Differential sorting of tyrosine kinases and phosphotyrosine phosphatases acting on band 3 during vesiculation of human erythrocytes

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One of the most intensively studied post-translational modifications of erythrocyte proteins is the phosphorylation of tyrosine residues of band 3, which is strictly regulated *in vivo* by PTKs (protein-tyrosine kinases) and PTPs (protein-phosphotyrosine phosphatases). Two PTKs (p72^{syk} and p56/53^{lyn}) and two PTP activities (PTP1B and SHPTP-2) have been immunologically identified so far in mature human erythrocytes. We have shown previously that band 3 undergoes tyrosine phosphorylation upon a decrease in cell volume, as occurs when erythrocytes treated with Ca²⁺/Ca²⁺ ionophore (A23187) lose KCl and release microvesicles. Similar levels of band 3 tyrosine phosphorylation in vesicles and in the parent cells are induced by this treatment. However, we have found that tyrosine phosphorylation of band 3 in vesicles is more stable than in whole erythrocytes. Examination of how the identified PTPs and PTKs are partitioned between the vesicles and the remnant cells during vesiculation reveals that PTP1B, unlike the PTKs, is retained entirely in the parent

cell compartment. Since a tight association between PTP1B and band 3 has been documented previously, we have investigated the partitioning of PTP1B and band 3 between the membrane and the membrane-skeletal fractions prepared from resting or Ca²⁺/A23187-treated cells. Our results rule out the possibility that the preferential retention of PTP1B within the cell was due to an increase in the amount of membrane-skeleton-associated band 3 (and of PTP1B) during the release of spectrin-free vesicles, suggesting a more complex modality of interaction of PTP1B with band 3 in the erythrocyte membrane. Analysis of erythrocytes of different cell ages revealed that PTP1B, unlike the other enzymes examined, was quantitatively conserved during erythrocyte aging. This suggests important roles for the down-regulation of tyrosine phosphorylation of band 3 in erythrocyte physiology, and for vesiculation as a mechanism of human erythrocyte senescence.

Key words: aging, erythrocyte, Lyn, PTP1B, SHPTP-2, Syk.

INTRODUCTION

The mammalian erythrocyte is a simplified cell type, lacking nucleic acids, the apparatus for protein synthesis and most of the cell signalling pathways that are essential in other cell types. Despite this simplification, mature erythrocytes retain a number of molecular components of signalling and/or regulatory pathways [1]. Of these, particular interest has been focused on erythrocyte protein kinases and phosphatases, because of their possible roles in the modulation of erythrocyte morphology, deformability and metabolic functions [2,3].

One of the most intensively studied post-translational modifications of erythrocyte proteins is the phosphorylation of tyrosine residues of band 3, which is strictly regulated *in vivo* through the concerted action of PTKs (protein-tyrosine kinases), and PTPs (protein-phosphotyrosine phosphatases). Two PTKs have been identified in mature erythrocytes: p72^{syk} and p56/53^{lyn} [4]. Soluble cytoplasmic, as well as membrane-bound, PTP activities have been documented [5–7]. A detailed study of the membrane-associated PTP activity of human erythrocytes has been conducted, with the most important component being identified as the non-receptor, non-SH2 (Src homology 2)-domain-containing PTP1B (or a closely related PTP), which appears to play a major role in down-regulating the tyrosine phosphorylation status of band 3 *in vivo* [8]. More recently, a previously described soluble PTP activity [9] has been ascribed to the SH2 domain-containing phosphatase SHPTP-2 [10].

We have shown that the tyrosine phosphorylation of band 3 is reversibly enhanced by a decrease in cell volume, as occurs in

hypertonic media [11] or when erythrocytes lose K⁺ and shrink in response to activation of the Gardos channel (Ca²⁺-dependent K⁺ channel) [12]. An increase in the intracellular Ca²⁺ concentration due to the Ca²⁺ ionophore A23187 also brings about cell crenation, echinocytosis and the release of microvesicles [13].

