Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites

Anna Maria Brunati, Luciana Bordin, Giulio Clari, Peter James, Manfredo Quadroni, Elisabetta Bariton, Lorenzo A. Pinna, and Arianna Donella-Deana

Introduction

Phosphorylation/dephosphorylation of protein tyrosine residues has been implicated in the regulation of several erythrocyte functions, including metabolism, membrane transport, cell volume, and cell shape. Protein tyrosine kinases identified to date in red cells include insulin receptor tyrosine kinase and the Syk and Lyn, the Y-phosphorylation of band 3 is only partially reduced. Indeed, the PP1-resistant phosphorylation of band 3 precedes and is a prerequisite for its coimmunoprecipitation with Lyn, which interacts with the phosphoprotein via the SH2 domain of the enzyme, as proven by binding competition experiments. Upon recruitment to primarily phosphorylated band 3, Lyn catalyzes the secondary phosphorylation of the transmembrane protein. These data are consistent with the view that band 3 is phosphorylated in intact erythrocytes by both PP1-resistant (most likely Syk) and PP1-inhibited (most likely Lyn) tyrosine kinases according to a sequential phosphorylation process. Similar radiolabeled peptide maps are obtained by tryptic digestion of 32P-band 3 isolated from either pervanadate-treated erythrocytes or red cell membranes incubated with exogenous Syk and Lyn. It has also been demonstrated by means of mass spectrometry that the primary phosphorylation of band 3 occurs at Y8 and Y21, while the secondary phosphorylation affects Y359 and Y904. (Blood. 2000;96:1550-1557)

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synergistic involvement of both Syk and Lyn in band 3 phosphorylation in isolated erythrocyte membranes. It has been demonstrated that the primary phosphorylation of band 3 catalyzed by p36\textsuperscript{Syk} (or p72\textsuperscript{Syk}) in erythrocyte membranes is a prerequisite for the association of added Lyn with the membranes and for the subsequent Lyn-catalyzed phosphorylation of different band 3 tyrosine residues. The present study shows that, in intact human red cells stimulated with pervanadate, band 3 undergoes sequential phosphorylation catalyzed by the concerted action of the Src-unrelated tyrosine kinase p72\textsuperscript{Syk} and the Src-related tyrosine kinase Lyn. Our findings suggest that, upon phosphorylation by p72\textsuperscript{Syk}, Y8 and Y21 act as docking sites for the SH2 domain of Lyn, which subsequently phosphorylates band 3 at additional secondary sites.

### Materials and methods

#### Materials

We purchased γ\textsuperscript{32}P\textsuperscript{ATP} and [\textsuperscript{32}P]Pi from Amersham (Little Chalfont, UK). PPI inhibitor and protease inhibitor cocktail were obtained from Calbiochem (Darmstadt, Germany) and Boehringer (Mannheim, Germany), respectively. Antiphosphotyrosine and anti-band 3 monoclonal antibodies were purchased from ICN Biotechnology (Irvine, CA) and Sigma (Dorset, UK), respectively. Anti-Lyn and anti-Syk polyclonal antibodies, raised against protein residues 44 to 63 and 257 to 352, respectively, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-GST antibody was purchased from Amersham Pharmacia Biotech. Recombinant glutathione-S-transferase (GST) and the Lyn SH2 domain fused with GST were expressed as previously described for c-Fgr SH2 domain. The phosphopeptides band 3 (1-24) and Src (523-533) and their unphosphorylated analogues were synthesized for c-Fgr SH2 domain. The phosphopeptides band 3 (1-24) and Src (523-533) and their unphosphorylated analogues were synthesized for c-Fgr SH2 domain. The phosphopeptides band 3 (1-24) and Src (523-533) and their unphosphorylated analogues were synthesized for c-Fgr SH2 domain.

#### Enzymes

Lyn and p36\textsuperscript{Syk} were isolated from human erythrocytes and further purified as previously described for rat spleen p36\textsuperscript{Syk} and Lyn.

