQY(*P*)EEIPIYL, where Y(*P*) is phosphotyrosine], pY505 [TEGQY(*P*)QPQPA], pY771 [SSNY(*P*)MAPYDNY], and pY536 [ESEY(*P*)GNITYPP], were kindly provided by Steven Shoelson (Joslin Diabetes Center, Harvard Medical School).

Analysis of Bound Proteins. After SDS/PAGE, proteins were electrotransferred to nitrocellulose membranes for immunoblot detection using 4G10 anti-phosphotyrosine monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse antibody. Antibody signals were developed with enhanced chemiluminescence (ECL kit; Amersham). For immune-complex kinase assays, immunoprecipitates were added to kinase buffer [50 mM Hepes, pH 7.5/15 mM $MgCl_2$ containing 10 μCi (370 kBq) of [γ-^{32}P]ATP per assay] and incubated at room temperature for 10 min. The reactions were stopped by denaturation in SDS/PAGE loading buffer. Proteins were resolved by SDS/PAGE, dried, and visualized by autoradiography.

Kinetics of binding of the SH2 domain to phosphotyrosyl peptides were measured by competition assay using surface plasmon resonance. The theory, operation, and immobilization chemistry have been described (23). The pY324 phosphopeptide was immobilized on a Biacore (Pharmacia) sensor chip surface, and 500 nM GST fusion protein was passed over the sensor chip. In subsequent injections, increasing concentrations of free phosphopeptides (10 nM to 100 μM for pY324 and 100 nM to 1 mM for pY505) were added to the 500 nM GST fusion protein in order to compete with the immobilized pY324. The GST fusion protein bound to the surface was displayed as a function of concentration of phosphopeptides used in competition.

RESULTS

p56^{lck} with Ser^{59} → Glu^{59} (S59E) Mutation Induces Different Tyrosine Phosphorylation of a Unique Set of Intracellular Proteins. Ser^{59} was mutated to Glu^{59} and to Ala^{59} (S59E and S59A) to mimic phospho- and non-phosphoserine, respectively. Wild-type and mutant p56^{lck}, expressed in CD4$^+$ HeLa cells, showed equivalent binding to CD4 (Fig. 1*A*) and had similar *in vitro* kinase activity measured either by autophosphorylation (Fig. 1*B*) or with enolase as an exogenous substrate (data not shown). The expression levels were comparable (Fig. 1*C*). The S59E mutant migrated at 61 kDa in SDS/polyacrylamide gels, whereas the wild-type p56^{lck} and the S59A mutant migrated at 56 kDa. Phosphorylation of Ser^{59} also induces a gel shift from 56 kDa to 61 kDa (18); the addition of a negative charge by the S59E mutation appears to mimic phosphorylation of Ser^{59}, at least in this respect.

Expression of wild-type p56^{lck} in CD4$^+$ HeLa cells followed by CD4 crosslinking induced tyrosine phosphorylation of a 95-kDa intracellular protein, in addition to autophosphorylation of p56^{lck} (Fig. 1*D*). A similar pattern was observed with S59A and S59E mutants. However, the latter mutant also induced constitutive tyrosine phosphorylations of 42-kDa and 80-kDa proteins in the absence of CD4 crosslinking, and it induced hyperphosphorylation of the 80-kDa protein upon CD4 crosslinking.

