Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the *src* family tyrosine kinase *lyn*

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Abstract The nitration of tyrosine residues in protein occurs through the action of reactive oxygen and nitrogen species and is considered a marker of oxidative stress under pathological conditions. The most active nitrating species so far identified is peroxynitrite, the product of the reaction between nitric oxide and superoxide anion. Previously, we have reported that in erythrocytes peroxynitrite irreversibly upregulates *lyn*, a tyrosine kinase of the *src* family. In this study we investigated the possible role of tyrosine nitration in the mechanism of *lyn* activation. We found that tyrosine containing peptides modelled either on the C-terminal tail of *src* kinases or corresponding to the first 15 amino acids of human erythrocyte band 3 were able to activate *lyn* when the tyrosine was substituted with 3-nitrotyrosine. The activity of nitrated peptides was shared with phosphorylated but not with unphosphorylated, chlorinated or scrambled peptides. Recombinant *lyn src* homology 2 (SH2) domain blocked the capacity of the band 3-derived nitrotyrosine peptide to activate *lyn* and we demonstrated that this peptide specifically binds the SH2 domain of *lyn*. We propose that nitropeptides may activate *src* kinases through the displacement of the phosphotyrosine in the tail from its binding site in the SH2 domain. These observations suggest a new mechanism of peroxynitrite-mediated signalling that may be correlated with the upregulation of tyrosine phosphorylation observed in several pathological conditions. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Tyrosine nitration; Tyrosine phosphorylation; Peroxynitrite; *lyn* tyrosine kinase; *Src* homology 2 domain

1. Introduction

The tyrosine kinases of the *src* family share a high degree of structural similarity. The *src* sequence consists of a poorly conserved N-terminal segment, two conserved domains termed *src* homology (SH)3 and SH2, followed by the catalytic domain SH1. The SH3 and SH2 domains play a dual role in *src* regulation, both being required to keep *src* kinases in an inactive state and to target the enzyme to specific substrates by directing protein–protein interactions. The catalytic activity of *src* kinases is modulated by phosphorylation of two tyrosine residues in two opposite ways. The autophosphorylation of Tyr 416 (*c-src* numbering), located inside the catalytic domain, correlates with enzyme activation, while the phosphorylation of C-terminal tyrosine (Tyr 527) gives rise to an inactive form of the enzyme. When the Tyr 527, located in the tail of *src* kinases, is phosphorylated it interacts intramolecularly with the SH2 domain and triggers an enzyme structure that forces the kinase to adopt a locked and downregulated conformation (for a review of *src* kinases see [1]) [2]. Disruption of this interaction may be one of the mechanisms for activation of *src* tyrosine kinases.

In human tissues, the nitration of tyrosine residues (addition of a $-NO_2$ group at position 3 of the aromatic ring) has been demonstrated under physiological conditions and has been shown to increase significantly in inflammatory conditions and degenerative diseases. Although multiple biochemical pathways responsible for tyrosine nitration have been proposed [3] so far, the most active nitrating species is peroxynitrite. Peroxynitrite is a potent oxidizing and nitrating species formed in a diffusion-limited radical–radical reaction between nitric monoxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet -}$) [4]. Peroxynitrite promotes the nitration of aromatic residues and tyrosines thus constitute key targets for peroxynitrite. In general, the oxidation of cysteines and tyrosines by $^{\bullet}NO$-related oxidants significantly modifies the enzymatic function of several target proteins [5–9], and it has also been shown that nitration of a tyrosine residue may downregulate phosphotyrosine-dependent signalling in cell-free systems and cell cultures [10,11]. Other groups as well as ourselves have reported that peroxynitrite upregulates the activity of some kinases [12–16], in particular tyrosine kinases of the *src* family in human pancreatic tumor tissues [9], erythrocytes [17] and synaptosomes [18].

We recently demonstrated that two *src* kinases found in erythrocytes, *lyn* and *hck*, are activated through different pathways involving cysteine-dependent or -independent processes [19]. We found that direct and irreversible activation of *lyn* by peroxynitrite occurred through a mechanism that cannot be explained by either cysteine or methionine oxidation. Since the activity of *src* kinases is regulated by the C-terminal tyrosine (Tyr 508 in the case of *lyn*), it is possible that peroxynitrite-induced modifications may interfere with kinase conformation, leading to its activation. MacMillan-Crow et al. [9] found an increase in tyrosine nitration and phosphorylation of *c-src* in human pancreatic ductal adenocarcinoma and in adenocarcinoma cell line treated with peroxynitrite. They suggest that the nitration of critical tyrosine residues

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Abbreviations: $^{\bullet}NO$, nitrogen monoxide; $O_2^{\bullet -}$, superoxide anion; MBP, myelin basic protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SH, *src* homology; GST, glutathione *S*-transferase

