Autophosphorylation Induces Autoactivation and a Decrease in the Src Homology 2 Domain Accessibility of the Lyn Protein Kinase*

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Lyn is a member of the Src family of protein-tyrosine kinases that can readily undergo autophosphorylation in vitro. The site of autophosphorylation is Tyr307, which corresponds to the consensus autophosphorylation site of other Src family tyrosine kinases. The rate of autophosphorylation is concentration-dependent, indicating that the reaction follows an intermolecular mechanism. Autophosphorylation results in a 17-fold increase in protein-tyrosine kinase activity. Kinetic analysis demonstrates that phosphorylation of a substrate peptide by Lyn following autophosphorylation occurs with a 63-fold decrease in $K_m$, but no significant change in $V_{max}$, suggesting that autophosphorylation relieves the conformational constraint that prevents binding of the substrate peptide to the active site of the kinase. Using a phosphotyrosine-containing peptide (pYEEI) that has previously been shown to bind to the Src homology 2 (SH2) domain of Src family tyrosine kinases with high affinity, we found that autophosphorylation results in a significant decrease in accessibility of the Lyn SH2 domain, indicating that conformational changes in the protein kinase domain induced by autophosphorylation can be propagated to the SH2 domain. Our study suggests that autophosphorylation plays an important role in regulating Lyn by modulating both its kinase activity and its interaction with other phosphotyrosine-containing molecules.

The protein products of the Src family of oncoproteins and proto-oncogenes are non-receptor protein-tyrosine kinases that are believed to play important roles in controlling the growth, proliferation, and differentiation of many cell types (see Ref. 1 for review). Src family kinases are highly homologous in structure; they all contain an N-terminal myristoylation domain, a unique domain, Src homology 2 (SH2) and Src homology 3 (SH3) domains, a protein kinase domain, and a C-terminal regulatory domain. Studies of transforming mutants of several Src family kinases have provided evidence that interactions between these domains are important in the regulation of kinase activity. Two major consensus tyrosine phosphorylation sites have been identified in Src family kinases: (i) the autophosphorylation site in the protein kinase domain and (ii) the tyrosine phosphorylation site in the C-terminal regulatory domain. Autophosphorylation correlates with activation of the kinases, while phosphorylation of the C-terminal regulatory tyrosine suppresses kinase activity (see Ref. 1 for review). The C-terminal Src kinase has been shown to phosphorylate several members of this family and is thought to play a critical role in regulating their activity (2).

Phosphorylation of the C-terminal tyrosine in Src family kinases negatively regulates their activity. Several lines of evidence suggest that this regulation is governed by an intramolecular interaction between the phosphorylated C-terminal tyrosine and sequences in the SH2 domain that somehow stabilizes the inactive conformation of the kinases (see Refs. 1 and 3 for review). In addition to binding to the C-terminal regulatory phosphotyrosine, the SH2 domain has also been shown to bind other phosphotyrosine-containing proteins (4). Interaction with exogenous phosphotyrosine-containing proteins is thought to play an important role in the cellular functions of the kinases (see Refs. 4 and 5 for review). Using combinatorial peptide libraries, structural determinants in phosphotyrosine-containing proteins necessary for high affinity binding to the SH2 domain of Src family kinases have been determined (6). Peptides displaying high affinity binding to the SH2 domain of Src family kinases invariably contain a phosphotyrosine followed C-terminally by two acidic amino acids and then a hydrophobic residue. A phosphopeptide, pYEEI, derived from the hamster polyoma virus middle T antigen contains all the structural features important for high affinity binding to SH2 domains of Src family kinases (7, 8). The structural basis of the high affinity interaction between the phosphopeptide and the SH2 domain was elucidated from the crystal structure of the pYEEI-Src SH2 domain complex (9). The crystal structure reveals two major binding pockets for pYEEI, one for the phosphotyrosine and the other for the more C-terminal Ile residue. The phosphotyrosine-binding pocket contains an Arg residue that forms hydrogen bonds with the phosphate moiety and two basic residues that bind to the aromatic ring of the phosphotyrosine through amino-aromatic interactions. The Ile-binding pocket contains several hydrophobic residues responsible for hydrophobic interactions with the Ile residue. In addition to the two binding pockets, electrostatic interactions between the two Glu residues of the peptide and several basic residues of the SH2 domain also contribute to the high affinity binding of the peptide in the SH2 domain of pp60c-Src (9).

