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# Phosphotyrosine phosphatases acting on band 3 in human erythrocytes of different age: PTP1B processing during cell ageing

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**Abstract**

The phosphorylation of tyrosine residues of human red blood cell (RBC) band 3 is regulated in vivo by constitutively active tyrosine-kinases (PTKs) and phosphotyrosine-phosphatases (PTPs), identified so far as, respectively, $p72^{syk}$ and $p56/53^{lyn}$, and PTP1B and SHPTP-2. Tyr-phosphorylation of band 3 increases upon reduction of cell volume as in hypertonic media or during $Ca^{2+}$-induced membrane vesiculation. We show here that old RBCs display higher Tyr-phosphorylation levels of band 3 than younger cells under hypertonic conditions, at least in part due to the reduced cell volume of old RBCs, a condition of lowered threshold for activation of volume-sensitive PTKs. We have also analysed the membrane-bound PTP activity and the relative abundance of PTP1B (as the main membrane-associated PTP) in RBCs of different age. Immunodetection of PTP1B in purified ghost membranes revealed that the catalytic, N-terminal domain of the PTP is partially cleaved, in an age-dependent manner, from the membrane-bound domain, and it is lost during the preparation of ghost membranes. This suggests that erythrocytes may undergo in vivo activation of the $Ca^{2+}$-dependent calpain system that proteolytically regulates PTP1B activity, as already documented for other cell types. On the other hand, the assay of the PTP activity that remains associated with the membranes of RBCs of different age indicated that the PTP undergoes oxidative inactivation that can be further differentiated into reversible and irreversible components.
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*Keywords:* Red blood cell ageing; PTP1B; Protein 4.1; Band 3; Vesiculation; Tyr-kinase

## 1. Introduction

Band 3 protein is the major substrate for constitutively active Tyr-kinases (PTK) and phosphoTyr-phosphatases (PTP) of the human red blood cell (RBC). Tyr-phosphorylation of band 3 rises above basal, undetectable levels upon erythrocyte deoxygenation [1], or cell shrinkage due to hypertonicity or opening of the $Ca^{2+}$-activated $K^+$ channel [2]. When evaluated in the total population of RBCs, the shrinkage-induced band 3 phosphorylation appears to depend mainly on the stimulation of PTK activity, likely due to $p72^{syk}$, rather than inhibition of PTP activity [2]. As described in this paper, PTK activity appears largely conserved throughout RBC life, as suggested by the fact that old RBCs display even higher phosphorylation levels of band 3 than younger ones when challenged with hypertonic treatments. The latter phenomenon could be due to several factors, of which we have considered in this work: the reduced volume and cell hydration of old RBCs (a condition of lowered threshold for activation of volume-sensitive PTKs), and a partial impairment, or quantitative loss, of PTPs. We have analysed the total PTP activity in purified ghost membranes, and the relative abundance of PTP1B, as the main membrane-associated PTP that dephosphorylates band 3 [3] in RBCs of different age.

## 2. Experimental

RBCs of different age were obtained, by centrifugation on discontinuous stractan density gradients, as previously detailed [4]. Six subpopulations of cells (F1 to F6) of the following densities (g/ml) were typically obtained: $1.075 < F1 < 1.090 < F2 < 1.095 < F3 < 1.101 < F4 < 1.103 < F5 < 1.110 < F6 < 1.137$. Each subpopulation was characterized for RBC and reticulocyte count, mean cell volume

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(MCV, fl), mean corpuscular haemoglobin concentration (MCHC, g/dl) and protein 4.1a/4.1b ratio as a density-independent parameter that increases with cell age [4]. The latter was measured after separation, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of membrane proteins from white ghost membranes that were prepared as detailed elsewhere [4] and were also used for the assay of total PTP activity as vanadate-sensitive, *p*-nitrophenylphosphate hydrolysing activity, in the absence or presence of 2 mM dithiotreitol (DTT) [3]. PTP1B was quantified, after SDS-PAGE of whole RBCs or white ghost membranes, by Western blotting and chemiluminescence detection (ECL; Amersham Biosciences, Freiburg, Germany). The primary antibody used was a monoclonal anti PTP1B, recognising an epitope in the catalytic, N-terminal domain of PTP1B (clone FG6-1G, Calbiochem, La Jolla, CA, USA) and thus able to detect both the native, membrane-bound form (50 kDa) and the cytosolic (42 kDa) fragment of PTP1B. Immunodetection of band 3 tyrosine phosphorylation was carried out, after SDS-PAGE and transfer to nitrocellulose of whole RBCs, with monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate, Miton Keynes, UK) followed by chemiluminescence detection.