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could essentially mimic tyrosine phosphorylation, resulting in kinase activation. This hypothesis is interesting and prompts investigation of the mechanism of *src* kinase activation by peroxynitrite, as well as the identification of nitrated tyrosine residue(s). Our hypothesis is that nitrotyrosine-containing peptides may activate *lyn* similarly to other specific phosphopeptides, which have been shown to activate *src* kinases through the displacement of phosphorylated tail from the SH2 domain [2]. To demonstrate this hypothesis we tested the ability of a set of SH2-specific peptides, whose phosphotyrosine was substituted with 3-nitrotyrosine, to modulate the activity of *lyn* immunoprecipitated from human erythrocytes. Our data show that nitrated peptides are able to activate *lyn* through an SH2-dependent mechanism and support the hypothesis that the nitration of specific tyrosine residues may confer a previously unrecognized gain-of-function.

2. Materials and methods

2.1. Materials

[γ^{32}P]ATP (>3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA, USA). Polyclonal anti-*lyn* and agarose-conjugated anti-*lyn* antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Trysacryl-immobilized Protein A from Pierce (Rockford, IL, USA); peroxidase-conjugated goat anti-rabbit antibodies from Bio-Rad (Hercules, CA, USA). Nitrocellulose was obtained from Shleicher and Schuell (Keene, NH, USA). All peptides were synthesized by Neosystem (Strasbourg, France). Recombinant glutathione *S*-transferase (GST)-fusion proteins: *lyn* 131–243 (SH2), *lyn* 27–131 (SH3) and *lyn* 1–243 (SH2/SH3) were purchased from Pharmingen (San Diego, CA, USA). Glutathione–Sepharose 4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Peptide nomenclature and sequences

High affinity phosphotyrosine peptide, pY; unphosphorylated peptide, Y; nitrotyrosine peptide, NO_2Y; chlorotyrosine peptide, ClY; band 3-derived phosphotyrosine peptide, b3-pY; band 3-derived unphosphorylated peptide, b3-Y; band 3-derived nitrotyrosine peptide, b3-NO_2Y. The sequence of Y, pY, NO_2Y and ClY peptides is: NH_2-Thr-Glu-Pro-Gln-Tyr-Glu-Glu-Ile-Pro-Ile-COOH in which the Tyr is unphosphorylated, phosphorylated, or substituted with 3-nitro- or 3-chloro-tyrosine, respectively. The sequences of scrambled peptides in which the Tyr is phosphorylated or substituted with 3-nitro-tyrosine, are: NH_2-Glu(Ac)-Gln-Glu-Pro-Tyr-Ile-Pro-Ile-Glu-COOH, Sc1 and NH_2-Tyr-Pro-Glu-Pro-Glu-Ile-Gln-Ile-Glu-COOH, Sc2. The sequence of the b3-Y, b3-pY and b3-NO_2Y peptides is: NH_2-Met-Glu-Glu-Leu-Gln-Asp-Asp-Tyr-Glu-Asp-Met-Met-Glu-Glu-Asn-COOH in which the Tyr is unphosphorylated, phosphorylated or substituted with 3-nitro-tyrosine, respectively.

2.3. Cell lysis and immunoprecipitation

Washed and packed erythrocytes (150 μl) were diluted with 300 μl of isotonic phosphate buffer, pH 7.4 and then lysed by the addition of 150 μl of 4×RIPA buffer (100 mM Tris, pH 7.5, 0.6 M NaCl, 4% Triton X-100, 4% Na-deoxycholate, 0.4% SDS, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 4 mM phenylmethanesulfonyl fluoride, 400 μM sodium orthovanadate, 40 μM phenylarsine oxide). After 10 min of incubation in ice, the lysate was centrifuged at 12 000×*g* for 10 min at 4°C. The supernatant was incubated for 1 h at 4°C in a rotating wheel with 30 μl of Trysacryl-immobilized Protein A, clarified by centrifugation and incubated for 3 h at 4°C in a rotating wheel with 20 μl Protein A–Trysacryl pre-adsorbed with the polyclonal anti-*lyn* antibody. The immunoprecipitate was collected by centrifugation in a microfuge, washed twice with 1× RIPA buffer and twice with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS). The immunoprecipitate was then suspended in 10 μl TBS containing the various peptides at the concentrations indicated, incubated at 4°C for 2 h. The SH2 competition assay has been performed adding peptides and GST-*lyn* SH2 at the same time. The samples were then subjected to a kinase assay as described below.

2.4. In vitro kinase assay

The immunoprecipitates were washed in the kinase buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MnCl_2$, 0.1 mM sodium orthovanadate) and the kinase assay was performed using [γ^{32}P]ATP (>3000 Ci/mmol) as previously described [19]. When indicated, 1 μg of myelin basic protein (MBP) was added to the reaction mixture as exogenous substrate. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the gels were then dried and exposed to X-ray film for autoradiography. The ^{32}P-labelled proteins were quantified by a phosphorimager instrument (Packard, Camberra, CO, USA).

