Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1745 (2005) 20–28
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Effector-induced Syk-mediated phosphorylation in human erythrocytes☆

Luciana Bordin[a], Florina Ion-Popa[a], Anna Maria Brunati[a], Giulio Clari[a,*], Philip S. Low[b]

[a]Department of Biological Chemistry, University of Padua, Viale G. Colombo 3, 35121 Padua, Italy
[b]Department of Chemistry, Purdue University, 1393 Brown Bldg, West Lafayette, IN 47907-1393, Italy

Received 28 July 2004; received in revised form 9 December 2004; accepted 28 December 2004
Available online 29 January 2005

Abstract

Band 3 (AE1), the most prominent polypeptide of the human erythrocyte membrane, becomes heavily tyrosine phosphorylated following treatment of intact cells with protein tyrosine phosphatase inhibitors such as diamide, pervanadate, vanadate, or *N*-ethylmaleimide (NEM). The mechanism underlying this tyrosine phosphorylation is thought to involve the sequential action of two protein tyrosine kinases, Syk ($p72^{syk}$) and Lyn ($p53/56^{lyn}$). While Lyn catalysed phosphorylation appears to be strictly dependent on prior phosphorylation of Tyr8 and 21 of band 3 by Syk, little is known about the mechanism of induction of Syk phosphorylation. Data presented here show that both the fraction of Syk that associates with the membrane and the extent of phosphorylation of band 3 differ in response to the above inhibitors. While diamide and NEM stimulate syk translocation to the membrane during their induction of band 3 tyrosine phosphorylation, pervanadate and vanadate induce no change in kinase distribution. Moreover, diamide and NEM-induced Syk recruitment to the membrane are phosphotyrosine independent and involve their preferential association with Triton X-100-insoluble membrane skeletons. Together these data reveal a complex process controlling the association and catalytic activity of protein tyrosine kinases syk and lyn with the human erythrocyte membrane.

Keywords: Syk; Tyrosine-phosphorylation; Human erythrocyte; Band 3

1. Introduction

The phosphorylation/dephosphorylation of protein tyrosine residues has been implicated in the regulation of several erythrocyte functions, including metabolism [1–6], membrane transport [7,8], and cell volume and shape [8–10]. In general, the tyrosine phosphorylation state of a protein in the red cell reflects a balance between the competing activities of protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) [11–14]. In the unstimulated human erythrocyte, PTP activity is very high relative to that of PTKs, resulting in a low basal phosphotyrosine level [13,14]. However, upon treatment with PTP inhibitors such as pervanadate, diamide, and NEM [14–17], a large increase in erythrocyte protein tyrosine phosphorylation is observed, with the multifunctional transmembrane protein, band 3, constituting the major site of phosphorylation [9,10,18–21].

Two well characterized protein kinases, Syk ($p72^{syk}$) and Lyn ($p53/56^{lyn}$), are thought to be responsible for the vast majority of band 3 tyrosine phosphorylation [19]. Protein tyrosine kinase Syk, which is comprised of two tandemly arranged Src homology 2 (SH2) domains adjacent to a tyrosine kinase domain, catalyses the so-called "primary phosphorylation" of band 3 at tyrosines 8 and 21 within the cytoplasmic domain of the polypeptide. Subsequently, Lyn, recruited to band 3 through an interaction between its SH2 domain and one of the aforementioned phosphotyrosines, catalyses the "secondary phosphorylation" of band 3 at Tyr359 and 904 [21]. The localization of Lyn next to its phosphorylation sites can be easily explained by its binding to phosphorylated tyrosines 8 and 21 of band 3, which are positioned in the crystal structure adjacent to both Tyr359 and the membrane-spanning domain of band 3 (i.e. the

☆ Supported by Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).
* Corresponding author.
E-mail address: labclari@mail.bio.unipd.it (G. Clari).

0167-4889/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2004.12.010

location of Tyr904 [22]). However, there is no equivalent information regarding the mechanism of the recruitment of Syk to its phosphorylation site on the membrane, raising the question regarding how syk-mediated phosphorylation of tyrosines 8 and 21 is regulated. Thus, although Syk's positioning in its signalling complexes in T cells, B cells and NK cells is mediated by binding to a bis-phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) [23–26], no such sequence has been reported in red cells. Further, Woodside and colleagues [27] have recently found that Syk may interact with the cytoplasmic domains of β-integrins in a phosphotyrosine independent manner, and even if mature human erythrocytes are devoid of integrins, a related binding site might be involved in Syk regulation in erythrocytes. In this paper, we have examined the association of Syk with the human erythrocyte membrane under a variety of conditions that activate the phosphorylation of band 3. We demonstrate that diamide, pervanadate, vanadate and *N*-ethylmaleimide (NEM) induce Syk-mediated phosphorylation of human erythrocyte band 3 to different extents and that Syk becomes tightly associated with the membrane following diamide and NEM treatment, but not following vanadate or pervanadate administration. We also report that Syk's tight association with the membrane is phosphotyrosine independent and that the recruited enzyme is preferentially localized to the Triton X-insoluble membrane skeletal fraction rather than the lipid bilayer.

2. Materials and methods

2.1. Materials

Anti-phosphotyrosine and anti-band 3 monoclonal antibodies were purchased from ICN Biotechnology and Sigma, respectively. Anti-Syk antibody was from Upstate Biochemicals and anti-actin was from Cedarlane. Diamide, vanadate, and *N*-ethylmaleimide (NEM) were purchased from Sigma, and PP2 and protease inhibitor cocktail were obtained from Calbiochem and Roche, respectively. Pure nitrocellulose membrane was purchased from Bio-Rad Laboratories.

2.2. Isolation of human erythrocytes

Human erythrocytes were prepared from fresh blood collected from healthy donors, as previously described [18].

