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The Lyn-catalyzed Tyr phosphorylation of the transmembrane band-3 protein of human erythrocytes

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Band-3 protein (approximately 95 kDa), the major and multifunctional transmembrane protein of human erythrocytes, has been shown to be phosphorylated by endogenous Tyr-protein kinases on different Tyr residues at its N and C cytoplasmic domains.

Both the added p36^{syk} (catalytic domain of p72^{syk}) and Lyn kinases are able to phosphorylate the isolated cytoplasmic domain of band 3 (cdb3), yielded by chymotryptic digestion of band 3 in the isolated membranes (ghosts).

However, the two Tyr-protein kinases exhibited different phosphorylation behaviours when added to the isolated erythrocyte membranes. More precisely, the added p36^{syk} markedly Tyr phosphorylates the band-3 protein, whereas the added Lyn phosphorylates it very poorly.

It is of interest that Lyn can associate with membranes and markedly phosphorylate band 3 when this latter protein has been previously phosphorylated by p36^{syk}, i.e. the p36^{syk}-catalyzed phosphorylation is proposed to be a prerequisite for the association of Lyn with the membrane (likely to band 3) and for the Lyn-catalyzed phosphorylation of different band-3 Tyr sites.

Keywords: p36^{syk}-catalyzed Tyr phosphorylation; Lyn-catalyzed Tyr phosphorylation; band 3; human erythrocytes.

For a long time, the major multifunctional transmembrane band-3 protein (approximately 95 kDa) of human erythrocytes has been found to be endogenously phosphorylated at its N-cytoplasmic domain (approximately 43 kDa), protruding into the cytosol as an extended flexible finger, not only on the Ser/Thr residues [1] by cAMP-independent casein kinase I [2–6], but also on Tyr residues [7–18] by Tyr-kinases.

Human erythrocyte Tyr-protein kinases, phosphorylating band 3 in the isolated ghosts or its isolated cytoplasmic domain of band 3 (cdb3), have been studied in several laboratories including our own [15, 16], where two Tyr-protein kinases have been isolated from human erythrocyte cytosol by DEAE-cellulose chromatography followed by Heparin-Sepharose chromatography.

While the most abundant cytosolic Tyr-protein kinase (approximately 36 kDa) [15] belongs to the Syk family, being reported to be yielded quite likely by proteolytic breakdown of its larger parent p72^{syk} in the human erythrocyte membranes [19] and other cells [20, 21], the other minor cytosolic Tyr-protein kinase [15] has been shown (by immuno-blotting assay) to belong to the Lyn family, as previously reported in [19].

This paper deals with the different properties of the two Tyr-protein kinases and, in particular, with the mechanism by which the p36^{syk}-catalyzed phosphorylation of band 3 in the isolated ghosts recruits band 3 for the Lyn-catalyzed phosphorylation of Tyr residue(s) not ^{32}P labelled by p36^{syk}.

In particular, the results reported here show that Lyn, when added to isolated human erythrocyte ghosts, can associate with membranes and markedly phosphorylate the band-3 cytoplasmic domain, only once this latter had been previously disposed by the p36^{syk}-catalyzed phosphorylation in the presence of unlabeled ATP.

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Abbreviations. PhMeSO$_2$F, phenylmethylsulphonyl fluoride; HS1, Pro-Glu-Gly-Asp-Tyr-Glu-Glu-Val-Leu-Glu; P-HS1, Tyr(P)-HS1; cdb3, isolated cytoplasmic domain of band 3; CKI, casein kinase I.

MATERIALS

Anti-Lyn was purchased from Santa Cruz Biotechnology; [γ-^{32}P]ATP was from Amersham International; DEAE-Sepharose and Heparine-Sepharose were from Pharmacia. The peptides Pro-Glu-Gly-Asp-Tyr-Glu-Glu-Val-Leu-Glu (HS1) and its Tyr(*P*)-phosphorylated derivative (P-HS1) were prepared as described in [22].