2.5. Solid phase binding assay

This procedure was used to detect the binding of biotin-labelled band 3-derived peptides with *lyn* kinase or purified GST fusion proteins containing *lyn* SH2 or SH3 domains. *lyn* immunoprecipitate, obtained from erythrocyte lysate using agarose-conjugated anti-*lyn* antibodies as described above, was incubated for 2 h at 4°C with 1 mM biotinylated peptides. After extensive washing in TBS, the *lyn* immunoprecipitate was suspended in 10 μl TBS-1%SDS, centrifuged and 5 μl of supernatant spotted on dry nitrocellulose paper. Alternatively, 5 μg of GST-*lyn* SH2, GST-*lyn* SH3 or GST alone were incubated for 2 h at 4°C with 1 mM biotinylated peptides. The complex was pulled out by adding 10 μl of glutathione–Sepharose beads (1 h at 4°C). The beads were then extensively washed with TBS and bound proteins were dissociated from beads by 1% SDS (10 μl). The beads were removed by centrifugation and 5 μl of the supernatant spotted on nitrocellulose. The membrane was then blocked with 3% bovine serum albumin/Tween 20–TBS for 2 h. To reveal the biotin-labelled proteins, the nitrocellulose was incubated for 1 h with avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA), and the spots containing the biotinylated peptide were visualized by enhanced chemiluminescence (ECL kit, Pierce). When indicated, the filters were also subjected to immunoblot analysis using polyclonal anti-GST antibodies or polyclonal anti-*lyn* antibodies, and the immunoreactivity detected by ECL.

2.6. Competition assay between band 3-derived peptides

lyn was immunoprecipitated from erythrocyte lysate using agarose-conjugated anti-*lyn* antibodies and incubated 2 h at 4°C with 2 mM biotin-labelled b3-pY peptide. After extensive washing in TBS, *lyn* immunoprecipitate was incubated for other 2 h at 4°C with b3-NO_2Y or b3-Y (0–2 mM) and the displacement of bound b3-pY from *lyn* was revealed in the supernatant after removal of beads by centrifugation. The 100% release was obtained by adding 1% SDS (10 μl) to b3-pY-bound *lyn* immunoprecipitate. The procedure to detect biotin-labelled peptide was carried out as described before. The intensity of the spots was quantified by densitometric analysis (GS-700 Imaging Densitometer, Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Activation of lyn by nitrotyrosine (NO_2Y) and phosphotyrosine (pY) peptides

Liu et al. [2] have identified a short phosphotyrosine-containing peptide (pY) that binds the *src* SH2 domain with high affinity (K_d = 40 nM). This peptide is a model peptide obtained from a library in which the C-terminal phosphotyrosine residues of *c-src* are partially randomized. As mentioned above, the SH2 domain is involved in negatively regulating *src* kinase activity through its intramolecular binding to a phosphorylated tyrosine in the tail of the kinase. The pY peptide competes for this intramolecular binding and thereby precludes the autoinhibition of *src* kinases, but the unphosphorylated peptide (Y) does not. We performed experiments in which the *lyn* kinase, immunoprecipitated from human erythrocytes, was incubated with peptides derived from pY. We used pY and peptides with the same amino acid sequence but with tyrosine modified to 3-nitrotyrosine (NO_2Y) or 3-chlorotyrosine (ClY). The latter tyrosine modification was

