Identification of the Site in the Syk Protein Tyrosine Kinase That Binds the SH2 Domain of Lck*

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The Syk protein tyrosine kinase (PTK) is expressed in many hematopoietic cells and is involved in signaling from various receptors for antigen and Fc portions of IgG and IgE. Upon cross-linking of these receptors, Syk is rapidly phosphorylated on tyrosine residues and enzymatically activated. We and others have found that the Lck kinase, a member of the Src family of PTKs, binds through its Src homology (SH) 2 domain to tyrosine phosphorylated Syk and to the related Zap kinase. Here we report that this interaction is direct and identify the two tandem tyrosines at the autophosphorylation site of Syk, Tyr\(^{318}\) and Tyr\(^{419}\), as the binding site for the SH2 domain of Lck. Mutation of either or both tyrosines to phenylalanines abrogated binding, while mutation of a second repetition of the motif at Tyr\(^{539}\) and Tyr\(^{540}\), or of the three tyrosines in the C terminus of Syk, did not. The SH2 domain of Lck bound the autophosphorylation site only when both Tyr\(^{318}\) and Tyr\(^{419}\) were phosphorylated. In intact cells the binding of the SH2 domain of Lck correlated with the ability of Syk to induce tyrosine phosphorylation of cellular proteins.

Phosphorylation of cellular proteins on tyrosine residues is an early event that follows triggering of a variety of transmembrane receptors on leukocytes. Protein tyrosine kinases (PTKs)\(^1\) of the Src family are thought to be key players in many of these signaling systems (1, 2). More recently, the two currently known mammalian members of the Syk family, Syk (3, 4) and Zap (5, 6), have also been found to participate in early signaling events in lymphocytes (5–15) and Syk also in other leukocytes (16–19) and in erythrocytes (20). In T cells, both PTKs are rapidly phosphorylated upon receptor stimulation, and at least Zap associates with the \(\zeta\) and CD3e chains of the T cell antigen receptor (TCR) complex in a tyrosine phosphorylation-dependent manner (5, 6, 10, 21–23). Although Syk can bind to phosphorylated receptor subunits in mast cells (18, 24), the mechanism and exact site(s) of Syk binding to the TCR are less clear, since receptor association can be detected in resting T cells in the absence of CD3 or TCRz phosphorylation (8).

It is clear from recent publications that the Syk and Src family PTKs interact and synergize in inducing substrate phosphorylation (6, 8, 10, 11, 25, 27–29),\(^2\) but the exact mechanism(s) or sequence of events remain incompletely understood. The reported physical association between the two classes of PTKs, which occurs in T cells following stimulation of the TCR, may offer some explanation. Indeed, Lck can associate through its SH2 domain with tyrosine phosphorylated Syk and Zap (8, 27).\(^2\) To further characterize this interaction, we have identified the site in Syk that binds the Lck SH2 domain with high affinity.

MATERIALS AND METHODS

Antibodies and Reagents—The preparation and characterization of the rabbit antiserum directed against Syk (residues 253 to 365) has been described earlier (8). The anti-Tyr(P) monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-CD3e (OKT3) was from Ortho. The H902 monoclonal antibody, recognizing the HIV-1\(^{TM}\)-derived sequence RIQPGPRAFVTIGKR (30) which is encoded as a N-terminal epitope tag by both the pTag5SRs and pTag-CMV-neo expression vectors (8, 25), is available from the AIDS Research and Reference Program (Bethesda, MD). The GST-SH2 domain of Lck (residues 121–224) construct is described elsewhere.\(^2\)

Site-directed Mutagenesis and Transfections—The Y518F, Y519F, Y539F, Y540F, Y622F, Y623F, Y624F, and K395R mutations were made by site-directed mutagenesis of syk in the pTag5SRs expression vector (8, 25) using the Transformer Site-Directed mutagenesis kit (Clontech, Palo Alto, CA), according to the manufacturer’s instructions. The disabling Y192F mutation of the SH2 domain of Lck is described elsewhere.\(^2\) Each mutation was verified by sequencing. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum and antibiotics, and were transfected by Lipofectamine as described previously (8, 25).