#### Isolation of human erythrocytes

Human erythrocytes were prepared from fresh blood collected from healthy donors as previously described.

#### Isolation and phosphorylation of erythrocyte membranes (ghosts)

Erythrocyte membranes were isolated from hemolyzed red cells as described elsewhere. Erythrocyte membranes (3 μg) were phosphorylated for 10 minutes at 30°C in 30 μL of an incubation mixture containing 50-mmol/L Tris-HCl, pH 7.5; 10-mmol/L MnCl\textsubscript{2}; 30-mmol/L γ\textsuperscript{32}P\textsuperscript{ATP} (specific activity, 1000 cpm/μmol); 200-μmol/L sodium orthovanadate (basal medium); and the indicated amount of either p36\textsuperscript{Syk} or Lyn tyrosine kinase.

#### Preparation of Syk-phosphorylated ghosts

Ghosts were phosphorylated by 150-mmol/L p36\textsuperscript{Syk} for 10 minutes in basal medium containing unlabeled adenosine triphosphate (ATP) instead of radiolabeled ATP. Syk-phosphorylated ghosts were separated from p36\textsuperscript{Syk}, ATP, and other reagents by centrifugation and washed twice as described elsewhere. Syk-phosphorylated ghosts were then secondarily 3P-phosphorylated by Lyn in the basal medium.

The reactions were stopped by addition of 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol followed by 5 minutes of treatment at 100°C. The solubilized membranes were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE).

#### Treatment of intact red cells and antiphosphotyrosine immunoblotting

Packed cells, prepared as above described, were resuspended (at 16% hematocrit) in buffer A (20-mmol/L Tris-HCl, pH 7.5; 150-mmol/L NaCl; 10-mmol/L KCl; 1-mmol/L MgCl\textsubscript{2}; 100-μg/mL streptomycin; 25-μg/mL chloramphenicol; 24-mmol/L glucose; and 1-mmol/L adenine). Resuspended cells (300 μL for each sample) were incubated for 30 minutes at 35°C in the absence or presence of pervanadate, prepared by mixing hydrogen peroxide (3 mmol/L) and sodium orthovanadate (2 mmol/L). When indicated, PPI inhibitor was added to the incubation mixture immediately after pervanadate addition. After incubation, each sample was centrifuged, and the packed cells were hemolyzed in 1.8 mL of a hypotonic buffer containing 5-mmol/L sodium phosphate, pH 8; 0.02% NaN\textsubscript{3}; 30-μmol/L phenylmethylsulphonylfluoride; 1-mmol/L sodium orthovanadate; and protease inhibitor cocktail. Membranes were separated from hemolysates by centrifugation (20 000g for 20 minutes), and an aliquot (3 μg) was solubilized in SDS-PAGE sample buffer (50-mmol/L Tris-HCl, pH 8.9, containing 5-mmol/L ethylenediaminetetraacetic acid (EDTA), 380-mmol/L glycine, 2% SDS, and 1% β-mercaptoethanol). After 5 minutes of treatment at 100°C, the solubilized membrane proteins were subjected to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and immunostained with anti-P-Y antibody.

#### Anti-Lyn, anti-Syk, and anti-GST immunoprecipitations

Packed membranes prepared from intact erythrocytes treated as above were extracted for 1 hour at 4°C with 20-mmol/L Tris-HCl, pH 7.5; 10% glycerol; 1% Nonidet P-40; 1-mmol/L EDTA; 50-mmol/L NaCl; 1-mmol/L sodium orthovanadate; and protease inhibitor cocktail. After centrifugation, the supernatants were incubated for 5 hours at 4°C with the appropriate antibody bound to protein A-Sepharose. The immune complexes were washed 3 times by centrifugation and resuspension in 50-mmol/L Tris-HCl, pH 7.5, containing protease inhibitor cocktail and 1-mmol/L sodium orthovanadate.

