EFFECT OF PHOSPHOLIPASE-A_2 ON SYK-MEDIATED PHOSPHORYLATION OF HUMAN ERYTHROCYTE MEMBRANE

LUCIANA BORDIN[1], FLORINA ION-POPA[1], ANNA MARIA BRUNATI[1], GIULIO CLARI[1]

Keywords erythrocytes, kinases, phosphorylation

Abstract. In human erythrocytes the regulation of Tyr-phosphorylation of membrane proteins, mainly band 3, involves two Tyr-protein kinases, Syk and Lyn, in a two steps mechanism. However, two isoforms of Syk are identified, p72 and p36, which is a product of proteolysis of the larger isoform. Comparing efficiency of both isoforms in phosphorylating band 3, $p36^{syk}$ results clearly much more effective than $p72^{syk}$. A reorganization of membrane lipidic structure induced by PLA_2 treatment can increase $p72^{syk}$-catalysed phosphorylation of band 3 up to four times than in the control. This suggests that tandem SH2 domains of $p72^{syk}$, lacking in p36 isoform, are responsible of the lower ability of kinase of catalysing band 3 Tyr-phosphorylation, probably due either to steric bulk, or to particular sequestering of the holoenzyme into membrane compartment.

INTRODUCTION

Phosphorylation/dephosphorylation of protein tyrosil residues has been implicated in the regulation of several erythrocytes functions including metabolism (Low 1987; Harrison 1991; Low 1993; De Neef 1996; Minetti 1996; Mallozzi 1997), membrane transport (De Franceschi 1997; Musch 1999), cell volume and shape (Musch 1999; Bordin 1995; Minetti 1998). The tyrosil phosphorylation state of a protein in the cell reflects the balance between the competing activities of the protein tyrosine kinases (PTK) and the protein tyrosine phosphatases (PTP). Normally, the activity of PTP is very high relative to that of PTK, thus resulting in the maintenance of very low basal levels of phosphotyrosine in the cell (Brautigan 1992; Mauro 1994; Walton 1993).

As in the case of other cells and proteins, Tyr-phosphorylation is markedly enhanced upon treatment with PTPase inhibitors such as pervanadate diamide and NEM (Singh 1995; Dhawan 1997; Natarajan 1997) that may trigger an increase of protein tyrosin phosphorylation, and in red blood cells the multifunctional transmembrane protein band 3 has been found to represent the major Tyr-phosphorylated protein (Bordin 1995; Minetti 1998; Brunati 1996; Harrison 1994; Tuy 1983; Brunati 2000).

In human erythrocytes two are the kinases involved in band 3 Tyr-phosphorylation, Syk and Lyn, belonging to the Syk and Src family, respectively (Harrison 1994).

Protein kinase Syk, which comprises two tandemly arranged Src homology 2 (SH2) domains and a tyrosine kinase domain, catalyses the so called “primary phosphorylation” of Tyr8 and 21, located in the cytoplasmic domain of band 3. Successively, Lyn recruited to band 3 through its SH2 domain recognizing one of these two P-tyrosines, catalyses the “secondary phosphorylation” of Tyr359 and 904 of band 3 (Brunati 2000).

What raises up from previous findings is that Lyn activity is strictly dependent on its recruitment to Syk-phosphorylated band 3 Tyr residues (Brunati 2000). However, what triggers/regulates Syk activity is still in processing.

Syk has been described to bind dis-phosphorylated immunoreceptor tyrosine-based activating motif (ITAM) of the cytoplasmic region of immunoreceptors such as T-cell receptor (TCR), B-cell receptor (BCR), Fc-receptors and NK cell receptors. This Syk recruitment, besides targeting its action results in improving/activating enzyme. Recently it has been found that Syk may interact with integrin cytoplasmic domains also in a phospho-tyrosine independent manner (Woodside 2001, Woodside 2002), thus introducing a novel paradigm for the regulation of Syk kinase.

