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Oxidized and poorly glycosylated band 3 is selectively phosphorylated by Syk kinase to form large membrane clusters in normal and G6PD deficient red cells

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Short Title: Tyr-phosphorylation of oxidized and poorly-glycosylated RBCs membrane band 3

Abbreviations used: **α Bd3**, anti-band 3 antibody; **α Tyr**, anti-phosphotyrosine antibody; **α Syk**, anti-Syk antibody; **BS³**, bis-sulfosuccinimidyl-suberate; **DTT**, dithiothreitol; **G6PD**, glucose-6-phosphate dehydrogenase; **α Lyn**, p53/56 Lyn antibody; **MW**, molecular weight; **MS**, mass spectrometry; **NAbs**, naturally occurring antibodies; **PMF**, peptide mass fingerprinting; **PTPs**, protein tyrosine phosphatases; **RBCs**, red blood cells; **SFKs**, Src family kinases; **Syk**, p72 Syk

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Oxidative events involving band 3 (Anion Exchanger 1) have been associated with red blood cells (RBCs) removal through binding of naturally occurring antibodies (NAbs), however, the underlying mechanism has been only partially characterized. In addition to inducing direct membrane protein oxidative modification, oxidative treatment, specifically triggers the phosphorylation of band 3 tyrosine residues. This study reports that diamide, a sulfhydryl group oxidant, induces disulfide cross-linking of poorly-glycosylated band 3 and that the oligomerised band 3 fraction is selectively tyrosine (Tyr-) phosphorylated both in glucose-6-phosphate dehydrogenase (G6PD) deficient and control erythrocytes. This phenomenon is irreversible in G6PD deficient RBCs while it is temporarily limited in control RBCs. Diamide treatment caused p72 Syk phosphorylation and translocation to the membrane. Diamide also induced p72 Syk co-immunoprecipitation with aggregated band 3. Moreover, following size exclusion separation of Triton X-100 extracted membrane proteins, Syk was found only in the high molecular weight (MW) fraction containing oligomerised / phosphorylated band 3. Src family inhibitors efficiently abrogated band 3 Tyr-phosphorylation, band 3 clustering and NAbs binding to the RBCs surface suggesting a causal relationship between these events. Experiments performed with the non-permeant cross-linker BS³ showed that band 3 Tyr-phosphorylation enhances its capability to form large aggregates. Our results suggest that selective Tyr-phosphorylation of oxidised band 3 by Syk may play a role in the recruitment of oxidized band 3 in large membrane aggregates that show high affinity to NAbs, leading to RBCs removal from circulation.

Key words: band 3, diamide, Tyr- phosphorylation, p72 Syk, Src family kinases.

INTRODUCTION

G6PD deficiency exerts a protective effect against infection by malaria [1] and causes a haemolytic anemia [2] that is triggered by exposure to oxidants. This protection is possibly mediated by increased susceptibility of G6PD deficient RBCs to membrane modification, leading to the phagocytosis of parasitized RBCs [3]. To explain the mechanisms of RBCs destruction and/or their splenic removal, band 3 modifications have been extensively studied in hereditary haemolytic disorders relating to increases in oxidative membrane damage, such as β -thalassemia, G6PD deficiency, sickle cell anemia and malaria infected RBCs [4-6]. Band 3 is the major integral protein of the RBC membrane, playing a crucial role in membrane functional organization, stability and erythrocytes volume regulation [7,8].

Previous studies have shown that -SH group reagents such as diamide can promote membrane oxidative damage leading to band 3 dimerization through disulfide cross-linking in a dose dependent manner [9, 10]. In addition, diamide induced oxidized/dimerized band 3 acquires the capability to bind anti-band 3 NAbs [4, 10, 11]. The same phenomenon was observed in the removal of either senescent erythrocytes or of pathological RBCs such as in β -thalassemias, G6PD deficiency, sickle cell anemia and malaria infected RBCs, all characterized by the presence of denatured haemoglobin products binding to the band 3 cytoplasmic domain and possibly inducing its oxidation [5, 6, 12-19].

In vitro studies have shown that diamide treatment increases the band 3 Tyr-phosphorylation state, suggesting a possible functional connection between membrane oxidative damage and modulation of signal transduction pathways involving kinases/phosphatases [20, 21]. Tyr-kinases Lyn, from the Src-family kinases (SFKs) and Syk have been seen to functionally interact with the cytoplasmic domain of band 3 in RBCs [21, 22]. Syk and Lyn act on different Tyr-residues of band 3 cytoplasmic domain and kinetic studies suggest that Syk action might be the primary Tyr-phosphorylative event [21-23]. Red cell oxidative treatment induces Syk phosphorylation [24], translocation to the red cell membrane [25], and its activation and association to band 3 [21, 26].

Increased band 3 Tyr-phosphorylation has been reported in G6PD deficient erythrocytes, treated with low diamide concentrations [27], confirming the functional relationship between RBC redox states and the regulation of band 3 Tyr-phosphorylation.

Since the changes in protein Tyr-phosphorylation state are dependent on the balance between kinases and phosphatases, studies on Tyr-phosphatases (PTPs) blocked by -SH groups reagents have been carried out, showing that PTPs are relevant to band 3 Tyr-phosphorylation events following oxidant treatment [28], band 3 being a substrate of the SHP-2 Tyr-phosphatase [20, 29, 30]. However, the relative importance of the oxidant activated Tyr-kinases and/or PTPs inhibition on band 3 Tyr-phosphorylation has not been clarified. Since band 3 possesses two reactive cysteines (Cys 201 and Cys 317) in its cytoplasmic domain, it can be disulfide cross-linked by oxidant RBCs treatment. Conformational changes of oxidatively modified band 3 has been hypothesized to explain changes to its cytoskeleton binding properties [9]. It is of interest to note that at the diamide concentrations needed to observe changes in band 3 Tyr-phosphorylation [25] a fraction of total band 3 molecules is oxidized and clustered to form high MW aggregates [10]. In the present study, we investigate whether oxidatively induced band 3 Tyr-phosphorylation could be involved in the genesis of the membrane modifications that cause G6PD deficient RBCs removal. In particular we studied the mechanisms that lead to the selective recruitment of oxidized band 3, to form the high MW band 3 aggregates, binding NAbs, observed in several haemolytic diseases [5, 12-14, 16, 17, 31].

EXPERIMENTAL

Reagents

Unless otherwise stated, all materials were obtained from Sigma.