Studies on the role of autophosphorylation in the regulation of the biological activity of products of the c-src and v-src genes, pp60c-Src and pp60v-Src, respectively, are well documented (see...
Regulation of Lyn Kinase Activity by Autophosphorylation

Ref. 1 for review). Mutation of the autophosphorylation site (Tyr416) of pp60c-Src generates a mutant displaying somewhat reduced kinase activity but maintaining full oncogenicity (10). Thus, autophosphorylation does not seem to play a very significant role in regulating the kinase activity and oncogenicity of pp60c-Src because it is not obligatory for kinase activity or transforming potential. A similar conclusion on the kinase activity of pp60c-Src can be made because nonphosphorylated pp60c-Src displays significant kinase activity, and autophosphorylation leads to a 1.5–2-fold increase only in its kinase activity (36). This is in contrast to insulin- and epidermal growth factor receptors which require autophosphorylation, not only for kinase activity, but also for normal biological responses. No detailed analyses of the effect of autophosphorylation on the regulation of the kinase activity and cellular functions of other members of the Src family have been documented.

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Construction of the Lyn Baculovirus Vector and Generation of the Recombinant Lyn Baculovirus

Site-directed mutagenesis (20) was used to introduce unique BamHI and BglII sites, respectively, in the 5' and 3' untranslated regions of the mouse lyn cDNA (21) for subcloning purposes. The modified full-length lyn cDNA was subcloned into the BamHI site of the baculovirus expression vector pVL41, a generous gift of Professor M. D. Summers. Spodoptera frugiperda 9 (Sf9) insect cells (Invitrogen Corp.) were co-transfected with wild-type baculoviral DNA and pVL41lyn by standard calcium phosphate transfection procedures (22). Recombinant Lyn baculovirus was purified by three rounds of plaque purification by direct visual screening. The titre of the recombinant Lyn baculovirus was determined, and immunoblotting using the anti-Lyn antiseraum (L40) (17) was used to optimize protein production following infection.

Purification of Lyn from Crude Cell Lysates of Sf9 Cells Infected with Recombinant Lyn Baculovirus

A large scale (2-litter) culture of Sf9 cells grown to a density of 8.1 × 10⁶ cells per ml were infected with recombinant Lyn baculovirus at a multiplicity of infection of 1.0. The cells were harvested 3 days after infection for protein purification. All the extraction and purification procedures were carried out at 4°C unless otherwise indicated. Cells were pelleted at 3,000 × g for 5 min, washed once with Grace's serum-free medium, and homogenized in buffer consisting of 25 mM Hepes, pH 7.0, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenate was clarified by centrifugation at 100,000 × g for 40 min. Recombinant Lyn was purified essentially as described in Ref. 13 with some modifications. Briefly, the homogenates were purified by sequential column chromatography steps on a Q-Sepharose ion exchange column, followed by an hydroxyapatite column, a phenyl-Sepharose column, and a Sephacryl-200 gel filtration column. The partially purified enzyme preparation was then applied to a Mono Q ion exchange column (Pharmacia) pre-equilibrated with column buffer consisting of 25 mM Hepes, pH 7.0, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After washing the column, bound proteins were eluted with a 30-mL linear gradient of 0–0.3 M NaCl. Immunoblot analysis using α-Lyn antibody and SDS-PAGE analysis followed by Coomassie Blue staining demonstrated that the recombinant Lyn preparation was highly purified and consisted of two major protein bands which corresponded to the 53- and 56-kDa forms of Lyn (Fig. 1A).

Protein Kinase Assay

The protein-tyrosine kinase activity of Lyn was determined by measuring incorporation of [γ-32P]ATP into [γ-32P]ATP into pYEEI peptide, a substrate derived from the cell cycle control kinase p34<sup>cd2</sup> which has been shown to act as a specific and efficient substrate for Src family tyrosine kinases in vitro (13, 30). Routine enzyme assays were carried out at 30°C in a 50-μL volume of kinase buffer (20 mM Tris-HCl, pH 7, 10 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 50 μM Na<sub>2</sub>VO<sub>4</sub>), 100 μM ATP (specific radioactivity, 300–400 cpm/pmol), and 300 μM [γ-32P]ATP (specific radioactivity, 300–400 cpm/pmol), and 300 μM [γ-32P]ATP (specific radioactivity, 300–400 cpm/pmol), and 300 μM [γ-32P]ATP (specific radioactivity, 300–400 cpm/pmol), and 300 μM [γ-32P]ATP (specific radioactivity, 300–400 cpm/pmol). The reaction was terminated by addition of 20 μL of 50% acetic acid. A 30-μL aliquot was spotted onto a phosphocellulose paper square which was subsequently washed six times in 0.3% H<sub>3</sub>P<sub>4</sub>O<sub>7</sub> once with acetone, and then dried. Radioactivity in the dried paper square was monitored by Cerenkov counting.
Monitoring the Time Course of Autophosphorylation and Change of Protein-tyrosine Kinase Activity of Lyn

The time course of autophosphorylation was conducted under two conditions which differ mainly in the ATP concentrations.

