Spontaneous Autophosphorylation of Lyn Tyrosine Kinase at both Its Activation Segment and C-Terminal Tail Confers Altered Substrate Specificity[†,‡]

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ABSTRACT: Two tyrosyl residues have been reported to play a crucial role in the regulation of protein tyrosine kinases of the Src family: autophosphorylation of Tyr416 (c-Src numbering) located in the catalytic domain correlates with enzyme activation, while Csk-mediated phosphorylation of the C-terminal tyrosine Tyr527 (c-Src numbering) gives rise to inactive forms of Src kinases. Here we show that the Src-related Lyn kinase undergoes spontaneous and stoichiometric autophosphorylation at both Tyr396 (homologous to c-Src Tyr416) and Tyr507 (homologous to c-Src Tyr527). Such a doubly autophosphorylated form of Lyn is hyperactive toward peptide substrates and insensitive to Csk-induced downregulation. In contrast, doubly autophosphorylated Lyn exhibits reduced activity toward protein substrates such as phospho-p50/HS1 (hematopoietic-lineage cell-specific protein) and p57/PDI (protein disulfide isomerase related protein), whose multiple sequential/processive phosphorylation relies on the accessibility of the SH2 domain of the kinase. These data disclose a novel conformation of Lyn that is catalytically active despite the presence of an intramolecular interaction between the phosphorylated tail and the SH2 domain. This enzyme conformation is expected to display a reduced oncogenic potential resulting from its defective recognition of a subset of protein substrates whose targeting is mediated by the Lyn SH2 domain.

Protein tyrosine kinases belonging to the Src family have been implicated in cellular responses to a variety of extracellular signals. Src PTKs[1] currently consist of at least nine members, c-Src, c-Yes, Fyn, Yrk, c-Fgr, Lyn, Lck, Hck, and Blk. They are mostly expressed in hematopoietic cells and share a high degree of structural similarity showing common domain architecture and regulatory mechanisms. Their sequence consists of a poorly conserved N-terminal segment (the "unique domain"), two conserved domains, SH3 and SH2, followed by the catalytic domain, SH1 [see Mustelin (1994) and Brown and Cooper (1996) for reviews]. SH2 and SH3 domains play a dual role in the Src kinases: either they function to target the enzymes to specific substrates directing protein−protein interactions (Pawson & Gish, 1992) or they are required for keeping the kinase in an inactive state (Sicheri et al., 1997; Xu et al., 1997). Two tyrosine residues are landmarks of the Src family. One is located inside the catalytic domain (Tyr416 in c-Src) and the other (Tyr527 in c-Src) is included in the carboxy-terminal tail of the cellular Src homologues.

Tyr-527, which is not present in the tail of the viral Src homologues, can be phosphorylated by a tyrosine protein kinase called Csk, which is responsible for the downregulation of the Src kinases (Okada et al., 1991; Bergman et al., 1992; Superti-Furga et al., 1993; Ruzzene et al., 1994). Src PTK SH2 domain interacts with the phosphorylated tyrosine of the tail, thus causing the kinase to adopt a conformation that displays a low enzyme activity (Cantley et al., 1991; Cooper & Howell, 1993). The solved crystal structures of the downregulated forms of Src and Hck make clear that this interaction does not seem to disturb directly the structure of the catalytic domain. The role of P-Tyr527/SH2 interaction may be to position the SH3 domain in such a way that it can bind the SH2−kinase linker and contact the small lobe of the kinase catalytic domain. Consequently helix αC of the small lobe forces the residue Glu310, which is probably critical for kinase activity, away from the active site (Sicheri et al., 1997; Xu et al., 1997).

Tyr416 and the homologous tyrosines of other Src PTKs constitute the main autophosphorylation site of these enzymes. This site is located within the activation segment of the catalytic domain close to the position of the γ-phosphate of ATP. The apparent flexibility of the activation segment in the vicinity of Tyr416 suggests that autophosphorylation could occur intramolecularly once the regulatory domains are not clamped in place (Sicheri et al., 1997); however, data supporting both intramolecular (Sugimoto et al., 1985; Feder & Bishop, 1990) and intermolecular autophosphorylation (Cooper & MacAuley, 1988; Azuma et al., 1991; Sotirellis et al., 1995; Barker et al., 1995) have been published. It has been found that autophosphorylation is required for the

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[1] Abbreviations: PTK, protein tyrosine kinase; SH3, SH2, and SH1, Src homology domains 3, 2, and 1; Csk, C-terminal Src kinase; p50/HS1, hematopoietic-lineage cell-specific protein; p57/PDI, protein disulfide isomerase related protein; ARH1, antigen receptor homology 1; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

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catalytic activity of Src PTKs (Kmiecik et al., 1988; Koegl et al., 1995; Brown & Cooper, 1996; Boerner et al., 1996; Weijland et al., 1996; Moarefi et al., 1997). This hypothesis is supported by the crystal structures of Lck active catalytic domain and downregulated Src and Hck (Yamaguchi & Hendrickson, 1996; Sicheri et al., 1997; Xu et al., 1997). Indeed autophosphorylation of Tyr416 in the activation segment would result in the reorganization of this sequence, which would favor the inward rotation of helix αC and consequent conversion of the active site to a catalytically competent form.

The Src-like PTK encoded by the *lyn* protooncogene comes in two sizes, 53 and 56 kDa, which are expressed in normal hematopoietic cells and in T lymphocytes transformed by HTLV-I and herpesvirus saimiri (Bolen et al., 1992). Lyn is thought to play an important role in relaying signals originating from cell surface receptor, such as B-cell receptor (BCR). In these cells Lyn is required for BCR-induced normal calcium mobilization, as well as for the increase in tyrosine-phosphorylated proteins induced by receptor ligation (Takata et al., 1994). HS1 (hematopoietic-lineage cell-specific protein) is one of these proteins (Yamanashi et al., 1993). p50/HS1 undergoes a sequential phosphorylation *in vitro*: previous phosphorylation catalyzed by either TPKIIB/p38^{syk} or p72^{syk} converts the protein into a good substrate for the Src kinases Lyn and c-Fgr (Brunati et al., 1995a; Ruzzene et al., 1996). It has also been demonstrated that Lyn can specifically phosphorylate *in vitro* a 57-kDa protein, which is related to protein disulfide-isomerase (p57/PDI). p57/PDI can be phosphorylated by Lyn according to a processive phosphorylation mechanism, which implies that a primary phosphorylation site of the substrate binds to the SH2 domain of the enzyme and triggers the phosphorylation at secondary site(s) (Donella-Deana et al., 1996).

In order to shed more light on the mechanism of Lyn regulation we have investigated the Lyn autophosphorylation reaction and both its effect on Lyn activity toward exogenous substrates and its downregulation by Csk-mediated phosphorylation.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was purchased from Amersham; enolase and chemicals were from Sigma. cdc2(6−20) (KVEKIGEGTYGVVYK), Lyn(392−398) (EDNEYTA), Lyn(487−511) (PTFDYLQSVLDDFYTATEGQYQQQP), and Lyn(487−511)^{Phe507} peptides and the phosphopeptides PEGDYpEEVLE and VIEDNEYpTAR were synthesized by the methods reported previously (Brunati et al., 1995b; Donella-Deana at al., 1996). Anti-FSO_2BzAdo antibody was kindly provided by Dr. P. J. Parker (Imperial Cancer Research Fund, London). Anti-Lyn, anti-c-Fgr, anti-Fyn, and anti-Syk IgGs, directed against protein residues 44−63, 47−68, 29−48, and 252−352, respectively, were from Santa Cruz Biotechnology. Anti-Csk antibody was raised against the sequence 48−64 of Csk (Brunati et al., 1992), and anti-phosphotyrosine antibody was from ICN Biotechnology.

