Regulation of the Lck SH2 Domain by Tyrosine Phosphorylation*

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Src homology 2 (SH2) domains bind to phosphotyrosine (Tyr(P)) residues in specific sequence contexts in other proteins and thereby mediate tyrosine phosphorylation-dependent protein-protein interactions. The SH2 domain of the Src family kinase Lck is phosphorylated at tyrosine 192 in T cells upon T cell antigen receptor triggering. We have studied the consequences of this phosphorylation on the properties of the SH2 domain and on the function of Lck in T cell activation. We report that phosphorylation at Tyr192 reduced the capacity of the isolated SH2 domain to bind a high affinity peptide ligand and Tyr(P)-containing cellular proteins. This effect was mimicked by mutation of Tyr192 to an acidic residue. In intact T cells, where Lck participates in T cell antigen receptor signal transduction in an SH2 domain-dependent manner, phosphorylation of Tyr192 correlated with reduced downstream signaling. Our results indicate that tyrosine phosphorylation of the SH2 domain of Lck terminates its high affinity binding to ligands, thereby negatively regulating its participation in T cell antigen receptor signaling. This represents a novel mechanism for the regulation of the function of SH2 domains.

SH22 domains are independently folded hemispherical units of ∼100 amino acid residues, which are found in many signaling proteins (1–5). Their physiological function is to bind Tyr(P) residues in specific sequence contexts in other cellular proteins, thereby facilitating the formation of tyrosine phosphorylation-induced multimeric protein complexes (1, 3).

The ligand-binding surface of the SH2 domain of the Lck nonreceptor protein tyrosine kinase contains two pockets, one for the Tyr(P) residue and another for the amino acid residue three positions C-terminal to it, the +3 amino acid (2, 5). Although the first pocket is well conserved among SH2 domains, the residues of the SH2 domain that form and surround the second pocket vary more. These differences determine the depth and properties of the pocket and thereby the preferred amino acid ligand (6, 7). In the case of the Lck SH2 domain, the optimal ligand is a Tyr(P) followed by two acidic residues followed by an isoleucine at position +3, a specificity largely determined by amino acid residues in β strands D and E and in the EF loop adjacent to the second pocket (6, 8). Recently, it was shown that a single amino acid substitution in the EF loop in the SH2 domain of c-Src (a T215W mutation) changed the ligand selection of the SH2 domain to that of the Grb2 SH2 domain (9), which has a tryptophan residue at the corresponding location in its EF loop. Conversely, a switch from tryptophan to threonine in the SH2 domain of Grb2 changed its ligand preference to that of the c-Src SH2 domain (9).

Here we describe a more physiological regulation of the function of an SH2 domain, namely the effect of phosphorylation of a highly conserved tyrosine residue, Tyr192, in the end of β strand E in the Lck SH2 domain. This phosphorylation event, which can be catalyzed by Syk and possibly Zap nonreceptor kinases, leads to a profound down-regulation of the ligand binding capacity of the SH2 domain. The consequences for the function of Lck as a signal transducer in T cell activation were studied.

MATERIALS AND METHODS

Cells and Reagents—Jurkat human T leukemia cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, l-glutamine, and antibiotics. COS-1 cells were kept at logarithmic growth in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, l-glutamine, and antibiotics. The rabbit antiserum against the protein tyrosine kinase Syk (residues 253–365) was described earlier (10). The anti-Lck antiserum, raised against residues 39–64 of the kinase, was as before (11). The 4G10 anti-Tyr(P) monoclonal antibody (mAb) was from Upstate Biotechnology (Lake Placid, NY), and the H902 mAb (12) is available from the National Institutes of Health AIDS Research and Reference Reagent Program (reagent number 521). Custom-made Tyr(P)-containing phosphopeptides were purchased from Research Genetics Inc. (Huntsville, AL).

cDNA Constructs, Mutagenesis, and COS-1 Cell Transfections—The mammalian expression plasmids pTag/CVM-neo and pTag/SMc, containing lck and syk, respectively, have been described before (10, 13). These constructs are based on the vectors pRC/CMV and pME18S, respectively, modified (14) to encode a His6 sequence and an epitope tag (RIQRGPGRAFVTIK) located at the N terminus of the expressed product and recognized by the H902 mAb (12).