Because of the delicate balance that exists under physiological conditions in the mature erythrocyte between PTPs and PTKs, we are investigating the role of band 3 tyrosine phosphorylation in the modulation of membrane stability during erythrocyte vesiculation. We show in the present paper that the PTKs and PTPs responsible for the modification of band 3 are sorted differentially between the spectrin-free vesicular compartment and the remnant cells during the vesiculation event. In particular, the major membrane-bound PTP of the human erythrocyte appears to be confined entirely to the remnant cells, and is not detected in the vesicles. Conversely, both kinases p72^{syk} and p56/53^{lyn}, and the recently described phosphatase SHPTP-2, do not follow an 'all-or-none' rule in partitioning between cells and vesicles, and are all detected, in variable amounts, in both compartments. The functional consequence of this differential sorting is that tyrosine phosphorylation of band 3 is reversible in cells, but stable in vesicles, due to the lack, or extremely low levels, of PTP activity in the vesicular compartment.

We have addressed the issue of how the complete exclusion of PTP1B from the vesicle membrane during vesiculation might originate. Previous studies suggested a prevalent association of PTP1B with band 3 [8], and the amount of band 3 relative to membrane lipids is similar [14–16] or slightly reduced [17] in vesicles with respect to the parent cell. Results suggest a complex

Abbreviations used: PTK, protein-tyrosine kinase; PTP, protein-phosphotyrosine phosphatase; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane; Ht, haematocrit; SH2, Src homology 2.

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interaction between PTP1B and the membrane, probably involving the sublocalization of PTP1B to microdomains, where it associates with band 3 or lipid molecules, mostly via hydrophobic interactions. The precise nature and arrangement of these regions and their fate during vesiculation need further study.

As they age *in vivo*, mammalian erythrocytes lose membrane surface area and decrease in size [18]. Membrane vesiculation has been suggested to occur *in vivo* [19], and to represent an important event in the senescence of erythrocytes [20,21]. We have studied the PTPs and PTKs in erythrocytes of different ages. The results obtained indicate that PTP1B is conserved quantitatively, on a per cell basis, during erythrocyte aging. This suggests that a mechanism similar to that of *in vitro* vesiculation is responsible for PTP sequestration within the cell membrane during the progressive decrease in cell size and surface extension that takes place as erythrocytes age *in vivo*. The peculiar behaviour of PTP1B observed in two different model systems of *in vitro* vesiculation and in erythrocytes of different ages further supports the notion that microvesiculation takes place during erythrocyte senescence.

EXPERIMENTAL

Antibodies and reagents

Mouse antibody against recombinant PTP1B of human origin, recognizing an epitope in the catalytic, N-terminal domain of PTP1B (clone FG6-1G), was from Calbiochem (La Jolla, CA, U.S.A.), and mouse monoclonal anti-phosphotyrosine antibody (clone 4G-10) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Polyclonal rabbit antibodies against p72^{syk} of human origin, recognizing an epitope in the N-terminus of Syk, against p56/p53^{lyn} of human origin, recognizing an epitope in the N-terminus of Lyn, and against SHPTP-2 of human origin, recognizing an epitope mapping in the C-terminus of SHPTP-2, were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies for Western blotting and Triton X-100 were from Bio-Rad, and chemiluminescence detection reagents (ECL-plus[®]) and Percoll[®] were from Amersham Biosciences. Polyarabinogalactan was obtained from Larex Inc. (St. Paul, MN, U.S.A.). Calcium ionophore A23187, bestatin and pepstatin A were from Calbiochem. Aprotinin, leupeptin, PMSF, Tos-Lys-CH₂Cl (tosyl-lysylchloromethane), Tos-Phe-CH₂Cl (tosylphenylalanylchloromethane) and n-octyl glucoside were from Sigma (St. Louis, MO, U.S.A.).