## 3. Results

For this study, six subpopulations of erythrocytes of different age were obtained, in each of several independent experiments, by density separation as previously described [4] and characterized for their cellular properties. It was in particular verified, by measuring the cell-age parameter protein 4.1a/4.1b ratio, that the separation technique provided a true enrichment in old RBCs in the densest cell fractions (Fig. 1).

Fig. 2 shows the immunodetection of Tyr-phosphorylated band 3 in erythrocytes of different age stimulated with hypertonic treatment. Old RBCs (F6) display higher phosphorylation levels of band 3 than younger cells (F1). This could be at least in part explained by the reduced cell volume of old RBCs and their dehydrated state (see MCV and MCHC plots in Fig. 1), which lower the threshold of a putative shrinkage-sensitive kinase [2]. To determine whether other factors could contribute to the phenomenon, we analysed the phosphotyrosine-phosphatase activity and the content of the main membrane-bound PTP in RBCs of different age. Total PTP activity was assayed in white ghost membranes under the assumption, previously supported by others [3], that membrane-bound PTP1B is the major PTP of human RBCs acting on band 3. As reported in Fig. 3 (empty circles), the PTP activity (operationally defined as the vanadate-sensitive *p*-nitrophenylphosphate hydrolysing activity) decreases from young to old RBCs, when normalized over total protein content of ghost membranes. A parallel assay was conducted under reducing conditions

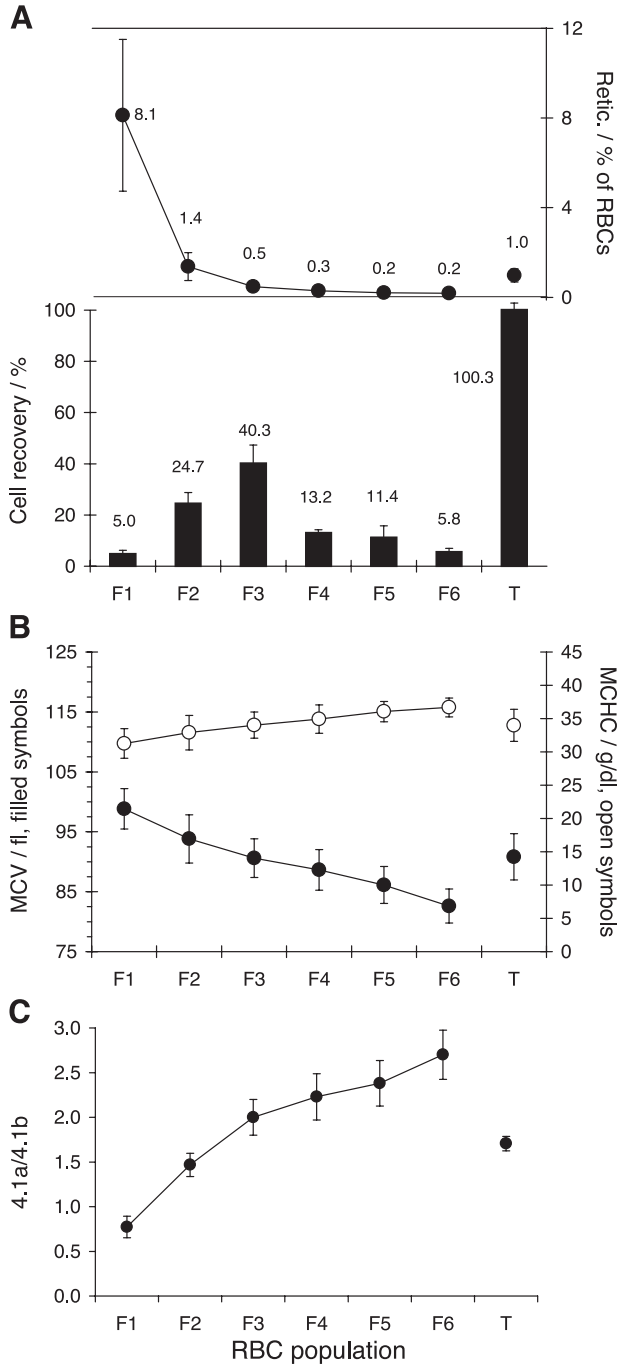


Fig. 1. Separation and characterization of erythrocytes of different age. (A) Recovery of RBCs (histogram) and reticulocytes (line), in subpopulations of increasing cell age/density from F1 to F6. (B) Mean cell volume (MCV/fl, filled circles) and mean corpuscular haemoglobin concentration (MCHC/g/dl, empty circles) of cells of different age/density. (C) The cell-age parameter 4.1a/4.1b was quantified after SDS-PAGE of RBC membrane proteins. All parameters are average of values measured in four independent experiments. Error bars represent standard deviation.