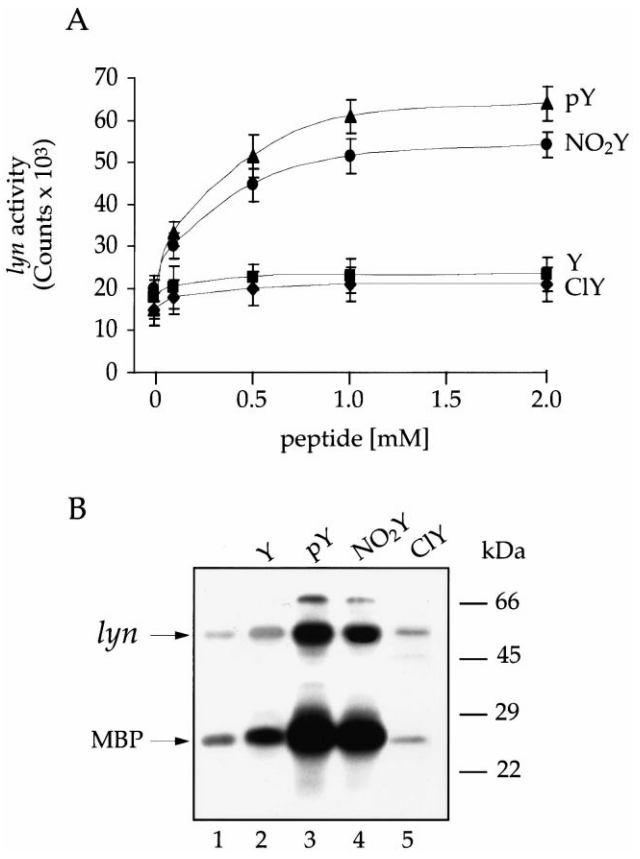

Fig. 1. Activation of *lyn* tyrosine kinase by pY and NO_2Y peptides. A: *lyn* immunoprecipitate, obtained from human erythrocytes, was incubated for 2 h at 4°C with 0.1–2 mM of non-phosphorylated (Y, ■), phosphorylated (pY, ▲), nitrated (NO_2Y, ●), and chlorinated (ClY, ♦) peptides. The nomenclature and peptide sequences are reported in Section 2. The samples were subjected to in vitro kinase assay with [γ^{32}P]ATP, and *lyn* activity was measured by quantification of ^{32}P incorporation into the protein using a phosphorimager Instrument. *lyn* activity was expressed as total counts (mean ± S.D. of three separate experiments). B: Autoradiography of the in vitro kinase assay of *lyn* immunoprecipitate treated with 1 mM of Y, pY, NO_2Y, and ClY (lanes 2–5, respectively) in the presence of the exogenous substrate MBP. Lane 1, *lyn* immunoprecipitate incubated without any peptide. The samples were resolved on 15% SDS–PAGE, dried and exposed to X-ray film. A representative of three experiments with consistent results is shown. The molecular mass markers in kDa are indicated on the right.

chosen because it is of possible biological relevance, being found in atherosclerotic tissues and considered a highly specific marker of hypochlorous acid formation by myeloperoxidase [20]. In agreement with previously published results obtained with *c-src* [2], the incubation of *lyn* with 0.1–2 mM pY peptide increased kinase autophosphorylation activity in a dose-dependent manner (Fig. 1A). Notably, the NO_2Y peptide also stimulated *lyn* activity dose-dependently, while ClY and Y peptides only minimally increased kinase autophosphorylation. Measurement of ^{32}P incorporation into the kinase revealed that pY and NO_2Y peptides at 1 mM respectively induced a 4.5-fold and 3.0-fold enhancement of *lyn* autophosphorylation activity. Fig. 1B shows the in vitro kinase assay of *lyn* treated with 1 mM Y, pY, NO_2Y, and ClY peptides (lanes 2–5, respectively) in the presence of the exogenous substrate MBP to demonstrate that pY and NO_2Y also greatly stimulated the ability of *lyn* to phosphorylate a substrate. Treatment of *lyn* with the unphosphorylated peptide (Y) at 1 mM only slightly increased kinase activity (Fig. 1B, compare lanes 2 and 1).

To investigate the importance of the specific amino acid sequence surrounding the tyrosine residue, peptides were synthesized with phosphotyrosine or nitrotyrosine in the fifth position (Sc1) or in the first position (Sc2), but with the remaining amino acids arbitrarily scrambled. The ability of scrambled peptides to modify *lyn* autophosphorylation is shown in Fig. 2 (lanes 5–8). The results indicate that when nitrated or phosphorylated, none of these peptides was able to activate the kinase. These results suggest that for NO_2Y the amino acid sequence surrounding the tyrosine residue (Glu at +1 and +2) confers the ability to activate *lyn*, a finding previously reported for pY also [2]. Bearing in mind these earlier results, it could therefore be hypothesized that the NO_2Y peptide is able to activate *lyn* through a mechanism that involves binding to the SH2 domain. The following experiments were performed to provide further proof of this hypothesis.

3.2. Activation of lyn kinase by b3-NO_2Y and b3-pY peptides derived from band 3

Recent data [21] have shown that, in human erythrocytes, Tyr 8 and Tyr 21 of the cytoplasmic domain of band 3 when phosphorylated by the non-*src* tyrosine kinase *syk*, act as a docking site for the SH2 domain of *lyn*, which subsequently phosphorylates band 3 at additional secondary sites (Tyr 359 and Tyr 904). It is therefore important to establish if a nitrotyrosine-containing peptide, whose sequence is expressed in proteins, could modify *lyn* kinase activity. Peptides corresponding to the first 15 amino acids of band 3 were synthesized and Tyr 8 was either phosphorylated or nitrated. With