Condition 1—Autophosphorylation of purified Lyn was conducted at 30°C in a volume of 90 µl containing 33 mM Tris-HCl, pH 7.7, 17 mM MgCl₂, 1.7 mM MnCl₂, 83 mM Na₂VO₃, and 164 nM Lyn. The reaction was initiated by the addition of 167 µM [γ-³²P]ATP (specific radioactivity 1,000–3,000 cpm/µmol). At the designated time intervals, 5 µl of the reaction mixture was removed and mixed with an equal volume of 5 × SDS-PAGE sample buffer containing 1.4 ml dithiobitol. The samples were boiled prior to analysis on a 10% polyacrylamide gel followed by autoradiography. The protein bands corresponding to the kinase were excised from the gel, and radioactivity in the excised protein bands was determined by scintillation counting. Stoichiometry of autophosphorylation was expressed as moles of PO₃⁻⁻ incorporated per mol of Lyn.

Condition 2—The reaction conditions were essentially the same as those described under Condition 1, except that the final [γ-³²P]ATP concentration was 100 µM.

To monitor the change in Lyn tyrosine kinase activity, autophosphorylation of the kinase was carried out under identical conditions as described above, except that 100 µl of reaction mixture was removed at 30°C in a volume of 90 µl containing 33 mM Tris-HCl, pH 7.7, 63 mM MgCl₂, 1.7 mM MnCl₂, 83 mM Na₂VO₃, and 164 nM Lyn. The reaction was initiated by the addition of 167 µM [γ-³²P]ATP (specific radioactivity 1,000–3,000 cpm/µmol). At the designated time intervals, 5 µl of the reaction mixture was added to a mixture containing 45 µl of kinase assay buffer, [Lys¹⁹]cdc2(6–20) peptide, and [γ-³²P]ATP to initiate phosphorylation of the [Lys¹⁹]cdc2(6–20) peptide. The peptide phosphorylation was allowed to proceed at 30°C for 5 min only to avoid any significant further autophosphorylation of Lyn. The assay conditions were the same as those detailed under Protein Kinase Assay.

Monitoring the Concentration-dependent Changes of Autophosphorylation and Protein-tyrosine Kinase Activity of Lyn

Autophosphorylation of purified Lyn was carried out in kinase buffer containing 100 µM [γ-³²P]ATP and Lyn. The amount of Lyn in the reaction was fixed at 18 pmol while its concentration was changed by varying the reaction volume. The reaction volumes and the corresponding Lyn concentrations were 100 µl, 75 µl, 50 µl, and 25 µl for 0.18 µM, 0.24 µM, 0.36 µM, and 0.72 µM, respectively. After a 4-min incubation at 30°C, the reaction mixture was subject to SDS-PAGE to determine the rate of autophosphorylation. Less than 10% of Lyn in the reaction mixture was phosphorylated at the end of the reaction.

Determination of the Kₘ for ATP and Vₘₐₓ of Lyn Autophosphorylation

The autophosphorylation reaction was carried out in kinase assay buffer, 162 µM Lyn, and [γ-³²P]ATP (specific radioactivity, 30,000–40,000 cpm/µmol) at concentrations of 5–200 µM. The mixture was incubated at 30°C for 5 min and stopped by the addition of SDS-PAGE sample buffer. The samples were boiled prior to analysis on a 10% polyacrylamide gel. The protein bands corresponding to Lyn were excised, and radioactivity associated with each band was determined by scintillation counting. The rate of autophosphorylation was expressed as PO₃⁻⁻ incorporated per min; less than 5% of Lyn was autophosphorylated.

For proteolytic digestion, the reaction mixture was first dialyzed against 2 × 2 liters of dialysis buffer overnight to remove free ATP. The dialyzed sample was concentrated and then alkylated by treatment with 4-vinylpyridine (2 µl per 100 µl of concentrated mixture) at 37°C for 3 h. After addition of 30 µg of BSA as carrier protein, the autophosphorylated Lyn was precipitated by incubating with 0.9 µl of ethanol at 20°C overnight and then washed with 1 µl of ethanol at −20°C. After removal of the residual ethanol by a Speed Vac, the precipitated proteins were exhaustively digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (1 mg/ml) in a volume of 150 µl at 37°C for 48 h. The tryptic phosphopeptide fragment was isolated by reverse phase HPLC before analysis by the two-dimensional thin layer electrophoresis-thin layer chromatography (TLC) procedure.