(DTT added) to reverse a possible partial damage of the oxidation-sensitive PTPs. As shown in Fig. 3 (filled circles), the reducing treatment increases the PTP activity in all subpopulations. The extent of the recovery is ap-

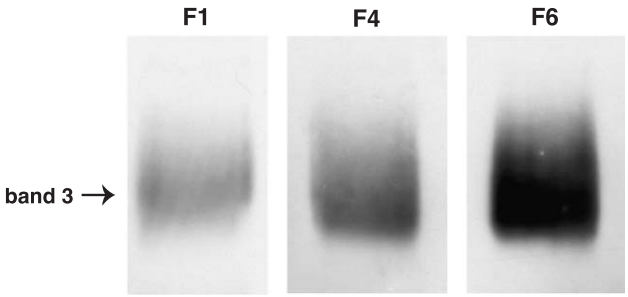


Fig. 2. Tyr-phosphorylated band 3 in RBCs of different age after hypertonic treatment. RBCs suspensions at 10% haematocrit were treated for 15 min at 37 °C in 5 mM phosphate buffer pH 7.4, 4 mM KCl, NaCl to 600 mosM total. After incubation, aliquots of the cell suspensions were treated with sample buffer and subjected to SDS-PAGE. Immunodetection of Tyr-phosphorylated proteins was carried out as described under Section 2. F1: young cells, F4: middle-age cells, F6: old cells. An equal number of cells is present in each lane. The immunoblot shown is representative of results obtained in four similar experiments.

proximately 20% of the activity under non-reducing conditions in young/middle-aged cells (F2–F3), while it decreases to approximately 10% in older cells (F6). The difference is statistically significant between F3 (representative of the most abundant population of cells) and F6 (population of 5% oldest cells). We interpreted this observation as indicative of a progressive increase, with cell age, of a subpopulation of phosphatase molecules that suffered from a damage that could not be restored by mild reducing treatments, probably a more severe oxidation of the active-site cysteine, or protein denaturation. We wondered if the decline in total PTP activity from young to old cells could be accounted for by a quantitative loss of PTP1B with ageing. PTP1B exists in vivo as two related polypeptides, a native 50-kDa form, membrane-bound by virtue of its C-terminal hydrophobic domain [5], and the catalytic, N-terminal domain, free in the cytosol. It has been described that in platelets the proteolytic cut of PTP1B is under control of the calpain system of $Ca^{2+}$-dependent proteases and that the event is associated with an increase in the activity of the released catalytic domain of PTP1B [6]. PTP1B was found in white ghost membranes from RBCs of different age, as the native 50-kDa form and, in lower amounts, also as the 42-kDa form, as shown in Fig. 4. The PTP1B decreases from young to old cells, due to the decrease in the 50-kDa form, without a corresponding increase in the 42-kDa form. The decrease mirrors the decline in total PTP activity measured in ghosts. However, when the quantification of PTP1B was repeated using whole erythrocytes instead of purified ghost membranes, a different result was obtained. As shown in Fig. 5, total PTP1B (50 + 42 kDa) keeps virtually constant throughout RBC ageing, while the relative abundance of its two components changes in an age-dependent manner, with a significant increase of the 42-kDa form at the expenses of the native, membrane-bound 50-kDa form. Most of the 42-kDa form could be lost during preparation of ghost membranes, which explains its quantitative decline in ghosts

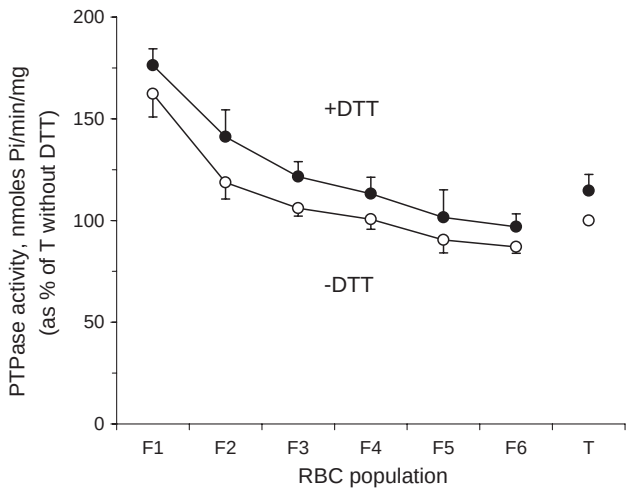


Fig. 3. PTP activity in ghost membranes from RBCs of different age. The *p*-nitrophenylphosphate-hydrolysing activity of ghost membranes was measured in the presence (filled circles) or absence (empty circles) of DTT. The measured values were referred to total protein content of the samples. Finally, the calculated value of the total population (T) (in the absence of DTT) was taken as the 100% to which all other values were referred. Data are average of values measured in three independent experiments.