SFK inhibitors PP1/PP2 were purchased from Calbiochem, Darmstadt, Germany. DC Protein Assay was purchased from Biorad, Hercules, California, USA; bis-sulfosuccinimidyl-suberate (BS³) and Chemiluminescence kit were purchased from Pierce Biotechnology, Rockford, USA; α Syk and α pTyr antibodies were purchased from Cell Signaling Technology, Inc, CA, USA.

Treatment of RBCs

Venous blood was taken from 6 normal and 5 G6PD deficient human subjects (Mediterranean variant, type II deficiency). G6PD deficient erythrocytes had a residual activity lower than 2 %. All samples were genotyped [32] and presented the mutation 563 C --> T. Blood was washed three times with phosphate buffer saline with glucose 5 mM at pH 7.4 (PBS-glucose) to obtain packed RBCs. RBCs were resuspended at 30% hematocrit in PBS-glucose. RBCs were incubated in the presence of 2mM diamide for 45 minutes (min) at 37°C, alternatively a time course from 0 to 90 min was carried out where necessary. Identical aliquots of RBCs were incubated for corresponding incubation periods at 37°C without diamide as control. In separate experiments, RBCs were incubated with o-vanadate 1mM for 1 hour at 37°C. When necessary RBCs were pretreated (before diamide treatment) with SFK inhibitors (10 μ M of PP1 and 10 μ M PP2) for 1 hour at 37°C in the dark. Each reaction was terminated by 3 washes with PBS-glucose. Control and diamide treated RBCs were incubated with increasing concentrations (0, 0.25, 0.50, 1 mM) of bis-sulfosuccinimidyl-suberate (BS³) for 15 min at 37°C. RBCs were washed 3 times in PBS-Tris 40 mM pH 7.4, and membranes were prepared. Solubilized membranes were frozen at -20°C.

RBCs membrane preparation

Packed RBCs from normal and G6PD deficient human subjects were lysed in hypotonic buffer (5 mM sodium phosphate, 1 mM EDTA, pH 8.0) containing protease inhibitor cocktail and, when necessary, phosphatase inhibitor cocktail. Membranes were washed as previously described [10]. Membrane alkali treatment to deplete cytoskeletal proteins was performed as previously described [10].

Electrophoresis and immunoblotting

Membrane protein content was quantified using DC Protein Assay and was solubilized in Laemmli Buffer [33] under reducing (2% DTT) or non-reducing conditions in a volume ratio of 1:1. SDS-PAGE analysis was conducted by heating the samples for 5 min at 100°C and loading 20 μ g membrane proteins on the 8% gel for protein staining by blue colloidal Coomassie [34].

For western blotting analysis, 30 μ g of proteins was loaded on each lane, transferred to nitrocellulose membranes and immunostained with α Syk and α pTyr, both diluted to 1:2000, using anti-mouse peroxidase labeled antibodies and detected by chemiluminescence according to the manufacturer's instruction (Pierce Biotechnology, Rockford, USA). For α Bd3 western blots 0.5 μ g of proteins was loaded and the α Bd3 was diluted to 1:50000 and detected by a chromogenic substrate as previously described [10].

α Syk immunoprecipitation

RBCs from normal and G6PD deficient human subjects were treated in the presence or absence of diamide and membranes were prepared as described above. 200 μ l of membrane proteins were solubilized for 15 min at 0°C with three volumes of 1% triton-X 100 in hypotonic buffer (5 mM sodium phosphate, 1 mM EDTA, pH 8.0). After centrifugation in refrigerated Eppendorf microfuge at 13.000 x g, supernatants were collected and incubated with rabbit α Syk cross-linked to protein A-Sepharose (50 μ l) via a bifunctional coupling reagent dimethylpimelidate (according to

“Antibodies: A Laboratory Manual”, by Edward Harlow) for 2 hours at 4 °C under gentle mixing. Beads were washed three times with 1% triton-X100 in PBS pH 7.4. Three volumes of Laemmli sample buffer containing 2% DTT (final concentrations) were added to packed beads, that were heated and subjected to SDS-PAGE and western blot analysis, with mouse α Bd3 and α pTyr.

Membrane proteins separated on Sepharose CL-6B

RBCs from normal and G6PD deficient human subjects were treated in the presence or absence of diamide and membranes were prepared and fractionated as previously described [10]. With minor modifications, 1ml membranes were solubilized in 2 ml extraction buffer (10 mM Hepes, 130 mM NaCl, 10 mM N-ethylmaleimide, 1mM EDTA, 1 mM PMSF, 1% triton-X100, pH 7.4), gently shaken for 10 min at 20°C and centrifuged at 13000 rpm for 5 min in an Eppendorf microfuge. The supernatant was applied to a 40 x 1 cm column filled with Sepharose CL-6B equilibrated with a solution containing 10 mM Hepes, 50 mM NaCl, 0.1 % triton-X100 (pH 7.4), at a flow rate of 1 ml/min. Constant flow was maintained using an HPLC pump. The effluent was collected in 1 ml fractions. Fractions were concentrated 10 fold and analysed for immunoblotting with α Bd3, α pTyr and α Syk as described above.

Treatment of control and diamide treated RBC membranes with control and diamide treated RBC cytoplasmic fractions

RBCs from 6 normal and 5 G6PD deficient human subjects were treated with or without diamide (see above). Packed RBCs were subjected to haemolysis by adding 5 volumes of 5 mM sodium phosphate, pH 8.0 in presence of protease inhibitor cocktail and centrifuged in refrigerated Eppendorf microfuge at 13.000 x g, at 4°C for 10 min, then the supernatant was collected to obtain the cytoplasmic fractions.

RBC membranes were prepared as described above and incubated for 10 min at 4°C with or without diamide 2 mM. Membranes were washed 3 times in 5 mM sodium phosphate pH 8, in the presence of protease inhibitor cocktail at 12.000 x g, at 4°C for 10 min. Membranes were collected and incubated with the cytoplasmic fractions in presence of ATP 50 μ M for 10 min at 37°C.

Finally, membranes were washed 3 times in hypotonic buffer pH 8, at 13.000 x g, at 4°C for 10 min in presence of protease inhibitor cocktail and phosphatase inhibitor cocktail. Membrane proteins were separated on SDS-PAGE and blotted onto nitrocellulose membrane as described above.

Measurement of membrane bound IgG

Membrane bound IgG were estimated using an immuno-enzymatic assay as previously described [6]. In brief, RBCs were shortly incubated with an anti human IgG antibody conjugated to alkaline phosphatase, membranes were prepared as usual and solubilised with 1% triton X100. Alkaline phosphatase activity was determined by colorimetric detection.

Measurement of RBC GSH concentrations.

GSH was measured in packed RBCs by colorimetric detection following protein precipitation as previously described [3].