Methods. p57/PDI and p50/HS1 were purified as described elsewhere (Brunati et al., 1995a; Donella-Deana et al., 1996). p50/HS1 was previously phosphorylated by p38^{syk} and was separated from the unphosphorylated form by MonoQ FPLC (Brunati et al., 1995a).

c-Fgr, Fyn, and Csk were purified from rat spleen as described elsewhere (Brunati et al., 1992; 1993). Lyn, constituted essentially by the p53 isoform, was purified from the heparin−sepharose eluted peak I following the procedure described in Donella-Deana et al. (1996).

Protein phosphorylation catalyzed by Lyn and Csk and Lyn autophosphorylation were performed at 30 °C, in 30 μL of medium containing 50 mM Tris-HCl, pH 7.5, 5 mM $MnCl_2$, 40 μM [γ-^{32}P]ATP (specific activity 1000−2000 cpm/pmol) and the indicated amount of enzyme and protein. c-Fgr and Fyn autophosphorylation and c-Fgr-catalyzed phosphorylation were performed in the same incubation mixture containing 5 mM $MgCl_2$ instead of $MnCl_2$. The reactions were stopped at the indicated time by addition of 2% SDS, and the samples were subjected to SDS−10% PAGE and allowed to run for a further 30 min at 200 V after the front reached the end of the gel. The degree of protein phosphorylation was evaluated either by analysis on a Packard Instant Imager or by autoradiography and counting of the identified radiolabeled bands.

The phosphorylation reactions of samples used for CNBr digestion were stopped with formic acid (70% final concentration) and the cleavage was performed as described elsewhere (Ruzzene et al., 1994).

Identification of Tyr396 as Autophosphorylation Site. Autophosphorylated Lyn (20 pmol) was digested with CNBr, resolved by SDS−PAGE, and transferred electrophoretically to nitrocellulose filter. The 8 kDa fragment localized by autoradiography was digested by trypsin as described elsewhere (Brunati et al., 1995a). The tryptic peptides were lyophilized and dissolved in 0.2 mL of aqueous trifluoroacetic acid [TFA, 0.1% v/v)] containing 5 μg of synthetic peptide VIEDNEYpTAR to be used as internal standard. The sample was then analyzed on a SuperPac Pep-S C_2/C_{18} reverse-phase column (4 × 250 mm, 5 μm particle size) using a Perkin-Elmer 410 LC BIO HPLC system. The column was eluted for 1 min with an aqueous solution of TFA (0.1%) and for 90 min with a linear gradient (from 0% to 40%) of TFA/acetonitrile (0.08%) in the aqueous TFA solution at a flow rate of 1 mL × min^{-1}. While the synthetic peptide was spectrophotometrically detected at 220 nm, the radioactivity of the fractions collected every 20 s was measured by liquid scintillation counting. Under these conditions the retention time of the phosphopeptide was 15.9 min.

Peptide phosphorylation was assayed in the above-described basal buffer supplemented with the indicated amount of enzymes and peptides. In the experiments containing the cdc2(6−20) peptide the reactions were stopped after 5 min of incubation by spotting 25 μL of the mixture onto P81 phosphocellulose papers, which were processed as described elsewhere (Glass et al., 1978). In the experiments containing Lyn(392−398) peptide, the reactions were terminated after 5 min of incubation by addition of 1 mL of 1 N HCl and processed as described elsewhere (Brunati et al., 1995b).

Kinetic parameters were measured by 10 min of incubation of the above-described basal buffer containing increasing concentration of peptide substrate (from 0.025 to 2 mM) and 60 nM Lyn. K_m and k_{cat} values were determined by double-reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis−Menten equation.

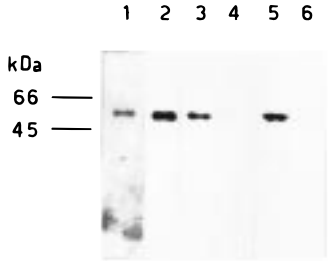


FIGURE 1: Analysis of the purified Lyn preparation: Coomassie brilliant blue stained SDS−PAGE of Lyn preparation (lane 1); SDS−PAGE autoradiography of 40 ng of Lyn autophosphorylated as described in Materials and Methods (lane 2); Western blots of Lyn with anti-Lyn (lane 3) and anti-Csk sera (lane 4); Western blot of Lyn with anti FSO_2BzAdo IgG performed either in the absence (lane 5) or in the presence (lane 6) of 3 mM ATP as described in Experimental Procedures. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

Protein concentration was determined either by the method of Bradford (1976) or by Coomassie blue staining of samples after SDS−PAGE followed by quantification by transmittance densitometry. Bovine serum albumin was used as a standard in both procedures.

Immunodetection with anti-FSO_2BzAdo IgG was performed by incubating 80 ng of Lyn fraction with 1 mM FSO_2-BzAdo and 10 mM $MnCl_2$ in the absence or presence of 3 mM ATP (preincubated for 5 min) at 30 °C for 1 h (Parker, 1993).

RESULTS

Mechanism of Lyn Autophosphorylation. Native Lyn, constituted essentially by the p53 isoform, purified from the particulate fraction of rat spleen to apparent homogeneity as indicated by Coomassie blue (Figure 1, lane 1) and silver staining (not shown), did not display any detectable immunoreactivity with the antisera raised against the following protein tyrosine kinases, which were originally present in the crude extract: Csk (Figure 1, lane 4), c-Fgr, Fyn, and Syk (not shown). Figure 1 also shows that the Lyn preparation was not contaminated by other protein kinases; in fact, only a single band that immunoreacted against anti-FSO_2BzAdo IgG (Parker, 1993) and displayed the same molecular mass of Lyn was evident (Figure 1, lane 5). FSO_2-BzAdo is an ATP analogue that covalently binds to the catalytic site of most protein kinases (Zoller & Taylor, 1979). The specific cross-reactivity of the Lyn band with anti-FSO_2-BzAdo serum was abolished when ATP was added to the reaction mixture before treatment with FSO_2BzAdo (Figure 1, lane 6). Moreover, the Lyn preparation did not react against anti-phosphotyrosine serum (not shown). This was likely due to dephosphorylation by protein tyrosine phosphatases, which were present in the first steps of the enzyme purification procedure. Figure 2 shows that the initial rate of Lyn autophosphorylation is parabolically dependent on the enzyme concentration and that a straight line is obtained when the initial rates of the reaction are plotted against the square of the protein concentration. These data are consistent with an intermolecular autophosphorylation mechanism. The time course of Lyn autophosphorylation is slow and reaches a stoichiometry of 1.6 ± 0.22 mol of P_i/mol of enzyme (mean + SE; $n = 6$) in 40 min (Figure 3A). Indeed, a stoichiometry of about 2 mol of P_i/mol of Lyn can be reached in 80 min of incubation (not shown).