Isolation of erythrocytes

Blood was obtained from normal volunteers and collected into 0.1 vol. of either 3.8% (w/v) sodium citrate aqueous solution or acid/citrate/dextrose (130 mM citric acid, 152 mM tri-sodium citrate and 112 mM glucose). After removal of plasma, cells were filtered through cellulose to remove leucocytes and platelets, as described in [22]. Purified erythrocytes were washed with buffer A (5 mM Hepes, 2 mM MgCl₂, 150 mM NaCl and 5 mM glucose, pH 7.4; 300–305 m-osmol/kg of water) containing 0.2 mM PMSF, and resuspended to 50% Ht (haematocrit) in the same buffer for subsequent experiments.

Ca²⁺-induced vesiculation of erythrocytes

To a 3% Ht erythrocyte suspension in buffer B (buffer A containing the protease inhibitors 65 μ M bestatin, 5.3 μ M pepstatin

A, 68 μ M Tos-Lys-CH₂Cl, 71 μ M Tos-Phe-CH₂Cl, 10 μ g/ml aprotinin and 160 μ M leupeptin) equilibrated at 37 °C was added CaCl₂ from a 250 mM solution to a final concentration of 0.3 mM under gentle stirring with a magnetic bar. Vesiculation was started by the addition of A23187 to a final concentration of 4 μ M from a 10 mM stock solution in DMSO. Samples were taken at various times for other analyses. Incubation was commonly conducted for 20 min, after which EGTA was added to a final concentration of 2.0 mM from a stock solution of 60 mM, pH 7.0, and cells were sedimented. The vesicle-containing supernatant was spun at 27 000 g for 20 min at 15 °C, and vesicles were carefully resuspended in buffer B and purified from contaminating erythrocytes and cell fragments on self-forming Percoll[®] gradients, as described previously [12]. Quantification of vesicles was routinely conducted by assaying the total protein content of a vesicle suspension by the Lowry method [23].

Vesiculation induced by nutrient deprivation

To obtain vesicles by a Ca²⁺-independent method, purified erythrocytes were washed in 10 mM glycyl-glycine/150 mM NaCl, pH 7.4, and suspended at 20% Ht in 50 mM glycyl-glycine, 5 mM KCl, 0.5 mM EDTA, NaCl up to a total osmolality of 295 m-osmol/kg of water and 50 μ g/ml ampicillin, pH 7.4, at 37 °C. The suspension (25–30 ml) was placed in a dialysis tube (cut-off 12 000–14 000 Da) and incubated for 24 h at 37 °C in a beaker containing 2 litres of the same buffer, under gentle agitation [15]. Then the cells were collected and sedimented. Vesicles were separated from the supernatant and purified from contaminating erythrocytes and cell fragments either by Percoll[®] gradient centrifugation as described above or by filtration through a 0.45 μ m-pore-size filter. Collected vesicles were processed as described for other vesicle samples.

Separation of erythrocytes of different ages

Erythrocytes were separated by centrifugation on discontinuous density gradients of polyarabinogalactan into six subpopulations of the following densities (g/ml): 1.075, 1.090, 1.095, 1.101, 1.103 and 1.110. Erythrocyte indices, and counts of the cells recovered in each fraction, were measured with a Cell-Dyn 4000 Hematology Analyser (Abbott Laboratories, Abbott Park, IL, U.S.A.). Ghost membranes were prepared from each subpopulation, and membrane proteins were separated by SDS/PAGE to quantify the ratio between the membrane proteins 4.1a and 4.1b as a parameter of cell age, as detailed elsewhere [24]. Whole cells were dissolved and subjected to SDS/PAGE and Western blotting as described below.