Band 3 MS analysis by MALDI-TOF

Band 3 gel slices from 1-DE coomassie stained gels were excised in control, G6PD deficient and diamide treated RBC membranes. Pieces of gel were destained by way of several washes in 5 mM NH_4HCO_3 /acetonitrile (50/50 v/v) and successively dried with pure acetonitrile. The gel slices were rehydrated for 45 min at 4°C in 20 μ l of a 5mM NH_4HCO_3 digestion buffer containing 10 ng/ μ l of trypsin. Excess protease solution was removed and the volume adjusted with 5 mM NH_4HCO_3 to cover the gel slices. Digestion was allowed to proceed overnight at 37°C [34].

Samples were loaded onto MALDI target using 1 μ L of the tryptic digests mixed 1:1 with a solution of alpha-Cyano-4-hydroxycinnamic acid (10 mg/mL in acetonitrile/trifluoroacetic acid 0.1%,

40/60). Band 3 N terminal phosphorylation was identified operating the instrument in linear modality according to the manufacturer's instructions. Phosphorylation was confirmed by phosphatase treatment according to a previously described method [35].

MS analysis of peptides of band 3 fractions sliced from 1-DE gel were performed with a MALDI-TOF Micro MX (Micromass, Manchester, UK) operating the instrument in reflectron modality according to the tuning procedures suggested by the manufacturer. Peak lists were generated with Proteinlynx Data Preparation using the following parameters: external calibration with lock mass using mass 2465,1989 Da of ACTH fragment 18-39 (sigma A8346) background subtract with adaptive mode, performing deisotoping with threshold 3%.

For peptide mass fingerprinting (PMF) analysis of MS spectra were converted into pkl files using Mass Lynx 4.0. Peak lists containing the 20 most intense peaks of the spectrum were sent to MASCOT PMF search (<http://www.matrixscience.com>) using the Swiss-Prot database (release 50.0 dating to 30-May-2006). Search settings allowed one missed cleavage with the trypsin enzyme selected, carboxymethylated cysteine as fixed modification and oxidation of methionine as potential variable modification and a peptide tolerance of 50 ppm. Only protein identifications with significant Mascot scores ($p < 0.05$) were taken in consideration.

RESULTS

Selective Tyr-phosphorylation of oxidized/oligomerized band 3 in control and G6PD deficient RBCs

Although published results report that diamide treatment induces band 3 oligomerisation and band 3 Tyr-phosphorylation, the temporal changes of these modifications and their causal relationships have never been established. Since, reduced glutathione (GSH) plays an important role as an antioxidant system depending on the NADPH function, we evaluated the levels of reduced GSH and band 3 oligomerisation states in control and G6PD deficient RBCs exposed to diamide treatment. In control and G6PD deficient RBCs GSH rapidly dropped after diamide treatment but only control RBCs were able to restore basal levels following 90 min of incubation (Fig. 1A).

In analogy with GSH levels variation, diamide treatment induced band 3 oligomerisation that was permanent in G6PD deficient RBCs and it was reversible in control RBCs (Fig. 1B, lanes 1, 2, 3 and lanes 7, 8, 9). To verify whether longer time was needed to reduce band 3 in G6PD deficient RBCs, we incubated the cells up to 12 hours, however, progressive band 3 oligomerisation was observed (data non shown). To assess whether changes in band 3 Tyr-phosphorylation might be affected by its oxidative modifications, we performed immunoblot analyses with specific *ap*Tyr antibodies under non reductive conditions. Diamide treatment caused marked Tyr-phosphorylation in correspondence with the oxidized, oligomeric band 3 region both in control and G6PD deficient red cells (Fig. 1B lanes 2, 5 and 8, 11). Only in control RBCs, after incubation for 90 min, band 3 reduction was associated with a net decrease in the band 3 Tyr-phosphorylation state (Fig. 1B lanes 3, 6 and lanes 9, 12). Reduction of disulfide bridges by DTT caused complete band 3 reduction and a parallel shift of the anti-phosphotyrosine signal to the monomeric band 3 region (Fig. 1C) indicating that oxidized band 3 is the site of tyrosine phosphorylation. G6PD deficient RBCs showed identical behaviour patterns (data not shown).

Since changes in protein phosphorylation states are dependent on the balance between cellular kinases and phosphatases, we evaluated the effects of Tyr-phosphatase (PTP) inhibition by *o*-vanadate. In control RBCs, *o*-vanadate treatment caused increases of monomeric band 3 Tyr-phosphorylation (Fig. 1D). No changes in the band 3 oxidative state were induced by *o*-vanadate (data not shown) suggesting that the selective Tyr-phosphorylation of oxidized band 3 induced by diamide cannot be related only to PTPs inhibition.

Under our experimental conditions the SFK inhibitors PP1 and PP2 reduced diamide induced band 3 Tyr-phosphorylation (Fig. 1E), showing that SFKs might be involved in band 3 phosphorylation

directly or indirectly through the modulation of p72 Syk [23]. G6PD deficient RBCs had identical behaviour (data not shown).

On the basis of these results, we conclude that, following diamide treatment only oxidized / oligomerised band 3 is Tyr-phosphorylated. This phenomenon is apparently due to changes of Tyr-kinases activity and / or substrate affinity because, following Tyr-phosphatases inhibition, band 3 was phosphorylated irrespective of its oxidation state.

Binding of Syk to modified band 3 in diamide treated RBCs

Since a small amount of Syk and Lyn had been previously reported to be associated with the RBCs membrane in steady state conditions [21, 25], we evaluated the amount of both kinases to the membranes of erythrocyte treated with diamide. We observed that Syk undergoes membrane translocation following diamide treatment (Fig. 2A, lane 2). It is of interest to note that Syk membrane association was evident in immuno-blots only when the samples were preliminarily reduced, indicating that following oxidant treatment Syk was most likely bound to membrane protein aggregates. In addition, in diamide treated RBCs, the bound membrane, Syk was Tyr-phosphorylated, suggesting that it had been activated (Fig. 2A, lane 4), we also blotted western-blot membranes with specific α Lyn antibody revealing the presence of Lyn constitutively bound to the membrane but no changes in the amount of Lyn membrane association were observed following diamide treatment (data not shown).