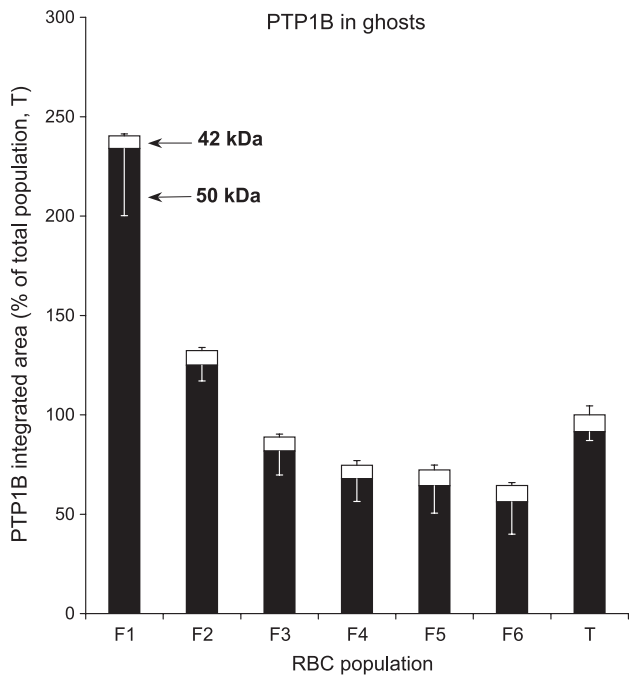


Fig. 4. PTP1B in white ghost membranes from erythrocytes of different age. Equal amounts of membrane, based on quantification of band 3, were loaded for each sample. The total amount of PTP1B (50 + 42 kDa form) of the total population of cells (T) was taken as 100%, and each PTP1B form from each other subpopulation was referred to this value. PTP1B 50 kDa, black bars; PTP1B 42 kDa, white bars. All parameters are average of values measured in three independent experiments.

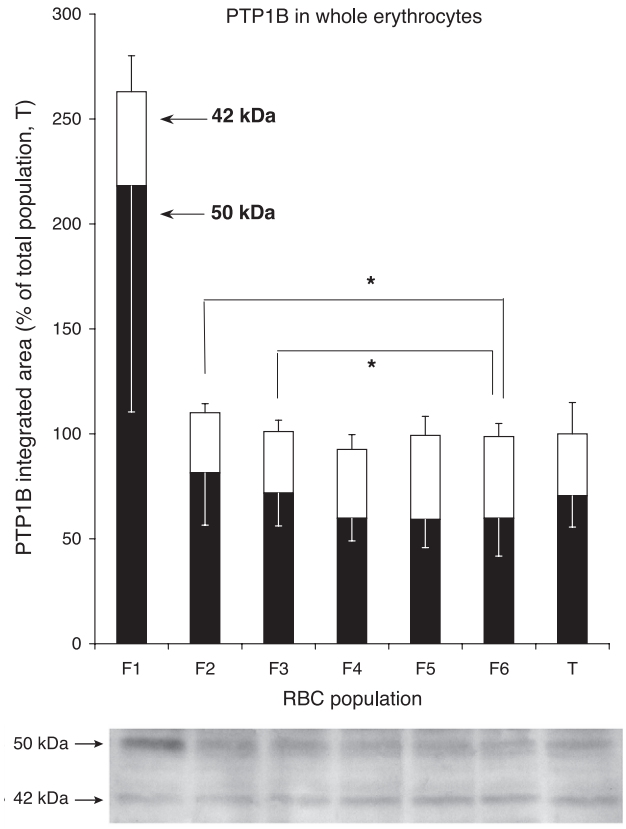


Fig. 5. PTP1B in erythrocytes of different age. PTP1B was quantified after SDS-PAGE of whole erythrocytes and immunodetection (representative blot shown at the bottom). An identical number of cells ($10^7$) from each subpopulation was loaded, based on cell count. The total amount of PTP1B (50 + 42 kDa) of the total population of cells (T) was taken as 100%, and each PTP1B form from each other subpopulation was referred to this value. PTP1B 50 kDa, black bars; PTP1B 42 kDa, white bars. All parameters are average of values from four independent experiments. Asterisks show statistical significance, to the paired *t*-test, for the difference in the amount of the 42-kDa fragment, between the populations indicated.