Mutagenesis of the tyrosine residue at position 192 of Lck to phenylalanine (Y192F) or glutamic acid (Y192E) was performed on the lck construct in pTag/CMV-neo using the Transformer kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions and verified by sequencing. In order to investigate the effects of Lck and its mutants in T cells, we deleted the N-terminal tag (to restore myristylation) and subcloned the constructs into the pEF-neo vector (15). The GST-SH2 domains of Lck were produced by polymerase chain reaction amplification of the desired region of the wild type, Y192F, and Y192E lck constructs (amino acids 121–224), using the proofreading Vent DNA polymerase (New England Biolabs, Beverly, MA). The polymerase chain reaction fragments were inserted in frame into the pGEX-3T prokaryotic expression vector (16), and the resulting GST-SH2 fusion proteins were purified by glutathione-Sepharose affinity chromatography. Transient transfection of COS-1 cells by lipofection was as before (10, 13).

Tryptic Peptide Mapping of in Vivo Labeled Lck—Jurkat T cells (10× 106 per sample) were labeled with 2 mCi/ml of [32P]orthophosphate-free
medium for 4 h, treated with 5 μg/ml of OKT3 mAb on ice for 15 min, washed, and incubated with 10 μg/ml Fab, goat anti-mouse Ig at 37 °C for 5 min. The cells were lysed under denaturing conditions (17) and Lck immunoprecipitated as before (10, 13). Control immunoprecipitates with normal rabbit serum did not contain the 56-kDa protein. Tryptic peptide mapping was as described (18), the peptides were separated by electrophoresis at pH 2, followed by ascending chromatography. The added Tyr192 peptide (NLDNGGFY*ISPR, 10 μg) was visualized by ninhydrin staining, and the 32P-labeled peptides were visualized by autoradiography for 15–20 h.

Binding Specificity of the Lck SH2 Domains—The ligand sequence specificity for the GST-SH2 domain constructs of Lck (wild type, Y192F, and Y192E) were determined using a degenerate phosphopeptide library as described (6, 7). The effects of phosphorylation of the Tyr192 residue on the binding of the SH2 domain of Lck to the high affinity Y*EEI sequence (6) were investigated after in vivo phosphorylation of Lck (wild type, Y192F, and Y192E) by Syk (wild type or kinase-dead) in COS-1 cells (13). Clarified lysates of syk and/or lck-transfected COS cells were mixed with 25 μl of Affi-Gel beads to which the phosphopeptides EPQY*EEIPIYLK or EPQY*EEFPIYLK had been coupled at 5 mg/ml. After 1 h the beads were washed extensively, bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE, and analyzed by H902 immunoblotting.

Erk2 Assay of Transiently Transfected JCaM1 Cells—20 × 10^6 JCaM1 cells were transiently transfected by electroporation with myc-tagged Erk2 in pEF-neo together with lck constructs in the same vector. After 24 h in culture, the cells were stimulated with OKT3 for 5 min, and the tagged Erk2 was immunoprecipitated with the 9E10 mAb (recognizing the myc-derived tag), and its activity toward myelin basic protein was assayed (15).

RESULTS

Lck Is Phosphorylated at Tyr^{192} in Triggered T Lymphocytes—Although phosphorylation of Lck at Tyr^{192} has been reported earlier (13, 19, 20), we first wanted to document this event following T cell antigen receptor (TCR) triggering. Lck was immunoprecipitated from metabolically 32P-labeled T cells stimulated with anti-CD3 mAbs for 5 min and analyzed by tryptic peptide mapping (Fig. 1). Phosphoamino acid analysis of the resulting radioactive spots (numbered 1–6 in Fig. 1) showed that peptides 1 and 2 contained only Tyr(P), and peptide 5 contained mainly phosphoserine (Ser(P)) and a trace of Tyr(P), whereas peptides 3 and 4 contained only Ser(P). Based on this result and our previous maps of Lck (21), we conclude that peptide 2 contains Tyr^{192}, the major in vivo tyrosine phosphorylation site (22), whereas Tyr^{204}, the autophosphorylation site (22) migrates in spot 5. The identified serine phosphorylation sites, such as Ser^{59}, Ser^{59} (23–25), and Ser^{194} (20) may indicate that it is relatively hydrophobic. Thus, the recovery of the peptide may be poor, perhaps also explaining the failure of some investigators to detect phosphorylation of this site (23–25).