#### Immune complex kinase assays

Tyrosine kinase assays of immune complexes formed with anti-Lyn and anti-Syk antibodies as described above were performed in basal medium containing either 300 ng of the cytoplasmic domain of band 3 or 200-μmol/L cdc2 (6-20) peptide, which served as exogenous substrates for Syk and Lyn, respectively. Following incubation for 10 minutes at 30°C phosphorylation of the cytoplasmic domain of band 3 was analyzed by SDS-PAGE followed by autoradiography. In the experiments containing cdc2 (6-20) peptide, the reactions were stopped by spotting 25 μL of the incubation mixture onto P81 phosphocellulose paper, which was then processed as described elsewhere.

#### 32P-peptide mapping of band 3 phosphorylated in vivo or in vitro

In vivo phosphorylation of band 3 was performed as follows: Packed erythrocytes (400 μL), prepared as described above, were preincubated in 3.6 mL of buffer B (buffer A without glucose and adenine) for 4 hours at 35°C to deplete endogenous ATP stores. The cells were then centrifuged at 750g for 3 minutes, resuspended in 2.1 mL of buffer A (16% hematocrit) containing carrier-free [\textsuperscript{32}P]Pi (11.1 MBq), and radiolabeled for 14 hours at 35°C, followed by incubation for 30 minutes at 35°C with pervanadate. Membranes were then isolated from the red cells and solubilized in SDS-PAGE sample buffer.

Band 3 was phosphorylated in vitro by incubating erythrocyte ghosts (15 μg) for 10 minutes at 30°C with p36\textsuperscript{Syk} and Lyn in the presence of γ\textsuperscript{32}P\textsuperscript{ATP} as described above and then solubilized in SDS-PAGE sample buffer. Solubilized in vivo or in vitro phosphorylated proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride; the membrane was then treated with 2-mol/L NaOH at 55°C.
for 1 hour to eliminate phosphoserines and phosphothreonines, and 32P-labeled band 3 was synthesized by autoradiography, excised, and digested with trypsin.12 The resulting peptides were separated in 2 dimensions on thin-layer cellulose plates by electrophoresis in 1% ammonium bicarbonate (pH 8.9) (1 hour, 1000 V) followed by ascending chromatography in a buffer containing 25% pyridine, 7.5% acetic acid, and 37.5% butanol.12

Identification of the band 3 residues either primarily phosphorylated by Syk or secondarily phosphorylated by Lyn