2.3. Anti-phosphotyrosine and anti-Syk immunoblotting

Packed cells, prepared as described above, were suspended (at 20% hematocrit) in buffer A (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 100 μg/ml streptomycin, 25 μg/ml chloramphenicol, 24 mM glucose and 1 mM adenosine). Suspended cells (250 μl for each sample) were incubated for 25 min at 35 °C alone or in the presence of increasing concentrations of diamide, NEM, vanadate, or pervanadate (prepared by mixing 3 mM hydrogen peroxide with 2 mM sodium orthovanadate; see Brunati et al. [21]). Where indicated, 5 μM PP2 inhibitor was added to the incubation mixture together with pervanadate, vanadate, diamide or NEM. After incubation, each sample was centrifuged and the packed cells were hemolysed in 1.5 ml of a hypotonic buffer containing 5 mM sodium phosphate, pH 8, 0.02% NaN_3, 30 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and protease inhibitor cocktail. Membranes were separated from cytosol by centrifugation (20,000×*g* for 20 min) and washed once in the same hypotonic buffer. Aliquots of membranes (10 μg) were then solubilized by adding 2% SDS and 1% β-mercaptoethanol (final concentrations) [18]. After treatment for 5 min at 100 °C, solubilized membrane proteins were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes and immunostained with anti-phosphotyrosine or anti-Syk antibodies. When required, the nitrocellulose paper was stripped and re-immunostained with anti-actin antibodies for use as a sample loading control.

2.4. Immunostaining

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

2.5. Anti-Syk immunoprecipitations (IP)

Packed membranes prepared from 1 ml intact erythrocytes (20% hematocrit) were treated as described above and then extracted for 1 h at 4 °C with buffer B containing 20 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate and the protease inhibitor cocktail. After centrifugation, supernatants were diluted 1:1 in 20 mM Tris–HCl, pH 7.5, 10 mM glycerol, 5 mM NaCl, 50 μM PMSF, and 10 mM *B*-mercaptoethanol, precleared with protein A-Sepharose, and then incubated for 5 h at 4 °C with anti-Syk antibodies bound to protein A-Sepharose. Immune complexes were washed three times in 50 mM Tris–HCl pH 7.5, 1 mM vanadate and protease inhibitor cocktail, and subjected to Western blot analysis by immunostaining with anti-band 3 or anti-phosphotyrosine antibodies.

2.6. Preparation of membrane skeletal and soluble fractions

Membranes, obtained from 200 μl treated and un-treated erythrocytes and recovered as described above, were extracted with 2 volumes buffer C containing 50 mM Tris, pH 7.5, 1% (final) Triton X-100, 1 mM vanadate and

protease inhibitor cocktail for 1 h at 4 °C. After the removal of an aliquot for Western blot analysis, the remainder was microfuged at 80,000×*g* for 40 min. Both supernatant, corresponding to the Triton-soluble fraction, and pellet, corresponding to the Triton-insoluble fraction (membrane skeleton), were then collected and the pellet was resuspended to the same soluble-fraction volume with buffer C. 10 μg of total membrane and the corresponding soluble and cytoskeleton fractions were then subjected to Western blot analysis and revealed with anti-phosphotyrosine or anti-Syk antibodies (or to SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining for use as a sample loading control).

3. Results

3.1. Effect of oxidizing and non-oxidizing protein tyrosine phosphatase inhibitors on band 3 tyrosine phosphorylation and Syk recruitment to the membrane

To explore the mechanism of $p72^{syk}$ regulation in human erythrocytes, we examined the effects of different compounds, known to induce band 3 tyrosine phosphorylation, on both syk association with the membrane and the level of band 3 tyrosine phosphorylation. Compounds employed to promote the tyrosine phosphorylation of band 3 included diamide (a thiol oxidizing agent), vanadate (a phosphate mimic that can inhibit a variety of phosphatases and ATPases), pervanadate (a mixture of vanadate and hydrogen peroxide), and *N*-ethylmaleimide (NEM, a thiol alkylating agent). As shown in Fig. 1A, pervanadate (0.5 mM, lane c) and NEM (1 mM, lane e) induce high levels of band 3 tyrosine phosphorylation, whereas diamide (2 mM, lane b) and vanadate (7 mM, lane d) exert a weaker effect. Surprisingly, at the same concentrations and under the same incubation conditions, syk association with the membrane is enhanced by diamide (lanes b and g) and NEM (lanes e and l), but not by pervanadate (lanes c and h) or vanadate (lanes d and i). Thus, while pervanadate stimulates band 3 tyrosine phosphorylation maximally, it does not induce syk association with the membrane, and although diamide induces band 3 phosphorylation minimally, it nevertheless promotes strong syk retention on the membrane. These data, therefore, suggest that tyrosine phosphorylation of band 3 is not dependent on syk binding to the membrane.

Although previous research [21] has suggested that syk phosphorylation of band 3 precedes and prepares band 3 for lyn binding and phosphorylation, it was still conceivable that some level of lyn phosphorylation might have been required to enable the syk binding/phosphorylation of band 3. To explore this possibility, we exploited the ability of PP2, a src family kinase inhibitor, to selectively inhibit lyn without blocking the activity of syk. By preventing any

