Deoxygenation and elevation of intracellular magnesium induce tyrosine phosphorylation of band 3 in human erythrocytes

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Abstract Deoxygenation increases the level of tyrosine phosphorylation of band 3 by ~25% in human red blood cells (RBCs), as determined by Western blotting. The effect is much more pronounced in osmotically shrunken RBCs or in the presence of vanadate. When the rise in intracellular free Mg^{2+} concentration in deoxygenated RBCs is simulated via clamping of the intracellular magnesium in oxygenated RBCs by ionomycin, band 3 phosphorylation is elevated by up to 10-fold. Phosphorylated band 3 is preferentially retained by RBC skeletons, after mild extraction with Triton X-100. Elevation of intracellular free Mg^{2+} leads to band 3 phosphorylation and is accompanied by rigidification of the membrane skeleton as determined by analysis of RBC membrane mechanical fluctuations. These findings suggest that the visco-elastic properties of human erythrocytes may be regulated by band 3 tyrosine phosphorylation.

Key words: Tyrosine kinase activation; Anion exchanger; Erythrocyte shrinkage; Mag-fura 2; Bending deformability; Blood circulation

1. Introduction

Band 3 of human erythrocytes is known to be phosphorylated at tyrosine residues [1–6]. Its phosphorylation state is determined by the balance between the activities of protein tyrosine kinases (PTKs) $p72^{syk}$ [4] and $p53/56^{lyn}$ [5] on the one hand and of phosphotyrosine phosphatase PTP1B [7] on the other hand. Oxidative stress and thiol alkylation were shown to elevate the level of tyrosine phosphorylation of band 3 in the intact red blood cells (RBCs) by inhibiting PTP [8], while a hyperosmotic stress and Ca^{2+} ions led to a similar result via PTK activation [6,9]. From in vitro studies, employing purified proteins and RBC ghosts, it is evident that band 3 PTKs may also be activated by Mn^{2+} and Mg^{2+} ions [10]. Whether activation also takes place in situ is still unknown. Since the intracellular free Mg^{2+} concentration is altered in a periodic way during oxygenation-deoxygenation of RBCs in circulation [11], we examined the possibility that the oxygenation status of RBCs modulates the phosphorylation of band 3.

It was shown that tyrosine phosphorylation affects the function of band 3 as an anion exchanger, leading to alterations in volume regulation of normal RBCs [9] and dehydration of sickle RBCs [12]. Tyrosine phosphorylation of this anion exchanger in cardiomyocytes was demonstrated to regulate intracellular pH [13]. Phosphorylation of the cytoplasmic domain of band 3 at tyrosine 8 inhibits binding of glycolytic enzymes and promotes their activity [3]. However, the consequence of tyrosine phosphorylation of band 3 on its function as a mechanical linker between the membrane and the underlying skeleton has not been investigated.

The present study examines the possible involvement of tyrosine phosphorylation of band 3 in the regulation of the mechanical properties of the RBC, suggesting an increased association between band 3 and the underlying skeleton as a possible mechanism for the decreased bending deformability [14] and filterability [15] following deoxygenation.

2. Materials and methods

2.1. Blood sampling

Blood samples (20 ml) from healthy volunteers were obtained by venipuncture and mixed immediately with the anticoagulant storage solution CPDA (15.6 mM citric acid, 89.6 mM sodium citrate, 225 mM glucose, 18 mM NaH_2PO_4, 1 mM adenine) in a 7:1 ratio. After centrifugation for 15 min at $200\times g$, 4°C, and removal of the plasma and buffy coat, RBCs were washed three times for 10 min at $1800\times g$ at 4°C in buffer A (25 mM HEPES pH 7.4, NaCl 135 mM and 10 mM glucose), followed by removal of the supernatant and upper 10% of erythrocytes each time. RBCs were resuspended in buffer A at 10% hematocrit. Deoxygenation of blood samples was performed, by flushing the RBC suspensions with pre-wetted nitrogen for 30 min, at 37°C. Hyperosmotic stress was applied at 37°C by employing buffer A, containing an additional amount of NaCl.

2.2. Clamping of intracellular free Mg^{2+} in RBCs

Erythrocytes were incubated for 30 min at 37°C in buffer A containing 5 μM ionomycin from Calbiochem (1000× stock solution in DMSO), 10 μM EGTA in the presence of variable amounts of $MgCl_2$ in the range of 0–10 mM.