Isolated membranes (60 µg) were phosphorylated, in a final volume of 500 µL, first by p36 syk and unlabeled ATP and then by Lyn and γ[32P]ATP as described above. Band 3 was then isolated by SDS-PAGE and digested first with cysteine bromide (1 mg/mL in 70% formic acid). The digest was dried in a Speed-Vac and then resuspended in 50 µL of 100-mmol/L ammonium acetate buffer, pH 8.0, containing 20 µg of sequencing-grade trypsin (Promega, Madison, WI) and incubated at 37°C for 6 hours. The digest was stopped by the addition of acetic acid to achieve a final pH of 3.0. The digest was then loaded onto an immobilized metal ion chromatography minicolumn. This was made by packing a 100-µL plastic pipette tip (with a plug of glass fiber at the end) with iminodiacetic acid-derivatized Sepharose (Amersham Pharmacia Biotech). The column was saturated with FeCl3, and the sample was loaded in 0.1-mol/L acetic acid. The column was washed with 20-bed volumes of 0.1-mol/L acetic acid. Elution was performed with 0.1% ammonium acetate, pH 8.0, containing 500-mmol/L Na2HPO4. The eluate was acidified by the addition of 10 µL of trifluoroacetic acid (TFA), and the digest was injected onto a 30-mm, 5-µm, C-18, 0.18 mm × 20 cm, reverse-phase microcolumn. A gradient was run from 100% A (0.1% TFA in water) to 70% B (0.07% TFA, 80% acetonitrile in water) over 1 hour. An ABI 120A syringe pump generated the gradient at 50-µL/min, which was split, giving a flow of 0.4 µL/min into the column. Individual peaks were collected manually (according to the UV trace) into 500-µL Eppendorf tubes containing a few beads of C-18 reverse-phase material to prevent sample loss. The radioactivity in each fraction was determined, and 5 phosphopeptide-containing fractions were obtained, 2 radioactive and 3 nonradioactive. The reverse-phase beads containing the peptides were packed individually into nanotips. The peptides were eluted with 80% acetonitrile and 5% acetic acid and were subjected to collision-induced dissociation in a Finnigan TSQ700 tandem quadrupole mass spectrometer using a collision energy of 10 to 30 eV and 1.8 × 10⁻²⁴ mmHg argon with a parent ion window of 2 to 3 mass units. The digest was then loaded onto an immobilized metal ion chromatography minicolumn. This was made by packing a 100-µL plastic pipette tip (with a plug of glass fiber at the end) with iminodiacetic acid-derivatized Sepharose (Amersham Pharmacia Biotech). The column was saturated with FeCl3, and the sample was loaded in 0.1-mol/L acetic acid. The column was washed with 20-bed volumes of 0.1-mol/L acetic acid. Elution was performed with 0.1% ammonium acetate, pH 8.0, containing 500-mmol/L Na2HPO4. The eluate was acidified by the addition of 10 µL of trifluoroacetic acid (TFA), and the digest was injected onto a 30-mm, 5-µm, C-18, 0.18 mm × 20 cm, reverse-phase microcolumn. A gradient was run from 100% A (0.1% TFA in water) to 70% B (0.07% TFA, 80% acetonitrile in water) over 1 hour. An ABI 120A syringe pump generated the gradient at 50-µL/min, which was split, giving a flow of 0.4 µL/min into the column. Individual peaks were collected manually (according to the UV trace) into 500-µL Eppendorf tubes containing a few beads of C-18 reverse-phase material to prevent sample loss. The radioactivity in each fraction was determined, and 5 phosphopeptide-containing fractions were obtained, 2 radioactive and 3 nonradioactive. The reverse-phase beads containing the peptides were packed individually into nanotips. The peptides were eluted with 80% acetonitrile and 5% acetic acid and were subjected to collision-induced dissociation in a Finnigan TSQ700 tandem quadrupole mass spectrometer using a collision energy of 10 to 30 eV and 1.8 × 10⁻²⁴ mmHg argon with a parent ion window of 2 to 3 mass units (50% half height) and a daughter ion resolution of 1 to 2 mass units.

Immunostaining

Proteins transferred to nitrocellulose membranes were incubated with the indicated antibodies followed by the appropriate biotinylated second antibody and developed using an enhanced chemiluminescence detection system (ECL, Amersham).

Results

In vivo and in vitro phosphorylation of human erythrocyte band 3 is synergistically catalyzed by Syk and Src-related tyrosine kinases

Consistent with previous data,12 addition of p36 syk, purified from human erythrocytes, to isolated red cell membranes (ghosts) induced a marked Y-phosphorylation of band 3 (Figure 1A, lane 3). In contrast, purified Lyn behaved as a very poor phosphorylating agent (Figure 1A, lane 2). However, preincubation of membranes with p36 syk and unlabeled ATP converted band 3 into an excellent substrate for Lyn (Figure 1B, lane 2). Prolonged direct incubation of the isolated membranes with Lyn alone did not abrogate the requirement for p36 syk as a priming agent (not shown). Similar results were obtained using p72src purified from rat spleen instead of erythrocyte p36 syk (not shown).