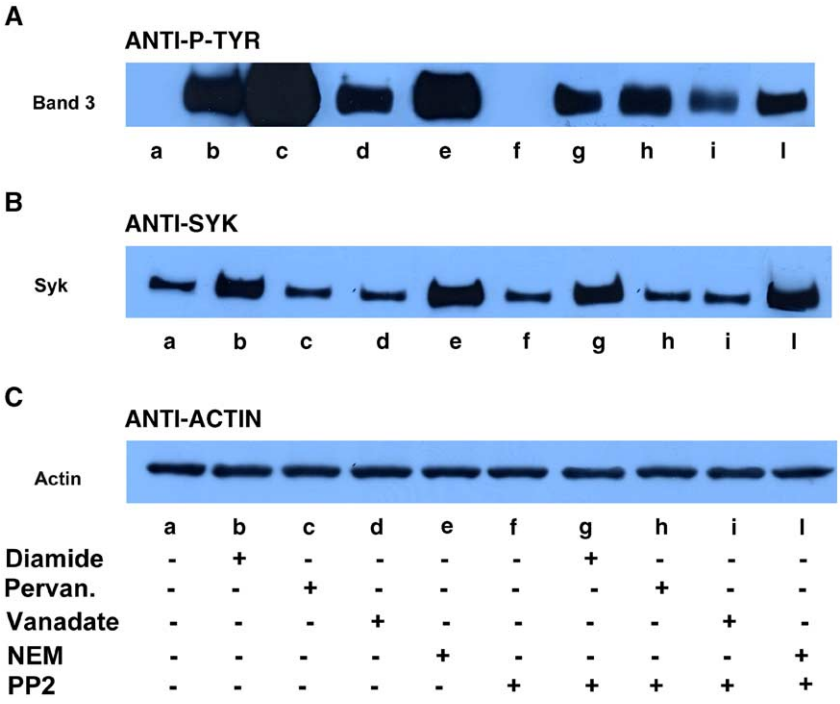

Fig. 1. Effect of different compounds on band 3 Tyr-phosphorylation (A) and Syk recruitment to membranes (B). Erythrocytes were treated with diamide 2 mM (lanes b and g), pervanadate 0.5 mM (lanes c and h), vanadate 7 mM (lanes d and i), or NEM (lanes e and l), in the absence (lanes a–e) or presence (lanes f–l) of 5 μM PP2. Lanes a and f represent the pattern from an RBC control. Membranes, obtained as described in the Materials and methods, were analysed by Western blot and revealed with anti-P-Tyr (panel A) and anti-Syk antibodies (panel B). In panel C we show the Western blot of panel B stripped and re-immunostained with anti-actin antibodies for loading control. Panels are representative of at least three separate experiments.

background phosphorylation of band 3 by lyn, we were able to ask whether syk binding and the phosphorylation of band 3 was in any way dependent on lyn. As seen in Fig. 1A (compare lanes g–l with lanes b–e), incubation with PP2 partially reduced band 3 tyrosine phosphorylation, but did not eliminate it (presumably due to its inhibition of lyn but not of syk). More importantly, PP2 had no effect on syk translocation to the membrane (compare Fig. 1B, lanes f–l

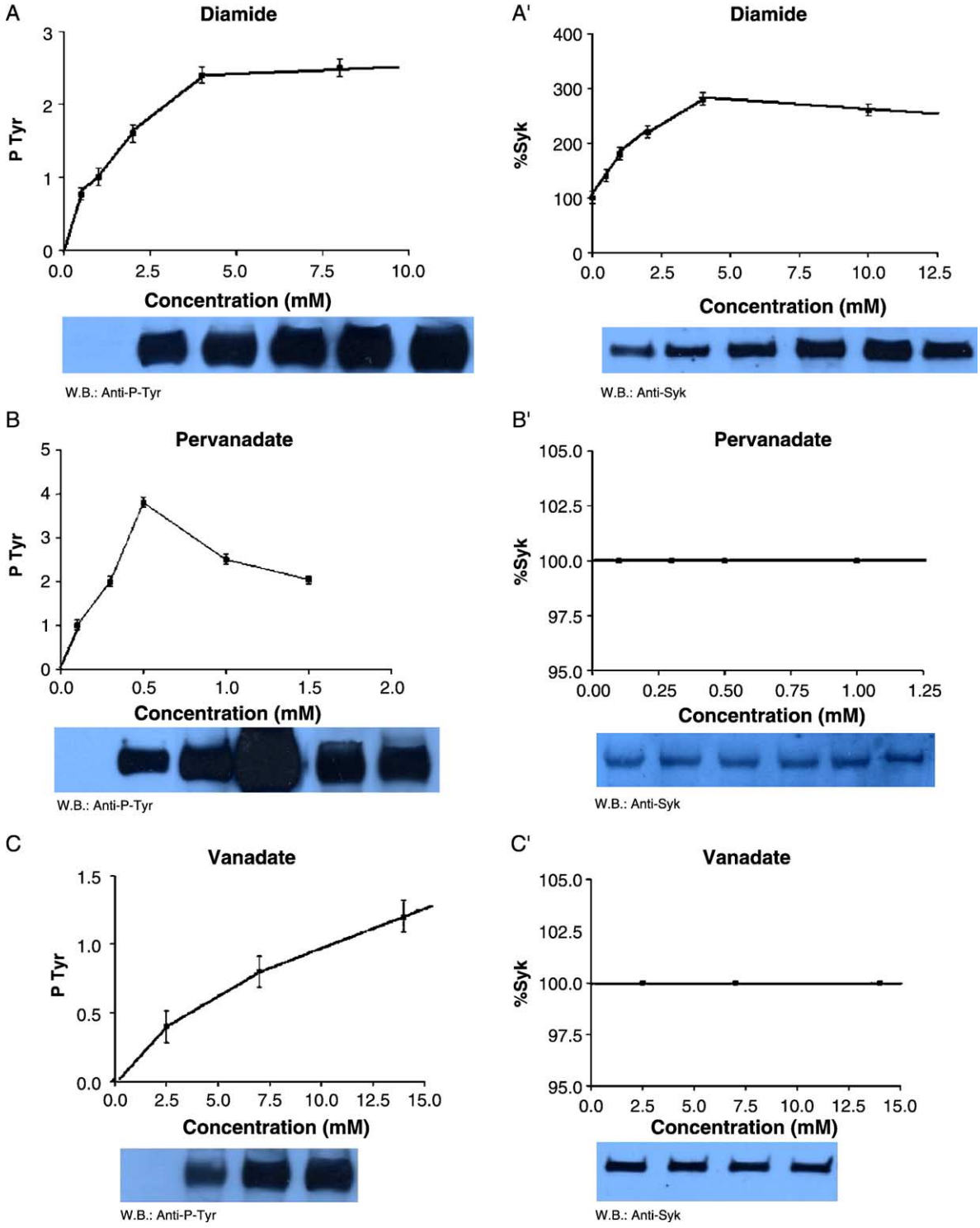

Fig. 2. Dose-dependent effect of diamide (A, A′), pervanadate (B, B′), vanadate (C, C′), and NEM (D, D′) on band 3 Tyr-phosphorylation and Syk recruitment. Erythrocytes were treated with increasing concentrations of each compound and membranes analysed by Western blot, were revealed with anti-P-Tyr (A, B, C, D) or anti-Syk (A′, B′, C′, D′) antibodies and the corresponding stains were counted in a densitometer. For the band 3-Tyr-P evaluation an arbitrary unit was chosen, whereas for the Syk recruitment, the amount of syk present in resting cells was used as 100% value.