For phosphopeptide mapping, each of the samples (the phospho-Lyn(391–400), the tryptic phosphopeptide fragment derived from autophosphorylated Lyn, and a mixture of both) was applied to a TLC plate. The first dimension was thin layer electrophoresis in a pH 3.5 buffer (pyridine, acetic acid, and H₂O in a ratio of 1:10:89) at 500 V for 2.5 h, and the second dimension was TLC in a buffer containing 1-butanol, pyridine, acetic acid, and H₂O in a ratio of 15:10:3:12. The radioactive spot on the TLC plate was located by autoradiography.

Preparation and Characterization of pYEEI Peptide Immobilized to a Solid Support

The purified pYEEI peptide was covalently coupled to Affi-Gel 15 agarose following the procedures detailed by the manufacturer (Bio-Rad). The degree of coupling of the peptide to Affi-Gel was determined and revealed a coupling density of 4.9 µmol of pYEEI peptide per ml of packed gel. The immobilized pYEEI peptide was diluted with the control gel (Affi-Gel 15 treated with ethanolamine to inactivate all the reactive groups) before use.

Characterization of Purified Recombinant Mouse Lyn—Recombinant mouse Lyn was purified from S9 insect cells infected with a baculovirus carrying the Lyn cDNA. Since our main objective was to investigate the role of autophosphorylation in regulation of Lyn kinase activity and conformation, it was imperative to ensure that the recombinant enzyme was not significantly tyrosine-phosphorylated prior to autophosphorylation in vitro. In order to purify dephosphorylated Lyn, we deliberately omitted phosphatase inhibitors from all buffers used for extraction and purification so that the recombinant Lyn could be fully dephosphorylated by the endogenous phosphatases in the crude cell lysate. An apparently homogeneous preparation of the recombinant enzyme was obtained after the final Mono Q anion exchange column step. The preparation contained two major proteins of 53 kDa and 56 kDa which corresponded to the previously characterized protein products of the mouse lyn gene (21) (Fig. 1A). The levels of tyrosine phosphorylation of the purified recombinant Lyn were assessed by immunoblot analysis using a monoclonal anti-phosphotyrosine antibody both before and after autophosphorylation. Only very weak anti-phosphotyrosine immunoreactivity was observed. The filter was probed with 3-µg Lyn (L40) antibody followed by horseradish peroxidase-conjugated sheep anti-rabbit IgG and developed using the enhanced chemiluminescence kit following the protocol detailed by the manufacturer. The concentrations of Lyn in the samples were determined by densitometry.

Preparation of the Phosphopeptide Standard for Mapping the Lyn Autophosphorylation Site

The Lyn(391–400) peptide was phosphorylated to 1 mol of PO₃⁻⁻ incorporated per mol of peptide by recombinant pp60 c-src. The phosphopeptide was purified by reverse phase HPLC under conditions described previously (14).

Determination of the Autophosphorylation Site of Lyn by Tryptic Phosphopeptide Mapping

Lyn (220 ng) was autophosphorylated to stoichiometry of 1 mol of PO₃⁻⁻ per mol of kinase by incubation for 1 h at 30°C in kinase buffer containing 50 µM [γ-³²P]ATP (5,000–10,000 cpm/µmol). The reaction was terminated by addition of 1 ml of dialysis buffer (10 mM NH₄HCO₃, pH 7.9, 0.1% SDS, and 20 mM β-mercaptoethanol). For proteolytic digestion, the reaction mixture was first dialyzed against 2 × 2 liters of dialysis buffer overnight to remove free ATP. The dialyzed sample was concentrated and then alkylated by treatment with 4-vinylpyridine (2 µl per 100 µl of concentrated mixture) at 37°C for 3 h. After addition of 30 µg of BSA as carrier protein, the autophosphorylated Lyn was precipitated by incubating with 0.9 µl of ethanol at 20°C overnight and then washed with 1 µl of ethanol at −20°C. After removal of the residual ethanol by a Speed Vac, the precipitated proteins were exhaustively digested with tosylphenylalanyl chloromethyl ketone-treated trypsin (1 mg/ml) in a volume of 150 µl at 37°C for 48 h. The tryptic phosphopeptide fragment was isolated by reverse phase HPLC before analysis by the two-dimensional thin layer electrophoresis-thin layer chromatography (TLC) procedure.
detected before autophosphorylation (Fig. 1c); however, incubation with MgATP for 1 h results in autophosphorylation of both the 53- and 56-kDa forms of recombinant Lyn (Fig. 1b and c). Prior to the addition of ATP, less than 1% of the purified preparation of Lyn was phosphorylated, suggesting that almost all the potential tyrosine phosphorylation sites, including the putative autophosphorylation site (Tyr397) and the C-terminal regulatory tyrosine (Tyr508), were in a fully dephosphorylated state.