In an attempt to confirm sequential phosphorylation of band 3 catalyzed by Syk and Lyn tyrosine kinases in intact cells, we stimulated the Y-phosphorylation of human erythrocytes by treating them with a combination of sodium orthovanadate and hydrogen peroxide (ie, pervanadate). Control and treated cells were then hemolyzed, and erythrocyte membranes were immediately isolated as described in “Materials and methods.” Antiphosphotyrosine immunoblotting revealed band 3 as the major Y-phosphorylated protein in membranes isolated from stimulated erythrocytes (Figure 1C, lane 2). The involvement of p72 src in the Y-phosphorylation of band 3 in erythrocytes activated by pervanadate has already been suggested.15 To verify the contribution of Lyn to band 3 phosphorylation in vivo, we carried out experiments with PP1, which is a highly potent inhibitor selective for Src family tyrosine kinases,31 including Lyn.32 Accordingly, PP1 proved unable to inhibit the in vitro phosphorylation of band 3 catalyzed

![Figure 1](http://www.bloodjournal.org) Y-phosphorylation of band 3 in isolated ghosts and in intact human erythrocytes. (A) Radioactive phosphorylation pattern of human erythrocyte membranes (ghosts) incubated with γ[32P]ATP either alone (lane 1) or in the presence of 35-mmol/L Lyn (lane 2) or 15-mmol/L p36 syk (lanes 3-6). Increasing concentrations of PP1 were present in the incubation medium of assays shown in lanes 4 to 6. (B) Radioactive phosphorylation pattern of erythrocyte ghosts, first phosphorylated by p36 syk in the presence of unlabeled ATP as described in "Materials and methods” and further incubated for 10 minutes in the presence of γ[32P]ATP without (lane 1) or with 35-mmol/L Lyn (lanes 2-5). Assays in lanes 3 to 5 were performed in the presence of increasing concentrations of PP1 added immediately before Lyn. γ[32P]ATP-labeled proteins were subjected to SDS-PAGE on 10% gels followed by autoradiography. (C) Anti-P-Y Immunostaining of erythrocyte membrane proteins phosphorylated in vivo. Intact human erythrocytes were incubated either in the absence (lane 1) or presence (lanes 2-5) of pervanadate as described in "Materials and methods.” Increasing concentrations of PP1 were present in the assays shown in lanes 3 to 5. After incubation, erythrocyte membranes were rapidly isolated, solubilized, and submitted to SDS-PAGE followed by transfer to a nitrocellulose filter. The filter was then immunostained with anti-P-Y antibody. Other experimental details are described in “Materials and methods.” Panels are representative of at least 6 different experiments.
by p36\textsuperscript{Y507} (Figure 1A, lanes 4-6). In contrast, secondary \textsuperscript{32}P-phosphorylation of band 3 catalyzed by Lyn was almost completely abolished in the presence of PP1 (Figure 1B, lanes 3-5). PP1 was also tested for its effect on pervanadate-stimulated \textsuperscript{32}P-phosphorylation in intact erythrocytes. As shown in Figure 1C (lanes 3-5), preincubation of red cells in the presence of both pervanadate and PP1 decreased the amount of phosphoryrosine detected in band 3. This result is consistent with the involvement of Lyn or another PP1-inhibited kinase in the phosphorylation of band 3 in intact erythrocytes. The residual \textsuperscript{32}P-phosphorylation of the protein observed even in the presence of 25-\textmu mol/L PP1 (Figure 1C, lanes 3-5) discloses the contribution of Src-unrelated protein kinases, as expected assuming that the sequential model of phosphorylation also takes place in intact cells.

We also tested the effect of PP1 on band 3 \textsuperscript{32}P-phosphorylation triggered by different stimuli.\textsuperscript{2,8} For this purpose, we treated intact erythrocytes for 30 minutes with diamide (1-3 mmol/mL), N-ethylmaleimide (2-4 mmol/mL), or with a hypertonic buffer (900 mOsm) and found that PP1 always inhibited partially the band 3 \textsuperscript{32}P-phosphorylation (not shown).