2.3. Measurement of intracellular free Mg^{2+} concentration in RBCs

Mg^{2+} concentration was measured using the fluorescent dye mag-fura 2 (Molecular Probes) and the ratio method as described in detail elsewhere (Barbul et al., in preparation). Briefly, erythrocytes were incubated in buffer A containing 10 μM mag-fura 2 acetoxymethylester (200× stock in DMSO), for 45 min at 37°C, washed from the non-trapped dye by buffer A (5 min at $200\times g$, 4°C) and stored on ice to prevent dye leakage before measurements. Dye-loaded RBCs (0.025–0.05% hematocrit) were introduced into a cuvette in 2 ml buffer A containing 1 mM $MnCl_2$ to quench the fluorescence of the dye that leaked to the extracellular medium. The fluorescence intensity (F) at 495 nm was measured when exciting at 341 nm and 371 nm and the ratio $R = F^{341}/F^{371}$ determined. Appropriate corrections were undertaken for the inner filter effect due to the different absorption of hemoglobin at the two excitation wavelengths. Control RBC suspension with the same optical density was used as a non-fluorescent control. Mg^{2+} concentration was calculated from the first-order binding reaction equation: $[Mg^{2+}] = K_d\ Q\ (R^*-R_{min})/(R_{max}-R^*)$ where R^* has been corrected for light absorption $R^* = R$ (Transmission^{371}/Transmission^{341}). The following parameters were determined from the titration curve of mag-fura-2: $K_d = [Mg^{2+}]$ at equal concentrations of bound and free indicator; R_{min} at zero $[Mg^{2+}]$; R_{max} at saturated $[Mg^{2+}]$ (more than 35 mM); $Q = F^{371}_{min}/F^{371}_{max}$.

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2.4. Preparation of RBC membranes and extraction of membrane proteins.

RBCs were lysed at 4°C by diluting 1:20 with buffer B (5 mM sodium phosphate, 1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1 mM PMSF, pH 8.0). White RBC ghosts were obtained by three successive washes in buffer B (20 000×*g* for 10 min at 4°C). Membrane proteins were extracted from the ghosts in buffer C (25 mM HEPES, 0.3 M NaCl, 0.3% Triton X-100, 1 mM EGTA, 0.1 mM PMSF, 0.1 mM sodium orthovanadate and 1 μg/ml aprotinin, pH 7.4) by gentle stirring at 0°C, for 45 min. Further centrifugation was carried out at 4°C for 45 min at 40 000×*g*. Both extract and pellet, or whole RBC ghosts, were mixed 3:1 (v/v) with Laemmli's SDS sample buffer [16], boiled and resolved in 10% SDS-PAGE (20–30 μg of protein per lane, determined by the Bradford method [17]).

2.5. Western blotting

The transfer to Hybond ECL nitrocellulose (Amersham) was followed by 1 h blocking in TTBS buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, 3% bovine serum albumin (BSA), pH 7.4). The blots were incubated with primary antibodies (Ab) for an additional hour, either with anti-phosphotyrosine PY-20, 1 μg/ml (Transduction Laboratories) or with anti-band 3, 1:1000 (Sigma). Blots were washed with TTBS containing 0.2% BSA and incubated 1 h with peroxidase-conjugated goat anti-mouse secondary antibodies, 1:40 000 (Jackson). The ECL detection system (Amersham) or enhanced system (Pierce) was applied. Blots were stained with Ponceau red (1:10 v/v water, Sigma). Films and blots were quantified by densitometry (Dinco and Rhenium BIS-303) employing Tina 2.10g software. Phosphotyrosine-incited signal was normalized to the protein amount in a specific band and is referred to as 'phosphotyrosine', in relative units (r.u.).

2.6. Immunoprecipitation of tyrosine-phosphorylated proteins

RBC membrane extract was incubated at 4°C with anti-phosphotyrosine PY-20 Ab (3.3 μg/ml) for 1 h and then with protein A-Sepharose overnight. The Sepharose pellet was washed three times in 25 mM HEPES buffer containing 0.2% Triton X-100, 75 mM NaCl, 0.1 mM sodium orthovanadate and 0.1 mM PMSF, solubilized in the sample buffer and electrophoresed. Precipitated proteins were analyzed by immunoblotting.