Preparation of membrane skeletons by detergent extraction of whole erythrocytes

Whole resting or Ca²⁺/A23187-treated erythrocytes were extracted [25] with Triton X-100, or n-octyl glucoside, which was described previously to better preserve the oligomeric state of band 3 than other non-ionic detergents [26]. Erythrocytes at 10% Ht in buffer A were treated with 0.3 mM Ca²⁺ and 5 μ M A23187 at 37 °C. At various times, aliquots were sampled and 2 mM EGTA (final concentration) was added. Cells were sedimented and supernatants discarded. Packed cells were resuspended at 50% Ht in buffer A. To this suspension was added an equal volume of ice-cold buffer A containing 4% (w/v) n-octyl glucoside or Triton X-100, 0.5 mM DTT, 40 μ M leupeptin, 30 μ M pepstatin

A and 0.46 mM PMSF. Samples were layered on a 35 % (w/v) sucrose solution in an ultracentrifuge tube and spun in a swinging-arm rotor at 98 000 *g* (r_{av}) for 90 min at 4 °C. Samples of the supernatants were collected and diluted SDS/PAGE sample buffer was added (see below). The pellets were resuspended in buffer A to give a final volume equal to the original volume of packed cells, and 0.5 vol. of SDS/PAGE sample buffer was added.

Preparation of ghost membranes

White ghost membranes were prepared as described previously [27]. Briefly, erythrocytes were lysed with 9 vol. of lysis buffer (5 mM Tris/HCl, pH 7.4, and 1 mM EDTA, plus the same protease inhibitors as in buffer B) at 4 °C. Membranes were collected by centrifugation at 25 000 *g* for 30 min at 4 °C, washed again with lysis buffer and once with lysis buffer containing 0.5 M NaCl ('high-salt' wash). This latter step was omitted for some samples (see the Results section). White ghosts were obtained by additional washes with lysis buffer.

Solubilization of samples for SDS/PAGE and Western blotting

Ghost membranes

Aliquots of white ghost suspensions were mixed with 0.5 vol. of SDS/PAGE sample buffer [50 mM Tris/HCl, 5 % (w/v) SDS, 35 % (w/v) sucrose, 5 mM EDTA, 200 mM dithiothreitol and 0.01 % Bromophenol Blue, pH 6.8].

Vesicles

Samples of vesicle suspensions of approx. 4 mg/ml total protein were dissolved with 0.5 vol. of SDS/PAGE sample buffer.

Whole erythrocytes

Samples from the dilute cell suspensions (3 % Ht) commonly used for erythrocyte treatments were centrifuged rapidly. Part of the supernatant was removed and the cells were resuspended in the remaining medium to 20 % Ht. To this was added 9 vol. of diluted SDS/PAGE sample buffer [1 vol. of SDS/PAGE sample buffer plus 1.7 vol. of 5 % (w/v) SDS in water].

Once SDS/PAGE sample buffer had been added, all samples were incubated for 15 min at 60 °C and then stored at -20 °C until analysis. SDS/PAGE and Western blotting were conducted by standard procedures as described previously [12]. Detection with chemiluminescence reagent was carried out according to the manufacturer's specifications.

RESULTS

Increased levels of band 3 tyrosine phosphorylation in vesicles are stable

When erythrocytes are induced to release microvesicles by Ca^{2+} /A23187 treatment, band 3 in the membranes of both cells and vesicles appears to undergo homogeneous tyrosine phosphorylation as a consequence of the stimulation of PTK activity in shrunken cells [11,12]. If staurosporine was added [11], after 20 min of Ca^{2+} /A23187 treatment, to inhibit the PTK activity that would otherwise be persistently stimulated in the irreversibly shrunken cells, and then the cells and vesicles were incubated separately, almost complete PTP-catalysed dephosphorylation of band 3 occurred in erythrocytes over 2 h (Figure 1, lanes 3–8).