This study is performed to evaluate the efficiency of the Tyr-phosphorylation of membrane band 3 by Syk, and deals with a comparison between the two isoforms of this enzyme present in human erythrocytes, p72 and p36, this latter being product by a proteolytic cleavage from the former larger enzyme (Zioncheck 1988). Besides, we report that $p72^{syk}$ exogenously added to membranes is retained while $p36^{syk}$ is promptly recovered in the supernatant. Moreover, changing membrane structure organization by PLA_2-catalysed lipid breakdown induces a net increase of membrane band 3 Tyr-phosphorylation by $p72^{syk}$, while no detectable variation of $p36^{syk}$ catalysis is detected, being maximum even in the absence of PLA_2 membrane treatment.

MATERIALS AND METHODS

Materials

PLA_2 was purchase by Calbiochem (LaJolla, CA), [-^{32}P]ATP was from Amersham Internation, protease inhibitor cocktail from Roche, and other reagents from Sigma.

Methods

Human erythrocytes were prepared by centrifugation (at 750 x g for 3 min) of fresh blood collected from healthy donors, and washed three times by centrifugation in buffer A (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 24 mM glucose, 25 g/ml chloramphenicol, 0.1 mg/ml streptomycin) and 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 (Brunati 1996).

PLA_2 treatment of isolated white ghosts

Aliquots (100 g) of isolated white ghosts were incubated for 20 min at 30°C in buffer Imidazole (0.5 M, pH 8.5) containing 1 mM $CaCl_2$, in the presence or absence of PLA_2 (20 units). After centrifugation (20 min at 13000 x g) membranes were recovered and washed three times in buffer B (25 mM Tris-HCl, pH 8, 1 mM vanadate and inhibitor cocktail) and resuspended in 0.5 M Tris-HCl, pH 7.5.

Purification of Tyr-protein kinases p72^{syk} and p36^{syk}

Tyrosine kinase p72^{syk} and its catalytic subunit p36^{syk} were isolated and purified from rat spleen as previously described (Brunati 1996).

Tyr-phosphorylation assays

Tyr-phosphorylation of membrane proteins was performed by separately incubating 10 g of PLA_2-treated and –untreated white ghosts at 30°C for the indicated time in 30 l of the reaction mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 20 M [-^{32}P]ATP ($3x10^6$ cpm/nmol) and 0.1 mM vanadate in the presence of p72^{syk} or its catalytic subunit p36^{syk} (70 units).

The reactions were stopped by addition of 2% SDS and 1% 2-mercaptoethanol (final concentration) followed by a 5-min treatment at 100°C. The solubilized membranes were analysed by 0.1% SDS/ 8% PAGE according to Laemmli (1970).

After electrophoresis, gels were stained with Coomassie brilliant blue according to (Laemmli 1970), submitted to 2 M NaOH treatment at 55°C for 1h and fixed again. Dried gels were autoradiographed at –80°C with intensifying screens.

1 unit Tyr-protein kinase was defined as the amount of enzyme catalysing the incorporation of 1 pmol ^{32}P into poly(Glu:Tyr 4:1) in 1 min under the above conditions (Brunati 1996).

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.

RESULTS AND DISCUSSION

We previously reported that red blood cell membranes undergo Tyr-phosphorylation of band 3 by specific catalysis of purified Syk, which belongs to the Tyr family kinase syk (Brunati 1996). Only following this primary phosphorylation, membranes can be sequentially phosphorylated by a second kinase, Lyn, which belongs to the src Tyr-protein kinase family, in the so called secondary phosphorylation (Brunati 1996), and this two-step mechanism has been demonstrated to be involved in the intact red blood cell Tyr-phosphorylation following oxidative, or hyperosmotic stress (Brunati 2000, Bordin 2002).