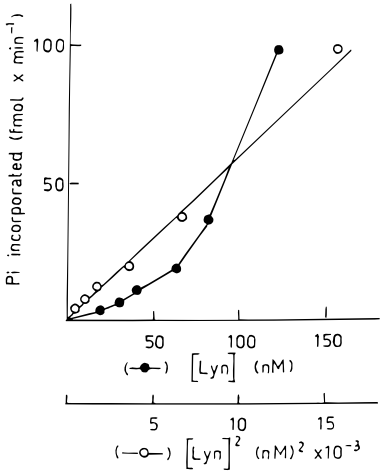


FIGURE 2: Dependence of Lyn autophosphorylation rate on the enzyme concentration. The amount of bound phosphate is plotted against either the protein concentration (●) or the square of this parameter (○). Experimental conditions are detailed in Materials and Methods. Data are means of five separate experiments performed with three different Lyn preparations. SE values were always less than 20%.

Identification of the Lyn Phosphoacceptor Sites. The high stoichiometry of Lyn autophosphorylation was somewhat intriguing and suggested that a second tyrosine besides the canonical autophosphorylation site (Tyr396) was affected. To shed light on this point, the radiolabeled CNBr fragments of the Src-like PTKs Lyn, c-Fgr, and Fyn autophosphorylated in the presence of [γ-^{32}P]ATP were isolated by SDS−PAGE and detected by autoradiography. As shown in Figure 4, the autophosphorylation of c-Fgr and Fyn, purified from spleen in a native dephosphorylated form, gave rise to a single radiolabeled band of about 10 kDa, which is known to contain the canonical autophosphorylation site Tyr400 [see also Ruzzene et al. (1994)] and Tyr420 of c-Fgr and Fyn, respectively (Figure 4, lanes 1 and 5). On the contrary, Lyn autophosphorylation, under basal conditions, produced two radiolabeled CNBr peptides. The molecular masses of the fragments (about 8 and 4 kDa) are consistent with the expected sequences containing the canonical autophosphorylation site (Tyr396 of p56/Lyn) and the Csk phosphorylation site (Tyr507 of p56/Lyn), respectively (Figure 4, lanes 2 and 3). Since the CNBr hydrolysis produces two fragments of about 8 kDa, in order to identify the autophosphorylated Lyn residue, the phosphopeptide VIEDNEYpTAR corresponding to the tryptic sequence containing the expected main autophosphorylated site (by homology with Src Tyr416) was synthesized. This phosphopeptide coeluted exactly with the single radioactive peak obtained by tryptic digestion of the 8 kDa CNBr fragment (Figure 5). The identification of Tyr507 as a second autophosphorylated site was based on the following evidence: (i) The 4 kDa CNBr fragment is expected to correspond only to the C-terminal part of the molecule, as also confirmed by the finding that an electrophoretically similar radiolabeled fragment was generated by Lyn phosphorylated by Csk (Figure 4, lane 4). (ii) As the 4 kDa fragment contains three tyrosyl residues (Tyr491, Tyr500, and Tyr507), which are present also in its tryptic derivative (487−511), we have synthesized this peptide and analyzed its kinetic constants for Lyn in comparison with those of an analogue in which the Tyr507 was replaced by

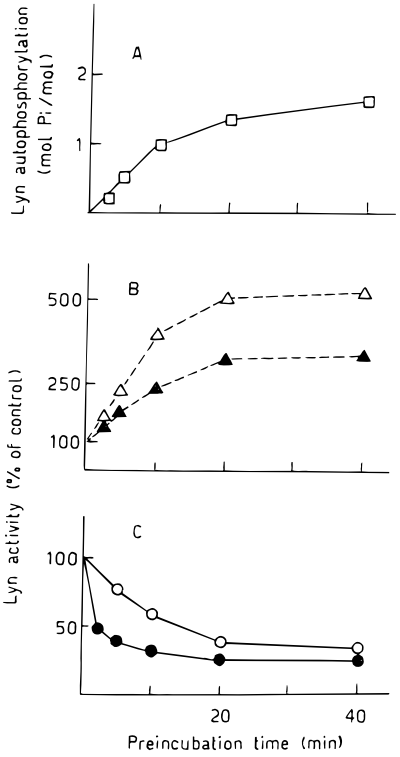


FIGURE 3: Tyrosine kinase activities of Lyn ensuing its autophosphorylation. Lyn (35 nM) was preincubated in the presence of [γ-^{32}P]ATP as described in Materials and Methods in five parallel sets of experiments. At the indicated time one set was stopped for SDS−PAGE analysis and Lyn autophosphorylation stoichiometry determination (A), while the other four experimental sets were subsequently incubated for 5 min in order to test the Lyn activity on the following substrates: 200 μM cdc2(6−20) (△) and 200 μM Lyn(392−398) (▲) peptides (B) or 70 nM p57/PDI (●) and 20 nM p50/HS1 (previously phosphorylated by Syk) proteins (○) (C). Activity of autophosphorylated Lyn is expressed as a percentage of the activity displayed by the unphosphorylated enzyme, i.e., preincubated without ATP. The experimental conditions adopted for the measurement of the enzyme activity, i.e., the short incubation time and the presence of exogenous substrates, ensured a negligible autophosphorylation of the enzyme unphosphorylated form as verified by SDS−PAGE. The initial rate of phosphate transfer to cdc2(6−20) and Lyn(392−398) peptides displayed by unphosphorylated Lyn was 5.3 pmol·min^{-1} and 2.8 pmol·min^{-1}, respectively. Results are means of six separate experiments performed with three different Lyn preparations. All calculated SE values are less than 20%.

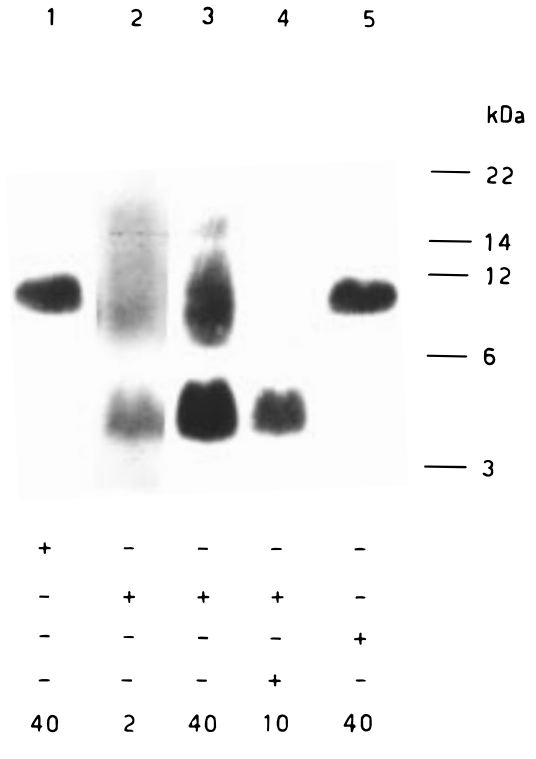


FIGURE 4: CNBr ^{32}P-peptide maps of autophosphorylated Lyn, c-Fgr, and Fyn tyrosine kinases. c-Fgr (60 nM) (lane 1), 90 nM Lyn (lanes 2−4), and 46 nM Fyn (lane 5) were autophosphorylated for the indicated time. In the experiment of lane 4, 60 nM Csk was also added to the incubation mixture. At the end of the incubation, each sample was digested with CNBr and the radiolabeled fragments were resolved by SDS−20% PAGE and evaluated by autoradiography and Instant Imager (Packard) analysis. Because of its relevance, lane 2 was overexposed in order to better highlight the radioactive CNBr fragments. The positions of the molecular mass markers (in kilodaltons) are indicated on the right. The autoradiographs are representative of six separate experiments performed with three different Lyn preparations and of three separate experiments performed with c-Fgr and Fyn.