In some of the recombinant Lyn preparations, a protein band corresponding to a 54–55-kDa form of Lyn also exists (Fig. 1c). This protein band does not cross-react with the anti-tyrosine antibody, indicating that it is not tyrosine-phosphorylated. However, upon incubation with potato acid phosphatase, this 54–55-kDa form of Lyn disappears, suggesting that it represents a serine/threonine-phosphorylated form of Lyn (data not shown).

Correlation between the Level of Autophosphorylation and [Lys19]cdc2(6–20) Peptide Kinase Activity of Lyn—We have demonstrated that Lyn purified from bovine spleen (14) and purified recombinant Lyn (Fig. 1) can undergo autophosphorylation. In order to elucidate the role of autophosphorylation in the regulation of Lyn kinase activity, we investigated the effect of autophosphorylation on the protein-tyrosine kinase activity of Lyn using [Lys19]cdc2(6–20) peptide as the substrate. This resulted in a gradual increase in phosphate incorporation until a stoichiometry of 1 mol of PO32− per mol of kinase was attained, suggesting that only one major autophosphorylation site exists per molecule of Lyn (Fig. 2b). Accompanying the increase in autophosphorylation, an increase in tyrosine kinase activity of up to 17-fold was observed, strongly supporting the notion that activation of Lyn is a consequence of autophosphorylation (Fig. 2c).

Intermolecular versus Intramolecular Mechanisms of Autophosphorylation—Autophosphorylation can occur via intra- or intermolecular mechanisms, and these two mechanisms are either independent of or dependent on kinase concentration, respectively. When the initial rates of autophosphorylation at different Lyn concentrations were measured, a concentration-dependent increase in autophosphorylation rate was noted, indicating that in vitro autophosphorylation of Lyn follows an intermolecular mechanism (Fig. 3). The observation is reminiscent of the autophosphorylation mechanism reported for epidermal growth factor receptor, insulin receptor, and pp60c-Src (11, 12, 24, 25).

Kinetic Analysis of the Autophosphorylation Reaction—It has been well documented that stimulation of several cell surface receptors in hematopoietic cells, including the B-cell receptor in B-lymphocytes, results in rapid tyrosine phosphorylation and activation of Lyn (15, 16, 31–34). In contrast, the rate of Lyn autophosphorylation is slow (Fig. 2b); a 45-min incubation was necessary to achieve a stoichiometry of 1 mol of PO32− per mol of Lyn. This prompted us to further investigate the kinetics of autophosphorylation.

The initial velocity of autophosphorylation was determined using 160 nM Lyn and various concentrations of ATP. Lineweaver-Burke analysis of the data shows that the Kₘ for ATP is 14 μM and the Vₘₐₓ of autophosphorylation at 160 nM Lyn is...
autophosphorylation is concentration-dependent, a higher concentration had bound to the active site. As we have shown that Lyn autophosphorylation is concentration-dependent, a higher 

\[ V_{\text{max}} \] value would have been attained if a higher Lyn concentration had been used in the assay.

It would be informative to know the \( K_m \) and \( V_{\text{max}} \) values for Lyn in the autophosphorylation reaction. However, since Lyn acts as both the enzyme and the substrate in the reaction, it is not possible to determine these kinetic parameters by conventional kinetic analysis.

The Catalytic Consequences of Autophosphorylation—The kinetic properties of Lyn before and after autophosphorylation were analyzed using \((\text{Lys}^{190}\text{cdc216-20})\) peptide as the substrate in the presence of 100 \( \mu \text{M} \) ATP. Autophosphorylation does not significantly change the \( V_{\text{max}} \) (1 \( \mu \text{mol} \) of phosphate incorporated/min/mg) of peptide phosphorylation by the kinase. The \( K_{\text{m}} \) of nonphosphorylated Lyn for the peptide substrate is extremely high (25 \( \mu \text{m} \)), suggesting that the active site of nonphosphorylated Lyn is essentially inaccessible to the substrate peptide. Upon autophosphorylation, the \( K_{\text{m}} \) of the kinase for the peptide decreases by 63-fold to 400 \( \mu \text{M} \), indicating that autophosphorylation renders the active site of the kinase more accessible to the exogenous peptide substrate, thus increasing the affinity of the kinase for its substrate.