Figure 1 Persistence of tyrosine phosphorylation of band 3 in vesicles released by Ca^{2+} /A23187-treated erythrocytes

A 3 % Ht suspension of erythrocytes in buffer A was treated for 20 min at 37 °C with 4 μM A23187/0.3 mM Ca^{2+} . Samples were taken at 0 and 20 min for immunodetection of Tyr-phosphorylated band 3 (lanes 1 and 2 respectively). After chelation of Ca^{2+} with EGTA (2 mM final), staurosporine (3 μM final) was added. Cells were sedimented and the vesicle-containing supernatant was incubated separately at room temperature. Cells were resuspended at 20 % Ht with buffer A in the absence of staurosporine and incubated for 2 h at room temperature (the binding of staurosporine to PTKs is stable and resistant to washes of cells, making further addition of the compound in subsequent steps unnecessary). Starting from this point, samples from the cell suspension were taken after 0, 30, 40, 50, 60 and 120 min (lanes 3–8 respectively) and processed for immunodetection of Tyr-phosphorylated band 3. At 120 min, a sample of vesicles was also processed for immunodetection (lane 9). The amount of cells present in lanes 1–8 corresponds to 2×10^7 erythrocytes. The amount of vesicles loaded contained the same amount of band 3 as in 2×10^7 erythrocytes. This was determined separately by densitometric quantification of band 3 in Coomassie Blue-stained gels loaded with vesicles and ghost membranes from a known amount of erythrocytes (results not shown). The data are representative of results obtained in four similar experiments.

Conversely, the level of band 3 tyrosine phosphorylation in vesicles was stable over the 2 h incubation period (Figure 1, lane 9). The reasons for the stable tyrosine phosphorylation of band 3 in vesicles, as opposed to cells, were investigated further.

PTP1B in purified ghost membranes and vesicles

In human erythrocytes, PTP1B (or a closely related PTP) has been identified as the major membrane-bound PTP, and appears to be tightly associated, both structurally and functionally, with band 3 [8]. We investigated whether the persistence of band 3 tyrosine phosphorylation in vesicles was due to the absence of PTP from vesicles, or to a compromised activity of the enzyme because of oxidation or proteolysis, or both. PTPs are in fact particularly prone to oxidation, due to a sensitive cysteine residue in the catalytic site [28]. On the other hand, it is known from studies conducted in platelets that, in response to platelet activation, the native form of PTP1B is cleaved, by a selective proteolytic cut, to give a catalytic domain (which is soluble in the cytoplasm) and a membrane-bound C-terminal domain [29,30]. As shown below, both forms of the enzyme are also found in human erythrocytes (we have reported on this aspect in more detail elsewhere [31]). It was therefore possible that the same proteolytic events occur in the erythrocyte in response to elevated intracellular Ca^{2+} levels, and that a mechanism exists, during the process of vesiculation, by which the PTP and/or its fragments are excluded from the vesicle compartment.

To address this issue, we performed immunodetection of the phosphatase using a murine monoclonal antibody directed against the catalytic domain of human PTP1B [8]. As PTP1B is tightly associated with the membrane under physiological conditions, we initially probed white ghost membranes prepared from resting or Ca^{2+} /A23187-treated erythrocytes, as well as purified whole vesicles released by treatment, for the presence of PTP1B. As shown in Figure 2 (upper panel), PTP1B appeared in ghost membranes of both resting (lanes 1 and 3) and stimulated (lanes 2 and 4) erythrocytes, but was completely absent from vesicles

Figure 2 Detection of PTP1B in erythrocyte membranes, vesicles and platelets

Erythrocytes (3% Ht suspension in buffer B) were incubated for 20 min at 37 °C with 4 μ M A23187/0.3 mM Ca^{2+} . After chelation of Ca^{2+} with EGTA, cells were sedimented and washed twice with buffer B, before membrane preparation. The vesicle-containing supernatant was processed for vesicle purification. White ghost membranes were prepared as described in the Experimental section, by omitting (lanes 1 and 2) or including (lanes 3 and 4) the high-salt wash step. Upper panel, immunodetection of PTP1B; lower panel, immunodetection of band 3, showing that equivalent amounts of band 3 were present in ghost and vesicle samples. Lanes 1 and 3, control erythrocyte membranes; lanes 2 and 4, Ca^{2+} /A23187-treated erythrocyte membranes; lane 5, vesicles; lane 6, whole lysate of resting platelets; lane 7, whole lysate of platelets activated with 1 unit/ml thrombin for 5 min at 37 °C under stirring. Ghost samples contained 16 μ g of membrane protein, equivalent to 4×10^7 original erythrocytes. Vesicle samples contained an equivalent amount of band 3 as in the samples of ghosts. Platelets were obtained from platelet-rich plasma of the same blood samples used for erythrocyte preparation. A platelet suspension (10^9 cells/ml) in 10 mM Hepes, 137 mM NaCl, 2.9 mM KCl and 12 mM NaHCO_3 , pH 7.4, was activated with thrombin (1 unit/ml) for 5 min at 37 °C under stirring [29,30]. Whole platelets were solubilized by adding to the suspension 0.5 vol. of SDS/PAGE sample buffer (see the Experimental section). The blot is representative of results obtained in three similar experiments.