This study is performed to evaluate the efficiency of the Tyr-phosphorylation of membrane band 3 by Syk, and deals with a comparison between the two isoforms of this enzyme present in human erythrocytes, p72 and p36, this latter being product by a proteolytic cleavage from the former larger enzyme (Zioncheck 1988). The first interesting finding is that when p72^{syk} is added to isolated red blood cell membranes, it is sequestered and no more released even after numerous washing, while p36^{syk} is promptly recovered in supernatant (data not shown).

When incubated with [γ-^{32}P]ATP and exogenous p36^{syk} (Fig. 1, lanes d, e), erythrocyte white membranes showed high level of band 3 Tyr-phosphorylation (lane e), not comparable to the level of ^{32}P-Tyr band 3 achieved by p72^{syk}-catalyzed reaction (lane c).

Fig. 1

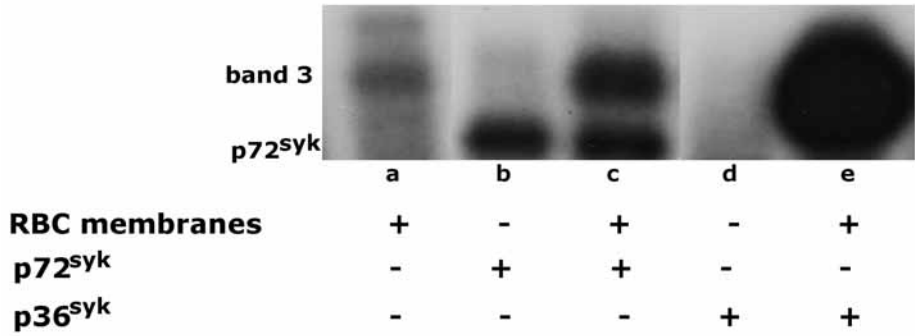

Fig. 1. Membrane band 3 Tyr-phosphorylation. 10 μg membranes, were incubated at 30°C for 5 min in the reaction medium containing [γ-^{32}P]ATP, alone (lane a), with p72^{syk} (lane c) or with p36^{syk} (lane e), all as described in Methods. Samples were analysed by SDS-PAGE, and gels were subjected to NaOH treatment and autoradiographed at –80°C for 16h.
Lanes b and d showed autophosphorylation level of p72^{syk} and p36^{syk}, respectively.
The figure is representative of three separate experiments.

Under the same conditions, p36^{syk} phosphorylates band 3 up to twenty times band 3 Tyr-phosphorylation achieved by p72^{syk}.
That this difference in band 3 Tyr-phosphorylation may be due to a partial inactivation of p72^{syk} is excluded since its autophosphorylation level (lane b), considered as a parameter of its activity, is very high. It may be that the larger steric bulk of the holoenzyme, or its particular sequestration into membrane compartment can prevent the phosphorylation of all band 3 target sites. On the other hand, p36^{syk}, being smaller in size and lacking of tandem SH2 domain, is able to easier phosphorylate all band 3 Tyr-residues.
In order to verify whether p72^{syk} efficiency can be improved by substrate rearrangement, we compare enzyme activities on different membrane preparations, i.e., native white membranes, and membranes treated with PLA_2. As shown in Fig. 2, while p36^{syk} does not considerably change its efficiency in Tyr-phosphorylating band 3, p72^{syk} catalysis is highly improved by this membrane treatment, reaching up to four times band 3 Tyr-phosphorylation level obtained in native membranes.