To evaluate whether native or oxidatively activated Syk possesses different degrees of affinity to native or oxidized band 3, we compared control or diamide treated membranes with the cytoplasmic RBC fractions obtained from control or diamide treated erythrocytes. Syk from untreated RBCs cytoplasm was able to bind to diamide treated membranes (Fig. 2B, lane 2) and at a much lower extent to control membranes (Fig. 2B, lane 1). The addition of diamide treated RBCs cytoplasm caused further increase in Syk binding to the diamide treated membranes (Fig. 2B, lane 4) and, to a minor extent, also to control membranes (Fig. 2B, lane 3). Under the same experimental conditions, we also measured band 3 and Syk Tyr-phosphorylation states. In parallel to Syk membrane translocation, we observed intense band 3 Tyr-phosphorylation only in diamide treated membranes (Fig. 2B, lanes 6, 8). Following cytoplasm diamide treatment, membrane association of Tyr-phosphorylated Syk was seen to increase (Fig. 2B, lane 7, 8), but Syk binding to untreated membranes caused small changes in band 3 Tyr-phosphorylation (Fig. 2B, lanes 7) indicating that Syk requires oxidized band 3 to show its maximal affinity and/or catalytic activity.

To further validate this hypothesis, we immunoprecipitated Syk with specific α Syk antibody from solubilized RBC membrane proteins. As shown in Figure 2C, lanes 2 and 4, in diamide treated RBCs, Syk was immunoprecipitated in association with phosphorylated band 3. It is interesting to note that when sample reduction was omitted, both immunoprecipitated Syk and band 3 were present only in high MW protein aggregates localised at the top of the gel (data not shown). Following 90 min incubation time we observed complete reversal of the diamide induced effects in control erythrocytes but not in G6PD deficient RBCs. (data not shown).

On the basis of these results, we conclude that selective phosphorylation of oxidized / oligomerized band 3 could be due to increased Syk affinity toward modified band 3.

Mapping of band 3 phosphorylation sites in diamide treated RBCs

Since Syk has been reported to specifically phosphorylate the tyrosine residues 8 and 21 of the band 3 cytoplasmic domain [22], we mapped the band 3 Tyr-phosphorylation sites in RBCs treated with diamide. Figure 3 shows the occurrence of double Tyr-phosphorylated (+160 Da) band 3 N terminal tryptic peptide. Since Tyr 8 and 21 are the only two Tyr-residues contained in the 6538 N terminal tryptic peptide, the 160 Da mass shift indicates their phosphorylation. MALDI-TOF analysis had to be performed by linear modality do to the high MW of the N-terminal peptide. The double phosphorylated 6698 Da peptide was absent in band 3 isolated from untreated RBCs (Fig. 3, CTRL) and in diamide treated RBCs in presence of either the SFK inhibitors PP1 and PP2 and after

phosphatase treatment of the sample (data not shown). The presence of the Tyr-phosphorylated tryptic peptide containing the putative Lyn substrate tyrosine 359 (from Tyr³⁴⁷ to Lys³⁶⁰) was also detected but, under our experimental conditions, its corresponding signal was weak and sporadic (data not shown). On the basis of these results, the involvement of Syk on the phosphorylation of oxidized band 3 was substantiated.

Preferential clustering clusterisation of oxidized / hyper-phosphorylated band 3

Previously work has shown that in diamide treated RBCs oxidized band 3 preferentially clusters in high MW aggregates [10] but the band 3 phosphorylation state was not taken into account in the formation of this aggregate. In the present report, size exclusion chromatographic fractions of solubilised membrane proteins were subsequently subjected to immuno-blot analysis to evaluate the occurrence of Tyr-phosphorylated band 3 and Syk in the high MW fractions (Fig. 4A, fractions 3 to 5). We observed a net prevalence of Tyr-phosphorylated band 3 and Syk in the high MW fraction, suggesting preferential aggregation of Tyr-phosphorylated band 3. The relevance of band 3 Tyr-phosphorylation for band 3 clustering was also supported by the observation that treatment with the SFK inhibitors PP1 and PP2, in addition to inhibiting band 3 phosphorylation also caused a decrease in diamide induced high MW band 3 aggregates (Fig. 4A). Marked differences were detectable after 90 min of incubation, when band 3 containing aggregates regained basal levels in control erythrocytes, but not in G6PD deficient erythrocytes (Fig. 4 B).

To investigate the hypothesis that band 3 phosphorylation may promote its clustering by weakening its cytoskeletal connections, we incubated diamide treated RBCs with increasing concentrations of the poorly membrane permeating cross-linkers BS³. Figure 5 shows that diamide pre-treatment caused enhancement of band 3 cross-linking even at low BS³ concentrations. As expected, at longer incubation times this phenomenon was reversed in control RBCs. On the basis of these results, we conclude that Tyr-phosphorylated band 3 is selectively recruited in high MW aggregates due to an apparent increase in its lateral mobility.

Naturally occurring IgG membrane binding

As previous studies had demonstrated that NABs binding to the RBCs surface depends on the band 3 oxidation/oligomerisation state [10, 36], we tested the effect of SFK inhibitors on NABs binding. Diamide treated RBCs were incubated with autologous serum to allow naturally occurring IgG binding to erythrocyte surface. As shown in figure 6, membrane IgG binding was occasional and transient in control RBCs, on the contrary, IgG binding was more sustained and progressively increasing in G6PD deficient RBCs. Following 45 min of diamide treatment, PP1 and PP2 treatment caused an average 1.92 ± 0.66 fold decrease IgG binding in control RBCs and 1.89 ± 0.78 fold decrease in G6PD deficient RBCs. Those results indicate that Tyr-phosphorylation apparently plays a role also in the formation of band 3 antigenic complexes.

Selective recruitment of poorly-glycosylated band 3 in diamide induced oligomers

As shown here (Fig.1B) and in an earlier report [10] the SDS-PAGE faster migrating band 3 fraction is seen to disappear following diamide treatment. To investigate the hypothesis that band 3 is preferentially aggregated we developed a method to estimate the rate of glycosylation of SDS-PAGE separated band 3 by measuring the relative amount of the tryptic fragments bound to the glycosidic moiety. Band 3 trypsin cleavage theoretically originates from different fragments containing the Arg⁶⁴⁶ residue linked to the glycosidic moiety. Mass spectrometry analysis showed the net prevalence of the 1564 Da peptide from Leu⁶³² to Arg⁶⁴⁶ (LSVPDGFKVSNSSAR). The mass of this peptide should increase following its glycosylation and it should be out of scale of the analytical setting used. Therefore, the amount of the 1564 Da peptide should be inversely related to the amount of glycosylated band 3 molecules. The band 3 gel region was cut into 3 parts (Fig. 7, fractions A,B,C). The ratio between the 1564 Da peptide and the 1490 Da band 3 peptide (from aminoacid 234 to 246, chosen as internal reference) decreased in tandem with the band

electrophoretic mobility in membranes of control RBCs shifting from 0.21 to 0.74. The 1564/1490 ratio shifted from the average ratio of 0.42 ± 0.08 in monomeric band 3, to 0.80 ± 0.13 in oligomeric band 3 in membranes of diamide treated RBCs (Fig. 7, Diamide), indicating the preferential oligomerisation of the less glycosylated band 3 fraction. No significant differences were observed between control and G6PD deficient erythrocytes incubated with diamide for 45 min (data not shown).