Construction of the Lyn Autophosphorylation Site—When fully autophosphorylated, purified recombinant Lyn could incorporate \( \text{PO}_4^{3-} \) to a stoichiometry of 1 mol of \( \text{PO}_4^{3-} \) per mol of kinase, indicating that autophosphorylation occurred exclusively at one site (Fig. 2). In order to further confirm that there was only a single autophosphorylation site and to identify this site, we performed phosphopeptide mapping on fully autophosphorylated Lyn. Exhaustive tryptic digestion of fully autophosphorylated Lyn yielded only one phosphopeptide fragment, confirming that Lyn autophosphorylates exclusively at one site (Fig. 4a).

The sequence of Lyn around tyrosine 397 is highly homologous to the sequence around the known autophosphorylation site in pp60c-Src (Fig. 5), strongly suggesting that Tyr397 is the autophosphorylation site in Lyn. A peptide derived from residues 391-400 of Lyn, Lyn(391-400), was synthesized and radiolabeled with 32P. The resulting phosphopeptide VIEDNE(pY)TAR was used as the standard for identification of the Lyn autophosphorylation site. Both the standard and the tryptic phosphopeptide fragment derived from Lyn migrated to an identical position in the two-dimensional phosphopeptide maps (Fig. 4, b and c), confirming that Tyr397 is indeed the autophosphorylation site.

Specificity of Lyn Binding to the Immobilized pYEEI Peptide—Recently, Payne et al. (8) and Songyang et al. (6) demonstrated high affinity binding of isolated recombinant SH2 domains of Src family kinases to the phosphopeptide pYEEI derived from the hamster polyoma virus middle T antigen (Fig. 5). Thus, the binding of Lyn to the immobilized pYEEI peptide is highly specific and occurs only when phosphorylated TyrEEI and other essential structural determinants are present.

Correlation between the Level of Autophosphorylation and Src Homology 2 Domain Accessibility of Lyn—Garcia et al. (26) demonstrated noncompetitive inhibition of pp60c-Src by the pYEEI peptide. Presumably, the inhibition is a result of binding of the pYEEI peptide to the SH2 domain of pp60c-Src. Moreover, interaction between the N-terminal region of the SH2 domain and a segment in close proximity to the autophosphorylation site of pp60c-Src has been reported (28). These data support the
notion that conformational perturbation of the SH2 domain of Src family kinases can be propagated to the kinase domain and alter their kinase activity. However, the effect of conformational changes induced by autophosphorylation in the kinase domain on the functions of the SH2 domain have never been documented. To this end, we investigated the effect of autophosphorylation-induced structural changes in the protein kinase domain on the conformation of the SH2 domain of Lyn.

Fig. 7 shows the time course of changes in PO32 incorporation, kinase activity, and the ability of Lyn to bind the immobilized pYEEI peptide. A significant decrease in the amount of Lyn bound to the immobilized pYEEI peptide (Fig. 7c and d) accompanied an increase in the degree of autophosphorylation (Fig. 7a) and kinase activity (Fig. 7b). Before autophosphorylation, the majority of Lyn in the assay mixture (30 ng (58%) out of a total of 52 ng of Lyn) was bound to the immobilized pYEEI peptide. After 60 min, when the kinase was fully autophosphorylated to a stoichiometry of 1 mol of PO32 incorporated per mol of kinase, the amount of kinase bound to the immobilized phosphopeptide dropped to 2.75 ng (only 5.3% of the total Lyn available). This suggests that autophosphorylation results in a dramatic decrease in the affinity of the SH2 domain of Lyn for the pYEEI peptide.

As mentioned under Characterization of Purified Recombinant Mouse Lyn, some of our purified Lyn preparations contain a 54–55-kDa form of Lyn which might arise as a result of serine/threonine phosphorylation in vivo. As indicated in Fig. 7, the 53-kDa, 54–55-kDa, and 56-kDa forms of Lyn can undergo autophosphorylation and the autophosphorylation-induced decrease in SH2 domain accessibility.