METHODS

Human erythrocytes were prepared by centrifugation (at 750×*g* for 3 min) of fresh blood collected from healthy donors. To minimize contamination by leucocytes and platelets, the packed red cells were washed three times by centrifugation in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 24 mM glucose, 25 μg/ml chloramphenicol, 0.1 mg/ml streptomycin (buffer A), discarding the buffy coat and the upper third of the packed red cell layer. The packed red cells were hemolysed and the membranes (ghosts) were prepared as previously described in [23].

Purification of Tyr-protein kinases (p36^{syk} and Lyn) and of band-3 proteolytic fragments. p36^{syk} and Lyn were isolated from human erythrocyte cytosol [15] and rat spleen [22], respectively.

The 40/45-kDa fragments of the cytoplasmic domain of band 3 protein (cdb3) were obtained by α-chymotrypsin-promoted di-

gestion of inverted membrane vesicles [23] derived from ghosts and isolated by DE 52 chromatography according to [24].

Tyr-phosphorylation assays. Tyr-phosphorylation of membrane proteins was performed by incubating separately 3 μg white ghosts, 0.2 μg isolated cytoplasmic domain of band 3 (cdb3) or 3 μg poly(Glu80Tyr20) at 30°C for 10 min in 30 μl reaction mixture containing 50 mM Tris/HCl, pH 7.5, 10 mM $MnCl_2$, 20 μM [γ-^{32}P]ATP (3×10^6 cpm/nmol) and 0.1 mM vanadate, in the presence of p36^{syk} (100 units), or Lyn (100 units).

When Lyn kinase activity was assayed on *P*-Tyr-membranes, white ghosts were first phosphorylated by p36^{syk} in the presence of 100 μM unlabeled ATP, recovered by centrifugation at 14000 rpm (Eppendorf centrifuge 5415C) for 10 min, and washed twice in 20 mM Tris/HCl, pH 8, containing 0.02% NaN_3 and 0.03 mM phenylmethylsulphonyl fluoride ($PhMeSO_2F$), then incubated in the presence of Lyn (100 units) under the same above conditions described for the Tyr phosphorylation of white ghosts.

The reactions were stopped by addition of 2% SDS and 1% 2-mercaptoethanol (final concentration) followed by a 5-min treatment at 100°C as described in [25]. The solubilized membranes were analyzed by 0.1% SDS/8% PAGE, essentially according to Laemmli [26].

After electrophoresis, gels were stained with Coomassie brilliant blue according to Laemmli [26], submitted to 2 M NaOH treatment at 55°C for 1 h as described in [25], and fixed again. Dried gels were then autoradiographed at −80°C with intensifying screens.

1 unit Tyr-protein kinase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol ^{32}P into poly(Glu80 Tyr20) in 1 min under the above conditions.

For the evaluation of ^{32}P incorporated into the different proteins, the corresponding radioactive bands were excised from the alkali-treated gels and radioactivity determined in a liquid scintillation counter.

The values of K_m were evaluated by the Lineweaver-Burk double-reciprocal plot. The values reported are the means of at least three separate experiments; the SE value was less than 15%.

[^{32}P]-Peptide mapping of band 3. Erythrocyte membranes, radiolabeled with ^{32}P by incubation in the presence of [γ-^{32}P]ATP with either p36^{syk} or Lyn (after p36^{syk}-catalyzed phosphorylation in the presence of unlabeled ATP), were subjected to SDS/PAGE.

The gels were transferred electrophoretically to nitrocellulose filters and the phosphorylated band 3 was localized by autoradiography. The ^{32}P-labeled band 3 was excised and tryptically digested on poly(vinylpyrrolidone)-treated nitrocellulose pieces, as described in [27], and the resulting peptides were separated by two-dimensional thin-layer electrophoresis at pH 8.9 (40 min at 1000V) followed by ascending chromatography in a buffer containing 25% pyridine, 7.5% acetic acid, 37.5% butanol [28].

Western blot of Lyn kinase. Lyn (20 μl), purified as described in [15], was subjected to 0.1% SDS/8% PAGE as described above.

Protein bands were transferred to nitrocellulose filters using a Hoefer apparatus at 400 mV for 2 h. The filters were treated for 1 h at room temperature with 3% bovine serum albumin in 10 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl. The antibody was used at a dilution of 1:200. Immunoreactive proteins were incubated with biotinylated donkey anti-(rabbit IgG) and detected by incubation with streptavidin−alkaline-phosphatase conjugate.