2.7. Measurement of cell membrane fluctuations

Cell membrane fluctuations (CMF) were assayed by point dark field microscopy, as previously described [14], with modifications. RBCs suspended in buffer A containing 1 mg/ml BSA were attached to the glass substrate in the experimental chamber. Time series of light intensity scattered from a small illuminated area (~0.25 μm^2) at the cell's edge were registered at a sampling rate of 75 Hz. The half-width of the amplitude distribution of light intensity fluctuations, normalized to the time-independent component, was averaged for four different points along the periphery of each cell is and referred to as 'CMF level'. Following the measurement of the CMF level under control conditions in buffer A, the chamber was opened and perfused with the appropriate medium to clamp intracellular Mg. The chamber was sealed and incubated at 37°C for 30 min. CMF were measured at the room temperature (~25°C).

3. Results

3.1. Tyrosine phosphorylation of band 3 protein is induced by deoxygenation of intact erythrocytes

The basal level of tyrosine phosphorylation of proteins in normal human RBCs is usually very low, making it difficult to determine the phosphorylation level by immunoblotting with anti-phosphotyrosine Ab. However, deoxygenation of intact erythrocytes led to a modest but significant ~25% elevation in tyrosine phosphorylation of band 3 protein, appearing as a broad band at ~95 kDa (Fig. 1). When tyrosine phosphatase activity is inhibited by sodium orthovanadate, or when RBCs are subjected to a hyperosmotic stress, the phosphorylation of this protein is readily seen. Deoxygenation under these conditions, causes an additional pronounced rise in phosphotyrosine content (Fig. 1). The identification of the phosphorylated protein as band 3 relies on previous literature [1–5]. This was further confirmed by stripping of the phosphotyrosine immunoblots which were then probed with anti-band 3 Ab, yielding the same optical density pattern in the 95 kDa region. In addition, RBC extracts were precipitated with anti-phosphotyrosine Ab, resolved by electrophoresis and examined with anti-band 3 Ab. This resulted in a clearly seen band at 95 kDa (data not shown). Thus, it may be suggested that band 3 (and its 60 kDa catabolic fragment) is the main target of tyrosine phosphorylation in the RBC, although phosphorylation of other minor proteins can not be ruled out.

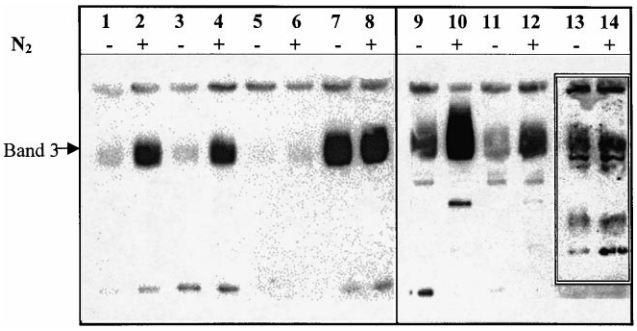

Fig. 1. Phosphotyrosine immunoblots of RBC membrane proteins obtained from oxygenated and deoxygenated cells. RBC suspension was perfused for 30 min, at 37°C, with nitrogen (+) or air (−). Perfusion buffer A contained: 30 μM sodium orthovanadate in the absence (lanes 1,2) or in the presence of 10 μM EGTA (lanes 3,4); 5 μM ionomycin, 3.6 mM $MgCl_2$ and 10 μM EGTA (lanes 7,8); 0.4 M NaCl (lanes 9,10); 0.3 M NaCl (lanes 11,12). In order to improve the visualization of the low level tyrosine phosphorylation in control cells (lanes 5,6) films were overexposed employing the Pierce enhanced chemiluminescence kit (inset, lanes 13,14).

Deoxygenation of RBC elevates tyrosine phosphorylation of band 3 by 2–3-fold in 0.3 and 0.4 M hypertonic NaCl solutions and up to 6-fold in vanadate-treated RBCs (Fig.