**DISCUSSION**

In the present study we have demonstrated that Lyn autophosphorylation correlates with an increase in its kinase activity. Our observations support the notion that autophosphorylation stabilizes the active conformation of the kinase and thereby leads to activation of the kinase. Autophosphorylation occurs exclusively at Tyr397. This agrees well with similar studies on other members of the Src family (see Ref. 1 for review). We have shown that Lyn autophosphorylation follows an intermolecular or trans-mechanism. Similar to Lyn, autophosphorylation of the insulin receptor which occurs by a trans-mechanism is a prerequisite for its activation (11). Comparison of the sequence surrounding the autophosphorylation site in the insulin receptor (Tyr1162) with that of Lyn reveals significant homology (Fig. 5) (1, 11). The similar enzymatic properties of these two kinases in addition to the sequence homology surrounding their autophosphorylation sites suggest that the two kinases follow similar molecular mechanisms of autophosphorylation and autoactivation.

Based upon the crystal structure of the insulin receptor tyrosine kinase domain (IRK), a model explaining how trans-autophosphorylation leads to activation of the insulin receptor has been postulated (11). In this model, the nonphosphorylated
IRK is locked in an inactive conformation in which the "self" autophosphorylation site (Tyr1162) is "engaged" in the substrate-binding region of the active site. Upon autophosphorylation, the phosphate moiety of Tyr(P)1162 of IRK is believed to electrostatically interact with Arg1131 in the catalytic loop. Presumably, such an interaction stabilizes the active conformation by "disengaging" Tyr(P)1162 from the substrate-binding region in the active site. Such a model can also be used to explain the kinetic consequences of Lyn autophosphorylation. The "self" Tyr397 of Lyn blocks the binding of substrate, and, as a result, nonphosphorylated Lyn is in an inactive conformation. This model is supported by the fact that the Km value of nonphosphorylated Lyn for the exogenous peptide substrate is extremely high (25 mM). Presumably, upon autophosphorylation, Tyr(P)397 is "disengaged" from the substrate protein-binding region in the active site. As a result, binding of substrate to the active site is allowed and the kinase is activated. This model is further substantiated by the 63-fold decrease in the active site more accessible to the kinase domain of Lyn and Tyr 527 of pp60c-Src for the substrate peptide after autophosphorylation. We postulate that Arg366 in the catalytic loop, homologous to Arg1131 of IRK, is the basic residue binding to Tyr(P)397 and in turn allowing binding of the substrate peptide to Lyn by disengaging Tyr(P)397 from the substrate-binding regions in the active site (Fig. 5). Confirmation of the putative Tyr(P)397, Arg366 interaction requires the elucidation of the crystal structure of autophosphorylated Lyn.

Similar studies of pp60c-Src shows that autophosphorylation led to a 2-fold increase in kinase activity while our study reports a 17-fold increase in the kinase activity of Lyn upon autophosphorylation (36). Autophosphorylation of pp60c-Src did not alter the Km value but caused a 2-fold increase in Vmax for its substrate protein, casein (36). This is in sharp contrast to Lyn where autophosphorylation alter the Km, but not the Vmax for its substrate peptide. Thus, despite the high degree of sequence homology between the protein kinase domains of pp60c-Src and Lyn, the extent and molecular mechanisms of autoactivation of these two kinases are quite different.

Previous studies have shown that Lyn is physically associated with a number of hematopoietic cell surface receptors including the B-cell receptor (15), Fcy-Receptor I (31), Fce-g receptor I (32), interleukin 7 receptor (33), and granulocyte colony-stimulating factor receptor (34). Stimulation of these cell surface receptors results in rapid phosphorylation and activation of Lyn in vivo (see Refs. 15, 16, and 36 for review), and it is therefore intriguing that the in vitro autophosphorylation of Lyn occurs at a very slow rate. It is possible that the physical association of Lyn with these receptors increases the effective concentration of Lyn available for autophosphorylation. Likewise, upon stimulation of the receptors, conformational changes originating from the receptors could be propagated to the kinase domain of Lyn and somehow render the active site more accessible to the trans-Tyr397 residue of the neighboring Lyn molecule.

There is a substantial body of evidence supporting the involvement of Src homology 2 (SH2) domains in the regulation of the activity of Src family kinases. The generally accepted model involves binding of the C-terminal phosphotyrosine (Tyr508 of Lyn and Tyr527 of pp60c-Src) to the SH2 domain which forces the kinase to assume an inactive conformation (29). Upon dephosphorylation of the C-terminal phosphotyrosine, its interaction with the SH2 domain is disrupted and this allows the kinase to assume the de-repressed conformation and undergo autophosphorylation (Fig. 8). The detailed structural basis for inactivation by such an interaction is not understood. In addition to the C-terminal phosphotyrosine-SH2 domain interaction, interaction between SH2 domains of Src family kinases with exogenous phosphotyrosine-containing proteins has been documented (see Refs. 15, 16, and 35 for review). Interaction of the SH2 domain of pp60c-Src with the exogenous pYEEI peptide inhibits its kinase activity. Furthermore, photoaffinity crosslinking of the SH2 domain of pp60c-Src with a pYEEI peptide analog partially inactivated the kinase (26). Based upon these observations, Garcia et al. (26) postulated that occupancy of the SH2 domain induces a conformational change that is transmitted to the kinase domain and attenuates the tyrosine kinase activity of pp60c-Src (26).