Synthetic P-Tyr-peptide experiments. When the Tyr-phosphorylated synthetic peptide, P-HS1, prepared as in [22], was tested for its inhibitory effect of Lyn binding to p36^{syk}-phosphorylated membrane proteins, increasing amounts of both unphosphorylated (HS1) and phosphorylated (P-HS1) peptide were added to the reaction mixture above described for the Tyr-phosphorylation assay, and containing p36^{syk}-phosphorylated white ghosts (1 μg).

When Lyn binding to p36^{syk}-phosphorylated membranes and, separately, to unphosphorylated membranes was investigated, increasing amounts of both HS1 and P-HS1 peptide were separately added to both membrane preparations containing Lyn (10 nmol), and incubated at 30°C for 10 min. The SDS/PAGE, Commassie brilliant blue staining and NaOH treatments were as described above, and the ^{32}P-labelled band 3, identified by autoradiography, was excised from alkali-treated gels and radioactivity determined in a liquid scintillation counter [25].

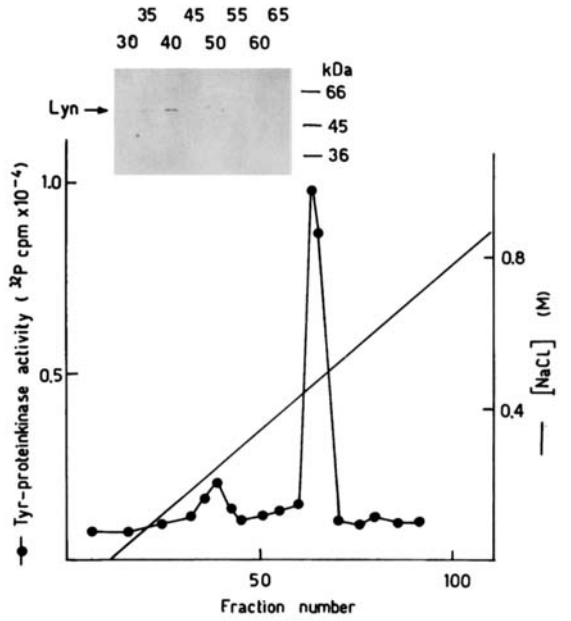

Fig. 1. Heparin-Sepharose chromatographic profile of protein kinase actvity displayed by DEAE-Sepharose peak of Tyr-protein kinase pooled as described in [15]. Tyr-protein kinase activity assayed on Poly (Glu80Tyr20) [15]. The inset shows the immunoblotting assay with anti-Lyn IgG. The numbers of the lanes correspond to those of the eluted fractions analyzed by the Western-blot procedure.

RESULTS

Chromatographic separation and identification of the two Tyr-protein kinases. When the ammonium sulfate fraction (60% saturation) from the red hemolysate supernatant of human erythrocytes was submitted to DEAE-Cellulose chromatography followed by Heparin-Sepharose chromatography under the conditions previously described [15], the elution profile of Tyr-protein kinase activity was obtained (Fig. 1).

The immunoblotting assay (inset of Fig. 1) shows that the minor Tyr-protein kinase (eluted at 0.2 mM NaCl) belongs to the Lyn family, whereas the more retarded 36-kDa Tyr-kinase (eluted at 0.48 mM NaCl) belongs quite likely to the Syk family as does the 36−40-kDa Tyr-kinases [20, 21], yielded by proteolytic breakdown of their larger parent p72^{syk}, which more recently [19] has been shown (by immunoblotting assay) to be present also in the human erythrocyte membranes.