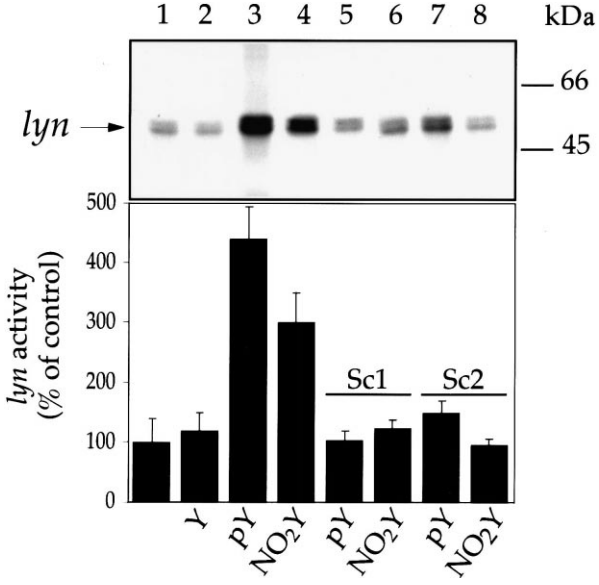

Fig. 2. Effects of nitrated and phosphorylated scrambled peptides Sc1 and Sc2 on *lyn* activity. *lyn* immunoprecipitate was incubated with the indicated peptides at a concentration of 1 mM. The sequences of Sc1 and Sc2 are reported in Section 2. The samples were subjected to in vitro kinase assay with [γ^{32}P]ATP and analyzed by 10% SDS–PAGE. *lyn* activity was measured as in the legend to Fig. 1. *lyn* activity was expressed as a percentage of the value in control sample (100%). Data are the mean ± S.D. of three separate experiments. The upper panel shows a representative autoradiography of the samples described.

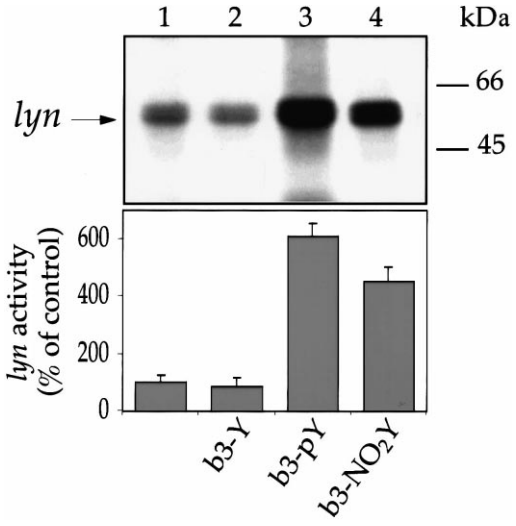

Fig. 3. Activation of *lyn* autophosphorylation activity by b3-pY and b3-NO_2Y peptides. *lyn* immunoprecipitate was incubated with 1 mM of the indicated peptides derived from the first 15 amino acids of band 3. The sequences of the peptides are reported in Section 2. The experimental procedure was as in Fig. 2.

respect to the sequence of pY peptide, these peptides maintain the residue Glu at +1 and possess a different acidic residue at +2 (Asp). The results, shown in Fig. 3, showed that both b3-pY and b3-NO_2Y peptides strongly stimulated *lyn* autophosphorylation activity. Interestingly, band 3-derived peptides possessed a higher capacity to activate *lyn* than either pY or NO_2Y (compare lanes 3 and 4 of Fig. 2 with lanes 3 and 4 of Fig. 3). Measurement of ^{32}P incorporation showed that the b3-pY and b3-NO_2Y peptides stimulated *lyn* autophosphorylation activity 6.5-fold and 4.5-fold, respectively.

3.3. *lyn* SH2 domain abolishes lyn activation induced by b3-pY and b3-NO_2Y

To test the possibility that kinase activation induced by b3-pY and b3-NO_2Y involves peptide binding to a regulatory domain of *lyn*, we used recombinant GST fusion proteins containing either *lyn* SH2 and/or *lyn* SH3 domains. These GST fusion proteins were added to *lyn* immunoprecipitate in the presence of b3-pY or b3-NO_2Y peptides. Interestingly, we found that GST fusion proteins containing *lyn* SH2 domain (*lyn* 1–243 and *lyn* 131–243, see Fig. 4A) abolished the kinase activation induced by b3-pY and by b3-NO_2Y, while GST-*lyn* 27–131, which contains only the *lyn* SH3 domain, did not (Fig. 4B). These results provide indirect evidence that b3-NO_2Y and b3-pY peptides may interfere with *lyn* activity by competing to bind the SH2 domain to the phosphorylated tail. As expected, neither the incubation of *lyn* with GST-*lyn* 131–243 without peptides (Fig. 4B, lane 6) or the treatment of *lyn* immunoprecipitate with GST protein had any effect on kinase activity (result not shown).