Yeast Two-Hybrid System—The Saccharomyces cerevisiae strain L40 (MATa, trp1, leu2, his3, lys2\(^{is2}\)::LexA-HIS3, ura3::LexA-lacZ) and the yeast expression plasmid pBTM116 were provided by A. Vojtek (Seattle, WA); the yeast expression vector pACTII was from Clontech. These cells and plasmids were described previously (31, 32). The full-length wild-type syk cDNA, as well as the Y518F/Y519F double mutant and the K395R mutant, encoding a kinase-inactive form of the enzyme, were inserted into the pBTM116 plasmid, containing a Trp\(^{+}\) selection marker, in frame with the DNA binding domain of LexA. The Lck SH2 domain constructs were obtained by polymerase chain reaction amplification of the SH2 domain (residues 121–224) from a wild-type and Y192E-mutated Lck,\(^2\) using the proofreading Vent DNA polymerase (New England Biolabs). The amplified fragments were inserted in frame with the Gal4 activation domain of the pACTII vector, which contains a LexA\(^{+}\) selection marker. Simultaneous transformation of L40 cells, maintained in standard conditions (33), with pBTM116 and pACTII-based constructs was performed as described (34, 35). Cotransformants were selected on Trp\(^{−}\)Leu\(^{−}\)plates. The transformants were finally tested for \(\beta\)-galactosidase activity, either by a color filter assay (36) or more frequently by a quantitative assay using 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside as a substrate, essentially as described elsewhere.\(^2\)

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1 The abbreviations used are: PTK, protein tyrosine kinase; Tyr(P), phosphorysine; TCR, T cell antigen receptor; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.

2 Couture, C., Songyang, Z., Jascur, T., Williams, S., Tailor, P., Cantley, L. C., and Mustelin, T. (1996) J. Biol. Chem. 271, in press.
Previously (34, 35). The results are expressed as units of β-galactosidase activity, as defined by Miller (37).

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that the SH2 domain of Lck could interact with either tyrosine-phosphorylated Syk or Zap, we felt that it was important to further demonstrate that this interaction was direct and did not require the participation of any lymphocyte-specific intermediary component. First, we used the yeast two-hybrid system developed by Fields and Song (42) to measure the interaction between the SH2 domain of Lck and various mutants of Syk. In this system, the first hybrid consisted of a fusion between the SH2 domain of Lck and Syk in the yeast two-hybrid system. The data represent the β-galactosidase activity in *S. cerevisiae* L40 reporter cells cotransformed with wild-type or Y192E-mutated SH2 domain of Lck (residues 121–224)/pACTII constructs together with either wild-type, Y518F/Y519F- or K395R-mutated syk cDNA constructs in the pBTM116 vector. Double transformants were isolated after growth on selective medium, and the β-galactosidase activity was measured in cell lysates using chlorophenol-red-β-D-galactopyranoside as a substrate. The values are expressed as Miller’s units of enzymatic activity (37) and represent the average (± S.D.) obtained from three distinct transformants. Similar results were obtained in two independent experiments. B, anti-Tyr(P) immunoblot of anti-Syk immunoprecipitates from yeast cells expressing either Lex-A (lane 1) or the Lex-A:Syk hybrid (lane 2). The migration of the Lex-A:Syk protein is indicated by an arrow.

RESULTS

Binding of Syk to the SH2 Domain of Lck in the Yeast Two-hybrid System—Although we and others have reported

**Fig. 1. A**, quantitative analysis of the interaction between the SH2 domain of Lck and Syk in the yeast two-hybrid system. The data represent the β-galactosidase activity in *S. cerevisiae* L40 reporter cells cotransformed with wild-type or Y192E-mutated SH2 domain of Lck (residues 121–224)/pACTII constructs together with either wild-type, Y518F/Y519F- or K395R-mutated syk cDNA constructs in the pBTM116 vector. Double transformants were isolated after growth on selective medium, and the β-galactosidase activity was measured in cell lysates using chlorophenol-red-β-D-galactopyranoside as a substrate. The values are expressed as Miller’s units of enzymatic activity (37) and represent the average (± S.D.) obtained from three distinct transformants. Similar results were obtained in two independent experiments. B, anti-Tyr(P) immunoblot of anti-Syk immunoprecipitates from yeast cells expressing either Lex-A (lane 1) or the Lex-A:Syk hybrid (lane 2). The migration of the Lex-A:Syk protein is indicated by an arrow.