Reconstitution of JCaM1 Cells by Lck Mutants—Tyr^{192} is located in the SH2 domain of Lck, and it has been demonstrated that this domain is critical for the participation of Lck in TCR signaling. For example, deletion of the SH2 domain rendered an active Lck incapable of augmenting TCR-induced tyrosine phosphorylation of cellular proteins and interleukin 2 production (26), although the kinase was still highly active (26, 27). Thus, we decided to test the potential role of Tyr^{192} phosphorylation in T cell activation by expressing wild type Lck or two point-mutants of it in the Lck-negative JCaM1 T cells,
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which do not respond to TCR/CD3 ligation unless Lck is re-expressed (28). As a read-out for TCR signaling, we used the TCR-induced activation of the MAP kinase pathway, which is an established integration point for signal transduction in T cells as well as other cells. In one Lck mutant phosphorylation at Tyr192 was prevented by mutation of Tyr192 to a phenylalanine (F) residue (Y192F mutant), and in the other Tyr192 was changed to a glutamic acid residue (Y192E mutant). These findings suggest that wild type Lck was phosphorylated at Tyr192 and that this event reduced anti-CD3-mediated Erk2 activation. In agreement with this notion, the Y192E-mutated Lck was much less efficient (156 ± 146% of resting samples, n = 3). These results have been obtained in several independent experiments and suggest that phosphorylation at Tyr192 may decrease the function of the SH2 domain, perhaps by reducing its ability to bind critical cellular ligands. We have also observed that an activated Y505F-mutated form of Lck can reconstitute Erk2 activation in JCaM1 cells to a similar extent as wild type Lck. In our experiments, the double Y192F/Y505F mutant was more efficient than Y505F Lck, and the Y192E/Y505F-Lck was as inefficient as the Y192E mutant (Fig. 2B). This shows that TCR-induced Erk2 activation is predominantly dependent on the function of the SH2 domain of Lck.

A Negative Charge at Position 192 Impairs the Function of the SH2 Domain—To analyze more directly the binding of ligands to the Lck SH2 domain, we generated glutathione S-transferase (GST) fusion proteins of the wild type Lck SH2 domain and the Y192F and Y192E point-mutated versions of it. These three GST fusion proteins and control GST were incubated (at 100 nM) with lysates of resting or pervanadate-treated Jurkat T cells, bound to glutathione-Sepharose, washed extensively, and probed for associating phosphoproteins by anti-Tyr(P) immunoblotting. As expected, the wild type Lck-SH2 bound many cellular proteins and the Y192F mutation had no detectable effect on this binding (Fig. 3). In contrast, the Y192E-modified SH2 domain bound much fewer cellular proteins from the same lysates (Fig. 3). Because the amount of GST fusion proteins was identical in each sample, it seems that introduction of a negative charge at position 192 in the Lck SH2 domain reduced the capacity of the domain to bind cellular Tyr(P)-containing proteins. It should be noted, however, that some Tyr(P)-containing proteins still bound to the Y192E-mutated SH2 domain but not to GST alone, suggesting that the Y192E mutation did not destroy the SH2 domain but instead altered or reduced its ability to bind to cellular ligands.

The Y192E Mutation Reduces High Affinity Ligand Selection—To determine whether the Y192E mutation affected the

![Fig. 2. Effect of Tyr192 phosphorylation on the function of Lck in intact T cells. A, OKT3-dependent activation of an epitope-tagged Erk2 in JCaM1 cells expressing wild type Lck or Y192F- or Y192E-mutated Lck. The values represent the percentage increase in tagged Erk2 activity after stimulation of the cells with OKT3 for 5 min compared with the Erk2 activity measured in resting cells from the same sample. Erk2 activity was measured in anti-tag immunoprecipitates by phosphorylation of MBP and quantitated by laser densitometry. Expression of the tagged Erk2 and Lck mutants were similar in all samples. The number of independent experiments is indicated. B, upper panel, autoradiogram of the Erk2 kinase assay in a similar experiment using the indicated Lck mutants. Lower panel, anti-Erk2 immunoblot of the same samples.](Image 366x582 to 497x729)

![Fig. 3. Binding of Tyr(P)-containing cellular proteins to the SH2 domain of Lck. Anti-Tyr(P) immunoblot of cellular proteins bound to GST alone (lanes 1 and 2) or to GST fusion proteins containing the wild type (lanes 3 and 4), Y192F-mutated (lanes 5 and 6) or Y192E-mutated (lanes 7 and 8) SH2 domain from lysates of 10 × 10^6 resting (lanes 1, 3, 5, 7 and 9) or pervanadate-treated (100 μM Na3VO4 and 0.3 mM H2O2 for 5 min (lanes 2, 4, 6, 8, and 10) Jurkat T cells. Lanes 9 and 10 represent the total cell lysates. Similar results have been obtained in three independent experiments.](Image 50x355 to 296x729)
selectivity or affinity of the SH2 domain, we subjected the wild type and mutated SH2 constructs to the phosphopeptide library selection technique (6, 7). When the specifically bound peptides were eluted and sequenced, we observed the usual selection of the wild type or Y192F-mutated Lck-SH2 domain for the Y*EEI sequence. Although the Y192E mutant SH2 domain could still select a Y*EEI sequence, the relative affinity for isoleucine at the +3 position was greatly reduced (Fig. 4). This result confirms that the point mutations in the SH2 domain did not destroy the ligand-binding ability of the SH2 domain and provides a likely explanation for the reduced binding of Tyr(P)-containing polypeptides to the Y192E-mutated SH2 domain shown in Fig. 3.