3.4. The b3-NO_2Y and b3-pY peptides specifically bind the SH2 domain of lyn

To support our hypothesis further, we used b3-pY and b3-NO_2Y peptides labelled with biotin at the N-terminal position, to demonstrate their binding to the *lyn* SH2 domain in a solid phase binding assay. We first tested the ability of these biotinylated peptides to activate *lyn* in the in vitro kinase

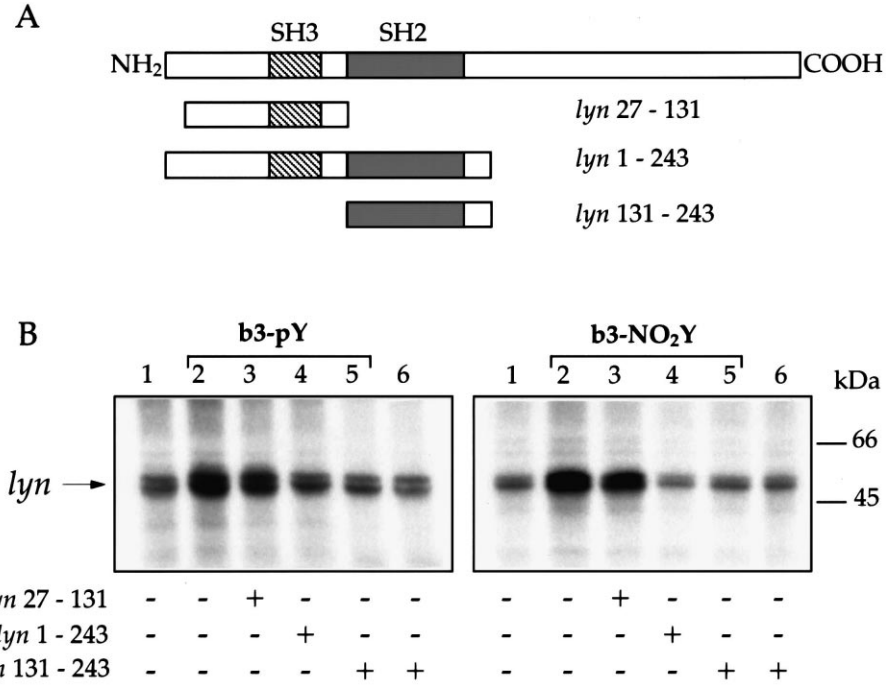

Fig. 4. *lyn* SH2 domain abolishes the activation of *lyn* induced by b3-pY and b3-NO_2Y peptides. B: *lyn* immunoprecipitate was incubated with b3-pY (left) or b3-NO_2Y (right) both at 1 mM in the presence of 1 mg/ml of purified GST-*lyn* 27–131 (lane 3), GST-*lyn* 1–243 (lane 4) or GST-*lyn* 131–243 (lane 5). Lane 1 represents *lyn* immunoprecipitate incubated alone and lane 2 the *lyn* immunoprecipitate incubated with b3-pY and b3-NO_2Y peptides. Lane 6, *lyn* immunoprecipitate incubated with 1 mg/ml of GST-*lyn* 131–243 without peptides. The samples were subjected to in vitro kinase assay with [γ^{32}P]ATP, and analyzed by 10% SDS–PAGE. The gels were dried and exposed to X-ray films for autoradiography. A representative of three experiments with consistent results is shown. The *lyn* protein regions used as GST fusion proteins are reported in A.

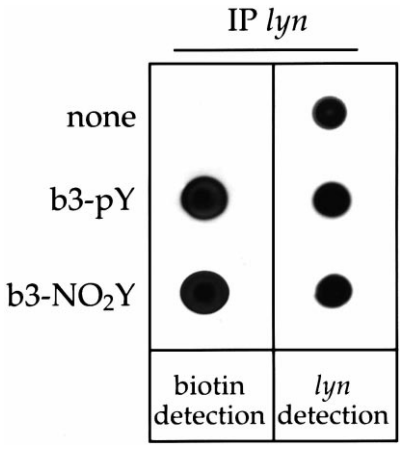

Fig. 5. Binding of b3-pY and b3-NO_2Y peptides to *lyn* tyrosine kinase. Solid phase binding assay of *lyn*, immunoprecipitated by agarose-conjugated anti-*lyn* polyclonal antibodies, with b3-pY and b3-NO_2Y peptides labelled with biotin. *lyn* immunoprecipitate was incubated for 2 h at 4°C with the biotinylated peptides (1 mM). Each samples (5 μl) was spotted on the nitrocellulose and binding was revealed by the ABC detection procedure (Vectastain ABC kit, Vector). The reaction was visualized on nitrocellulose by ECL kit (Pierce) (left panel). The same nitrocellulose was then probed with anti-*lyn* polyclonal antibodies and the immunoreactivity detected by ECL to verify that comparable amounts of *lyn* were present in each spot (right panel). A representative of three experiments with consistent results is shown.

assay and found that both biotinylated and non-biotinylated peptides activated *lyn* in a similar manner (data not shown). As shown in Fig. 5, when the immunoprecipitate of *lyn* was treated with biotinylated peptides (1 mM), as described in Section 2, the biotin detection revealed a very intense reaction