Phe. While the native sequence was rather efficiently phosphorylated by the enzyme [k_{cat} 33 min^{-1} and K_m 810 μM) the derivative was only slightly affected.

The autophosphorylation at Tyr507 was detectable even at short incubation times (Figure 4, lane 2) and different ATP concentrations (5−100 μM) (not shown); its relative amount invariably ranged between 42% and 57% of the total radioactivity incorporated into the two sites. In other words we were unable to detect any appreciable autophosphorylation of Tyr396 without a concomitant and nearly equivalent radiolabeling of Tyr507. In contrast, when Lyn was incubated in the presence of Csk for 10 min, the expected radiolabeled band of 4 kDa was found (Figure 4, lane 4). The low amount of radioactivity (about 13%, slightly visible in lane 4 of Figure 4; see, however, the following Figure 10) incorporated at Tyr396 contained in the 8 kDa fragment suggested that, under these conditions, the Lyn autophosphorylation was almost completely prevented by the down-regulating enzyme (Figure 4, lane 4). In agreement with this interpretation, the catalytic activity displayed by Lyn on cdc2(6−20) peptide under these conditions was drastically reduced, resulting in about 25% that of the control performed in the absence of Csk (not shown), and was consistent with the presence of the Csk-induced downregulated enzyme conformation (Brown & Cooper, 1996; Sicheri et al., 1997; Xu et al., 1997).

Role of Autophosphorylation in the Regulation of Lyn Kinase Activity. Biochemical and crystallographic studies have indicated that Src kinases are positively regulated upon autophosphorylation at a single site located in the activation segment (Kmiecik et al., 1988; Koegl et al., 1995; Brown & Cooper, 1996; Boerner et al., 1996; Yamaguchi & Hendrickson, 1996; Moarefi et al., 1997). Lyn autophosphorylates at two sites that have been reported to regulate the enzyme in opposite ways. To elucidate the functional effects of such a double autophosphorylation we investigated the catalytic activity of autophosphorylated Lyn on both peptide and protein substrates. Lyn preincubation with ATP resulted in an increased activity on the peptide substrates Lyn(392−398) and cdc2(6−20) (Figure 3B). In particular, 20 min of autophosphorylation under the conditions reported in Materials and Methods promoted a decrease of the K_m value for

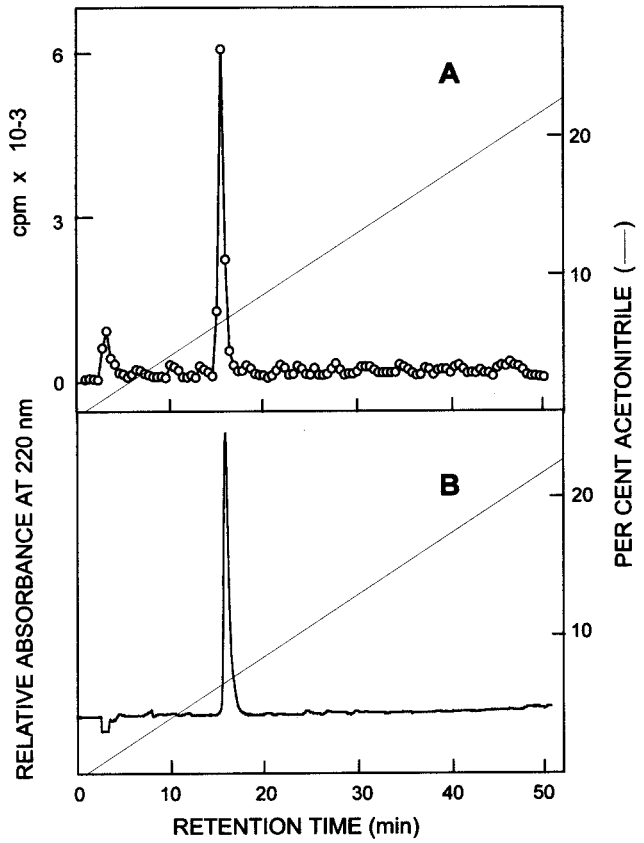


FIGURE 5: Identification of Tyr396 as an autophosphorylated site. The 8 kDa CNBr radioactive fragment obtained by autophosphorylated Lyn was digested with trypsin, mixed with the synthetic phosphopeptide VIEDNEYpTAR, and analyzed by reverse-phase HPLC as described in Materials and Methods. The radiolabeled ^{32}P-phosphate content of eluate was measured by liquid scintillation counting of 0.3 mL fractions (A), while the synthetic phosphopeptide content was spectrophotometrically measured at 220 nm (B).

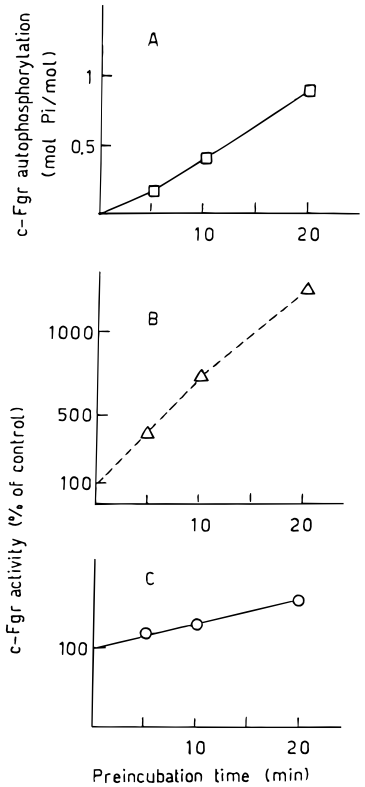


FIGURE 6: c-Fgr tyrosine kinase activities following the enzyme autophosphorylation. c-Fgr (30 nM) was autophosphorylated in three parallel sets of experiments performed as in Figure 4. c-Fgr autophosphorylation stoichiometry (A) and c-Fgr activity on cdc2-(6−20) (B), and p50/HS1 (C) are shown. Results are means of three separate experiments with SE values always less than 15%.

cdc2(6−20) from 860 to 200 μM, accompanied by a slight increase in the k_{cat} (from 41.2 to 53.7 min^{-1}). By contrast to the peptide substrates, two protein substrates, namely, phospho-p50/HS1 and p57/PDI, susceptible to Lyn-catalyzed sequential and processive phosphorylation, respectively (Brunati et al., 1995a; Ruzzene et al., 1996; Donella-Deana et al., 1996), were much less readily phosphorylated by the autophosphorylated Lyn than by the nonautophosphorylated enzyme (Figure 3C). These data indicate that, while autophosphorylation renders the catalytic site of Lyn more competent to phosphorylate peptide substrates, it decreases the phosphorylability of protein substrates whose recruitment and phosphorylation have been shown to be mediated by the SH2 domain of the enzyme (Brunati et al., 1995a; Ruzzene et al., 1996; Donella-Deana et al., 1996). Figure 6B shows that the autophosphorylation of c-Fgr, which occurs only at Tyr400, also correlates with a remarkable increment of activity toward the peptide cdc2(6−20) but is not associated with any decrease in activity on protein substrate; the phosphorylation of p50/HS1 in fact slightly increased (Figure 6C).