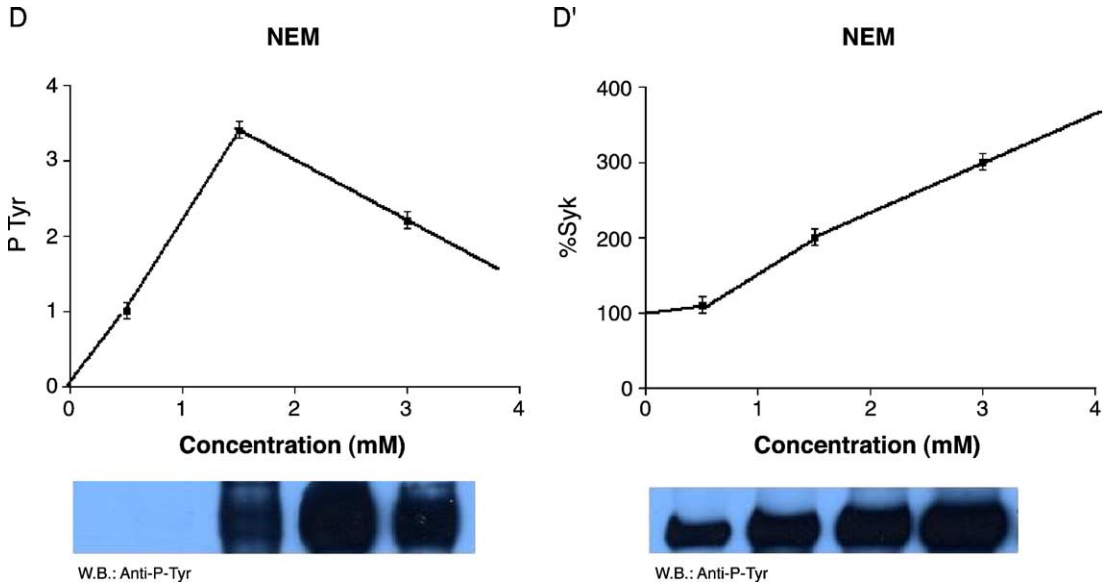

Fig. 2 (*continued*).

in the presence of PP2 with lanes a–e in the absence of PP2). These data thus preclude any requirement for lyn phosphorylation of tyrosines 359 and 904 in the recruitment of syk to the erythrocyte membrane.

To more thoroughly investigate any possible relationship between band 3 tyrosine phosphorylation and Syk association with the membrane, we evaluated the concentration dependence of each of the above four compounds on both enrichment of syk on the membrane and band 3 phosphorylation. As shown in Fig. 2, each of the four effectors displays a different concentration dependence in its effect on band 3 tyrosine phosphorylation and Syk enrichment on the membrane. Thus, pervanadate (panels B and B′) and NEM (panels D and D′) induce tyrosine phosphorylation to similar levels, but differ greatly in their abilities to promote Syk retention on the membrane, since pervanadate does not alter Syk distribution, whereas NEM promotes the highest level of enzyme recruitment among all compounds tested. Further, vanadate (panels C and C′) induces significantly less band 3 tyrosine phosphorylation than pervanadate, but is indistinguishable in its inability to stimulate Syk translocation, whereas diamide (panels A and A′) appears to display a simple saturation behavior in its impact on both band 3 phosphorylation and Syk binding to the membrane. Besides emphasizing that each of the above stimulants of erythrocyte tyrosine phosphorylation behaves differently, these findings confirm the hypothesis that there is no essential link between band 3 tyrosine phosphorylation and Syk recruitment to the membrane.

3.2. Reversibility of band 3 Tyr-phosphorylation and Syk translocation

Because the erythrocyte tyrosine phosphorylation thought to regulate erythrocyte processes is reversible (i.e. glycolysis, membrane transport, and cell volume/shape control), the question naturally arose whether the translocation of syk to the membrane might also reverse following the removal of the added stimulant. Although NEM was already known to mediate irreversible alkylation of membrane thiol groups, diamide was considered potentially reversible, since it is often used as a surrogate for inducing physiological disulfide bond formation. In order to explore this latter possibility, RBCs were treated with 2 mM diamide and examined for both the quantity of membrane-associated syk and the level of band 3 tyrosine phosphorylation as a function of time in the presence or absence of dithiothreitol. As seen in Fig. 3, band 3 tyrosine phosphorylation was observed to decrease dramatically following the removal of diamide and the re-incubation of the cells for 20 min in buffer A (lane c). In fact, when incubation in the absence of diamide was continued for 90 min (lane e), phosphorylation was observed to return to its basal level (lane a). Further, when 2 mM DTT was added after the removal of diamide, band 3 phosphorylation returned to the control level within the initial 20 min of re-incubation (lane d). Thus, diamide-induced tyrosine phosphorylation is slowly reversible (~90% in <20 min) in the absence of exogenous reducing power, but rapidly reversible in its presence.