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functionally impaired SH2 construct (the Y192E mutant), which shows that the interaction required the function of the SH2 domain. In agreement with this notion, the Syk hybrid was found to contain Tyr(P) when expressed in S. cerevisiae (Fig. 1B). A kinase-deficient form of Syk (K395R mutant) was unable to interact with either the wild-type or Y192E-mutated SH2 domain. When the tyrosine residues corresponding to the autophosphorylation site of Syk, Tyr\(^{518}\), and Tyr\(^{519}\), were mutated to phenylalanine residues, the interaction with the wild-type SH2 domain was reduced to less than 5% compared to that observed between the wild-type Syk and the Lck SH2 domain construct. Although the interaction between the wild-type SH2 domain of Lck and the Y518F/Y519F-mutated Syk seems to be marginal, it is nonetheless reproducible and may indicate the presence of one or several other binding sites. If so, these sites may be of lower affinity or their phosphorylation may depend on the primary autophosphorylation site of Syk, Tyr\(^{518}\), and Tyr\(^{519}\), which affects catalytic activity (25). No interaction could be detected between the SH2 domain of Lck and Zap in this system (not shown), presumably due to the inability of Zap to autophosphorylate.

**The Interaction between Syk and the SH2 Domain of Lck Requires an Intact Primary Autophosphorylation Site**—We have earlier reported that tyrosine phosphorylation of Syk (when expressed in COS cells) was reduced by 90% when both of the two tyrosines in the conserved autophosphorylation sites of PTKs, Tyr\(^{518}\), and Tyr\(^{519}\), were mutated to phenylalanines (25). In addition to this mutant, we also generated two other tyrosine-to-phenylalanine mutants of Syk. The first of these, Y539F/Y540F, was mutated at a second NYYK motif only 21 amino acids downstream of the NYYK motif containing Tyr\(^{518}\) and Tyr\(^{519}\). The second, Y622F/Y623F/Y624F, was mutated at the three tyrosines in the extreme C terminus of Syk. This site was chosen because the sequence following the tyrosines, DVVN, makes this site a possible Lck SH2 binding site. When expressed in COS cells all these Syk mutants contained Tyr(P) (25) (data not shown).

Wild-type Syk and its point-mutated versions were expressed in COS cells alone or in combination with Lck (to potentially increase phosphorylation of the binding site). After 48 h the cells were lysed, and the clarified lysates were incubated with 100 nM of recombinant GST fusion protein containing the SH2 domain of Lck (Fig. 2, upper panel) or control GST (Fig. 2, lower panel) for 1 h, followed by glutathione-Sepharose beads for 1 h. After washing the beads extensively, the bound proteins were eluted and analyzed by anti-Tyr(P) immunoblotting.

In these experiments, wild-type Syk expressed alone bound well to the GST-SH2 domain construct (Fig. 2, lane 2), but no Tyr(P)-containing protein of 72–74 kDa could be precipitated from lysates of cells transfected with Y518F/Y519F-mutated syk (Fig. 2, lane 3). In contrast, Syk molecules containing the Y539F/Y540F or Y622F/Y623F/Y624F mutations bound well to the SH2 domain (lanes 4 and 5), indicating that neither Tyr\(^{539}\)/Tyr\(^{540}\) nor the C-terminal Tyr\(^{622}\)/Tyr\(^{623}\) residues are required for binding. Co-expression of Lck did not affect the binding (lanes 6–10). This experiment is in agreement with the data obtained in the yeast two-hybrid system and indicates that the autophosphorylation site of Syk needs to be intact for the creation of a binding site for the SH2 domain of Lck.