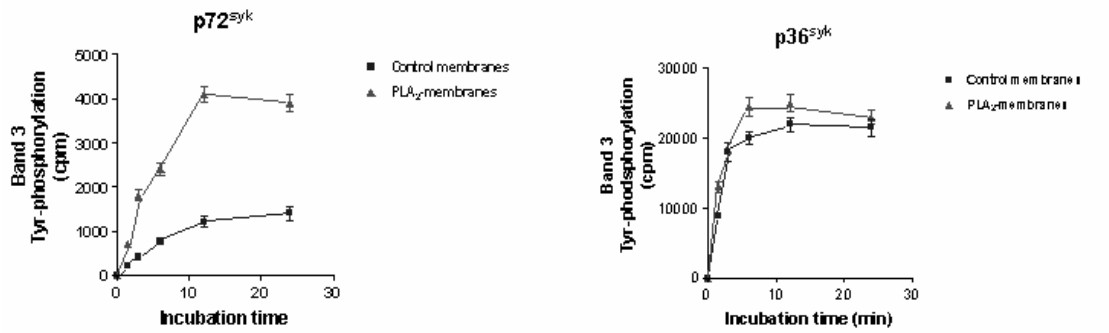

Fig. 2. **Effect of PLA_2 on $p72^{syk}$ and $p36^{syk}$-catalyzed Tyr-phosphorylation of membranes**. Membranes (10 μg) were pre-incubated with (or without, control membranes) PLA_2 and subsequently incubated with $p72^{syk}$ or $p36^{syk}$ for the indicated times, as described in Methods. Samples were analysed by SDS-PAGE, and gels were subjected to NaOH treatment. ^{32}P-Tyr-band 3 was identified by autoradiography, excised from the gel and counted in a liquid scintillation counter.
Data are expressed as means ±SEM (n=5).

In this line of evidence, PLA_2-catalysed lipid breakdown of membranes induces both such a rearrangement of membrane lipid constituents as to set free the kinase, and/or allowed band 3 to reorganize within lipid bilayer in a such way as to be more accessible to the activity of $p72^{syk}$.
Membrane set-up has to be considered a significant parameter of the intact erythrocyte correct functions. We have previously demonstrated that in particular conditions, such as those evidenced in the pathology of Glucose-6-Phposhate Dehydrogenase deficiency (G6PDd), human erythrocyte membranes show differences which can be highlighted by increased level of band 3 Tyr-phosphorylation induced by oxidative as well as hyperosmotic stresses (Bordin 2005). If in pathological conditions an increased is involved, in more physiological conditions, as in pregnancy, a decrease of band 3 Tyr-phosphorylation is observed under those same conditions of stress (Bordin in press), thus further confirming that i) rearrangement of membranes is determinant for the Tyr-P level of the cell; ii) P-Tyr level can be an useful method to evaluate the state of the cell.

REFERENCES

Bordin L., Brunati A. M., Donella-Deana A., et al. Band 3 is an anchor protein and a target for SHP-2 tyrosine phosphatases in human erythrocytes. Blood 2002; 100: 276-282.
Bordin L, Clari G, Moro I, et al. Functional link between phosphorylation state of membrane proteins and morphological changes of human erythrocytes. Biochem. Biophys. Res. Commun. 1995; 213: 249-257.
Bordin L., Quartesan S., Zen F., Vinello F., Clari G. Band 3 Tyr-phosphorylation in human erythrocytes from non-pregnant and pregnant women. Biochim. Biophys. Acta (in press).
Bordin L., Zen F., Ion-Popa F., Barbetta M., Baggio B., Clari G. Band 3 Tyr-phosphorylation in normal and glucose-6-phosphate dehydrogenase-deficient human erythrocytes. Mol. Membr. Biol. 2005; 22:411-420.
Brunati A.M., Bordin L., Clari G., et al. Sequential phosphorylation of protein band 3 by Syk and Lyn tyrosine kinases in intact human erythrocytes: identification of primary and secondary phosphorylation sites. Blood 2000; 96: 1550-1557.
Brautigan D.L. Great expectation: protein tyrosine phosphatases in cell regulation. Biochim. Biophys. Acta 1992; 1114: 63-77.