Phosphorylation of cdb3. The two Tyr-protein kinases (p36^{syk}, and Lyn), when separately incubated with cdb3 in the presence

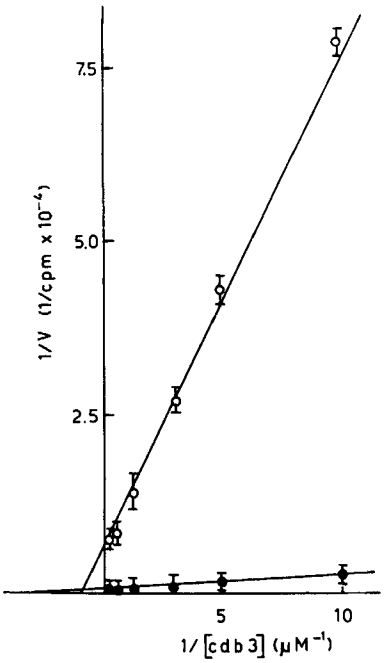

Fig. 2. Lineweaver-Burk plot showing the cdb3 Tyr-phosphorylation rate by Tyr-protein kinases p36^{syk} (filled circles) and Lyn (empty circles) as a function of the cdb3 concentration.

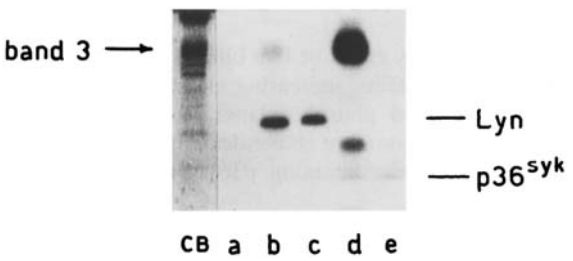

Fig. 3. Phosphorylation patterns of erythrocyte membrane proteins by Tyr-protein kinase p36^{syk} and Lyn. Human erythrocyte ghosts (3 μg) were incubated alone (lane a, control) at 30°C for 10 min or in the presence of Lyn (lane b) or p36^{syk} (lane d) in the incubation mixture described in the Methods. Lanes c and e show the autophosphorylation pattern (controls) of Lyn (lane c) and p36^{syk} (lane e). The samples were analyzed by 0.1% SDS/8% PAGE (see Methods) and the gels were subjected to NaOH treatment as described in the Methods. Lane CB shows the NaOH-treated Coomassie brilliant blue stained gel of membrane proteins. Autoradiograms were exposed for 6 h.

of [γ-^{32}P]ATP, are able to phosphorylate the cdb3 to different extents, Lyn exhibiting much lower affinity (apparent K_m = 1.03 μM) than p36^{syk} (apparent K_m = 0.3 μM).

Phosphorylation of band 3 in the isolated membrane (ghosts). When the two Tyr kinases, compared on the basis of the same concentration of kinase units, defined as described in the Methods, were separately added to the isolated membranes (ghosts) in the presence of [γ-^{32}P]ATP, phosphorylation patterns were obtained (Fig. 3).

Band 3, while being markedly Tyr-phosphorylated by p36^{syk} (Fig. 3, lane d), is phosphorylated very poorly by Lyn (lane b). What is of interest is that this latter kinase becomes able to bind to the membranes (as judged by the comparative immunoblotting assay (Fig. 4, lanes d and e) and to markedly phosphorylate band 3 (Fig. 5, lane b), only when the membranes are previously phosphorylated by p36^{syk} (in the presence of unlabeled ATP (Fig. 5, lane b).