**Direct Binding of the Lck SH2 Domain to Syk**—To investigate the potential binding of the Lck SH2 to Tyr\(^{518}\)/Tyr\(^{519}\) of Syk in more detail and eliminate the possibility of intermediate molecules, we created the single mutants Y518F and Y519F. When expressed in COS-1 cells, and compared to the wild-type and Y518F/Y519F-mutated enzymes, these two single mutants contained intermediate levels of Tyr(P) and were only marginally active in vivo (Fig. 3A). Anti-Tyr(P) immunoblotting of anti-Syk immunoprecipitates from these cells showed that the Y518F mutant of Syk contained 52.0 ± 16.5% (n = 3) and the Y519F mutant 43.8 ± 18.3% (n = 3) as much Tyr(P) as wild-type Syk, whereas the Y518F/Y519F mutant contained only 3–10% as much Tyr(P) as the wild-type enzyme (Fig. 3B), as reported earlier (25). This suggests, but does not prove, that Syk is predominantly phosphorylated at both residues when expressed in COS cells.

Far-Western probing of the nitrocellulose filters with the GST-Lck SH2 followed by anti-GST revealed a reactive band (often a doublet) comparable in size to Syk only in the lysates containing wild-type Syk (Fig. 4A). Immunoblots of the same filter with the anti-tag monoclonal antibody H902 showed that at least the lower component of the doublet co-migrated with Syk. The upper band may be a hyperphosphorylated, but much less abundant, form of Syk or another cellular protein that is phosphorylated only in the presence of wild-type Syk. The Lck SH2 domain did not bind to any proteins in cells expressing the Y518F, Y519F, or Y518F/Y519F mutants of Syk (Fig. 4A, lanes 3–5). These proteins, however, were expressed at the same levels as wild-type Syk (lower panel), and they all contained Tyr(P) (Fig. 3A). Similar results were obtained in several experiments, and co-expression of Lck did not affect the result. The identification of this SH2 domain-reactive species as Syk was accomplished by repeating the far-Western probing experiment on anti-Syk immunoprecipitates obtained from Syk-transfected COS-1 cells. Once again, we detected a 72-kDa band in the precipitates obtained from wild-type Syk-expressing cells (Fig. 4B, lane 2), but not from cells expressing the Y518F- and/or Y519F-mutated enzymes (Fig. 4B, lanes 3–5). This confirms that the wild-type Syk molecule can interact directly with the SH2 domain of Lck and suggests that phosphorylation at both Tyr\(^{518}\) and Tyr\(^{519}\) is required for the binding to occur.
Both Tyr518 and Tyr519 Need to be Phosphorylated for SH2 Domain Interaction—Although our results demonstrate that the SH2 domain of Lck can interact directly with Syk, and that this interaction dependson an intact autophosphorylation site, they do not exclude the possibility that the SH2 domain of Lck interacts with another site, the phosphorylation of which depends on the full enzymatic activity of Syk and prior phosphorylation of both Tyr518 and Tyr519. To directly address this question, we generated GST fusion proteins containing the Syk-derived sequence surrounding the wild-type or mutated autophosphorylation site, residues 506–531 of the porcine enzyme (Fig. 5A). In order to generate tyrosine-phosphorylated versions of these constructs, we subjected each GST fusion protein to an extended in vitro kinase reaction using a recombinant purified Lck enzyme. When the phosphorylated constructs were subjected to tryptic peptide mapping, the wild-type protein generated three phosphopeptides (Fig. 5B), one of which, peptide 2, was not present in the maps derived from any mutant constructs. The lower panel is an anti-tag immunoblot of the same samples as in A. The lower panel is an anti-tag immunoblot of the same anti-Syk immunoprecipitates obtained from the same samples as in A. The lower panel is an anti-tag immunoblot of the same anti-Syk immunoprecipitates obtained from the same samples as in A.