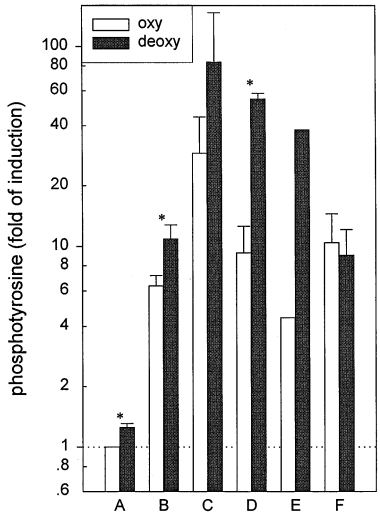

Fig. 2. The level of band 3 tyrosine phosphorylation in oxygenated and deoxygenated erythrocytes. A: control ($n=9$); B: 0.3 M NaCl ($n=4$); C: 0.4 M NaCl ($n=3$); D: 30 μM sodium orthovanadate ($n=4$); E: 30 μM sodium orthovanadate and 10 μM EGTA ($n=2$); F: 3.6 mM $MgCl_2$, 5 μM ionomycin and 10 μM EGTA ($n=7$). Data are expressed as mean ± S.E.M.. $^*P<0.05$, significant difference from the oxygenated pair by Student's *t*-test.

2). Since vanadate is known to also inhibit ATPases, which may lead to the elevation of intracellular free Ca^{2+} in RBCs, some experiments were performed in the presence of EGTA in the extracellular medium. Under these conditions, deoxygenation causes an even larger, 9-fold increase, in band 3 phosphotyrosine content.

3.2. Elevation of intracellular free Mg^{2+} increases tyrosine phosphorylation of band 3

Direct measurements of intracellular free Mg^{2+} concentration ($[Mg^{2+}]_{in}$) in RBCs, based on mag-fura 2, by the fluorescence ratio method, show that free Mg^{2+} concentration in normal RBC increases from 0.38 ± 0.04 mM (mean ± S.E.M.) in the oxygenated RBCs to a value of 0.69 ± 0.06 mM in deoxygenated ones. In order to examine the hypothesis that the elevation of $[Mg^{2+}]_{in}$ is responsible for the enhanced phosphorylation of band 3, $[Mg^{2+}]_{in}$ was clamped at different values, with ionomycin, in the presence of various extracellular Mg^{2+} concentrations ($[Mg^{2+}]_{out}$). The phosphorylation level of band 3 tyrosine and $[Mg^{2+}]_{in}$ were monitored in parallel (Fig. 3A,B). Intracellular free Mg^{2+} was found to stimulate band 3 phosphorylation in a dose-dependent manner. At 3.6–10 mM $[Mg^{2+}]_{out}$, when $[Mg^{2+}]_{in}$ reached the saturation level of 1.2 mM, an elevation of 10.4 ± 4.0-fold (mean ± S.E.M.) of band 3 phosphorylation was observed. RBC deoxygenation, under these Mg clamping conditions, did not cause any additional significant changes in the phosphorylation level of band 3.

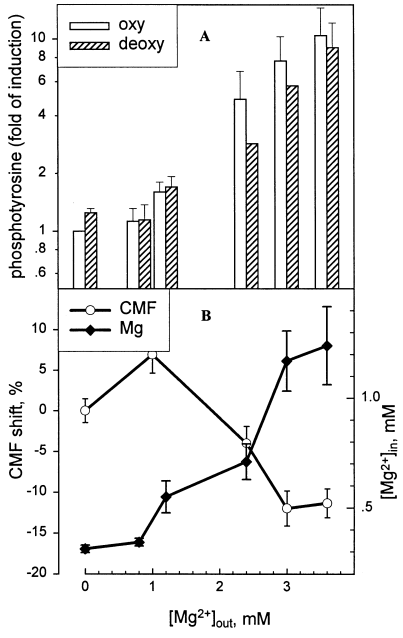

Fig. 3. Band 3 tyrosine phosphorylation, intracellular free Mg^{2+} concentration and RBC membrane fluctuations as functions of Mg concentration in the extracellular medium. RBC $[Mg^{2+}]_{in}$ is clamped at different levels by 5 μM ionomycin in buffer A containing 10 μM EGTA and appropriate extracellular Mg^{2+} concentrations. A: Band 3 phosphotyrosine content. RBCs were incubated for 30 min at 37°C while perfusing with air (oxy) or nitrogen (deoxy). B: Membrane fluctuations (CMF) and intracellular free magnesium concentration (Mg). CMF were measured before and after loading RBCs with Mg^{2+}. The relative shift of CMF level is plotted against Mg^{2+} concentration in the extracellular buffer; 20–30 cells were measured per point. Data are expressed as mean ± S.E.M. Differences from the control are significant for $[Mg^{2+}]_{out}$ exceeding 1 mM by Student's *t*-test with $P < 0.05$.