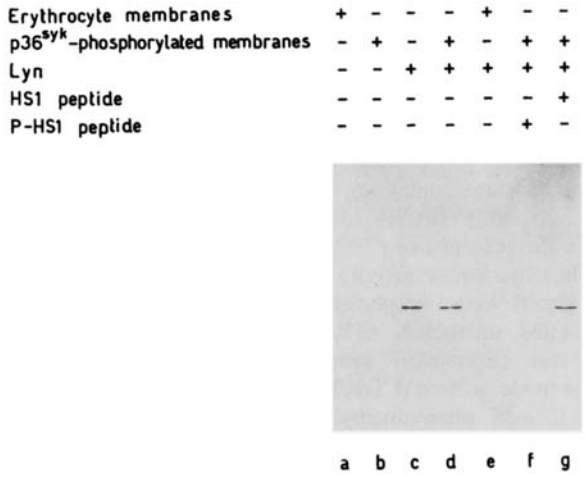

Fig. 4. Binding of Lyn protein kinase to erythrocyte membranes. 3 μg untreated membranes (lanes a, e) or p36^{syk}-phosphorylated membranes (lanes b, d, f, g) were incubated at 30°C for 10 min in the absence (lanes a, b) or presence (lanes d–g) of Lyn protein kinase. Lanes f and g show Lyn protein kinase binding to p36^{syk}-phosphorylated white ghosts in the presence of the phosphorylated (P-HS1) (lane f) and unphosphorylated synthetic peptide (HS1) (lane g). Anti-Lyn control is shown in lane c. The incubation was stopped by centrifugation as described in the Methods. The resuspended samples were analyzed by 0.1% SDS/8% PAGE and Western blotting as described in the Methods.

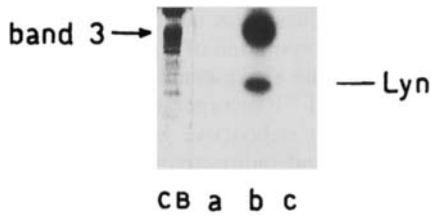

Fig. 5. Lyn-catalyzed phosphorylation of erythrocyte membranes previously phosphorylated by p36^{syk}. 3 μg white ghosts, once phosphorylated by p36^{syk} in the presence of unlabeled ATP, were incubated in the absence (lane a) or presence (lane b) of Lyn protein kinase, as dscribed in the Methods. Lane c shows the autophosphorylation of Lyn incubated alone (indicated by the arrow). Lane CB shows the NaOH-treated Coomassie brilliant blue stained gel of membrane proteins. Autoradiograms were exposed for 60 min.

The p36^{syk}-catalyzed phosphorylation of band 3 appears, therefore, to be a prerequisite for both the association of Lyn with membranes and for the Lyn-catalyzed phosphorylation of band 3. Moreover, these p36^{syk}-catalyzed and Lyn-catalyzed Tyr phosphorylations most likely involve different sites of band 3, as indicated by the comparison of the ^{32}P-labeled peptide maps (Fig. 6) obtained from the trypsin-catalyzed proteolysates of band 3 from the ghosts separately Tyr phosphorylated by added p36^{syk} (Fig. 6A) or by added Lyn kinase after the p36^{syk}-catalyzed phosphorylation in the presence of unlabeled ATP (Fig. 6B).

It is to be stressed that both the association of Lyn-kinase with membranes (Fig. 4, lane d) and the Lyn-catalyzed phosphorylation of band 3 (Fig. 7, empty circles) are inhibited by adding Tyr(*P*)-synthetic peptide (P-HS1; Fig. 4, lane f and Fig. 7, filled circles) previously phosphorylated by p36^{syk} [22], its target Tyr site being surrounded by acidic amino acid residues such as Tyr8, Tyr21 and Tyr904 of band 3.

Such a Tyr(*P*)-peptide, by reacting with the SH2 domain of Lyn [22], inhibits both the binding of this kinase to the previously p36^{syk}-phosphorylated membrane band 3 (Fig. 4, lane f)