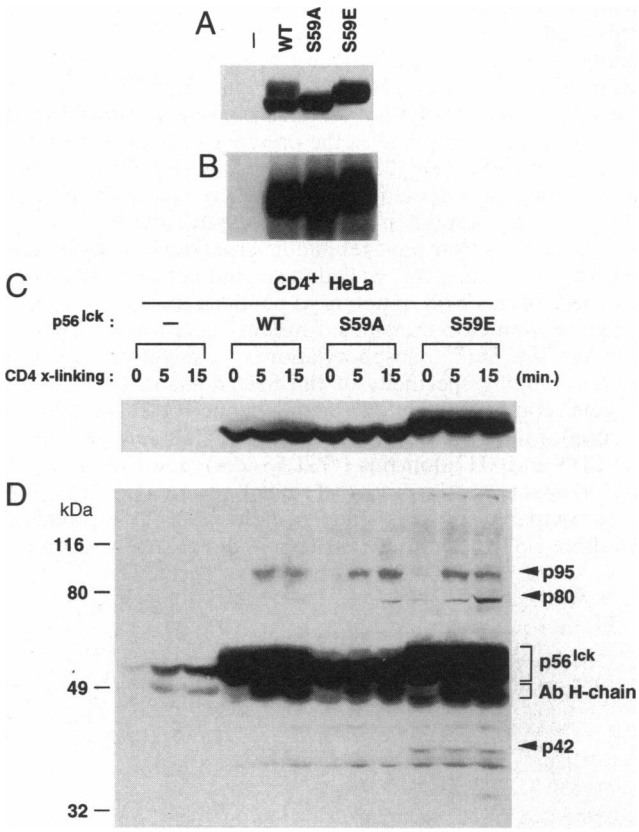

FIG. 1. Effects of S59E mutation of p56^{lck} on tyrosine phosphorylation of cellular proteins. (*A*) Wild-type (WT) and S59A and S59E mutants of p56^{lck} were expressed in CD4$^+$ HeLa cells. CD4 and bound p56^{lck} were coimmunoprecipitated from lysates of untransfected and transfected cells with the monoclonal OKT4 antibody, separated by SDS/8% PAGE, electrotransferred to nitrocellulose, and developed by immunoblotting with anti-Lck antibody. The two bands seen with wild-type are its Ser^{59}-phosphorylated (upper band) and nonphosphorylated (lower band) forms (18). (*B*) From another aliquot of the same cell lysates, p56^{lck} was immunoprecipitated with anti-Lck antibody. The immunoprecipitates were incubated with kinase buffer containing [γ-^{32}P]ATP and separated by SDS/PAGE; the signal was detected by autoradiography. (*C*) Untransfected and transfected cells were activated by crosslinking with OKT4 antibody and goat anti-mouse IgG for the specified time and lysed. Total cell lysates were separated by SDS/8% PAGE, electrotransferred to nitrocellulose membrane, and developed by anti-Lck immunoblotting. (*D*) The same blot from *C* was stripped of anti-Lck antibody and reprobed with the 4G10 anti-phosphotyrosine antibody. p56^{lck} and 95-, 80-, and 42-kDa tyrosine-phosphorylated protein bands are marked. Ab H-chain, antibody heavy chain.

It has been reported that phosphorylation of Ser^{59} by MAP kinase *in vitro* leads to a small decrease in catalytic activity in an *in vitro* kinase assay (19). The present study also found little effect of Ser^{59} modification on *in vitro* kinase activity (Fig. 1*B*). This suggests that the change in tyrosine phosphorylation of intracellular proteins caused by expression of the S59E mutant (Fig. 1*D*) may be due to changes in the ability of p56^{lck} to interact with other proteins, rather than to changes in the catalytic activity of p56^{lck}. Since SH2 domains bind to phosphotyrosyl proteins and can protect the bound phosphotyrosine from protein-tyrosine-phosphatase activity, the possible involvement of Ser^{59} modification in SH2 domain function was further studied.

Ser^{59} Region Affects Binding of the SH2 Domain to Phosphotyrosyl Proteins. GST fusion proteins (Fig. 2*A*) containing the unique 66-residue N-terminal region (GST.1–77), SH3 domain (GST.65–123), N terminus plus SH3 domain (GST.1–

123), and only the SH2 domain (GST.119–224) of p56^{lck} were expressed in *Escherichia coli*, purified, and examined for their ability to bind to phosphotyrosyl proteins. Anti-phosphotyrosine immunoblot analysis showed that only GST.119–224 precipitated phosphotyrosyl proteins from a v-*src*-transformed HeLa cell lysate prepared in the presence of the phosphatase inhibitor Na_3VO_4 (Fig. 2*B*). Binding of these proteins to the SH2 domain was specifically blocked by competition with phosphotyrosyl peptide pY324 (see *Materials and Methods*), a peptide derived from hamster middle-sized tumor antigen that binds tightly to the p56^{lck} SH2 domain, but not by competition with several other phosphotyrosyl peptides (Fig. 2*B*) (12, 23).