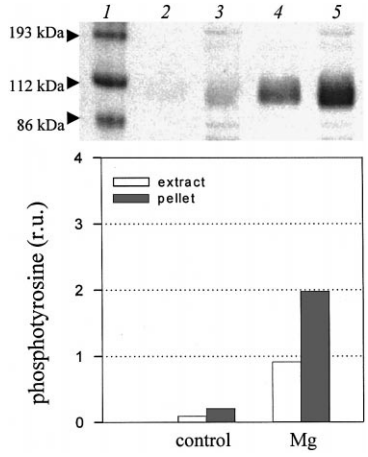

Fig. 4. Extractability of tyrosine-phosphorylated band 3 from control and Mg-clamped RBCs. Top: Tyrosine phosphorylation pattern of erythrocyte band 3 protein, immunoblotting with anti-phosphotyrosine Ab. Lane 1:- molecular weight standards; lanes 2,3: control; lanes 4,5: RBCs, incubated for 30 min at 37°C with 5 μM ionomycin, 3.6 mM Mg^{2+} and 10 μM EGTA. Lanes 2,4: extracts; lanes 3,5: Triton X-100-insoluble pellets. Bottom: Distribution of phosphorylated band 3 between Triton X-100-soluble and -insoluble fractions of RBC membranes.

3.3. Elevation of intracellular free Mg^{2+} leading to band 3 phosphorylation is accompanied by rigidification of RBCs

Bending deformability of the RBC's membrane skeleton was determined by measuring the CMF level in intact RBCs. Band 3 phosphorylation was stimulated by elevating $[Mg^{2+}]_{out}$ in the presence of an ionophore, and CMF were measured in the same cells before and after the stimulation. Significant abolition of erythrocyte membrane fluctuations is registered at external Mg^{2+} concentration exceeding 2.4 mM

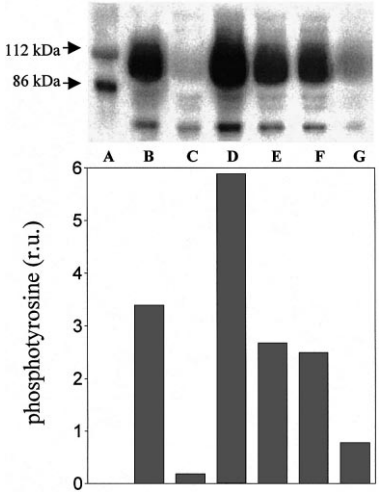

Fig. 5. Reversibility of band 3 tyrosine phosphorylation in situ. A: standard proteins; B: RBCs exposed to 0.3 M NaCl; C: control, oxygenated RBCs in a medium with normal tonicity; D: RBCs were exposed to 0.3 M NaCl and deoxygenated simultaneously; E: cells from the previous lane (D) were reoxygenated in the same 0.3 M NaCl solution; F: erythrocytes loaded with Mg^{2+} in buffer A containing 3 mM $MgCl_2$ and 5 μM ionomycin; G: cells from F whose intracellular magnesium content was reduced by incubation in buffer A containing 2 mM EDTA, 5 μM ionomycin and 10 μM EGTA. All incubations were carried at 37°C for 30 min.

(Fig. 3B), under conditions of pronounced elevation of band 3 phosphorylation (Fig. 3A).

3.4. The association of phosphorylated band 3 with the skeleton in control and Mg-clamped RBCs

The strength of the association between band 3 and RBC's skeleton was probed by extracting the membrane skeleton with a Triton X-100-containing buffer. The phosphorylated band 3 was shown to distribute unequally between the insoluble skeletons and extracts. The proportion of tyrosine-phosphorylated protein remaining in association with other skeletal proteins exceeds by 2-fold the phosphorylated component in the extractable fraction both in control and Mg-stimulated erythrocytes (Fig. 4).

3.5. Deoxygenation and Mg-induced phosphorylations are reversible

The phosphorylation of band 3 was found to decline almost to its initial level under hyperosmotic conditions when RBCs, exposed to both hypertonicity and deoxygenation, were reoxygenated (Fig. 5, lanes D,E). A similar situation was observed when RBCs were first incubated with 1.2–3 mM Mg^{2+}, in the presence of ionomycin, and then were transferred to a solution containing EDTA and ionomycin which led to the decrease of $[Mg^{2+}]_{in}$ (Fig. 5, lanes F,G).