DISCUSSION

Physiological senescent RBCs or pathological erythrocytes such as in G6PD deficiency, β -thalassemias and malaria are possibly removed by peripheral circulation following the binding of denatured haemoglobin molecules (haemichromes) to the cytoplasmic domain of the integral membrane protein band 3 [4-6]. Interestingly, G6PD deficiency as well as β -thalassemias provide protection against malaria and are characterised by enhanced haemichrome formation induced by malaria parasite growth [6]. Haemichromes produced in different pathological situations are able to bind and oxidise the band 3 cytoplasmic domain inducing the formation of large band 3 clusters [5]. It must be underlined that band 3 clusters cause RBCs recognition by way of a mechanism involving anti-band 3 NABs binding to the RBCs surface [4, 37, 38].

Since band 3 is normally linked to the cytoskeleton by junctional complexes, the formation of large band 3 aggregates should be restrained by such interactions unless they are weakened by regulatory mechanisms. As a matter of fact ankyrin docks in close proximity to the band 3 cytoplasmic domain Tyr-phosphorylation sites, and non-phosphorylated band 3 appears to be less prone to forming high MW aggregates [39, 40]. Furthermore, hyper-phosphorylated band 3 has been found to be associated with G6PD deficiency [27], deoxygenated sickle cells [26, 41] and senescent RBCs [30], all clinical conditions characterized by increased RBCs oxidative stress and band 3 clusters formation.

In the present paper, we observed selective phosphorylation of oxidized / oligomeric band 3 both in diamide treated control and G6PD deficient RBCs. Control and G6PD deficient RBCs, showed differential band 3 phosphorylation time courses in close correspondence with GSH.

The band 3 cytoplasmic domain appears to be one of the principal substrates of Tyr-kinases, such as the SFKs Lyn and Syk [23]. Even if possible inhibition of Tyr-phosphatases might contribute to enhancing band 3 Tyr-phosphorylation [29, 40], here, we show that diamide causes selective Tyr-phosphorylation of the oxidized / oligomerised band 3 fraction, while Tyr-phosphatase inhibition by *o*-vanadate causes widespread band 3 Tyr-phosphorylation irrespective of its oxidation state [29, 41]. These findings indicate a primary increase of Tyr-kinase activity and/or affinity for oxidatively modified band 3 while phosphatases inhibition seems to play a secondary role in enhancing kinases selective action on oxidized band 3.

Naturally occurring IgG binding to diamide treated RBCs proceeded in a parallel fashion with band 3 phosphorylation and clustering in control and G6PD deficient RBCs. Band 3 phosphorylation, clustering and IgG binding were all inhibited by SFK inhibitors PP1 and PP2, indicating a causal role of band 3 Tyr-phosphorylation.

In diamide treated RBCs, we observed the preferential oligomerisation / phosphorylation of the less glycosylated band 3 fraction supporting the hypothesis that, in RBCs, only a fraction of band 3 molecules is prone to being oxidatively cross-linked and Tyr-phosphorylated. Previous reports indicated that band 3 shows an increased tendency to cluster in congenital dyserythropoietic anemia type 2 characterised by band 3 under-glycosylation and haemolytic anemia [42, 43].

Our data, therefore, indicate that two band 3 post-translational modifications modulate the clusterisation capability of band 3: glycosylation appears to be a restraint to its oxidative cross-linking and while Tyr-phosphorylation appears to facilitate oxidatively modified band 3 clusterisation. However, it is of importance to note that in the present report, we only studied the early events that followed sulfhydryl groups oxidation while, in pathological conditions, more

complex membrane modifications, involving haemoglobin denaturation products and additional phosphorylative events, may occur. Our results highlight also the need to further investigate the molecular details of the erythrocyte Tyr-kinases oxidative activation and phosphorylative regulation of band 3 – cytoskeleton interactions. In summary, our findings provide new evidence to explain the formation of band 3 clusters observed in different physiological and pathological situations associated with RBCs removal, shifting from the classical view of a passive role of damaged membrane components to the activation of a regulatory mechanism involving Src kinase as an upstream regulator of Syk through its affinity change to modified band 3 (Fig. 8). Taking in account that haemichromes bound band 3 molecules become quickly oxidized [5], band 3 Tyr-phosphorylation may represent a mechanism for their selective recruitment in large membrane clusters.

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Figures and Legends:

Fig 1 A

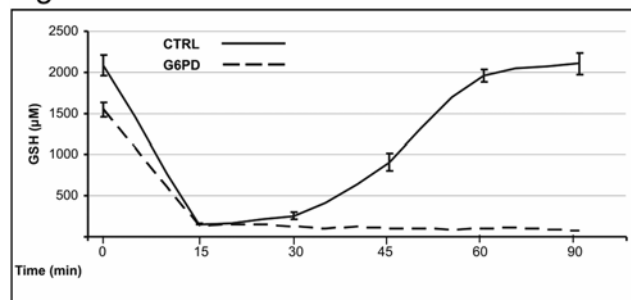


Fig 1

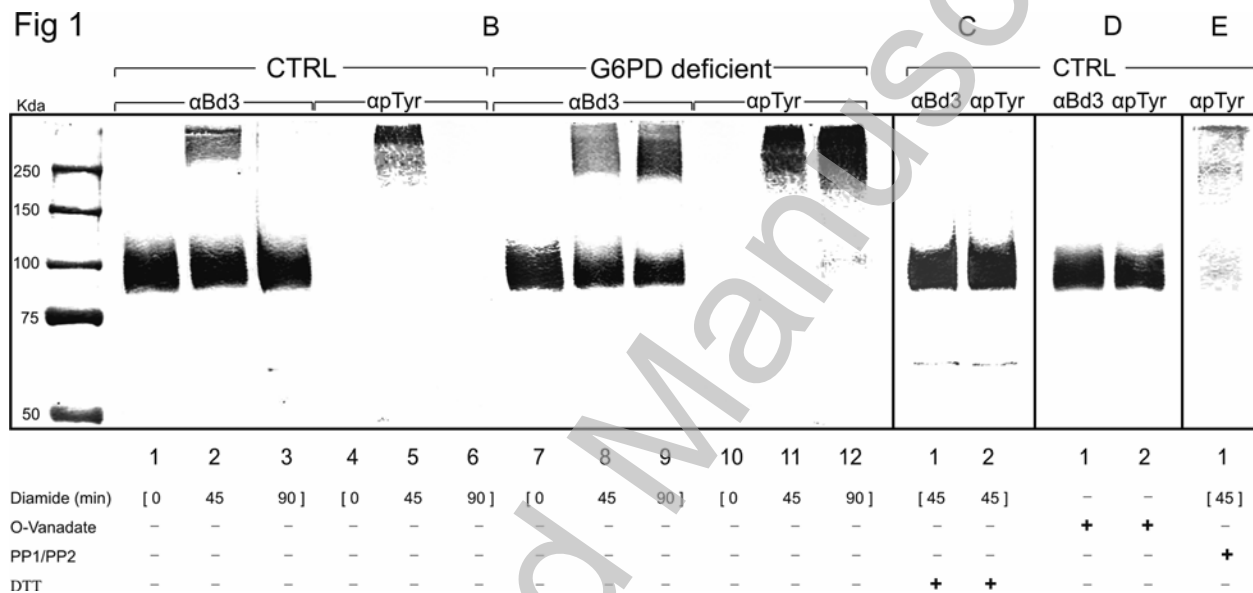


Figure 1(A,B,C,D,E) Selective Tyr-phosphorylation of oxidized / oligomerized band 3 in control and G6PD deficient RBCs

Western blotting of SDS-PAGE separated RBCs membrane proteins; Band 3 and phospho-tyrosine residues were stained by specific antibodies (α Bd3, α pTyr). (A) GSH levels were measured in control (CTRL) and G6PD deficient (G6PD) RBCs following the addition of diamide at time 0 (values are expressed as μ Moles in packed RBCs. Mean of 3 independent experiments \pm SD).

(B) Membrane proteins of control (CTRL) and G6PD deficient RBCs were incubated with diamide for 0, 45 and 90 min. Membranes were solubilized in absence of reducing agent (DTT); (C) membrane proteins of control RBCs treated with diamide and solubilized in presence of DTT; (D) membrane proteins of control RBCs treated with o-vanadate in absence of DTT; (E) membrane proteins of control RBCs treated with PP1/PP2 and diamide for 45 min in absence of DTT. Independent experiments were performed a minimum of three times using blood samples from different donors.

Fig 2

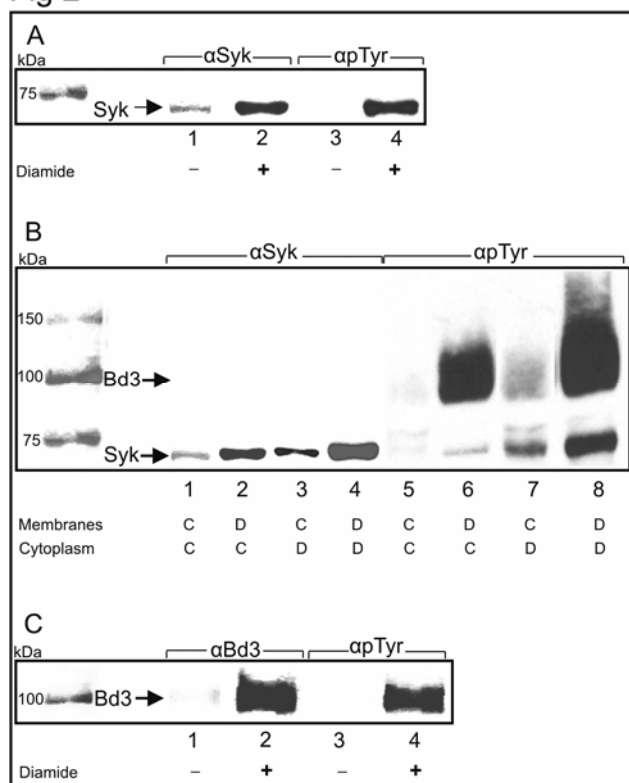


Figure 2 Modification of Syk binding properties and activity induced by oxidised band 3

Western blotting of SDS-PAGE separated RBC membrane proteins; Syk and phospho-tyrosine residues were stained by specific antibodies (α Syk, α pTyr). **A**) Membrane proteins of control and diamide treated RBCs, solubilized in presence of reducing agent (DTT); **B**) Control RBC membranes (C) and diamide treated membranes (D) were incubated with the cytoplasmic fraction (Cytoplasm) of control and diamide treated RBCs. **C**) α Syk immuno-precipitated fractions of membrane proteins of control and diamide treated RBCs were analysed by α Bd3 and α pTyr western blots, under reducing conditions. Experiments were performed a minimum of three times using blood samples from different donors.

Fig 3

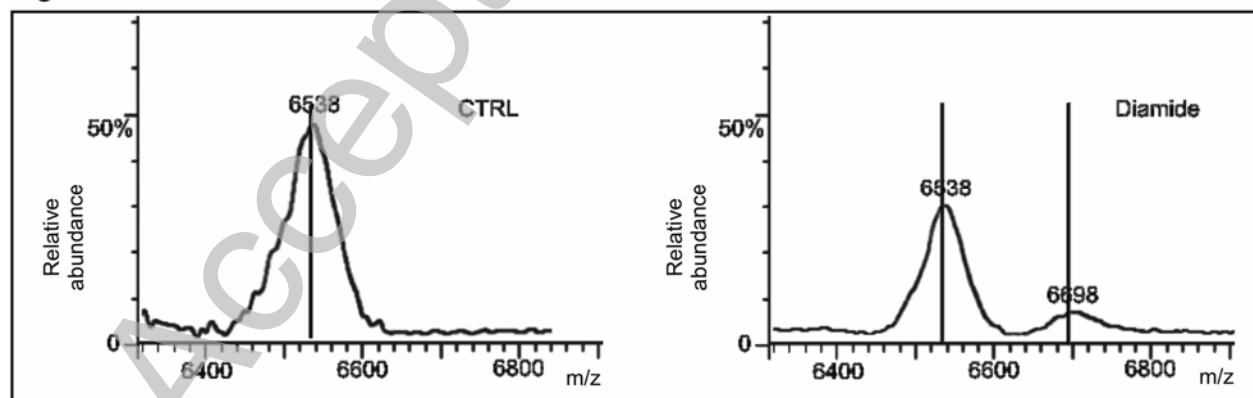


Figure 3 Mapping of band 3 cytoplasmic domain phospho-tyrosine residues revealed the involvement of 8 and 21 Tyr residues.