Lysate from CD3-crosslinked Jurkat T cells was used to see whether the Ser^{59} phosphorylation site might modify the protein binding specificity of the SH2 domain. GST fusion proteins containing the SH2 domain alone (GST.119–224), a portion of the unique N-terminal region including Ser^{59} plus the SH3 and SH2 domains (GST.53–224), and the SH3 and SH2 domains lacking the N-terminal region (GST.65–224) were compared for their binding to phosphotyrosyl proteins. All three GST fusion proteins precipitated common phosphotyrosyl proteins at 30, 50–80, and 120 kDa (Fig. 3*A*). However, both GST.119–224 and GST.65–224 preferentially bound to 60- and 95-kDa proteins whereas GST.53–224 bound uniquely to a 100-kDa protein and possibly to a 45-kDa protein in the GST fusion protein area (Fig. 3 *A* and *B*). Thus, the phosphotyrosyl protein binding specificity of the SH2 domain was modified by aa 53–64 of p56^{lck}.

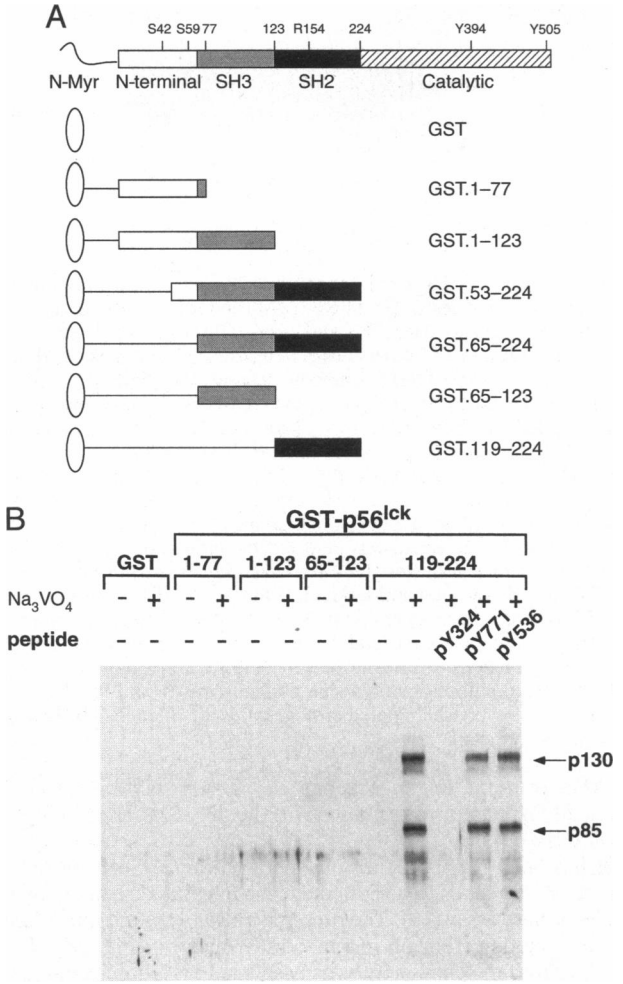

FIG. 2. Binding of phosphotyrosyl proteins to GST fusion proteins containing the Lck SH2 domain. (*A*) Construction of GST fusion proteins containing regions of p56^{lck}. N-Myr, *N*-myristoyl. (*B*) v-*src*-transformed HeLa cells were lysed in the presence or absence of 1 mM Na_3VO_4 and 1 mM NaF. Cell lysates were incubated with GST fusion proteins. Competition of phosphotyrosyl protein binding to GST.119–224 by phosphotyrosyl peptides was performed by adding 10 μM pY324, pY771, or pY536 to incubation mixture. Proteins bound to GST fusion proteins were precipitated with 10 μl of glutathione-Sepharose beads, washed, separated by SDS/8% PAGE, and visualized by anti-phosphotyrosine immunoblotting.