(lane 5). Ghost membranes were prepared by two different procedures, omitting (lanes 1 and 2) or including (lanes 3 and 4) a 'high-salt' wash in the preparation, to respectively maintain or release components bound to the membrane by electrostatic interactions; the two methods gave similar results.

It should be emphasized that equivalent amounts of band 3 protein (and thus, approximately, of membrane area) were present in ghost and vesicle samples (Figure 2, lower panel).

The band present in the anti-PTP1B immunodetection of the vesicle sample (Figure 2, upper panel, lane 5) between the native form of PTP1B (50 kDa) and its breakdown product (42 kDa) is not related to PTP1B. In fact, it is probably an artefact of the chemiluminescence detection and/or is due to non-specific binding of the antibodies to the blot, since a band in the same region was also visible when vesicle samples were probed with different primary antibodies (results not shown). However, this band was detected only in vesicles from Ca^{2+} -loaded erythrocytes

Figure 3 PTP1B, unlike SHPTP-2, p72^{syk} and $\text{p56/53}^{\text{lyn}}$, is excluded from the vesicle compartment

(A) Erythrocytes were treated with 4 μ M A23187/0.3 mM Ca^{2+} for 20 min at 37 °C to induce vesiculation. Samples were taken at 0 min (lane 1) and 20 min (lane 2) for immunodetection of PTP1B, SHPTP-2, p72^{syk} and $\text{p56/53}^{\text{lyn}}$ in erythrocytes. Lane 3: vesicles released in the incubation medium, purified from contaminating erythrocytes and cell fragments, were processed for immunodetection of the same enzymes. (B) Immunodetection of PTP1B, SHPTP-2, p72^{syk} and $\text{p56/53}^{\text{lyn}}$ in erythrocytes and vesicles released by nutrient deprivation of cells for 24 h at 37 °C. Lane 1, control cells; lane 2, nutrient-deprived erythrocytes; lane 3, purified vesicles. The amount of cells present in each lane corresponds to 10^7 erythrocytes. The amount of vesicles loaded contained the same amount of band 3 as 10^7 erythrocytes. Data are representative of three similar experiments.

(see below), suggesting that it might be derived from Ca^{2+} -dependent proteolysis and/or transglutaminase-mediated cross-linking [13] of other polypeptides, almost certainly haemoglobin monomers.

It appears that PTP1B in Ca^{2+} -loaded erythrocytes undergoes the same proteolytic cut (Figure 2, upper panel, lanes 1 and 3 compared with lanes 2 and 4) that takes place in platelets upon activation with agonists such as thrombin (lanes 6 and 7), as described previously [29,30].

The lower-molecular-mass form of PTP1B produced by this proteolytic event did not seem to accumulate in erythrocyte ghosts, probably because it is lost (although not entirely; see Figure 2, lanes 1–4) during the preparation of ghost membranes [31]. Proteolysis does not affect all PTP1B molecules; in fact, a considerable amount of the native form is still visible in ghosts obtained from erythrocytes treated for 20 min with Ca^{2+} /A23187. Most importantly, both forms of PTP1B appear to be completely excluded from the vesicle compartment.