Brunati A.M., Bordin L., Clari G., et al. The Lyn-catalyzed Tyr-phosphorylation of the transmembrana band 3 protein of human erythrocytes. Eur. J. Biochem. 1996; 240: 394-399.
De Franceschi L, Fumagalli L, Olivieri O et al. Deficiency of Src family kinases Fgr and Hck results in activation of erythrocyte K/Cl cotransport. J. Clin. Invest. 1997; 99: 220-227.
Dekowski S.A., Rybicki A., Drickamer K. A tyrosine kinase associated with the red cell membrane phosphorylates band 3. J. Biol. Chem. 1983; 258: 2750-2753.
De Neef R.S., Hardy-Dessources M.D., Giraud F. Relationship between type II phosphatidylinositol 4-kinase activity and protein tyrosine phosphorylation in membranes from normal and sickle red cells. Eur. J; Biochem. 1996; 235: 549-556.
Dhawan S, Singh S, Aggarwal B-B. 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. 1997; 27:2172-2179.
Harrison M.L., Isaacson C.C., Burg D.L., et al. Phosphorylation of human erythrocyte band 3 by endogenous p72^{syk}. J. Biol. Chem. 1994; 269: 955-959.
Harrison M.L., Rathinavelu P., Arese P., et al. Role of band 3 tyrosine phosphorylation in the regulation of erythrocyte glycolysis. J. Biol. Chem. 1991; 266: 4106-4111.
Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) 1970, 227: 680-685.
Low P.S., Allen D.P., Zioncheck T.F., et al. Tyrosine phosphorylation of band 3 inhibits peripheral protein binding. J. Biol. Chem. 1987; 262: 4592-4596.
Low P-S, Rathinavelu P, Harrison M-L. Regulation of glycolysis via reversible enzyme binding to the membrane protein band 3. J. Biol. Chem. 1993; 268: 14627-142631.
Mallozzi G.,Di Stasi A.M.M., Minetti M. Peroxinitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. FASEB J. 1997; 11: 1281-1290.
Mauro L.J., Dixon J.E. ‘Zip codes’ direct intracellular protein tyrosine phosphatases to correct cellular ‘address’. Trends Biochem. Sci. 1994; 19: 151-155.
Minetti G., Piccinini G., Balduini C., et al. Tirosin phosphorylation of band 3 protein in Ca^{++}/A23187-treated erythrocytes. Biochem. J. 1996; 320: 445-450.
Minetti G, Seppi C, Ciana A, et al. Characterization of the hypertonically induced tyrosine phosphorylation of erythrocyte band 3. Biochem. J. 1998; 335: 305-311.
Musch M.W., Hubert E-M, Goldstein L. Volume expansion stimulates p72^{syk} and p56^{lyn} in skate erythrocytes . J. Biol. Chem. 1999; 274: 7923-7928.
Natarajan V, Scribner W.M., Vepa S. Phosphatase inhibitors potentiate 4-huydroxynomenal-induced phospholipase D activation in vascular endothelial cells. Am. J. Respir. Cell Mol. Biol. 1997; 17: 251-259.
Singh S, Aggarwal B-B. Protein-tyrosine phosphatase inhibitors block tumor necrosis factor dependent activation of the nuclear transcription factor NF-Kappa B. J. Biol. Chem. 1995; 270: 10631-10639.
Tuy F.D.P., Rosenfeld C., Kahn A. High tyrosine kinase activity in normal non proliferating cells. Nature 1983; 305: 435-438.
Walton K.M., Dixon J.E. Protein tyrosine phosphatases. Annu. Rev. Biochem. 1993; 62: 101-120.
Woodside D.G., Obergfell A., Leng L., et al. Activation of Syk protein kinase through interaction with integrin cytoplasmic domains. Curr. Biol. 2001, 11: 1799-1804.
Woodside D.G., Obergfell A., Talapatra A., et al. The N-terminal SH2 domains of Syk and ZAP-70 mediate phosphotyrosine-independent binding to integrin cytoplasmic domains. J. Biol. Chem. 2002; 277: 39401-39408.
Zioncheck T.F., Harrison M.L., Isaacson C.C., Geahlen R.L. Generation of an active protein-tyrosine kinase from lymphocytes by proteolysis. J. Biol. Chem. 1988, 263: 19195-19202.

1 - Dipartimento di Chimica Biologica, Università di Padova, Viale G. Colombo, 3, 35121 Padova