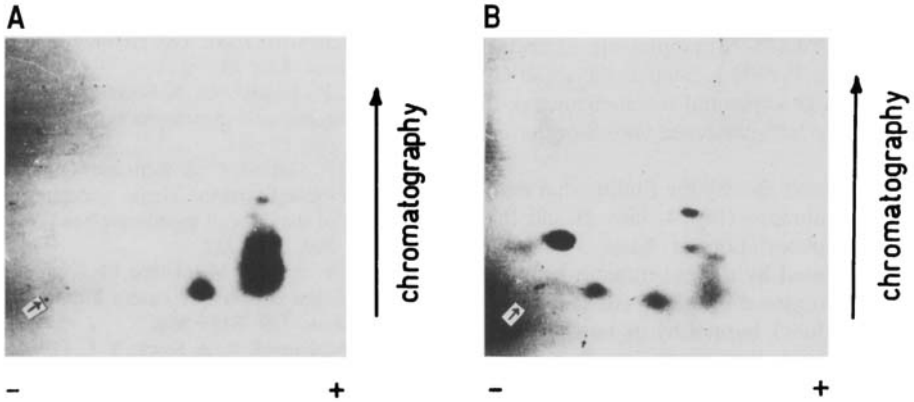

Fig. 6. Electrophoretic pattern of ^{32}P-peptides from tryptically digested membrane band 3 phosphorylated by p36^{syk} (A), and by Lyn protein kinase (B) after p36^{syk}-catalyzed phosphorylation. (A) 10 μg untreated isolated white ghosts were phosphorylated by added p36^{syk} as described in the Methods. (B) The same amount of membrane proteins were phosphorylated by p36^{syk} in the presence of unlabeled ATP, then by Lyn protein kinase in the presence of [γ-^{32}P]ATP, as described in the Methods. The samples were analyzed by SDS/PAGE as described in the Methods. After SDS/PAGE, the slab gels were electrophoretically transferred to nitrocellulose membrane (as described in the Methods) and the excised band 3 was tryptically digested. The resulting [^{32}P]-peptides were separated by two-dimensional thin-layer chromatography as described in the Methods.

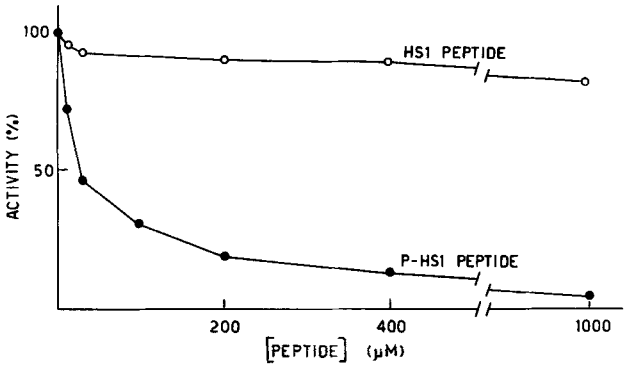

Fig. 7. Inhibition of Lyn-catalyzed phosphorylation of P-Tyr-membranes by added HS1 and P-HS1. The membranes, phosphorylated by p36^{syk} kinase in the presence of unlabeled ATP (see Methods), were phosphorylated by added Lyn protein kinase in the presence of [γ-^{32}P]ATP and increasing amounts of HS1 (empty circles) and P-HS1 (filled circles) peptide, as described in the Methods. After SDS/PAGE, band 3 was excised from the NaOH-treated gels and counted for radioactivity determined in a liquid scintilation counter.

and the Lyn-catalyzed phosphorylation of band 3 (Fig. 7, filled circles), whereas the same unphosphorylated synthetic peptide (HS1; Fig. 4, lane g and Fig. 7, empty circles) was inefficient.

DISCUSSION

Band-3 protein (approximately 95 kDa), the major membrane-spanning protein of human erythrocytes, is phosphorylated not only on Ser/Thr residues by cAMP-independent casein kinase, CKI [1–6], but also on Tyr residues by Tyr-protein kinases [7–18].

Recently, the endogenous Tyr phosphorylation of band 3 in the isolated ghosts incubated with [γ-^{32}P]ATP (in the presence of vanadate and other inhibitors of *P*-Tyr-protein phosphatases) has been reported [17, 18] to involve not only Tyr8 and Tyr21 at the extreme terminus of the N-cytoplasmic domain (as previously reported [7]), but also Tyr904 of the C-terminal region and, to a lesser extent, other Tyr sites, including Tyr359, located near the junction between the membrane-spanning segment of band 3 and its N-cytoplasmic domain (approximately 45 kDa) protruding into the cytosol as an extended flexible polyanionic finger.

The results reported here show that the isolated cytoplasmic domain of band 3 (cdb3, 40/45 kDa), obtained by α-chymotrypsin-catalyzed proteolysis of band 3 in the isolated ghosts, may be Tyr phosphorylated not only by added p36^{syk} [15] (generally considered [21] to be the catalytic fragment yielded by proteolysis of the membrane-bound larger parent p72^{syk} [19]) but also by added Lyn.