Similarly, both GST.119–224 and GST.53–224 precipitated 130- and 80-kDa phosphotyrosyl proteins from v-*src*-transformed HeLa cell lysate (Fig. 3*C*). However, GST.53–224 bound to a 70-kDa phosphotyrosyl protein (pp70) much more efficiently (Fig. 3 *C* and *D*). The effect of Ser^{59} modification on this altered specificity was measured by using two mutant GST.53–224 proteins, GST.53–224.S59A and GST.53–224.S59E. Like GST.53–224, GST.53–224.S59A bound almost equivalent amounts of pp80 and pp70. GST.53-224.S59E, however, bound much less pp70 than pp80, and in that sense had a specificity similar to that of the SH2 domain alone (GST.119–224). Deletion of aa 53–64 (GST.65–224) resulted in the same effect—i.e., much less binding of SH2 domain to pp70 (Fig. 3*D*).

Changes in Affinity of the SH2 Domain for Phosphotyrosyl Peptide Containing C-Terminal Tyr^{505} of p56^{lck}. Changes in protein binding properties of the SH2 domain produced by the N-terminal region of p56^{lck} were further confirmed by competition assays measuring the relative affinities of two phosphopeptides, pY324 [EPGY(*P*)EEIPIYL] and pY505 [TEGQY(*P*)QPQPA, derived from the p56^{lck} C-terminal phosphorylation site], using surface plasmon resonance (ref. 23; Fig. 4). Peptide pY324 bound ≈3-fold more strongly to GST.53–224 (ID_{50} = 1 μM) than to GST.119–224 (ID_{50} = 3.4 μM). On the other hand, pY505 bound to GST.53–224 (ID_{50} = 93 μM) much more weakly than to GST.119–224 (ID_{50} = 16 μM) (Fig. 4 *A* and *B*). A comparison of relative affinities shows that pY324 bound 5-fold better than pY505 to the SH2 domain in GST.119–224 (Fig. 4 *A* and *C*). However, pY324 bound to GST.53–224 90-fold better than did pY505 (Fig. 4 *B* and *C*). This is particularly interesting since removal of the sequence surrounding Ser^{59} (GST.65–224) reduced the preference for pY324 over pY505 back to 7-fold (Fig. 4*C*), a value comparable to the SH2 domain alone (GST.119–224). Additionally, GST.53–224.S59E displayed a relatively low preference (20-fold) for pY324 over the pY505 peptide (Fig. 4*C*). In summary, the SH2 domain alone (aa 119–224) and SH3 and SH2 domains together (aa 65–224) show relatively little selectivity for pY324 over pY505. The selectivity is dramatically increased by adding a fragment of the N-terminal region that includes Ser^{59} (aa 53–64) to the SH3 and SH2 domains together. However, the increment of the selectivity is lost when the S59E mutation is introduced to mimic phosphorylation. These experiments thus illustrate the important role of Ser^{59} in modifying the selectivity of the SH2 domain for phosphotyrosyl peptides.

DISCUSSION

The optimal sequence for binding to the isolated p56^{lck} SH2 domain (GST.119–224) is Glu-Glu-Ile at the three positions following the phosphotyrosine residue (12). This preference is mediated in part by a well-defined hydrophobic pocket that binds Ile at the third position following the phosphotyrosine (15). Thus, the small portion of the unique N-terminal region containing Ser^{59} may affect this binding pocket in some manner, leading to the differences in the binding specificity of the p56^{lck} SH2 domain that are seen in the context of GST.53–224. If this hypothesis is correct, the unique N-terminal region and the SH2 domain must be near each other despite the insertion of the SH3 domain between them in the primary sequence of the protein. In a recently solved crystal structure of SH3 and SH2 domains of p56^{lck} together, the most N-terminal residue of the SH3 domain is close to the hinge region between the SH3 and SH2 domains (24). This result indicates that Ser^{59} may well be in close proximity