Our observation that autophosphorylation of Lyn leads to a decrease in the accessibility of its SH2 domain to the immobilized pYEEI peptide provides evidence for the propagation of conformational changes from the kinase domain to the SH2 domain of Lyn. How would the propagation of conformational changes occur and what is the structural basis dictating the functional interaction between the kinase domain and the SH2 domain of Lyn? Using the crystal structure of the catalytic subunit of cAMP-dependent protein kinase as the template, Veron et al. (27) revealed a putative helix motif (the A-helix motif) in Src family kinases by homology modelling; this A-helix motif forms the basis of a hypothetical model for the cross-talk between the kinase domain and the SH2 domain documented for pp60c-Src and Lyn. In this model, the A-helix motif serves as a linker between the catalytic core and the SH2 domain of Src family kinases (27). This hypothetical A-helix motif can potentially interact with essential amino acid residues in the catalytic loop as well as residues in close vicinity to the autophosphorylation site. Presumably, these interactions allow propagation of the autophosphorylation-induced conformational changes from the protein kinase domain through the A-helix motif to the SH2 domain which may account for the decreased SH2 domain accessibility of autophosphorylated Lyn to the pYEEI peptide (Fig. 6).

Unlike pp60c-Src, Lyn has kinase activity upon autophosphorylation (36). Autophosphorylation of Lyn at Tyr397 gives rise to a fully activated tyrosine kinase (conformation 3). When phosphorylated, the phosphate moiety of Tyr(P)1162 of IRK is believed to electrostatically interact with Arg1131 in the catalytic loop and somehow render the kinase domain of Lyn and Tyr 527 of pp60c-Src for the substrate peptide after autophosphorylation. We postulate that Arg366 in the catalytic loop, homologous to Arg1131 of IRK, is the basic residue binding to Tyr(P)397 and in turn allowing binding of the substrate peptide to Lyn by disengaging Tyr(P)397 from the substrate-binding regions in the active site (Fig. 5). Confirmation of the putative Tyr(P)397, Arg366 interaction requires the elucidation of the crystal structure of autophosphorylated Lyn.

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GTpase activator protein, mitogen-activated protein kinase (MAP kinase), and phospholipase C-γ are among the proteins that are rapidly tyrosine-phosphorylated following stimulation of the B-cell receptor (see Ref. 15 for review). These proteins are believed to play essential roles in transducing signals initiated by stimulation of the B-cell receptor (37). When phosphorylated, these phosphoproteins can bind to the SH2 domain of Lyn, thereby providing a means of transmitting signals via Lyn that have initiated from the B-cell receptor. The binding site for...
these phosphoproteins has been mapped to the unique and SH2 domain of Lyn (37). Our observation that autophosphorylation decreases the accessibility of the SH2 domain of Lyn to the pYEEI peptide suggests that autophosphorylation may modulate binding of Lyn to phosphotyrosine-containing molecules in vivo.

From our data, we postulate that Lyn exists in at least three hypothetical conformational states in vivo (Fig. 8). The inactive form of Lyn is represented by conformation 1. In this conformation, the C-terminal regulatory tyrosine is phosphorylated, presumably by C-terminal Src kinase or a related kinase (38). Interaction between the C-terminal phosphotyrosine and the SH2 domain suppresses the kinase activity and prevents its SH2 domain from binding to exogenous tyrosine-phosphorylated protein molecules. Dephosphorylation of the C-terminal phosphotyrosine by an as yet unidentified phosphatase allows the kinase to assume a derepressed conformation which displays low or no kinase activity (conformation 2). Owing to its ability to bind the pYEEI peptide, Lyn in this conformation can potentially bind tyrosine-phosphorylated proteins. The kinase can then be activated by autophosphorylation giving rise to the fully active form of the enzyme (conformation 3). In this conformation, Lyn is capable of phosphorylating its protein substrates but our data suggest that its accessibility to phosphotyrosine-containing proteins is greatly reduced.

Since our observation suggests that autophosphorylation is obligatory for autoactivation of Lyn, dephosphorylation of Tyr(P)397 of Lyn has not been identified. Identification of this phosphatase will be important for understanding how the activity of this enzyme is regulated.

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