Detection of PTPs and PTKs in whole-cell lysates and vesicles

As some proteolytic products of PTPs possibly present in the cell cytoplasm might escape detection when purified white membranes are probed, subsequent experiments were designed to optimize the detection of PTP1B in whole erythrocytes. Moreover, the PTKs p72^{syk} and $\text{p56/53}^{\text{lyn}}$, and the soluble PTP recently identified as SHPTP-2, were examined in whole erythrocytes and vesicles. Figure 3(A) shows both the native and the 42 kDa form

of PTP1B in whole-erythrocyte lysates from resting and Ca^{2+} -loaded cells (lanes 1 and 2 respectively). The levels of both forms, in resting cells and after Ca^{2+} treatment, displayed inter-individual variability; however, no trace of either polypeptide was ever found in vesicle samples (Figure 3A, lane 3).

SHPTP-2 was detected, although in trace amounts, in vesicles. Both PTKs examined were found in both resting and Ca^{2+} /A23187-treated cells, and in the vesicles released during treatment (Figure 3A). The same number of resting and Ca^{2+} -treated cells was present in each lane. The vesicle sample contained the same amount of band 3 as the sample of resting cells (results not shown). Although quantitative details are not reported here, inspection of Figure 3(A) clearly reveals that whereas p56/53^{lyn} was, to a good approximation, equally represented in cells and vesicles relative to band 3 (Figure 3A, lanes 1 and 3), p72^{syk} appeared to be retained in the cell compartment to a greater extent, suggesting that different mechanisms may regulate the partitioning of the two kinases during vesiculation.

Properties of microvesicles released by Ca^{2+} -independent treatment

To determine whether the confinement of PTP1B to the cell compartment occurs only in Ca^{2+} -treated erythrocytes, as a consequence of Ca^{2+} -triggered processes (such as Ca^{2+} -dependent transglutaminase-mediated polypeptide cross-linking, proteolysis, or a combination of the two), or is a more general feature of this protein, we examined the behaviour of PTP1B and of the other proteins under study in an experimental model of vesiculation based on nutrient deprivation of the cells [15]. Under our experimental conditions, the amount of membrane released as microvesicles from Ca^{2+} /A23187-treated erythrocytes was up to 10 % of parent cell membranes, while the corresponding value for nutrient-deprived erythrocytes was approx. 5 % (results not shown). Figure 3(B) shows that vesicles released by the Ca^{2+} -independent method, like Ca^{2+} -dependent vesicles, did not contain PTP1B (lane 3). The absence of the artefactual band between the 50 kDa and 42 kDa forms of PTP1B (Figure 3A, lane 3 and Figure 2, lane 5) confirms that this signal in Ca^{2+} -dependent vesicles was due to Ca^{2+} -stimulated processes, such as cross-linking of haemoglobin subunits, giving rise to covalently bound molecular aggregates that interfere with chemiluminescence detection.

Both kinases, i.e. p56/53^{lyn} and p72^{syk}, and the soluble phosphatase SHPTP-2 were also present in vesicles from nutrient-deprived erythrocytes, confirming that the mechanism for the partitioning of these molecular species is similar in Ca^{2+} -dependent and Ca^{2+} -independent vesiculation.

PTPs and PTKs in human erythrocytes of different ages

PTPs and PTKs were analysed in erythrocytes of different ages, obtained by high-resolution density separation [24]. Six subpopulations of increasing cell age were typically obtained, and the following parameters were evaluated in each subpopulation: cell recovery, reticulocyte count and the density-independent cell-age parameter, protein 4.1a/4.1b ratio (Figure 4). As shown in Figure 5(A), PTP1B appeared to be retained in similar amounts in erythrocytes of progressively increasing age, except for the higher levels detected in the subpopulation of very light/young erythrocytes, which was also highly enriched in reticulocytes (Figure 4, middle graph, F1). Equal numbers of cells from each subpopulation of erythrocytes, including the total population, were examined. As older erythrocytes are characterized by a lower