To learn whether syk recruitment to the membrane exhibits the same reversibility, the same membrane preparations were also immunoblotted for syk content. As seen in panel B, lanes a–f, syk association with the membrane conforms roughly with the kinetics of band 3 tyrosine phosphorylation, displaying nearly complete reversal by 20 min in the absence of dithiothreitol and complete reversal by either 90 min in the absence of dithiothreitol or 20 min in its presence. When a higher concentration of diamide (4 mM) was examined (Fig. 3′), diamide removal and DTT addition succeeded in dephosphorylating band 3 as seen with 2 mM

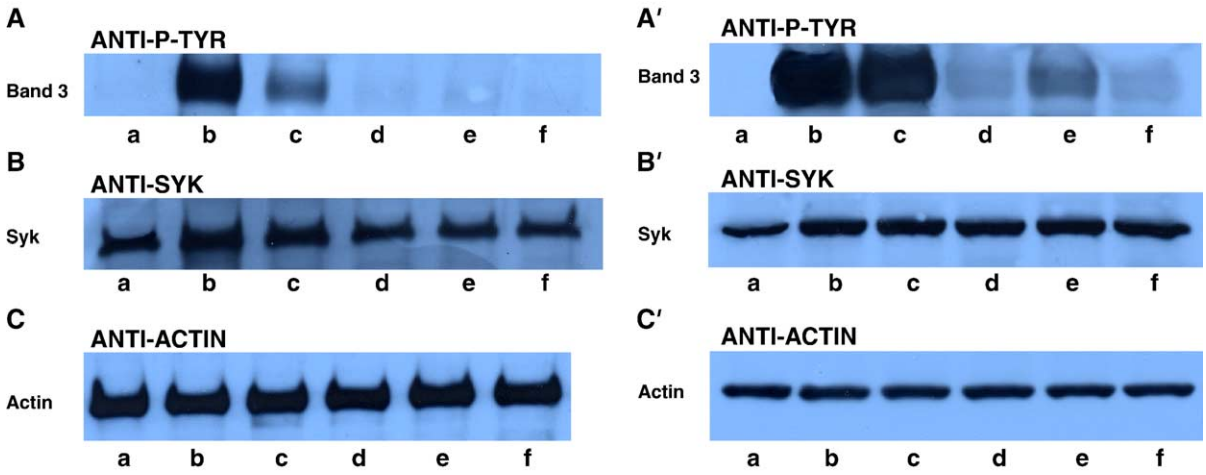

Fig. 3. Effect of band 3 Tyr-phosphorylation reversing conditions on Syk recruitment. Erythrocytes were treated with 2 mM (Fig. 3) or 4 mM (Fig. 3′) diamide for 25′ at 35 °C and immediately processed (lanes b), or washed and re-incubated in buffer A (lanes c and e), or in buffer A supplemented with DTT (lanes d and f) up to 20′ (lanes c and d) or 90′ (lanes e and f). Lanes a represent pattern obtained from RBC control. Membranes were analysed by Western blot and revealed with anti-P-Tyr (panel A and A′) or anti-Syk (panel B and B′). In panels C and C′ we show Western blot of panel B and B′ stripped and re-immunostained with anti-actin antibodies for loading control. Panels are representative of at least three separate experiments.

diamide, but the diamide-induced increase in Syk association with the membrane was not reversed, thus further confirming the hypothesis that diamide-induced syk recruitment does not follow the band 3 tyrosine phosphorylation. Further, it may be suggested that high diamide concentrations, besides reversibly inhibiting the PTP-ase activity, may induce an irreversible alteration in RBC membrane proteins that prevents diamide-recruited syk from being released.

3.3. Partial characterization of the membrane binding sites for syk

Because the only detectable substrate of $p72^{syk}$ in human erythrocytes is band 3 [19], it seemed reasonable to postulate that the binding site for syk on the membrane might involve a cytoplasmically exposed region of band 3. To examine this possibility, syk was immunoprecipitated from Nonidet P-40 solubilized red cell membrane extracts, and the resulting immune complexes were immunoblotted for the presence of band 3. As revealed in Fig. 4, the amount of band 3 that was co-precipitated with syk was essentially the same in both control extracts (lanes a) and extracts from diamide (lanes b) or NEM (lanes c) treated cells. Since diamide treatment roughly doubles the amount of syk associated with the membrane, these data suggest that band 3 does not constitute the new docking site for the diamide/NEM recruited syk. In fact, since essentially the same amount of band 3 is co-pelleted with syk from both stimulated and unstimulated membranes alike, it would appear that a subpopulation of Syk must constitutively bind band 3, regardless of the amount of stimulant added, and that when additional Syk is recruited to the membrane following effector addition, it does not bind to band 3. Besides, the anti-phosphotyrosine staining of the same panels definitively excludes the phosphotyrosine residues of band 3 as the new syk docking site, since band 3 in the control does not present any phosphorylation compared to the phosphorylated band 3 induced by diamide and NEM (lane a compared to lanes b and c, respectively).

Moreover, what is relevant is that diamide and NEM seem to be able to activate the enzyme as indicated by its tyrosine phosphorylation (Fig. 4B, lanes b and c compared to the total absence of syk phosphorylation of the control

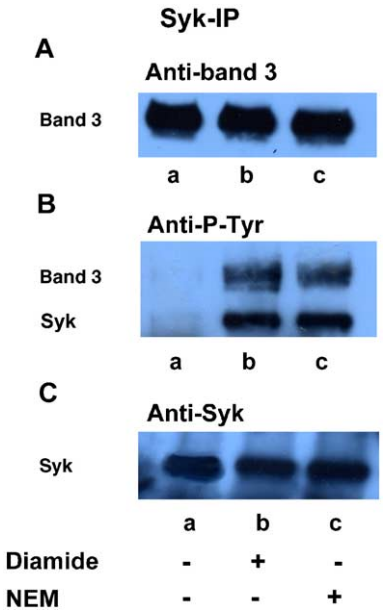

Fig. 4. Syk immunoprecipitation. Erythrocytes were treated in the absence (lane a) or presence of 4 mM diamide (lanes b) or 1.5 mM NEM (lanes c), and membranes, obtained as described in the Materials and methods, were solubilized and immunoprecipitated in the presence of anti-Syk antibodies. Samples were subjected to Western blot and immunorevealed with anti-band 3 (panel A), anti-P-Tyr (panel B) or anti-Syk antibodies (panel C). Panels are representative of at least seven separate experiments.

lane a), which has been described to be representative of syk activation [28]. It should also be noted that these two compounds induce a similar activation of syk, thus confirming previous data obtained with pervanadate [19,21] and under oxidative stress conditions [29], but not with peroxynitrite which, besides being an oxidant similar to pervanadate and diamide, is able to induce nitrotyrosine formation, leading to partial syk inhibition [30].