Band 3 was excised from Coomassie stained gels in control (CTRL) and diamide (Diamide) treated RBC membranes. Gel slices were subjected to trypsin digestion and analysed by a MALDI-TOF in linear modality in the expected MW range of the band 3 N terminal tryptic peptide (expected MW:

6538 Da, expected MW of the double phosphorylated peptide: 6698 Da). Experiments were performed a minimum of three times using blood samples from different donors.

Fig 4 A

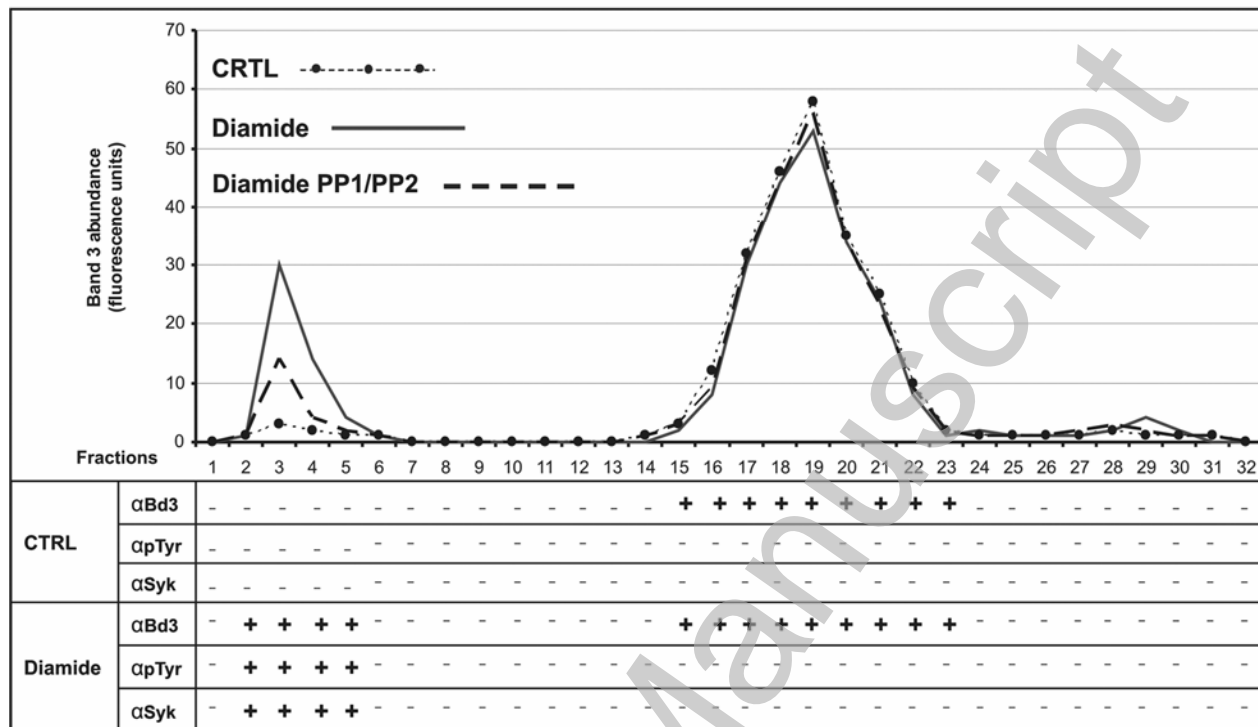


Figure 4A Identification of Syk and Tyr-phosphorylated / oxidised band 3 in the high MW aggregates separated by size exclusion. Inhibition of aggregates formation by Syk inhibitor PP1 / PP2

RBC membranes proteins were extracted by 1% triton X100 from control RBCs, and RBCs treated with diamide and diamide in presence PP1/PP2. Band 3 was labelled by eosine maleimide and separation was performed on a CL6B column. Band 3 abundance was expressed as fluorescent units. Fractions were further analysed by western blot with α pTyr, α Syk and α Bd3 antibodies. Positivity was marked (+). Experiments were performed a minimum of three times using blood samples from different donors .

Fig 4 B

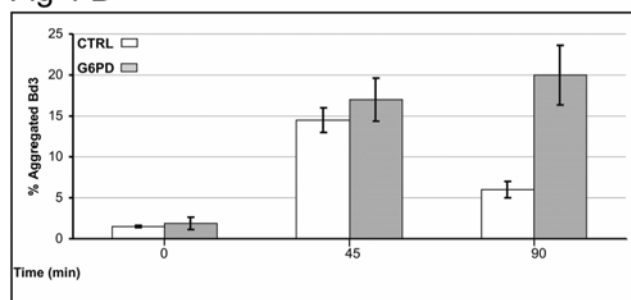


Figure 4B Time course of Band 3 content in high MW aggregates following diamide treatment

Band 3 content in high MW aggregates in Control (CTRL) and G6PD deficient erythrocytes detected after 0, 45 and 90 min of incubation with diamide 2 mM. Band 3 fluorescence was measured following its labelling by eosine maleimide and expressed as a percentage of its total amount using blood samples from 3 different control and 3 G6PD deficient donors (Mean of 3 independent experiments \pm SD)

Fig 5

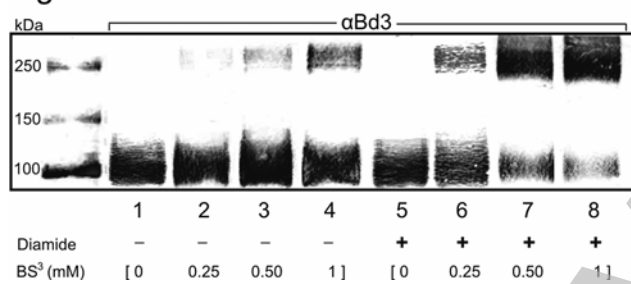


Figure 5 Increased tendency of band 3 cross-linking by BS³ following diamide treatment

Western blotting of SDS-PAGE separated RBC membrane proteins; band 3 was stained by α Bd3 antibody. RBCs were treated with diamide (30 min, 1mM) and then with BS³ (30 min) at different concentrations. Experiments were performed a minimum of three times using blood samples from different donors.

Fig 6

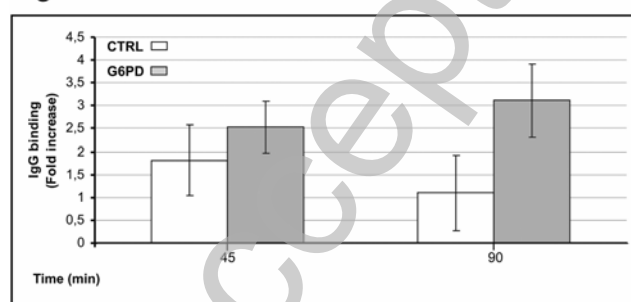


Figure 6 Time course of Naturally Occurring Antibodies (IgG) following diamide treatment.