Activation of p72\textsuperscript{Syk} and Lyn by pervanadate treatment of intact erythrocytes

Pervanadate treatment of intact red cells correlates with an activation of p72\textsuperscript{Syk}.\textsuperscript{11} This is also demonstrated by the immunoprecipitation experiments shown in Figure 2A, carried out using extracts of membranes isolated from control and treated cells prepared with Nonidet P-40 as described in "Materials and methods." After centrifugation, the extracted proteins were immunoprecipitated with anti-p72\textsuperscript{Syk} antibodies, and p72\textsuperscript{Syk} activity was tested in vitro toward the cytoplasmic domain of band 3. p72\textsuperscript{Syk} activity present in the anti-p72\textsuperscript{Syk} immunoprecipitates (IP) from stimulated cells was more than 3-fold higher relative to the basal activity found in untreated cells (compare histograms 1 and 2 in Figure 2A). As expected, PP1 treatment of erythrocytes did not affect p72\textsuperscript{Syk} activity (Figure 2A, histograms 3-5).

Using assays similar to those described above, we also performed anti-Lyn IP and Lyn activity was tested in vitro toward the synthetic peptide cdc2 (6-20), a substrate specific for Src kinases. While Lyn activity in control erythrocytes was low, it was greatly enhanced (by about 7-fold) in IP from pervanadate-treated cells (compare histograms 1 and 2 in Figure 2B). As expected, PP1 treatment of erythrocytes almost completely prevented phosphorylation of the peptide by the immunoprecipitated Lyn (Figure 2B, histograms 3-5). The high extent of Lyn activity found in pervanadate-treated erythrocytes (Figure 2B, histogram 2) is consistent with the presence of the doubly autophosphorylated (at Y396 and Y507), hyperactive Lyn conformation\textsuperscript{20} due to the blockage of phosphoryrosine phosphatase activities.

These results show that both p72\textsuperscript{Syk} and Lyn are activated by pervanadate treatment, i.e., under conditions that trigger band 3 \textsuperscript{32}P-phosphorylation. This finding, in conjunction with the effect of the Src specific inhibitor PP1, supports the view that p72\textsuperscript{Syk} is probably the tyrosine kinase responsible for the band 3 \textsuperscript{32}P-phosphorylation that is resistant to PP1 inhibition in vivo. Moreover, comparison of data presented in Figures 1C and 2B suggests that Lyn might be the Src kinase inhibited by PP1 and involved in band 3 \textsuperscript{32}P-phosphorylation.

Recruitment of Lyn to Y-phosphorylated band 3 in pervanadate-treated cells

According to the sequential model of band 3 phosphorylation, p72\textsuperscript{Syk} generates the docking sites for the binding of Lyn to the membranes and subsequent phosphorylation of additional band 3 tyrosine residues (Figure 1B).\textsuperscript{12} To assess the actual occurrence of an interaction between Lyn and band 3 in vivo, we assayed for the presence of band 3 in the anti-Lyn IP obtained from intact red cells as described in Figure 2B. Figure 3A shows that Lyn communoprecipitated with band 3 when Y-phosphorylation was stimulated by pervanadate treatment (compare lanes 1 and 2 in Figure 3A), irrespective of the presence of the Lyn-specific inhibitor PP1 (Figure 3A, lanes 3-5).

Immunostaining with anti-P-Y antibody of anti-Lyn IP obtained from stimulated cells shows that the band 3 species communoprecipitated with Lyn was Y-phosphorylated (compare lanes 1 and 2 in Figure 3B). Phosphotyrosine was also detectable in band 3 communoprecipitated from PP1-treated erythrocytes, in accordance with the view that the primary phosphorylating agent is not Lyn but rather a Src-unrelated kinase that is also activated by pervanadate treatment. Syk is therefore the first-choice candidate to perform this task.