In order to assess the occurrence of interactions between the C-terminal phosphorylated residue of doubly autophosphorylated Lyn and its SH2 domain, we performed peptide competition experiments using the phosphopeptide PEGDYpEEVLE derived from p50/HS1 protein and including the Src SH2-recognition motif, Tyr-P/acidic/acidic/hydrophobic residue (Songyang et al., 1994). As shown in Figure 7A, the SH2-specific PEGDYpEEVLE phosphopeptide inhibited the processive phosphorylation of p57/PDI, thus confirming that this process is mediated by the SH2 domain. However, the previous autophosphorylation of Lyn counteracts such an inhibition by the phosphopeptide, since the p57/PDI phosphorylation, albeit low, catalyzed by autophosphorylated Lyn was not affected by the phosphopeptide (Figure 7A). This finding is consistent with the view that Lyn autophosphorylation brings about inaccessibility to the enzyme SH2 domain, giving rise to a Lyn form that is able to catalyze the primary phosphorylation of p57/PDI but unable to trigger the SH2-mediated phosphorylation at the protein secondary sites. At variance with the inhibitory effect displayed by the phosphopeptide on the Lyn activity measured on p57/PDI, a negligible effect was found on the enzyme activity toward the pentadecapeptide cdc2(6−20) (Figure 7B). This is consistent with the notion that the phosphorylation of the peptide substrates is not a processive event requiring the availability of Lyn SH2 domain. Accordingly, also, the enhanced activity induced by Lyn autophosphorylation on cdc2(6−20) peptide was not affected by the presence of PEGDYpEEVLE phosphopeptide (Figure 7B).

Effect of Lyn Autophosphorylation on the Enzyme Downregulation Induced by Csk. Considering the above-reported data, which show that Lyn undergoes spontaneous autophosphorylation not only at Tyr396 but also at Tyr507, which is the specific target for Csk, and that this doubly phosphorylated form of Lyn results hyperactive in the canonical (nonprocessive/sequential) phosphorylation assays, it was

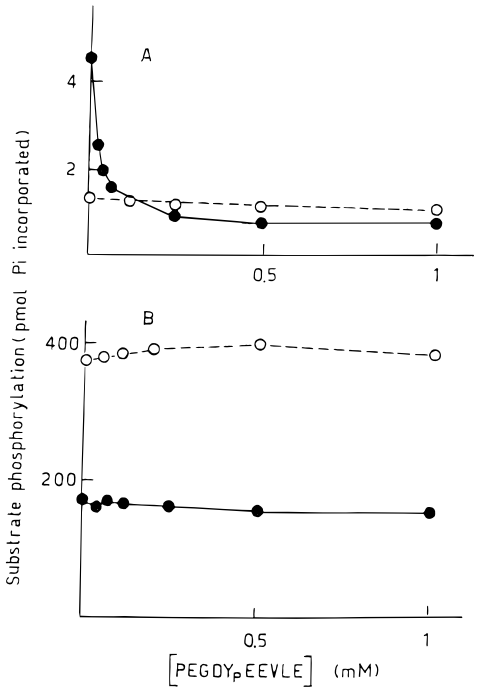


FIGURE 7: Effect of the phosphopeptide PEGDYpEEVLE on the activity displayed by either native or autophosphorylated Lyn toward peptide and protein substrates. Lyn (25 nM) was first preincubated (20 min) in the absence (●) or presence (○) of 40 μM [γ-^{32}P]-ATP. Autophosphorylated Lyn contained about 0.8 mol of Pi/mol located at Tyr507. The samples were then incubated with the indicated concentrations of PEGDYpEEVLE phosphopeptide (3 min) and finally the Lyn activity was tested by 10 min of incubation with either 70 nM p57/PDI (A) or 200 μM cdc2(6−20) peptide (B) as detailed in Materials and Methods. The different concentrations of the substrates were chosen on the basis of the different K_m values displayed by the two substrates. Under these experimental conditions, while the extent of p57/PDI phosphorylation in the absence of PEGDYpEEVLE is more than 2 mol of P_i/mol of protein, the extent of the peptide phosphorylation reaches the value of about 0.03 mol of P_i/mol of peptide. Values are means of four separate experiments. SE values were always less than 16%.

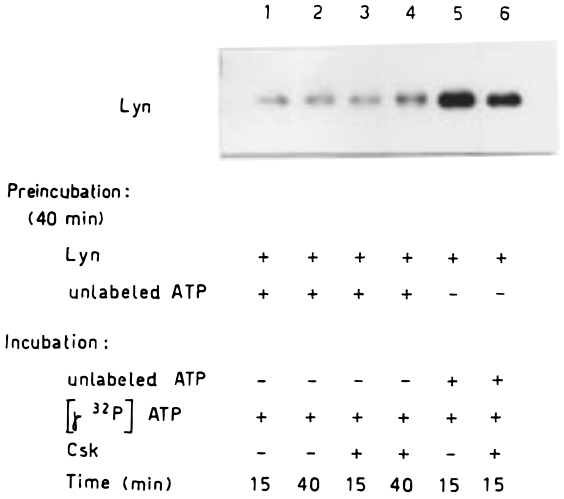


FIGURE 8: Activity of Csk on the native and autophosphorylated Lyn. Lyn (50 nM) was preincubated for 40 min either in the presence (lanes 1−4) or absence (lanes 5 and 6) of 40 μM unlabeled ATP. Incubations were then carried out for the indicated time after addition of unlabeled ATP to the experiments of lanes 5 and 6, 40 nM Csk to the experiments of lanes 3, 4, and 6, and carrier-free [γ-^{32}P]ATP to all samples. The incubations were stopped at the indicated time and the samples were subjected to SDS−PAGE followed by autoradiography. The autoradiography is representative of three separate experiments.