FIG. 3. In vivo activity and tyrosine phosphorylation of autophosphorylation site-mutated Syk. A, tyrosine phosphorylation of cellular proteins from COS-1 cells transfected with 5 μg of either empty pTag/SRα vector (lane 1), wild-type syk (lane 2), Y518F-syk (lane 3), Y519F-syk (lane 4), or Y518F/Y519F-syk (lane 5). Cell lysate samples were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the 4G10 anti-Tyr(P) monoclonal antibody. The lower panel is an anti-tag immunoblot of the same samples and shows the relative expression of each mutant. B, anti-Tyr(P) immunoblot of anti-Syk immunoprecipitates obtained from the same samples as in A. The lower panel is an anti-tag immunoblot of the same anti-Syk immunoprecipitates.

FIG. 4. Binding of the Lck SH2 domain to wild-type Syk, but not to the autophosphorylation site mutants of Syk. A, Lck SH2 far western on cell lysate samples obtained from COS cells transfected with 5 μg of empty pTag/SRα vector (lane 1), wild-type syk (lane 2), Y518F-syk (lane 3), Y519F-syk (lane 4), or Y518F/Y519F-syk (lane 5). The lower panel is an anti-tag immunoblot of the same samples, and shows that each construct was properly expressed. B, Lck SH2 far-western analysis on anti-Syk immunoprecipitates obtained from the same COS cell lysates as in A. The lower panel is an anti-tag immunoblot of the anti-Syk immunoprecipitates and shows that each Syk mutant was efficiently precipitated.

FIG. 5. The Lck SH2 domain binds to the autophosphorylation site of Syk only when both Tyr518 and Tyr519 are phosphorylated. A, amino acid sequence of the GST fusion proteins encompassing the autophosphorylation site region (amino acids 501–531) of wild-type Syk, Y518F-Syk, Y519F-Syk, and the Y518F/Y519F-Syk double mutant. Note that the only Syk-derived tyrosine residues are Tyr518 and Tyr519. B, tryptic peptide maps of the GST fusion fragments after in vitro phosphorylation by purified recombinant Lck in presence of γ-32P]ATP. After SDS-PAGE, transfer to nitrocellulose and autoradiography, the bands corresponding to the constructs (30 kDa) were excised and treated with trypsin and finally subjected to two-dimensional separation on cellulose-coated plates. The sample origin is in the lower left corner of each panel. Spot 1 corresponds to the singly phosphorylated peptide, whereas spot 2 corresponds to a doubly phosphorylated peptide. Spot 3 is found in all the maps and must therefore be derived from the GST fusion partner. C, Lck SH2 domain far-Western probing of the phosphorylated constructs. The GST fusion peptides were phosphorylated in vitro by recombinant Lck in presence of 1 mM ATP prior to electrophoresis and transfer to nitrocellulose. To avoid detection of the GST moiety of the fusion proteins, the GST-SH2 domain was first immobilized on glutathione-Sepharose and saturated with anti-GST monoclonal antibodies. After washing, the GST-SH2-anti-GST complex was eluted from the beads with 20 mM reduced glutathione and used to probe the nitrocellulose membrane. The left panel shows the result of the far-Western probing, the right panel is an anti-GST immunoblot showing equivalent amounts of the GST fusion protein.
of the mutated fusion proteins. This peptide corresponds to the doubly phosphorylated autophosphorylation peptide. Peptide 1 was present in all maps, except that of the Y518F/Y519F mutant, and thus corresponds to a singly phosphorylated peptide. Peptide 3, present in all maps, represents a phosphorylation site located within the GST portion of the fusion molecules. This shows that the wild-type GST-Syk peptide can be phosphorylated on both Tyr492 and Tyr493 by Lck in vitro. To determine whether the SH2 domain of Lck binds to the autophosphorylation site of Syk and whether it requires phosphorylation of both Tyr492 and Tyr493, we performed a far-Western probing experiment on each of the constructs phosphorylated in vitro by Lck. We found that only the wild-type (doubly phosphorylated) construct was recognized by the SH2 domain of Lck (Fig. 5C, lane 1), whereas the Y518F or Y519F single mutants or the Y518F/Y519F double mutant of this construct were not (Fig. 5C, lanes 2–4).