Peptide maps of band 3 phosphorylated in vitro or in vivo: identification of the protein residues phosphorylated by p36\textsuperscript{Syk} or Lyn in isolated erythrocyte membranes

We compared the \textsuperscript{32}P-peptide maps of band 3 obtained either from pervanadate-treated erythrocytes incubated in the presence of...
suggests the involvement of the SH2 domain of Lyn in the interaction between the enzyme and its substrate. This concept is also supported by the sequences containing the Syk-phosphorylated residues identified in vitro, MEELQQEYEY, and EENLEQEEEY, which exhibit a Src SH2-recognition motif. Our hypothesis was validated by competition experiments performed with band 3 phosphorylated both in vitro and in vivo.

Assays performed using synthetic peptides and isolated erythrocyte membranes revealed that peptide band 3 (1-26), phosphorylated at Y8 and Y21, but not its unphosphorylated derivative, prevented Lyn-mediated secondary phosphorylation of the transmembrane protein in a dose-dependent manner (Figure 6A).

The interaction between Lyn and band 3 is mediated by the SH2 domain of the kinase

The importance of primary Y-phosphorylation of band 3 in the recruitment of Lyn to the transmembrane protein (Figure 3B)
phosphorylated tyrosines of band 3, recombinant GST-SH2 fusion protein prevented the secondary phosphorylation of the transmembrane protein mediated by Lyn.

The specific involvement of the SH2 domain in the recruitment of Lyn to band 3 is also validated by competition experiments performed with band 3 phosphorylated in intact cells. Figure 7A shows the coimmunoprecipitation of phospho-band 3 with Lyn in anti-Lyn IP obtained from pervanadate-stimulated erythrocytes. The presence of increasing concentrations of recombinant Lyn SH2 domain, added during the immunoprecipitation, progressively hindered the interaction between the primarily phosphorylated protein and Lyn (Figure 7A, lanes 2-5). The same conclusion is supported by the experiments shown in Figure 7B. Human red cells treated as described in Figure 2B were immunoprecipitated with anti-GST antibody in the presence of GST-SH2 domain. Upon Y-phosphorylation by pervanadate treatment of intact cells, band 3 coimmunoprecipitated with recombinant Lyn (GST)/SH2 domain (compare lanes 6 and 7 in Figure 7B). The same interaction was detected in IP obtained from cells stimulated in the presence of the Src inhibitor PP1 (Figure 7B, lanes 8-10), thereby confirming the hypothesis that band 3 phosphorylation catalyzed by a Src-unrelated tyrosine kinase is a prerequisite for the binding of Lyn to the protein.

Discussion

It has previously been reported that p72syk may associate with band 3 and has been suggested that this tyrosine kinase is potentially involved in the pervanadate-induced Y-phosphorylation of band 3. The present experiments validate the hypothesis that activation of p72syk induces the phosphorylation of specific tyrosine residues of band 3 (Figures 1, 3, and 4). Moreover, we present the first direct evidence that, in pervanadate-stimulated red cells, a Src-related kinase is activated and involved in the phosphorylation of band 3, as judged by its reduced Y-phosphorylation brought about in vivo by PP1, an inhibitor specific for Src kinases (Figures 1C and 2B). The following evidence suggests that Lyn is the most likely candidate, among Src kinases, to phosphorylate the band 3: (1) Lyn is the only Src kinase we could purify in substantial amount from erythrocytes, (2) Lyn activity is highly increased in parallel with pervanadate-induced band 3 Y-phosphorylation (Figure 2B), (3) 32P-radiolabeled peptide maps of band 3 phosphorylated either in intact erythrocytes or in cell membranes by exogenous p36syk and Lyn are similar (Figure 4), and (4) other authors found that Lyn activity, at variance with that of Src and Fyn, is enhanced under conditions that also induce an increase of band 3 Y-phosphorylation.

Figure 5. Mass spectrometric sequencing (MS/MS) of the phosphopeptide-containing Y359. The radioactive fraction containing the 2+ ion m/z 843 isolated by immobilized metal affinity chromatography and reverse-phase chromatography was subjected to sequence analysis by collisionally activated dissociation using a triple quadrupole mass spectrometer. The resultant MS/MS spectrum is shown with the sequence ions labeled as indicated.