expectable that autophosphorylated Lyn would have been refractory to both Csk phosphorylation and downregulation. This prediction was confirmed by the findings that (i) Lyn preincubated for 40 min with unlabeled ATP-Mn^{2+} was very weakly phosphorylated by Csk (compare lanes 1 and 2 with lanes 3 and 4 of Figure 8) and (ii) while the autophosphorylation of native Lyn (Figure 8, lanes 5 and 6) and its activity on both p57/PDI (Figure 9A) and cdc2(6−20) peptide (Figure 9C) were readily inhibited by Csk, the effect of the latter enzyme on the activity of previously phosphorylated Lyn was greatly reduced (Figure 9B, D). The Csk-induced inhibition evident on both peptide and protein substrates (Figure 9) suggests that the well-known tail-bite conformation induced by Csk (Sicheri et al., 1997; Xu et al., 1997) locks the enzyme in a conformation in which the catalytic domain is incompetent on both kind of substrates. The remarkable Csk-induced inhibition of Lyn activity on peptide substrate (Figure 9C) was somewhat surprising since the Csk-catalyzed phosphorylation of Lyn takes place simultaneously to the Lyn autophosphorylation, which should prevent the inactivation by Csk and enhance the enzyme activity (see above). To gain further information on this point we performed time course phosphorylation experiments with Lyn preincubated with [γ-^{32}P]ATP-Mn^{2+} either in the absence or presence of Csk. Figure 10A shows that the strong Csk-induced inhibition, which was found at short incubation times, was overcome by the increased degree of Lyn autophosphorylation that takes place by extending the incubation time. Actually the separation and quantification of radiolabeled CNBr fragments (Figure 10B) provided evidence that the phosphorylation by Csk (which generates Lyn molecules monophosphorylated at the C-terminal site) proceeded faster than Lyn autophosphorylation (which generates doubly phosphorylated Lyn molecules). This finding accounts for the predominance of the Csk-induced inhibition whenever both processes occur in parallel. Our data also show that Lyn molecules phosphorylated at Tyr507 by Csk and downregulated can autophosphorylate at Tyr396 and consequently rescue their catalytic activity.

DISCUSSION

In the present study we have investigated the mechanism and some possible functional consequences of Lyn PTK autophosphorylation. This event seems to occur through an intermolecular mechanism and it appears to be a relatively slow process (Figures 2 and 3A). An unexpected outcome of our study is that Lyn autophosphorylation occurs at two different sites: not only at Tyr396, which is the canonical autophosphorylation residue (Figure 5), but also at Tyr507, which is the tyrosine specifically affected by Csk. This identification is supported by the following considerations: (i) the 4 kDa CNBr fragment containing this site matches only with the C-terminal part of Lyn and comigrates exactly with the radioactive fragment containing the site phosphorylated by Csk (Figure 4, lane 4); (ii) the hindered Csk-catalyzed phosphorylation of the previously autophosphorylated Lyn (Figure 8, lanes 3 and 4) can be explained by assuming that the Csk-phosphorylatable site is not more available; (iii) the synthetic peptide Lyn(487−511), which corresponds to the Lyn C-terminal tryptic sequence and

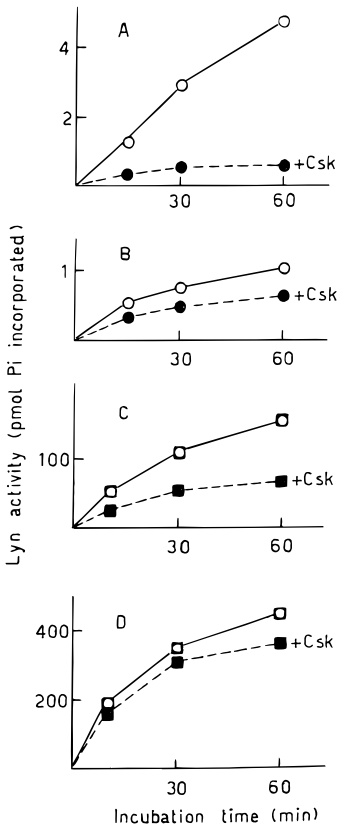
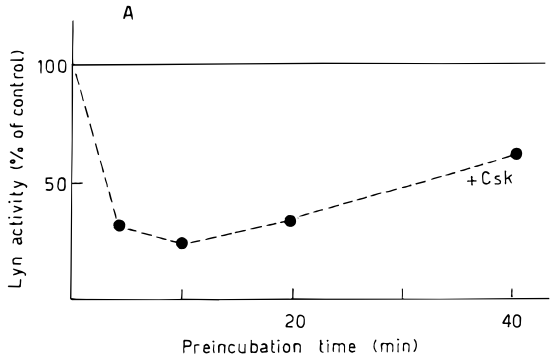


FIGURE 9: Effect of Lyn autophosphorylation on the enzyme inhibition induced by Csk. Lyn (30 nM) activity was assayed either on 70 nM p57/PDI (A and B) or 40 μM cdc2(6−20) peptide (C and D) after 20 min of enzyme preincubation without (A and C) or with 40 μM ATP (B and D), respectively. The incubations were performed for the indicated time either in the absence (○, □) or in the presence (●, ■) of 20 nM Csk. In control experiments Csk did not display any activity on either cdc2(6−20) peptide or p57/PDI. Results are means of four separate experiments performed with two different Lyn preparations, with SE always less than 16%.



B

Preincubation time (min)	5		10		20		40	
Csk	−	+	−	+	−	+	−	+
Lyn phosphoryl. (mol Pi/mol)	0.8	0.4	1.2	0.7	1.7	1.0	1.8	1.4
^{32}Pi incorporated (%)								
8 kDa (Y369)	48	10	46	19	47	29	48	38
4 kDa (Y507)	52	90	54	81	53	71	52	62

FIGURE 10: Time course of activity (A) and phosphorylation of Lyn (B) incubated in either the presence or absence of Csk. Lyn (100 nM) was preincubated for the indicated time either in the absence (—) or in the presence (●) of 100 nM Csk. An aliquot was then withdrawn from each sample and its Lyn activity, which was evaluated by 5 min of incubation with 40 μM cdc2(6−20) peptide substrate, is shown in panel A. The values reported for the experiments with Csk are expressed as a percentage of those found in the parallel experiments performed in the absence of Csk. In panel B is reported the total amount of $^{32}P_i$ incorporated in the phosphorylated Lyn and that found in the CNBr fragments (expressed as a percentage of the total radioactivity incorporated in the two bands). The Lyn fragments, which were obtained by CNBr digestion of the remaining sample aliquots, were resolved by SDS−20% PAGE and quantified by Instant Imager (Packard) analysis. Values are means of three separate experiments.

contains three tyrosyl residues, was efficiently phosphorylated by Lyn. By contrast, the peptide derivative containing Tyr507 replaced by Phe was only slightly phosphorylated by the enzyme. The two sites autophosphorylate simultaneously at about the same rate under all the conditions tested. Under the same conditions the native dephosphorylated forms of c-Fgr and Fyn, two other Src-like PTKs, autophosphorylate exclusively at the expected main autophosphorylation site (Tyr400 and Tyr420, respectively) (Figure 4; Ruzzene et al., 1994). Evidence has already been presented suggesting that some Src family PTKs might use their C-terminal tyrosine as a secondary site of autophosphorylation. Nevertheless, most results of which we are aware were obtained using either overexpressed Src kinases phosphorylated *in vivo* in cells containing Csk (Cooper & MacAuley, 1988; Jove et al., 1987; Abrams & Zhao, 1995) or recombinant enzymes containing the main autophosphorylatable tyrosine replaced by phenylalanine (Jullien et al., 1994; Osusky et al, 1995). Recently a truncated form of Src has been described that, incubated under different experimental conditions, gives rise to enzyme forms that are differently phosphorylated on Tyr338, on the main autophosphorylation site, and on the C-terminal tyrosine (Boerner et al., 1996). The capability of native Lyn to undergo autophosphorylation at both regulatory sites, under basal conditions, was also found *in vitro* with a human recombinant Lyn preparation (Abrams & Zhao, 1995). However in this paper no information was provided about the effect of this abnormal autophosphorylation on the catalytic activity. Sotirellis et al. (1995), on the contrary, reported that a mouse recombinant Lyn underwent intermolecular autophosphorylation exclusively at the main autophosphorylation site. They also reported that the canonical autophosphorylation correlates with an increase of Lyn kinase activity tested toward a peptide substrate. The different autophosphorylation pattern of mouse recombinant Lyn raises the possibility that some recombinant enzymes could behave differently from the native forms. In this respect a similar discrepancy, though in opposite direction, has been found between native and recombinant Csk: while the former was found to be typically devoid of autophosphorylation activity (Okada et al., 1991; Bergman et al., 1992; unpublished data), the latter autophosphorylated on one or more tyrosine residues both *in vitro* and *in vivo* (Bougeret et al., 1993; unpublished data).