3.4. Membrane location of diamide and NEM recruited syk

To partially localize the membrane docking site of the NEM/diamide-recruited syk, membranes from diamide or NEM treated erythrocytes, recovered as previously described, were extracted with 1% Triton X-100 (see Materials and methods) and centrifuged to separate the membrane skeletal fraction from the detergent-soluble fraction, which were found to contain 65% and 35% of

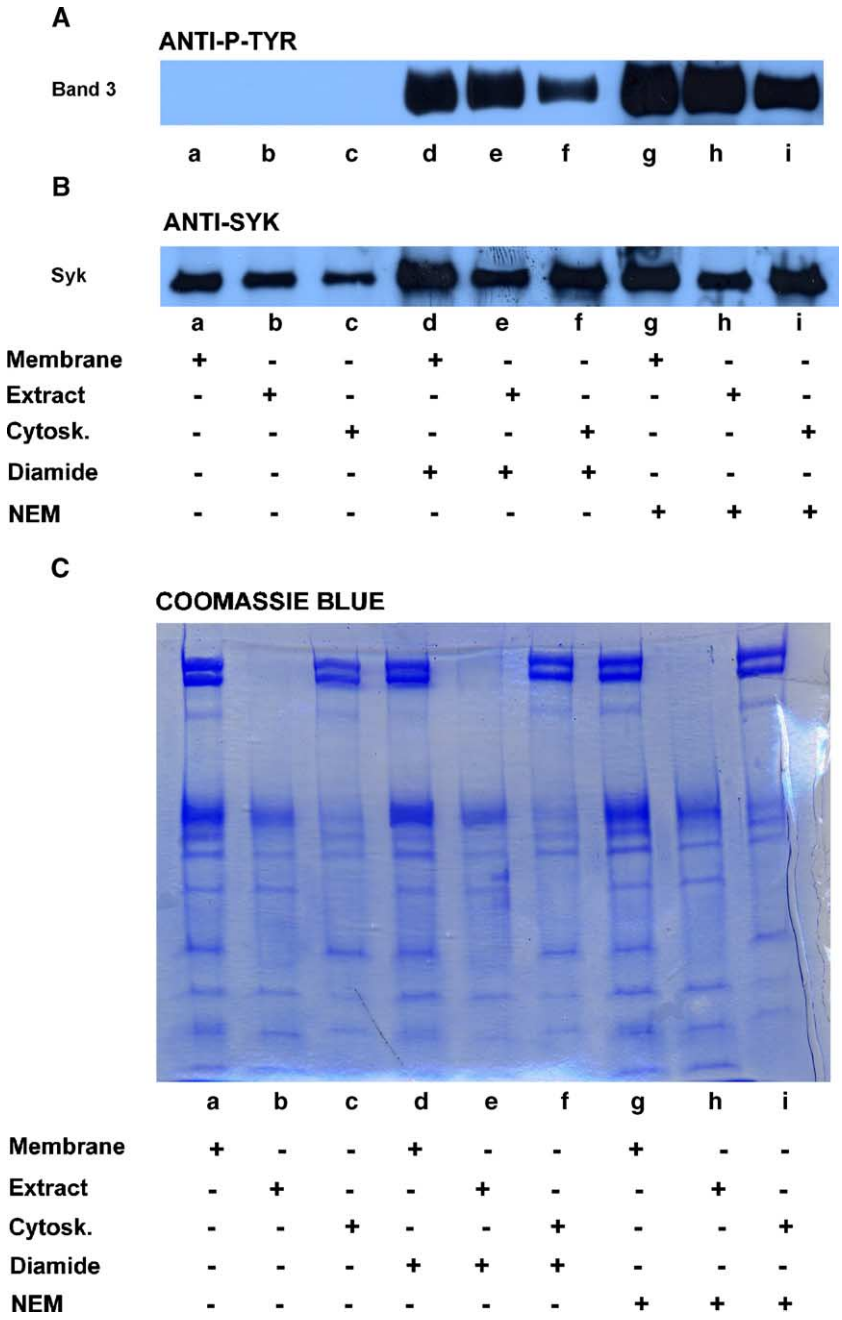

Fig. 5. Distribution of recruited Syk to membranes. Erythrocytes were treated with diamide 2 mM (lanes d–f) or NEM 1 mM (lanes g–i) and membranes, obtained as described before, were extract with Triton X-100 1%. Membranes (lanes a, d, g), and extract (soluble) (lanes b, e, h), or cytoskeletal (insoluble) (lanes c, f, i) fractions, collected by ultracentrifugation (see Materials and methods) were analysed by Western blot and revealed by anti-P-Tyr (panel A) or anti-Syk (panel B) antibodies. Lanes a–c represent patterns obtained by RBC control. Pattern 5C represent the corresponding Coomassie blue of the above patterns for loading and control. The figure represents three separate experiments.

the total membrane band 3, respectively, as evaluated by densitometry (using UN-SCAN-IT software). Both fractions were then analysed by Western blotting using anti-phosphotyrosine (panel A) or anti-Syk (panel B) antibodies to reveal the corresponding antigens. As shown in Fig. 5B, diamide (lanes d–f) and NEM (lanes g–i) recruited syk (about 80% more than the control) preferentially to the membrane skeletal fraction (compare lanes c, f and i), with little net recruitment to the soluble fraction (compare lanes b, e, and h). Taken together with the data in Fig. 5A, these data suggest that syk may associate with two sites on the membrane, one that is detergent-soluble and one that is not. This conclusion is in agreement with the amount of Syk immunoprecipitated in both treated and untreated cells (Fig. 4, panel C) that did not increase after cell treatment. In fact, immunoprecipitation experiments were carried out with membrane extracts, containing the detergent-soluble syk that does not increase following diamide/NEM treatments, while the detergent-insoluble syk counterpart, which is sensitive to the effector-induced translocation, cannot be immunoprecipitated.