(Fig. 4) and the decrease in total PTP activity with cell age (Fig. 3).

## 4. Discussion

The higher levels of Tyr-phosphorylated band 3 induced by hypertonicity in old RBCs compared to younger cells seem to depend, at least in part, on the reduced cell volume and hydration of senescent RBCs, which lower the threshold for activation of volume sensitive PTK(s) [2]. Results obtained in this paper suggest that a partial impairment of the membrane-bound PTP activity could also contribute to the observed phenomenon. Most of this activity has been ascribed to PTP1B [3], which on the one hand seems to undergo a progressive proteolytic processing during ageing and, on the other, seems to suffer from inactivation due to oxidative damage. PTP assay in purified ghost membranes may not reflect the true state of PTP1B in the cell, due to the loss of the PTP1B's 42 kDa domain during ghost preparation, as described here. A different method for assaying total PTP activity in whole cells is being developed for addressing this issue more precisely. Nevertheless, assay of PTP activity in ghosts, which reflects mainly the residual membrane-bound 50-kDa form of PTP1B, showed that reducing agents could increase total PTPase activity, indicating that an oxidative damage affects the PTPs during ageing, part of which is reversible (in vivo reduction could be sustained by intracellular GSH), while part is irreversible and mostly affects older RBCs. The age-dependent proteolytic processing of PTP1B may be a marker of activation events of the $Ca^{2+}$-dependent calpain system, which appears to be responsible for the cleavage of PTP1B in other cell types [6]. The accumulation of the 42-kDa fragment of PTP1B should be accompanied by an increase in PTP activity (as observed in platelets), which should favour the dephosphorylation of band 3 in old cells. This is opposed to what we have observed, suggesting that up-regulation of PTP1B may not follow its proteolytic processing in the human RBC. In this cell type, the prevailing effect, after the cleavage of PTP1B's 42-kDa domain, could be the dissociation of the phosphatase from its major substrate, band 3, which in turn would lead to increased net tyrosine phosphorylation of band 3. Interestingly, a similar model of $Ca^{2+}$-dependent uncoupling of the band 3-PTP1B association has been recently proposed, which, while not contemplating the proteolytic processing of PTP1B, is compatible with the results described here [7].

An increased $Ca^{2+}$ content in dense RBCs with respect to light cells has been documented [8], although the unequivocal demonstration of stable increases in $Ca^{2+}$ content in old RBCs of well-defined cell-age is still lacking. Evidence for differences in the $Ca^{2+}$-extrusion capacity between young and old RBCs is also sparse and controversial [9]. Possible alterations of $Ca^{2+}$ homeostasis in RBC ageing have to be taken into account [10], given the importance of $Ca^{2+}$ in several aspects of RBC physiology, such as regulation of Gardos channel, $Ca^{2+}$-pump, calpain and calmodulin, and membrane vesiculation. Stable increases in intracellular $Ca^{2+}$ may not necessarily represent the key demonstration of a role for $Ca^{2+}$ in RBC senescence: accumulation of $Ca^{2+}$ could be transient, and rapidly reverted by the $Ca^{2+}$-ATPase. Such transients could however be sufficient to trigger events like the opening of the $Ca^{2+}$-activated $K^+$ channel, membrane vesiculation and, as shown here, the cleavage of PTP1B. The accumulation of PTP1B 42 kDa in old RBCs may therefore represent the irreversible mark of transient increases in intracellular $Ca^{2+}$ that may occur during RBC ageing.

Increased levels of band 3 Tyr-phosphorylation have been detected in some hemoglobinopathies [11,12] such as sickle cell disease and β-thalassemia. The phenomenon has been interpreted as the result of oxidation-dependent PTP inhibition in the pro-oxidant environment favoured by abnormal, unstable haemoglobins. However, other factors could be at play, for example increased dehydration of

RBCs [13], which also would favour band 3 Tyr-phosphorylation. It would be interesting to verify whether, under the hypothesis presented here, the proteolytic processing of PTP1B occurred also in these abnormal RBCs where $Ca^{2+}$ homeostasis is likely altered [14].

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