In the present study peptide substrates proved to be useful for monitoring the basal activity of just the Lyn catalytic

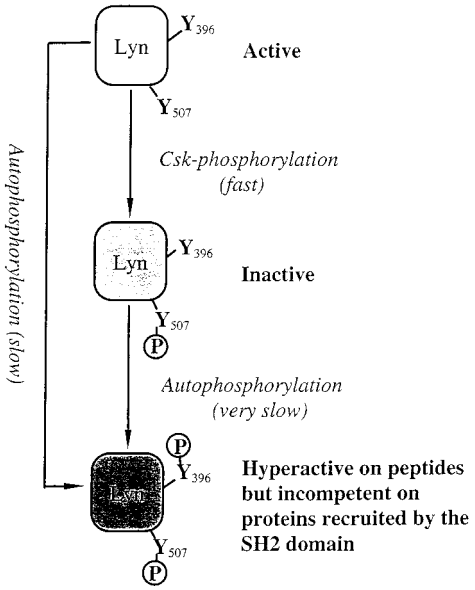


FIGURE 11: Regulation of Lyn activity by phosphorylation.

domain as opposed to protein substrates, whose targeting may also depend on the availability of other enzyme domains. The enzyme activity data of the variably (auto)phosphorylated forms of Lyn, tested on peptide and protein substrates, together with the effect of a phosphopeptide that can obliterate the enzyme SH2 domain, are consistent with the existence of at least three variably active conformational forms of native Lyn as depicted in Figure 11. The entirely dephosphorylated form of the enzyme used for our *in vitro* experiments is catalytically active on both peptide and protein substrates. It should be noted in this respect that this basal activity of dephosphorylated Lyn is monitored in the absence of any simultaneous autophosphorylation (Figure 3), strengthening the concept that unphosphorylated Lyn is indeed intrinsically active.

When Lyn is monophosphorylated at its C-terminal Tyr507 residue by Csk, it loses its catalytic activity toward both peptide and protein substrates (Figure 9). This probably occurs through the same conformational changes recently outlined by solving the crystal structures of two other Src kinases, c-Src and Hck, crystallized in their inactive, C-terminally phosphorylated form (Sicheri et al., 1997; Xu et al., 1997).

Upon autophosphorylation Lyn gives rise to doubly autophosphorylated molecules (at both Tyr396 and Tyr507), which not only possess an active catalytic domain, as shown by their increased affinity toward peptide substrates, but also are refractory, as expected, to Csk downregulation (Figures 3B and 9). The hyperactivity of this conformation toward peptide substrates indicates that the beneficial effects of Tyr396 autophosphorylation overcome the negative ones due to Tyr507 autophosphorylation. Crystal structures of inactive Src and Hck and the active catalytic domain of Lck suggest that the conformation of helix αC in the catalytic domain small lobe is coupled to the phosphorylation state of the autophosphorylation site located in the activation segment (Yamaguchi & Hendrickson, 1996; Sicheri et al., 1997; Xu et al., 1997). Accordingly, we could presume that the phosphorylation of Lyn Tyr396 could stabilize the full active conformation of the helix αC, allowing the catalytic domain to adopt a form highly competent for phosphotransfer. Indeed, autophosphorylated Lyn displays an efficiency on cdc2(6−20) peptide about 6-fold higher than that shown by the unphosphorylated form. These results are in agreement with data showing that the C-terminally phosphorylated and downregulated Lyn, Src, and Hck can rescue their catalytic activity on peptide substrates following autophosphorylation at the tyrosine located in the activation segment (see Figure 10 of this paper; Boerner et al., 1996; Moarefi et al., 1997) as depicted in Figure 11. While autophosphorylation stimulates Lyn activity toward peptide substrates, it actually prevents the phosphorylation of protein substrates such as phospho-p50/HS1 and p57/PDI, whose multiphosphorylation occurs through sequential/processive modules requiring the accessibility of the SH2 domain (Brunati et al., 1995a; Ruzzene et al., 1996; Donella-Deana et al., 1996). The inaccessibility of Lyn SH2 domain promoted by autophosphorylation (Figure 7A) supports a Lyn conformation, in which the phosphorylated tail interacts intramolecularly with the enzyme SH2 domain even though the molecule is autophosphorylated in Tyr396 and retains catalytic activity. The implication of the phosphorylated tail in the attainment of the doubly phosphorylated Lyn conformation is also supported by the finding that c-Fgr, which, under basal conditions, undergoes autophosphorylation only at the canonical site (Figure 4), is not downregulated in its activity on phospho-p50/HS1 protein substrate (Figure 6).

Our data would indicate that, in the doubly autophosphorylated Lyn conformation, the phosphorylation of exogenous targets that interact only with the enzyme catalytic domain can occur even though SH2 and SH3 regulatory domains are presumably clamped as a consequence of P-Tyr507/SH2 domain interaction. On the contrary, in the case of processive/sequential protein phosphorylation, the doubly phosphorylated Lyn has lost its catalytic competence, presumably due to its inability to recruit the previously phosphorylated substrates at its SH2 domain, which is already occupied by its phosphorylated C-terminal tyrosine. Consequently, the inaccessibility of SH2 domain promoted by autophosphorylation may have remarkable consequences on Lyn targeting and signal transduction, despite the fact that, unlike in the case of Lyn phosphorylated by Csk, this form of enzyme is still catalytically active. The actual occurrence in intact cells of this bisphosphorylated form of Lyn, downregulated toward some protein substrates, is supported by the observation that in CD45-deficient B cells both Tyr396 and Tyr507 of Lyn were hyperphosphorylated at resting state (Katagiri et al., 1995; Yanagi et al., 1996). Moreover, in agreement with our data, in these mutant cells containing doubly phosphorylated Lyn, the BCR-induced protein tyrosine phosphorylation was severely compromised (Yanagi et al., 1996). The authors suggested that CD45 could mediate *in vivo* the dephosphorylation of both positive and negative regulatory sites of Lyn, which in fact was almost completely dephosphorylated in normal cells at resting state.

From our data we can conclude that Lyn differs from other Src-like PTKs with respect to both the sites affected by autophosphorylation and the functional consequences of autophosphorylation. At variance with other Src kinases, Lyn autophosphorylation is not strictly required for enzyme activation (see Figure 6; Kmiecik et al., 1988; Koegl et al., 1995; Brown & Cooper, 1996; Boerner et al., 1996; Weijland et al., 1996; Moarefi et al., 1997). Indeed, autophosphoryl-

ation seems to constitute a doubly functional regulatory device that may downregulate the enzyme by preventing its SH2-mediated binding to the previously phosphorylated protein substrates and stimulate activity towards targets that interact only with its catalytic site. One of these targets could be the Ig-α and Ig-β ARH1 motifs, the phosphorylation of which by Lyn has been demonstrated to initiate by association of the substrate with the N-terminal part of the enzyme (Pleiman et al., 1994). However, further studies are necessary to better define the phosphorylation mechanism of the Lyn potential physiological substrates.