Phosphorylation at Tyr192 Also Reduces High Affinity Binding to Y*EEI—Having seen these striking effects of the Y192E mutation, we wanted to examine whether phosphorylation at Tyr192 had the same effect as its substitution with glutamic acid. Because addressing this question with the type of experiments shown in Figs. 3 and 4 would require milligram quantities of fully tyrosine phosphorylated GST-SH2 protein, we decided to use another approach. The synthetic peptide EPQY*EEIPTYL was coupled to Affi-Gel beads and used as a high affinity ligand for the SH2 domain of intact Lck expressed in COS cells. When these beads were incubated with such cell lysates, washed, and analyzed for the presence of Lck, it was obvious that wild type as well as Y192F-mutated Lck bound well, whereas Y192E-mutated Lck bound much less despite similar amounts of protein in the lysates (Fig. 5A). Co-expression of Syk, which phosphorylates Lck at Tyr192 in intact COS cells (13), changed this profile; the binding of wild type Lck was now much reduced (compare second and fifth lanes), whereas Y192F-mutated Lck still bound equally well (compare third and sixth lanes). Because the Y192F mutation does not per se affect the SH2 domain (Fig. 3), this result indicates that phosphorylation of wild type Lck at Tyr192 abolished the high affinity of the SH2 domain for the ligand peptide. This event apparently reduces the binding to the high affinity peptide as much as the Y192E mutation. This decreased binding did not occur when the wild type Lck was co-expressed with a kinase-inactive Syk (Fig. 5B) indicating that phosphorylation of Tyr192 depends on the catalytic activity of Syk. As an additional control, co-expression of Syk had no effect on the low affinity binding to another peptide in which the +3 amino acid was changed to a phenylalanine (not shown). These results show that phosphorylation of Y192, as well as the Y192E mutation, cause a substantial reduction in the binding of Tyr(P)-containing ligands by the Lck SH2 domain.

**DISCUSSION**

Post-translational Regulation of SH2 Domains: A General Mechanism?—Taken together, our results indicate that the ligand binding properties of the Lck SH2 domain can be post-translationally regulated by reversible phosphorylation of a tyrosine residue close to the +3 amino acid binding pocket. This may represent a mechanism by which the interactions of the Lck SH2 domain with certain cellular ligands are terminated. A similar observation has recently been made for the SH2 domain of c-Src (5) it seems plausible that this phenomenon is common to all Src family kinases, which all have an equivalent of Tyr192. In addition, many other SH2 domains have a tyrosine at or close to this location in beta-strand E. In one case, namely Zap, phosphorylation has been mapped to this site, Tyr69, in the N-terminal SH2 domain (29). Thus, it will be interesting to see if this is a widely used mechanism of regulation of SH2 domains.

Consequences of SH2 Domain Regulation for Src-like Kinases—In the case of Src-like kinases, the SH2 domain not only plays a role in interacting with other proteins but also participates in the regulation of the kinase activity of these enzymes by intramolecularly binding the negative regulatory Tyr(P) residue in the C terminus of these kinases (reviewed in Ref. 30). Thus, it would be predicted that a Tyr192 phosphorylation-induced decrease in ligand binding by the SH2 domain would lead to activation of the kinase. This is in agreement with our previous findings (13) and with our observation that the Y192E-mutated Lck is more active than the wild type enzyme both in intact COS cells and in vitro (not shown). In addition,
there is an additional mechanism by which Tyr$^{192}$ phosphorylation could lead to activation of the kinase. It was recently reported (31) that the suppressed state of Lck may involve the formation of a dimer, in which the SH2 domain of one molecule forms a stable complex with the C-terminal phosphopeptide and the SH3 domain of the other Lck molecule. In this interaction, Tyr$^{192}$ in the SH2 domain of one Lck molecule fits into the ligand binding groove of the SH3 domain of the other Lck molecule. Because the phosphorylation of Tyr$^{192}$ introduces a hydrophilic and negative charge onto the otherwise hydrophobic phenolic ring of Tyr$^{192}$, it is very likely that phospho-Tyr$^{192}$ would be excluded from the hydrophobic ligand binding groove of Lck at Tyr$^{192}$ also may have a positive effect on the signaling events that are only dependent on the enzymatic activity of Lck and not its SH2 domain. Such pathways remain to be identified.

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