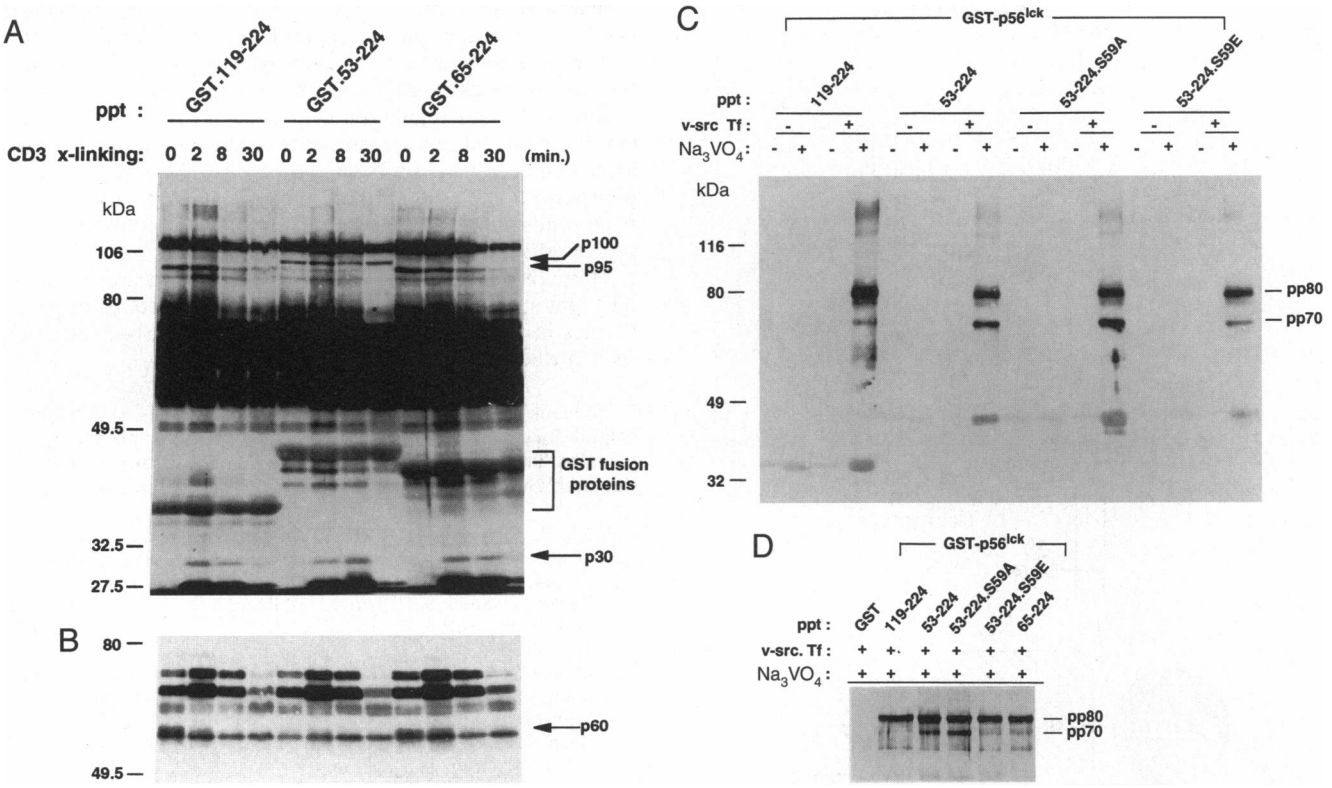

FIG. 3. Effects of Ser[59] modification in the unique N-terminal region of p56[lck] on binding of phosphotyrosyl proteins to its SH2 domain. (*A*) Lysates of CD3–CD4 cocrosslinked Jurkat T cells were incubated with GST fusion proteins. Bound proteins were immunoblotted with anti-phosphotyrosine antibody. After treatment with ECL reagent (Amersham), the blot was exposed to film for 10 sec. Proteins binding in common or specifically to GST.119–224, GST.53–224, and GST.65–224 are marked by arrows. (*B*) The same immunoblot as *A* was exposed for 1 sec. (*C*) GST fusion proteins containing the SH2 domain alone (GST.119–224) or in the context of aa 53–224 or aa 53–224 with Ser[59] mutated to either Ala or Glu (53–224.S59A and 53–224.S59E, respectively) were incubated with lysates of v-*src*-transformed (v-src Tf +) or untransformed HeLa cells in the presence or absence of phosphatase inhibitors (Na_3VO_4 and NaF). Bound phosphotyrosyl proteins were detected by immunoblotting with anti-phosphotyrosine antibody. (*D*) Phosphotyrosyl proteins bound to GST.119–224, GST.53–224, GST.53–224.S59A, GST.53–224.S59E, and GST.65–224 were compared. All lysates were from v-*src*-transformed HeLa cells and were incubated with GST fusion proteins in the presence of phosphatase inhibitors (Na_3VO_4 and NaF).