Although Lyn autophosphorylation can counteract the Csk activity, Lyn may still be downregulated by Csk *in vitro* since its C-terminal tyrosine phosphorylation catalyzed by Csk is faster than its concomitant autophosphorylation at both Tyr396 and Tyr507 (Figure 10). However, *in vivo* the regulation by autophosphorylation might assume a significant physiological role under particular conditions: (i) when the rate of Lyn autophosphorylation is increased, e.g., by polybasic compounds (Donella-Deana et al., 1992), and (ii) whenever Csk is not targeted to places where Src-like kinases are present and therefore it cannot downregulate the activity of these enzymes (Howell & Cooper, 1994).

Our results also indicate that, in signal transduction studies, the kind of substrate used for monitoring the Lyn activity, as well as the incubation time, may critically affect the data obtained.

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REFERENCES

Abrams, C. S., & Zhao, W. (1995) *J. Biol. Chem. 270*, 333−339

Azuma, K., Ariki, M., Miyauchi, T., Usui, H., & Takeda, M. (1991) *J. Biol. Chem. 266*, 4831−4839

Barker, S. C., Kassel, D. B., Weigl, D., Huang, X. Luther, M. A., & Knight, W. B. (1995) *Biochemistry 34*, 14843−14851

Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P., & Alitalo, K. (1992) *EMBO J. 11*, 2919−2924

Boerner, R. J., Kassel, D. B., Barker, S. C., Ellis, B., DeLacy, P., & Knight, W. B. (1996) *Biochemistry 35*, 9519−9525

Bolen, J. B., Rowley, R. B., Spana, C., & Tsygankov, A. Y. (1992) *FASEB J. 6*, 3403−3409

Bougeret, C., Rothhut, B., Jullien, P., Fischer, S., & Benarous, R. (1993) *Oncogene 8*, 1241−1247

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254

Brown, M. T., & Cooper, J. A. (1996) *Biochim. Biophys. Acta 1287*, 121−149

Brunati, A. M., Guillaume, A., Marin, O., Donella-Deana, A., Cesaro, L., Bougeret, C., Fagard, R., Benarous, R., Fischer, S., & Pinna, L. A. (1992) *FEBS Lett. 313*, 291−294

Brunati, A. M., James, P., Donella-Deana, A., Matoskova, B., Robbins, K. C., & Pinna, L. A. (1993) *Eur. J. Biochem. 216*, 323−327

Brunati, A. M., Ruzzene, M., James, P., Guerra, B., & Pinna, L. A. (1995a) *Eur. J. Biochem. 229*, 164−170

Brunati, A. M., Donella-Deana, A., Ruzzene, M., Marin, O., & Pinna, L. A. (1995b) *FEBS Lett. 367*, 149−152

Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281−302

Cooper, J. A., & MacAuley, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4232−4236

Cooper, J. A., & Howell, B. (1993) *Cell 73*, 1051−1054

Donella-Deana, A., Marin, O., Brunati, A. M., & and Pinna, L. A. (1992) *Eur. J. Biochem. 204*, 1159−1163

Donella-Deana, A., James, P., Staudenmann, W., Cesaro, L., Marin, O., Brunati, A. M., Ruzzene, M., & Pinna, L. A. (1996) *Eur. J. Biochem 235*, 18−25

Feder, D., & Bishop, J. M. (1990) *J. Biol. Chem. 265*, 8205−8211

Glass, D. B., Masaracchia, R. A., Feramisco, J. R., & Kemp, D. E. (1978) *Anal. Biochem. 87*, 566−575

Howell, B. W., & Cooper, J. A. (1994) *Mol. Cell. Biol. 14*, 5402−5411

Jove, R., Kornbluth, S., & Hanafusa, H. (1987) *Cell 50*, 937−943

Jullien, P., Bougeret, C., Camoin, L., Bodeus, M., Durand, H., DiSanto, J. P., Fischer, S., & Benarous, R. (1994) *Eur. J. Biochem. 224*, 589−596

Katagiri, T., Ogimoto, M., Hasegawa, K., Mizuno, K., & Yakura, H. (1995) *J. Biol. Chem. 270*, 27987−27990

Kmiecik, T. E., Johnson, P. J., & Shalloway, D. (1988) *Mol. Cell Biol. 8*, 4541−4546

Koegl, M., Courtneidge, S. A., & Superti-Furga, G. (1995) *Oncogene 11*, 2317−2329

Moarefi, I., La-Fevre-Bernt, M., Sicheri, F., Huse, M., Lee, C-H., Kuriyan, J., & Miller, T. (1997) *Nature 385*, 650−653

Mustelin, M. (1994) *Src Family Tyrosine Kinases in Leukocyte*, R. G. Landes Co., Austin, TX.

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., & Nakagawa, H. (1991) *J. Biol. Chem. 266*, 24249−24252

Osusky, M., Taylor, S. J., & Shalloway, D. (1995) *J. Biol. Chem. 270*, 25729−25732

Parker, P. J. (1993) *FEBS Lett. 334*, 347−350

Pawson, T., & Gish, G. D. (1992) *Cell 71*, 359−362

Ruzzene, M., James, P., Brunati, A. M., Donella-Deana, A., & Pinna, L. A. (1994) *J. Biol. Chem. 269*, 15885−15891

Ruzzene, M., Brunati, A. M., Marin, O., Donella-Deana, A., & Pinna, L. A. (1996) *Biochemistry 35*, 5327−5332

Sicheri, F., Moarefi, I., & Kuriyan, J. (1997) *Nature 385*, 602−609

Songyang, Z., Shoelson, S. E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X. R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R. A., & Cantley, L. C. (1994) *Mol. Cell. Biol. 14*, 2777−2785

Sotirellis, N., Johnson, T. M., Hibbs, M. L., Stanley, I. J., Stanley, E., Dunn, A. R., & Cheng, H.-C. (1995) *J. Biol. Chem. 270*, 29773−29780

Sugimoto, Y., Erikson, E., Graziani, Y., & Erikson, R. L. (1985) *J. Biol. Chem. 260*, 13838−13843

Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A., & Draetta, G. (1993) *EMBO J. 12*, 2625−2634

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., & Kurosaki, T. (1994) *EMBO J. 13*, 1341−1349

Weijland, A., Neubauer, G., Courtneidge, S. A., Mann, M., Wierenga, R. K., and Superti-Furga, G. (1996) *Eur. J. Biochem. 240*, 756−764

Xu, W., Harrison, S. C., & Eck, M. J. (1997) *Nature 385*, 595−602

Yamaguchi, H., & Hendrickson, W. A. (1996) *Nature 384*, 484−489

Yamanashi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., & Yamamoto, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3631−3635

Yanagi, S., Sugawara, H., Kurosaki, M., Sabe, H., Yamamura, H., & Kurosaki, T. (1996) *J. Biol. Chem. 271*, 30487−30492

Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363−8368

BI971332S