However, the two Tyr-kinases, when added to the isolated ghosts, exhibit a different phosphorylation behaviour, i.e. p36^{syk} markedly Tyr phosphorylates the transmembrane band-3 protein, whereas Lyn can phosphorylate it very poorly, if at all (Fig. 3). What is of interest is that Lyn can markedly phosphorylate band 3 only if the band 3 has been previously phosphorylated by p36^{syk} (in the presence of unlabeled ATP; Fig. 5). More precisely (Fig. 6B), the electrophoretic behaviour indicates that the spot more prevalently phosphorylated by Lyn (Fig. 6B) is less negatively charged than the spots phosphorylated by p36^{syk} (Fig. 6A), migrating less than these latter spots towards the anode. This suggests that its target ^{32}P-labeled Tyr is surrounded by less negatively charged residues. However, this finding does not rule out the possibilty that Lyn can also phosphorylate other common sites which, under our experimental conditions, might not be ^{32}P labeled by Lyn, being already blocked by non-radioactive phosphate due to the prior p36^{syk}-catalyzed phosphorylation with unlabeled ATP.

Taking into account that p36^{syk} from human erythrocyte cytosol [15, 16] and from rat spleen [22, 29] has been found to preferentially phosphorylate Tyr residues surrounded by acidic amino acid residues, it seems reasonable to suggest that this latter kinase, when added to isolated ghosts, selectively phosphorylates the Tyr8, Tyr21 and Tyr904, located at the extreme N- and C-terminal regions, respectively, of band 3 and optimally suited for the p36^{syk} kinase, being surrounded by acidic amino acid residues.

Since *P*-Tyr904 of band 3, unlike Tyr8 and Tyr21, is flanked by the specific sequence Tyr-Asp-Glu-Val, which is among the recognition motifs preferred by Lyn (belonging to the Src family) for the binding of its SH2 domain [30], it is tempting to suggest that this p36^{syk}-catalyzed phosphorylation of Tyr904 residue may predispose band-3 protein for the binding of Lyn through its SH2 domain and, consequently, for its Lyn-catalyzed phosphorylation to occur.

This idea is suggested also by the finding that the Lyn-catalyzed phosphorylation of the isolated N-cytoplasmic extended finger of band 3 (cdb 3), lacking Tyr904 located at the small C-terminal tail, does not exhibit the sequential mechanism, i.e. it is not increased by the previous $p36^{syk}$-catalyzed phosphorylation (unpublished results).

Moreover, this idea is suggested also by the finding that both the binding of Lyn to the membranes (Fig. 4, lane f) and the sequential Lyn-catalyzed phosphorylation of band 3 (Fig. 7, filled circles) are strongly inhibited by a Tyr-synthetic peptide, Tyr(*P*)-HS1, previously phosphorylated by $p36^{syk}$ (its target Tyr being surrounded by acidic residues), but not by its unphosphorylated counterpart HS1. However, the possibility that other acidic Tyr-synthetic peptides, with the acidic primary sequence identical to that surrounding Tyr8 and/or Tyr21, lacking Val at the +3 position, may inhibit the binding of Lyn to band 3, cannot be ruled out. Such an SH2-domain-Tyr(*P*)-mediated mechanism may allow the Lyn to approach and phosphorylate more internal Tyr sites, and, in particular, Tyr359 (located near the junction between the N-cytoplasmic domain and the membrane-spanning domain) flanked by non-acidic residues even at the −1 position, as preferred by Lyn [31]

Alternatively, however, it might be that the $p36^{syk}$-catalyzed phosphorylation of Tyr8 and Tyr21 of the N-terminal cytoplasmic domain and of Tyr904 of the C-terminal domain, by increasing the electrostatic repulsion between these two terminal tails of band 3 and/or between the terminal tails of band 3 and the negatively charged membrane inner leaflet, promotes a process of peptide chain folding, thus modifying the conformational structure of the extended flexible cytoplasmic domain (approximately 43 kDa) in such a way as to allow the accessibility of Lyn to Tyr359 for its phosphorylation to occur.

In conclusion, this paper shows that Lyn is able to phosphorylate the transmembrane band 3, but that such a phosphorylation requires $p36^{syk}$ phosphorylation for preparing such a transmembrane protein to be phosphorylated by Lyn. Such a sequential interrelation between these two enzymes present in human erythrocytes may provide further insights into the mechanism operating in band 3 Tyr phosphorylation.

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