4. Discussion

The flux of ions across the human erythrocyte membrane [7,8], the rate of erythrocyte glycolysis [2,3], and the volume and shape of the human red cell [8–10] have all been reported to respond to changes in erythrocyte protein tyrosine phosphorylation. Because changes in tyrosine phosphorylation in other cell types has frequently been associated with the translocation of the responsible protein tyrosine kinase to a macromolecular signalling complex on the membrane, it seemed reasonable to inquire whether increases in erythrocyte phosphotyrosine content might similarly involve the translocation of the kinase to a new signalling site on the membrane. Recognizing that tyrosine kinase signalling in the human erythrocyte begins with the phosphorylation of band 3 by $p72^{syk}$ and then proceeds with activation and phosphorylation by $p53/56^{lyn}$ [21], we undertook to evaluate whether the translocation of syk might be central to the initiation of tyrosine kinase signalling in the human red cell. Surprisingly, under our conditions of membrane isolation, no correlation was found between syk translocation and membrane protein phosphorylation. Thus, whereas pervanadate was observed to constitute the most potent inducer of membrane tyrosine phosphorylation (followed by NEM, diamide and finally vanadate), pervanadate was unable to promote any change in syk association with the membrane. In contrast, whereas NEM and diamide were clearly less effective in promoting band 3 tyrosine phosphorylation, they were considerably more effective in stimulating syk accumulation on the membrane. Thus, in contradistinction to the situation in other cells, where syk translocation is critical to its participation in cell receptor signalling [23–26], the translocation of syk in the erythrocyte does not appear to constitute an essential step in phosphorylating band 3.

Conceivably, the NEM/diamide triggered movement of syk to the membrane might be involved in positioning the kinase to regulate changes in the membrane skeleton. Thus, NEM and diamide do not significantly increase syk binding to the detergent extractable fraction, but they do significantly elevate syk content in the membrane skeleton (Fig. 5).

Whereas membrane localization of syk in B cells is believed to occur as a consequence of the association of its tandem SH2 domains with proximal phosphotyrosines on the B cell receptor [25], several lines of evidence suggest that membrane binding of syk in both stimulated and unstimulated erythrocytes is unrelated to membrane phosphotyrosine content. Thus, syk is already partly membrane localized in unstimulated erythrocytes, which have virtually nondetectable levels of phosphotyrosine (Fig. 1). Further, the inhibition of lyn's catalytic activity has no effect on the intracellular distribution of syk (Fig. 1), suggesting that lyn provides no phosphotyrosine sites for syk binding. Third, the immunoprecipitation of syk (Fig. 4) co-pellets a population of band 3 with a significantly lower phosphotyrosine content than the residual band 3 in the same membranes. Thus, it would appear that the docking site for syk on red cell membranes is not phosphotyrosine dependent.

What then promotes the membrane association of syk in NEM treated erythrocytes? Disulfide bond formation can be ruled out, since treatment with NEM actually blocks sulfhydryls and prevents disulfide bridging rather than promotes their formation. SH3 domain mediated interactions are also not likely responsible, since syk has neither an SH3 domain nor a proline-rich sequence capable of interacting with one. Since the translocation is readily reversible and occurs in Ca^{2+}-free buffers, transglutaminases and other covalent bridges are also not likely involved. Thus, by process of elimination, we speculate that some type of protein conformational change in the membrane skeleton must generate a new or more accessible binding site that is otherwise occluded or inaccessible in non-stimulated cells, and to which Syk remains tightly associated even under membrane isolation conditions that might elute some enzymes from the membrane. Perhaps a site similar to that described by Woodside et al. [27,31], who characterized a Syk-integrin β interaction that requires no tyrosine phosphorylation but still involves an association with the N-terminal SH2 domain, is involved. In this line of evidence, while in other cells the activation step involves receptor modification, in human erythrocytes it might be ascribed to the alterations induced in the membrane by the protein tyrosine phosphatase inhibitors.

In conclusion, pervanadate, vanadate, diamide and NEM all induce the phosphorylation of erythrocyte membrane proteins, predominantly band 3, by $p72^{syk}$. Whereas NEM and diamide also promote syk translocation to the mem-

brane skeleton, vanadate and pervanadate induce no kinase redistribution. The syk binding site in resting cells resides predominantly on the cytoplasmic domain of band 3, and both this site and the inducible site in the membrane skeleton are not dependent on SH2 domain–phosphotyrosine interactions. The physiological functions of syk signalling in erythrocytes still remain to be elucidated.

Acknowledgment

Grateful thanks to Mr. Dario Spinello for supplying fresh blood from volunteers.

References

[1] P.S. Low, D.P. Allen, T.F. Zioncheck, P. Chari, B.M. Willardson, R.L. Geahlen, M.L. Harrison, Tyrosine phosphorylation of band 3 inhibits peripheral protein binding, J. Biol. Chem. 262 (1987) 4592–4596.

[2] M.L. Harrison, P. Rathinavelu, P. Arese, R.L. Geahlen, P.S. Low, Role of band 3 tyrosine phosphorylation in the regulation of erythrocyte glycolysis, J. Biol. Chem. 266 (1991) 4106–4111.

[3] P.S. Low, P. Rathinavelu, M.L. Harrison, Regulation of glycolysis via reversible enzyme binding to the membrane protein band 3, J. Biol. Chem. 268 (1993) 14627–142631.

[4] R.S. De Neef, M.D. Hardy-Dessources, F. Giraud, Relationship between type II phosphatidylinositol 4-kinase activity and protein tyrosine phosphorylation in membranes from normal and sickle red cells, Eur. J. Biochem. 235 (1996) 549–556.

[5] G. Minetti, G. Piccinini, C. Balduini, C. Seppi, A. Brovelli, Tyrosine phosphorylation of band 3 protein in Ca^{++}/A23187-treated erythrocytes, Biochem. J. 320 (1996) 445–450.

[6] G. Mallozzi, A.M.M. Di Stasi, M. Minetti, Peroxinitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3, FASEB J. 11 (1997) 1281–1290.