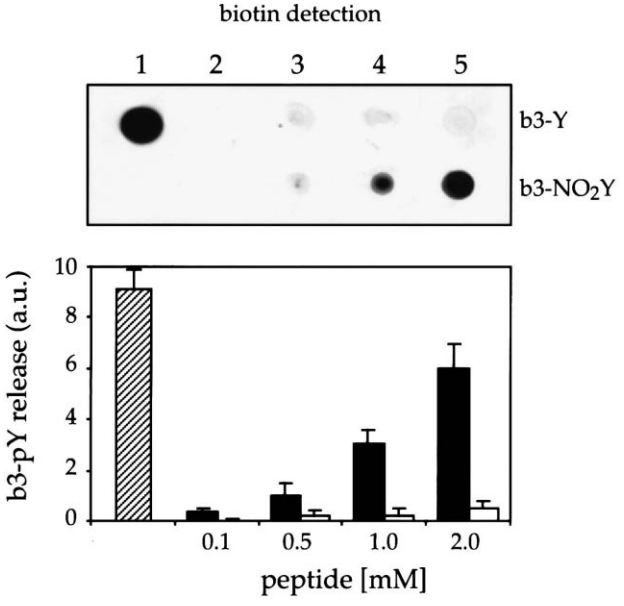

Fig. 6. Competition assay between b3-pY and b3-NO_2Y peptides. Biotinylated b3-pY (2 mM) was firstly bound to *lyn* immunoprecipitate and then the peptide was released from the kinase by b3-NO_2Y, b3-Y (lanes 2–5, black and white columns, respectively) or 1% SDS (lane 1 and hatched column). The presence of biotinylated b3-pY in the supernatant was visualized on nitrocellulose by ABC detection procedure and ECL (upper panel). The spots were quantified by densitometric analysis (bottom panel).

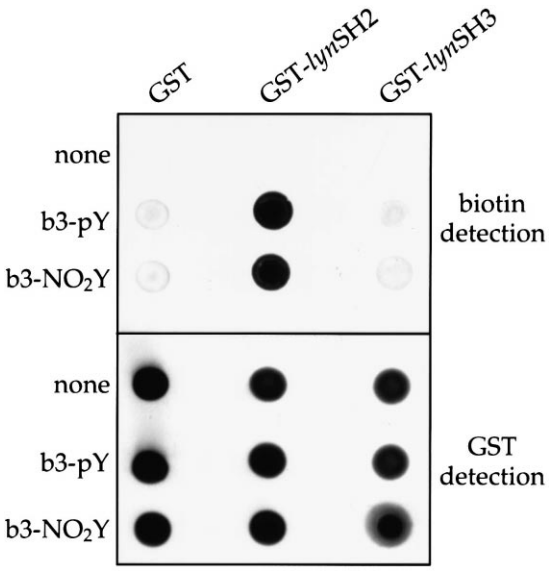

Fig. 7. Binding of b3-pY and b3-NO_2Y peptides to *lyn* SH2 domain. Solid phase binding assay of purified recombinant GST-*lyn* 131–243 (SH2), GST-*lyn* 27–131 (SH3), and GST alone with biotin-labelled b3-pY and b3-NO_2Y peptides (1 mM). The reaction was carried out for 2 h at 4°C and the GST complexes were then linked by glutathione–Sepharose beads (10 μl). 5 μl of each sample was spotted on the nitrocellulose and the binding between GST fusion proteins and biotinylated peptides was revealed as described in Fig. 5 (upper panel). To verify that comparable amounts of GST were present in each spot, the same nitrocellulose was probed with anti-GST polyclonal antibodies and the immunoreactivity detected by ECL (bottom panel). A representative of three experiments with consistent results is shown.

in the spots corresponding to samples treated with b3-pY and b3-NO_2Y peptides. When polyclonal anti-*lyn* antibodies were applied on the same nitrocellulose we found that all the samples contained comparable amounts of protein (Fig. 5). In addition, to ensure that the agarose-conjugated anti-*lyn* antibodies used to immunoprecipitate *lyn* from erythrocytes did not bind the biotinylated peptides, we performed control experiments in which agarose-conjugated anti-*lyn* antibodies (without *lyn* kinase) were treated with biotinylated peptides (1 mM) and the samples processed as described before. We detected no reaction in these samples (results not shown).

We also performed a solid phase binding assay to demonstrate whether the band 3-derived nitropeptide may compete with the phosphopeptide bound to *lyn*. We found that b3-NO_2Y, but not b3-Y, induced the displacement of b3-pY from *lyn* in a dose-dependent manner (Fig. 6, lanes 2–5). The displacement, however, occurred at relatively high concentration of nitropeptide suggesting a lower efficiency with respect to the phosphopeptide.

These results clearly demonstrate a binding of b3-pY and b3-NO_2Y to the *lyn* kinase, but we did not know exactly which domain of *lyn* was involved. To resolve this question, we incubated both the GST fusion proteins (*lyn* 131–243 or *lyn* 27–131) and GST alone with biotinylated peptides (1 mM). After the addition of glutathione–Sepharose beads to separate the GST-containing complexes, we identified the domain implicated in the binding using ABC staining. As shown in Fig. 7, biotin detection unambiguously demonstrates that the *lyn* SH2 domain (*lyn* 131–243) binds both b3-pY and b3-NO_2Y peptides with the same efficiency and that the bind-

ing was specific, since neither *lyn* SH3 (*lyn* 27–131) or GST alone showed any reaction. By applying anti-GST polyclonal antibodies on the nitrocellulose used in biotin detection we showed that all the samples contained comparable amounts of proteins (Fig. 7, bottom).