**DISCUSSION**

The published investigations concerning the physical interaction between Syk or Zap and the Src-family kinase Lck all support the conclusion that the SH2 domain of Lck is involved in this interaction (8, 27, 28). However, the assumption that this interaction is direct and does not require any intermediate components needed to be addressed. In this report, we show that the interaction between Syk and the SH2 domain of Lck can occur in a system (the yeast two-hybrid system) devoid of other lymphocyte-specific components. Furthermore, the binding of the SH2 domain depended on the catalytic activity of Syk itself, indicating that the binding site is likely to be an auto-phosphorylation site for Syk, an assumption that is verified by our experiments. The inability of the Y518F/Y519F-mutated Syk to interact with the SH2 domain of Lck in intact cells (Fig. 1) or in solution (Fig. 2) further suggests that the conserved autophosphorylation site of Syk, Tyr492 and Tyr493, might be the target of the Lck SH2 domain. The sequence at this region is ADENY*Y*KAQTHG. This sequence is not a perfect match with the predicted specificity of Src family SH2 domains (43), 1 position would be 3 amino acid binding pocket of the Lck-SH2 domain. This event regulates the function of the SH2 domain.

The receptor-induced tyrosine phosphorylation sites in Syk in activated lymphocytes have not yet been identified, but Zap was recently reported to be predominantly phosphorylated in triggered T cells at the two tyrosines that correspond to Tyr492 and Tyr493 (44, 45). In the case of Zap, however, phosphorylation of both Tyr492 and Tyr493 clearly depends on the presence of Src-family kinases. Indeed, Lck can phosphorylate Tyr493 in vitro, an event that augments the enzymatic activity of Zap, which in turn autophosphorylates on Tyr492 (44–46). Therefore, one expects the SH2 domain of Lck to be unable to bind Zap unless it has been first phosphorylated by Lck. This is supported by our finding that the SH2 domain of Lck did not interact with Zap in the yeast two hybrid system, or when expressed in COS-1 cells in the absence of Lck (not shown). In contrast, Syk was able to bind to the SH2 domain of Lck under the same conditions. This suggests that, in absence of Lck, Syk is capable of autophosphorylating at both Tyr492 and Tyr493, as reported (25), whereas Zap is unable to phosphorylate the corresponding Tyr492 and Tyr493 residues (44). This may well be the reason why the activation of Syk is independent of Lck or other Src-family kinases in a variety of systems (8, 11, 25, 47). Nevertheless, the SH2 domain-dependent recruitment of Src-family kinases by Syk might be a critical event in receptor-mediated lymphocyte activation (28). In B cells, the integrity of the autophosphorylation site of Syk (or the corresponding site in Zap) is required for B cell antigen receptor-mediated signaling (48, 49). Since the enzymatic activity of the Y518F/Y519F mutant of Syk is reduced by only 40%, the inability of this mutant to activate downstream signaling events in B cells (48), T cells, or COS cells (25) (Fig. 2), possibly stems from the absence of Syk/Src-family kinase interactions.

In addition to Lck, it was recently reported that two other molecules could interact with Syk through SH2-dependent mechanisms. First, the C-terminal SH2 domain of PLC-γ1 was shown to interact with Tyr492 and/or Tyr493 of the human Syk enzyme (corresponding to Tyr491 and Tyr492 of porcine Syk), located between the C-terminal SH2 domain and the kinase domain of Syk (26, 50). Second, we have found that the SH2 domain of the Vav proto-oncogene product binds to Tyr491 of Syk, both in vitro and in vivo. Importantly, binding of either SH2 domain to their respective binding sites was largely dependent on prior phosphorylation of Tyr492 and Tyr493 (or Tyr492 and Tyr493 of the human sequence), although these two residues did not participate in the intermolecular interaction per se (26). Furthermore, all three SH2 domain-containing ligands of Syk have been shown to be (25, 26), or are likely to be (51), substrates for Syk. In the case of Lck, Syk has been shown to phosphorylate the Tyr492 residue (25), located near the +3 amino acid binding pocket of the Lck-SH2 domain. This event regulates the function of the SH2 domain.

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Identification of the Site in the Syk Protein Tyrosine Kinase That Binds the SH2 Domain of Lck

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