to the SH2 domain, and may explain how it can easily contact and affect the SH2 domain.

Recently, enhanced binding affinity of the v-Crk SH2 domain to phosphotyrosyl proteins by addition of the 27-aa region located immediately N-terminal of the SH2 domain was observed (17). In addition, SH2 domains of p59[fyn] and p60[src] in the context of SH3 and SH2 domains together showed higher binding affinity to phosphotyrosyl proteins when compared with the isolated SH2 domains (16). For p56[lck], the unique N-terminal region rather than the SH3 domain affected

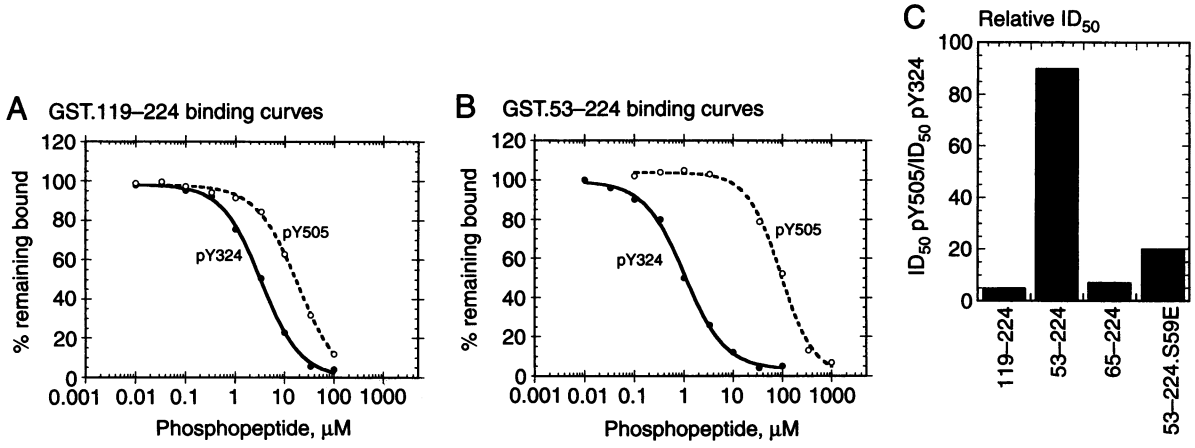

FIG. 4. Relative affinities of GST fusion proteins containing the SH2 domain of p56[lck] for phosphotyrosyl peptides pY324 and pY505. (*A*) GST.119–224 (500 nM) was preincubated with various concentrations of peptides and injected over a Biacore sensor chip surface containing immobilized pY324. The GST.119–224 bound to the surface was taken as a percentage of the amount bound at 0 μM competing phosphopeptide and plotted as standard sigmoidal curves. (*B*) The same experiment as *A* was done for GST.53–224 and plotted with the same scale as in *A*. (*C*) Relative preference of GST fusion proteins containing Lck 119–224, 53–224, 53–224.S59E, and 65–224 for the pY324 over the pY505 peptide was calculated as ID_{50} (pY505)/ID_{50} (pY324). Each experiment was done more than three times and the error range was within 5%.