IgG membrane binding in control and G6PD deficient after 45, 90 min of incubation with diamide. IgG were measured following the labelling by anti-Human IgG and expressed as fold increase in compared to untreated RBCs. Experiments were performed using blood samples from 3 different control and 3 G6PD deficient donors (Mean of 3 independent experiments \pm SD)

Fig 7

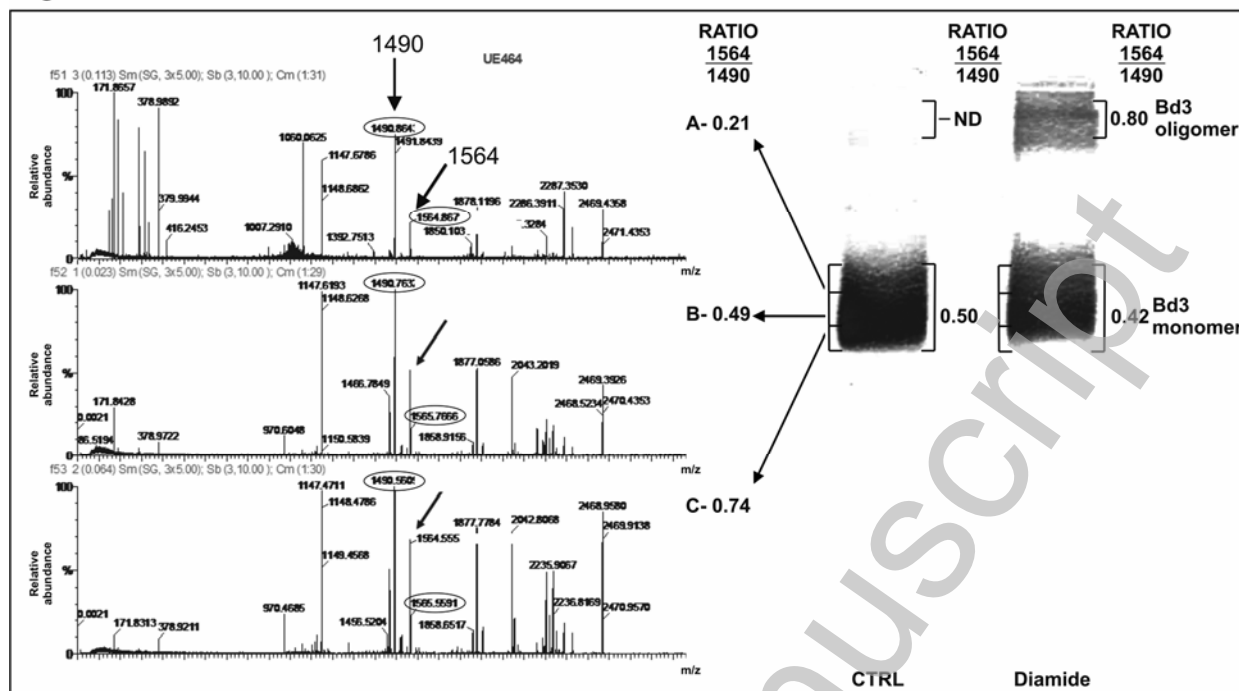


Figure 7 Selective oligomerisation of deglycosylated band 3 following RBCs diamide treatment

Untreated (CTRL) and diamide treated (Diamide) RBC membranes were depleted of cytoskeletal proteins by alkali treatment, proteins were separated by SDS-PAGE under non-reducing conditions and stained by comassie blue. A, B, C represent the MS spectra of the tryptic digests obtained from 3 sequential gel slices excised from the band 3 region. The fragment containing the band 3 glycosylic moiety binding residue (Arg⁶⁴⁶) has a MW of 1564 Da. The amount of the 1564 peptide was normalised with the 1490 band 3 tryptic peptide and expressed as a ratio between the height of the two peaks. ND: no signal was detected. Experiments were performed a minimum of three times using blood samples from different donors.

Fig 8

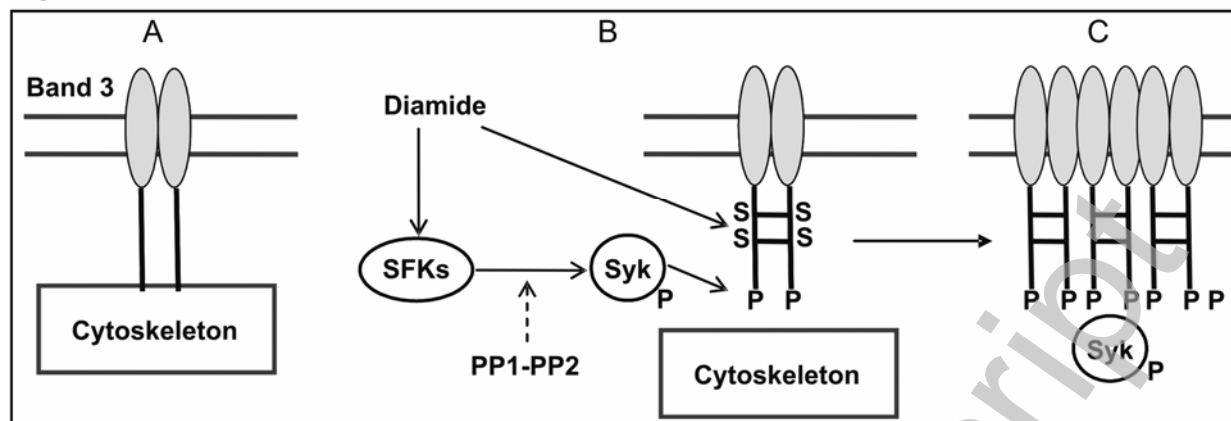


Figure 8 Hypothetic mechanism of band 3 clustering

A) Under basal conditions, band 3 is prevalently de-phosphorylated and associated to the cytoskeleton

B) Diamide treatment induces band 3 disulfide cross-linking and Syk phosphorylation. Syk binds to band 3 and catalyzes band 3 cytoplasmic domain Tyr-phosphorylation.

C) Tyr-phosphorylated band 3 has increased tendency to form large clusters, due to its decreased cohesion with the cytoskeleton

P, phosphoryl group

S-S, disulfide bonds