Figure 6. Specific inhibition of band 3 secondary phosphorylation by either band 3 (1-26) phosphopeptide or recombinant Lyn SH2 domain. The ability of Lyn to phosphorylate band 3 following phosphorylation by p36syk with unlabeled ATP was measured in the presence of 32P[γATP and increasing concentrations of either the indicated synthetic phosphopeptides and their unphosphorylated homologues (A) or recombinant GST and GST-SH2 domain (B). Incubations were carried out for 5 minutes under conditions described in "Materials and methods." The samples were submitted to SDS-PAGE, and the radioactivity incorporated into band 3 was evaluated either by analysis on a Packard Instant Imager or by autoradiography and scintillation counting of the identified radiolabeled bands. Lyn activity is expressed as the percentage of the control values obtained in the absence of effectors. Reported values represent means of 4 separate experiments, with SE indicated by vertical bars.
primary phosphorylation of band 3 catalyzed by p72syk has already been demonstrated in vitro for the hematopoietic lineage-specific protein HS1.40,41 Here we present the first in vivo evidence of a protein sequentially phosphorylated and propose that Syk or related kinases may play a general role in generating docking sites for Src SH2 domains in proteins, thereby converting them into good substrates for Src tyrosine kinases.

We also suggest that the involvement of a Src kinase in band 3 Y-phosphorylation might be a general theme in human erythrocytes. In fact, we found that PP1 inhibits also the band 3 Y-phosphorylation induced by cell treatment with the oxidizing agents diamide and N-ethylmaleimide or cell exposure to hypertonic conditions.

Band 3 is a multifunctional transmembrane protein, which serves as anion transporter, anchor for cytoskeleton, hemoglobin, and glycolytic enzymes, as well as a senescence antigen.34 The 43 kd N-terminal domain of band 3 protrudes into the cytosol as a flexible finger and contains the binding sites for cytoskeletal proteins and glycolytic enzymes, whereas the intramembrane domain mediates anion transport.34 Because Syk and Lyn phosphorylate band 3 at both cytosolic and membrane domains, Y359 and Y904 have been identified as the band 3 residues specifically phosphorylated by Lyn. While Y359 is located at the junction between the N-terminal cytosolic domain and the transmembrane domain of band 3, Y904 is situated at the C-terminal end of the protein. Most likely, the phosphorylation of these residues by Lyn is not directly dictated by local specificity determinants but by conformational features. The observation that the addition of either band 3 (1-26) phosphopeptide or the recombinant SH2 domain of Lyn prevents the band 3 secondary phosphorylation catalyzed by Lyn indicates that these conformational requirements rely primarily on the interaction between phospho-band 3 and the SH2 domain of Lyn. This hypothesis is validated by the finding that specific sequences are not stringently required for site recognition by Src kinases, apart from the frequent presence of a hydrophobic residue at position n-1.36,37,39

A sequential process of phosphorylation synergistically mediated by Syk and Src tyrosine kinases has already been demonstrated in vitro for the hematopoietic lineage-specific protein HS1.40,41 Here we present the first in vivo evidence of a protein sequentially phosphorylated and propose that Syk or related kinases may play a general role in generating docking sites for Src SH2 domains in proteins, thereby converting them into good substrates for Src tyrosine kinases.

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Band 3 is a multifunctional transmembrane protein, which serves as anion transporter, anchor for cytoskeleton, hemoglobin, and glycolytic enzymes, as well as a senescence antigen. The 43 kd N-terminal domain of band 3 protrudes into the cytosol as a flexible finger and contains the binding sites for cytoskeletal proteins and glycolytic enzymes, whereas the intramembrane domain mediates anion transport. Because Syk and Lyn phosphorylate band 3 at both cytosolic and membrane domains, Y-phosphorylation/dephosphorylation is likely involved in the regulation of several erythrocyte functions (ie, glycosylation, cell shape, cytoskeleton movements, and anion transport).

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References

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