[7] L. De Franceschi, L. Fumagalli, O. Olivieri, R. Corrocher, C.A. Lowell, G. Berton, Deficiency of Src family kinases Fgr and Hck results in activation of erythrocyte K/Cl cotransport, J. Clin. Invest. 99 (1997) 220–227.

[8] M.W. Musch, E.M. Hubert, L. Goldstein, Volume expansion stimulates $p72^{syk}$ and $p56^{lyn}$ in skate erythrocytes, J. Biol. Chem. 274 (1991) 7923–7928.

[9] L. Bordin, G. Clari, I. Moro, F. Dalla Vecchia, V. Moret, Functional link between phosphorylation state of membrane proteins and morphological changes of human erythrocytes, Biochem. Biophys. Res. Commun. 213 (1995) 249–257.

[10] G. Minetti, C. Seppi, A. Ciana, C. Balduini, P.S. Low, A. Brovelli, Characterization of the hypertonically induced tyrosine phosphorylation of erythrocyte band 3, Biochem. J. 335 (1998) 305–311.

[11] P. Boivin, C. Galand, The human red cell acid phosphatase is a phosphotyrosine protein phosphatase which dephosphorylates the membrane protein band 3, Biochem. Biophys. Res. Commun. 134 (1986) 557–564.

[12] G. Clari, A.M. Brunati, V. Moret, Membrane-bound phosphotyrosil–protein phosphatase activity in human erythrocytes. Dephosphorylation of membrane band 3 protein, Biochem. Biophys. Res. Commun. 142 (1987) 587–594.

[13] L. Bordin, A.M. Brunati, A. Donella-Deana, B. Baggio, A. Toninello, G. Clari, Band 3 is an anchor protein and a target for SHP-2 tyrosine phosphatase in human erythrocytes, Blood 100 (2002) 276–282.

[14] Y. Zipser, A. Piade, N. Kosower, Erythrocyte thiol status regulates band 3 phosphotyrosine level via oxidation/reduction of band 3-associated phosphotyrosine phosphatase, FEBS Lett. 406 (1997) 126–130.

[15] S. Singh, S.B.B. Aggarwal, Protein-tyrosine phosphatase inhibitors block tumor necrosis factor dependent activation of the nuclear transcription factor NF-kappa B, J. Biol. Chem. 270 (1995) 10631–10639.

[16] S. Dhawan, S. Singh, B.B. Aggarwal, Induction of endothelial cell surface adhesion molecules by tumor necrosis factor is blocked by protein tyrosine phosphatase inhibitors: role of the nuclear transcription factor NF-kappa B, Eur. J. Immunol. 27 (1997) 2172–2179.

[17] V. Natarajan, W.M. Scribner, S. Vepa, Phosphatase inhibitors potentiate 4-huydroxynomenal-induced phospholipase D activation in vascular endothelial cells, Am. J. Respir. Cell Mol. Biol. 17 (1997) 251–259.

[18] A.M. Brunati, L. Bordin, G. Clari, V. Moret, The Lyn-catalyzed Tyr-phosphorylation of the transmembrane band 3 protein of human erythrocytes, Eur. J. Biochem. 240 (1996) 394–399.

[19] M.L. Harrison, C.C. Isaacson, D.L. Burg, R.L. Geahlen, P.S. Low, Phosphorylation of human erythrocyte band 3 by endogenous $p72^{syk}$, J. Biol. Chem. 269 (1994) 955–959.

[20] F.D.P. Tuy, C. Rosenfeld, A. Kahn, High tyrosine kinase activity in normal non-proliferating cells, Nature 305 (1983) 435–438.

[21] A.M. Brunati, L. Bordin, G. Clari, P. James, M. Quadroni, E. Baritono, L.A. Pinna, A. Donella-Deana, Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites, Blood 96 (2000) 1550–1557.

[22] D. Zhang, A. Kiyatkin, J.T. Bolin, P.S. Low, Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3, Blood 96 (2000) 2925–2933.

[23] V. Chitu, R. Fajka-Boja, G.K. Toth, G. Varadi, Z. Hegedus, A. Franko, K.S. Szucs, E. Monostori, Comparative study on the effect of phosphorylated TCR zeta chain ITAM sequences on early activation events in Jurkat T cells, Peptides 22 (2001) 1963–1971.

[24] L.P. Kane, J. Lin, A. Weiss, Signal transduction by the TCR for antigen, Curr. Opin. Immunol. 12 (2000) 242–249.

[25] M. Merchant, R.G. Caldwell, R. Longnecker, The LMP2A ITAM is essential for providing B cells with development and survival signals in vivo, J. Virol. 74 (2000) 9115–9124.

[26] L.L. Lanier, A.B.H. Bakker, The ITAM-bearing transmembrane adaptor DAP12 in lymphoid and myeloid cell function, Immunol. Today 21 (2000) 611–614 (Review).

[27] D.G. Woodside, A. Obergfell, L. Leng, J.L. Wilsbacher, C.K. Miranti, J.S. Brugge, S.J. Shattil, M.H. Ginsberg, Activation of Syk protein kinase through interaction with integrin β cytoplasmic domains, Curr. Biol. 11 (2001) 1799–1804.

[28] O. El-Hillal, T. Kurosaki, H. Yamamura, J.-P. Kinet, A.M. Scharenberg, Syk kinase activation by a src kinase-initiated activation loop phosphorylation chain reaction, Proc. Natl. Acad. Sci. 94 (1997) 1919–1924.

[29] S. Qin, T. Kurosaki, H. Yamamura, Differential regulation of oxidative and osmotic stress induced syk activation by both autophosphorylation and SH2 domains, Biochemistry 37 (1998) 5481–5486.

[30] C. Mallozzi, A.M.M. Di Stasi, M. Minetti, Activation of src tyrosine kinases by peroxynitrite, FEBS Lett. 456 (1999) 201–206.

[31] D.G. Woodside, A. Obergfell, A. Talapatra, D.A. Calderwood, S.J. Shattil, M.H. Ginsberg, The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin β cytoplasmic domains, J. Biol. Chem. 277 (2002) 39401–39408.