4. Discussion

About a two-fold rise of $[Mg^{2+}]_{in}$ accompanies the deoxygenation process as demonstrated by previous [11] and present data. When this concentration jump is simulated by clamping $[Mg^{2+}]_{in}$ at different values, in the range of 0.3–1.2 mM, it incites tyrosine phosphorylation of band 3 protein in a dose-dependent manner. Though both stimuli induce tyrosine phosphorylation of band 3, the effect induced by elevation of $[Mg^{2+}]_{in}$ to its level in deoxygenated RBCs is higher compared to the effect of the deoxygenation itself. The observed difference may be attributed to competitive binding between PTK and hemoglobin to the site at the cytoplasmic domain of band 3. The acidic amino-terminal portion of the band 3, which is known to bind glycolytic enzymes and hemoglobin [3], also associates with tyrosine kinase which can be displaced by an excess of glyceraldehyde 3-phosphate dehydrogenase [2]. Since deoxy-hemoglobin has a much higher affinity for band 3 than its oxy-form, one may expect a competition between PTK and deoxyhemoglobin for the cytoplasmic tail of band 3 to attenuate the phosphorylation of the later one. This attenuation does not occur in oxygenated RBCs, where the phosphorylation level of band 3 depends only on the balance between activities of tyrosine kinase and tyrosine phosphatase. Therefore, ionophore-induced elevation of $[Mg^{2+}]_{in}$ in oxygenated RBCs will lead to a higher phosphorylation of band 3. This suggestion is supported by the finding of increased tyrosine phosphorylation of band 3 in hemoglobinopathies [18], where abnormal binding of hemoglobin to band 3 takes place.

The mechanisms of deoxygenation and Mg-stimulated tyrosine phosphorylations of band 3, as well as hypertonicity- [9] and Ca-induced [5] ones, are still unclear. The results emerging from the deoxygenation experiments, performed in the presence of vanadate, suggest that tyrosine kinase activation rather than phosphatase suppression is responsible for the net increase of band 3 phosphorylation. It is important to point out that the differences in band 3 tyrosine phosphorylation between normal and sickle erythrocytes can be noticed only in the presence of sodium orthovanadate [18] or upon deoxygenation [12]. Protein tyrosine kinase $p72^{Syk}$ was proposed to be responsible for the hypertonically induced phosphorylation of band 3 [9], although traditional inhibitors of Syk were ineffective in the case of intact RBCs [9,12]. One may anticipate that the 50% elevation of $[Mg^{2+}]_{in}$ in osmotically shrunken RBCs (Barbul et al., in preparation) will activate PTKs. Thus, it is expected that band 3 becomes more accessible for the kinase due to the hyperosmotic-induced elevation of the ionic strength in the cytosol, taking into account the electrostatic nature of binding of hemoglobin and glycolytic enzymes to the cytoplasmic domain of band 3 [19]. Deoxygenation in a hyperosmotic medium is anticipated to cause a further rise of $[Mg^{2+}]_{in}$ with subsequent elevation of band 3 phosphotyrosine content.

Our study suggests that tyrosine phosphorylation of band 3 leads to the strengthening of its association with the spectrin network. First of all, phosphorylated band 3 preferentially remains in Triton X-100-insoluble skeletal fraction. Secondly, phosphorylation of band 3 in intact RBC, caused by elevation of $[Mg^{2+}]_{in}$, is accompanied by a significant decrease of cell membrane fluctuations. The observed decrease in CMF reflects the rigidification of the membrane skeleton [20,21]. This rigidification can, in principle, be caused both by band 3 phosphorylation and by a possible direct effect of Mg^{2+} on the cytoskeleton.

Since changes in the level of band 3 phosphorylation in the intact RBC, induced by physiological stimuli (deoxygenation and hypertonicity), were shown to be reversible, it may be assumed that the system responsible for band 3 tyrosine phosphorylation functions under physiological regulation. The presence of a complex machinery for the phosphorylation of band 3, involving among others active PTKs and PTP, evinces the notion that tyrosine phosphorylation in human RBCs is not just a leftover of their precursor stem cell, but may play a role both in blood circulation and in the removal of senescent RBCs.

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