4. Discussion

This study provides the first evidence of a role of nitrotyrosine as a potential activator of tyrosine kinases of the *src* family. We show that *lyn*, a *src* tyrosine kinase isolated from human erythrocytes, may be upregulated by nitrotyrosine-containing peptides. The activation of *lyn* was characteristic of phosphotyrosine- and nitrotyrosine-containing peptides, since a peptide with the same amino acid sequence but containing unmodified tyrosine or chlorotyrosine did not affect *lyn* activity (Fig. 1).

The amino acid sequence of the nitrated peptide was identical to that of the high affinity SH2-binding phosphotyrosine peptide (pY), described by Liu et al. [2], and, as previously demonstrated for pY, the amino acids surrounding the nitrotyrosine are critical. In fact, two scrambled peptides, in which the acidic amino acids at +1 and +2 were substituted, lost the ability to activate *lyn* (Fig. 2).

The SH2 domain of *src* kinases is involved in negatively regulating kinase activity, which it does by binding with the phosphorylated tyrosine in the tail of the kinase; pY competes for this intramolecular binding. Our results suggest that substitution of phosphotyrosine with nitrotyrosine can cause *lyn* activation through the same mechanism and, thus, that nitration can mimic phosphorylation. Tyrosine nitration mimics phosphorylation with respect to the charge of the aromatic residue, since the addition of $-NO_2$ to position 3 of the aromatic ring lowers the p*K*a of the tyrosine hydroxyl group to 7.2 [22] and, thus, at slight alkaline pHs adds a negative charge.

We recently reported that peroxynitrite treatment of erythrocytes induced the activation of *lyn* through an irreversible mechanism [19]. As one of the major modifications induced by peroxynitrite is the nitration of tyrosine residues, the present results suggest that *lyn* upregulation may occur through the nitration of a tyrosine residue(s) in a substrate with high affinity for the SH2 domain. Although several nitrated substrates can in principle perform this function, we suggest that in erythrocytes a likely candidate may be band 3, the major tyrosine phosphorylation site of these cells. This hypothesis is based on two observations. First, we have shown that peroxynitrite treatment of human erythrocytes resulted in tyrosine nitration of the 22 kDa cytoplasmic N-terminal domain of the anion channel band 3 [23] and, second, we show in this study that the nitropeptide b3-NO_2Y, corresponding to the first 15 amino acids of band 3 which include one of the major phosphorylation sites of band 3 (Tyr 8), is able to activate *lyn* strongly in a manner comparable to that of b3-pY (Fig. 3). Moreover, the exogenous addition of the SH2 domain of *lyn* abolished b3-pY- and b3-NO_2Y-dependent activation of *lyn* (Fig. 4), and we also demonstrated that the SH2 domain is specifically involved in the binding of either b3-pY or b3-NO_2Y (Fig. 7). These results thus suggest that not only phosphorylation but also nitration of the cytoplasmic N-terminal domain of band 3 can cause *lyn* activation through a SH2-dependent mechanism and, since nitration is irreversible, at least in erythrocytes (our unpublished results), *lyn* upregulation was not downregulated by glucose metabolic recovery [19]. Furthermore, the finding that a nitrated substrate with SH2 binding affinity acquires the ability to activate a *src* kinase suggest that other substrates apart from band 3 may have the same property as demonstrated by our results with the NO_2Y peptide.

The hypothesis that nitration may be the mechanism through which *src* kinases are activated has recently been suggested [9]. These authors showed that when *c-src* is extracted from human pancreatic adenocarcinomas, it is both activated and nitrated, leading to the hypothesis that the nitration of critical tyrosine residues within *c-src* might alter kinase regulation, thereby increasing its activity. Although nitration of the regulatory tyrosine in the tail would be expected to downregulate the kinase (as suggested by the interchangeability of pY and NO_2Y peptides), it remains possible that the same *c-src* kinase possesses a tyrosine residue(s) that, when nitrated, may induce kinase activation through still uncharacterized intramolecular or intermolecular mechanisms.

Several recent reports have shown that peroxynitrite can also alter other signal transduction processes, resulting in the activation of mitogen-activated protein kinases [15] or in the inhibition of phosphotyrosine- and phosphoinositide-dependent signalling [12,24]. However, the upregulation of *src* kinases mediated by nitrating agents may induce rapid and uncontrolled cell growth and transformation, particularly in tissues overexpressing the inducible nitric oxide synthase. We therefore suggest that tyrosine nitration may represent a gain-of-function in the regulation of *src* kinases and may play a significant role in several pathological conditions.

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