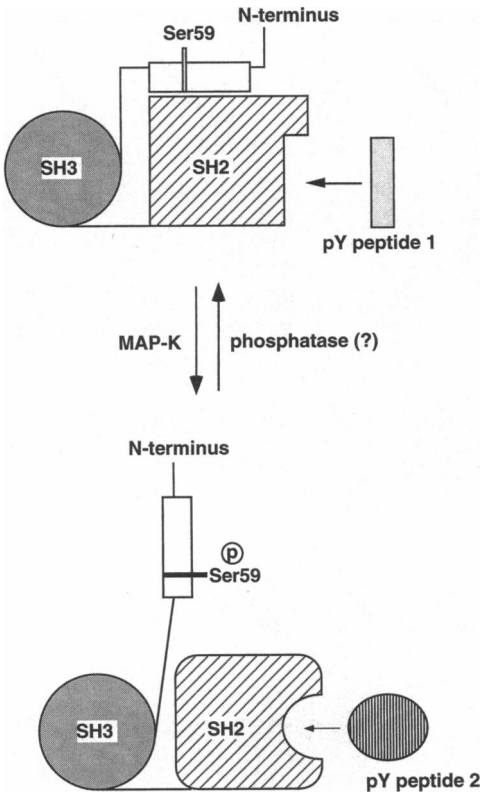

FIG. 5. Two states of the SH2 domain in p56^{lck}, modulated by phosphorylation of Ser^{59}, may show altered specificity for phosphotyrosyl (pY) peptides. MAP-K, MAP kinase.

the binding properties of its SH2 domain, as described in this paper. Together these results indicate that the context of the SH2 domain in relation to other portions of the protein can be a determinant of the specificity of protein–protein interactions directed by the SH2 domain.

Particularly, the SH2 domain of p56^{lck} may be reversibly regulated by phosphorylation in the N-terminal unique region. The S59E mutation and the Ser^{59} phosphorylation cause similar gel retardation of p56^{lck} (Fig. 1; ref. 18) and change the binding properties of the SH2 domain in GST.53–224 (Figs. 3 and 4). Thus, phosphorylation and dephosphorylation of Ser^{59} may regulate the equilibrium between two different states of the SH2 domain (Fig. 5). p56^{lck} is functionally located upstream of MAP kinase activation during T-cell receptor signaling (25), and the activated MAP kinase retrophosphorylates Ser^{59} of p56^{lck} (ref. 18; unpublished work). Thus the changed binding properties of the p56^{lck} SH2 domain caused by Ser^{59} phosphorylation would be important in the signaling cascade later than MAP kinase activation. These would include coupling to different signaling pathways by alternative intermolecular interactions using the changed specificity of the SH2 domain as well as changes in intramolecular regulation of p56^{lck} itself.

That MAP kinase is only transiently phosphorylated and activated upon CD3 crosslinking (26) indicates that activation of inhibitory mechanisms for MAP kinase follows immediately. In addition to induction of an "off signal"—i.e. expression of MAP kinase-specific phosphatase (27)—feedback suppression of an "on signal" where activated MAP kinase phosphorylates and suppresses its upstream kinases may occur. Indeed Raf-1 and MAP kinase kinase were phosphorylated by MAP kinase, resulting in reduction of their kinase activities (28, 29). Since p56^{lck} is directly involved in activation of the Ras pathway (25), which results in activation of MAP kinase, Ser^{59} phosphorylation of p56^{lck} by the activated MAP kinase may also reflect part of a feedback inhibitory mechanism for this pathway.

In this regard, increased selectivity of the SH2 domain in GST.53–224 toward phosphopeptide pY505 by S59E mutation (and presumably by Ser^{59} phosphorylation) indicates a potential role of the changed binding specificity of the SH2 domain in intramolecular regulation of p56^{lck}. In the activated T-cell environment, where tyrosine phosphatases are also active, increased binding affinity of the p56^{lck} SH2 domain toward phosphorylated Tyr^{505} would protect the phosphotyrosine from phosphatase activity and shift the equilibrium to a repressed form of p56^{lck}.

Thus, the SH2 domain of p56^{lck} apparently exists in two different states that may support multiple functions of p56^{lck}. Studies in intact cells using the mutants described here may shed further light on these roles.

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