Figure 4 Separation of erythrocytes into subpopulations of different cell ages

Erythrocytes were fractionated into six subpopulations of increasing cell density from fraction 1 (F1) to fraction 6 (F6) by centrifugation on polyarabinogalactan discontinuous density gradients. The relative abundance of cells in each subpopulation is shown in the histogram. The percentage of reticulocytes compared with erythrocytes was evaluated in each subpopulation (middle graph). The enrichment of older cells in fractions of increasing density was confirmed by measuring the cell-age parameter (4.1a/4.1b protein ratio) (upper graph). T, total population of cells. All parameters are averages of values measured in five independent experiments; error bars represent the S.D.

surface area extension (17 % decrease in the 1 % densest/oldest erythrocytes with respect to the unfractionated population of cells [18]), a decline in membrane-associated proteins, such as PTP1B, would be expected under the conditions described. Instead, stable levels of PTP1B, in particular the absence of a difference in the amount of PTP1B detected in the total population of cells compared with the oldest cell fraction (F6), suggest preferential retention of the phosphatase within the cell compartment during erythrocyte aging.

Conversely, both kinases examined, i.e. p72^{syk} and p56/53^{lyn}, displayed a progressive and continuous decline from young to old cells (Figures 5C and 5D). The difference between the total population of erythrocytes and the population of oldest cells (F6) was particularly evident. The average decrease in the content per cell was $64 \pm 8\%$ for p56/53^{lyn} and $47 \pm 13\%$ for p72^{syk} (mean $\pm$ S.D., $n = 5$) from T (total population) to F6. This profile may be interpreted as being the result, at least in part, of the parallel loss of membrane (at a constant haemoglobin content per cell [32]) and of membrane-associated PTKs during erythrocyte aging. Interestingly, the decline of p56/53^{lyn} appeared to be more pronounced than that of p72^{syk}, again suggesting different modes of interaction of the two kinases with the membrane.

The phosphatase SHPTP-2 displayed an intermediate behaviour between the pronounced decline of the kinases and the stability

Figure 5 PTPs and PTKs in human erythrocytes of different ages

Erythrocyte subpopulations of increasing cell age from F1 to F6 were obtained in the experiments reported in Figure 4. Whole lysates from the same number of cells (10^7) were resolved by SDS/PAGE and blotted with anti-PTP1B (A), anti-SHPTP-2 (B), anti-p72^{syk} (C) and anti-p56/53^{lyn} (D) antibodies, followed by chemiluminescence detection. Quantitative analysis of the individual proteins was performed by densitometry of a digital image of the films. For each experiment and each protein examined, a ratio was calculated between the integrated area of the protein band for a given subpopulation of cells and the integrated area for the total population of cells (T). Ratios measured in five different experiments were then averaged and displayed as histograms; error bars represent the S.D.

of PTP1B in erythrocytes of different ages, with a decrease of $17 \pm 8\%$ from T to F6 ($n = 5$; see Figure 5B).

Partitioning of PTP1B between the membrane and the membrane skeleton during vesiculation

In an attempt to explain the absence of PTP1B from vesicles, we investigated whether increasing amounts of the phosphatase could associate with the membrane skeletal component of Ca^{2+} -treated cells, thus being excluded from the budding vesicles, which have been characterized as being membrane-skeleton-free. Whole resting or Ca^{2+} /A23187-treated erythrocytes were extracted with non-ionic detergents at 4°C . Two different detergents were chosen that have been described previously to solubilize band 3 to different extents: Triton X-100 and n-octyl glucoside [8,25]. This procedure should allow us to detect any modifications in the interactions of band 3 with the membrane skeleton induced by Ca^{2+} /A23187 treatment of the cells, and to analyse possible recruitment of PTP1B to the skeletal component. Samples of raw cell lysates, membrane skeletons and detergent-soluble material, in amounts equivalent to 10^7 original packed erythrocytes, were examined for the presence of PTP1B and band 3 by immunodetection with the respective antibodies.

Figure 6(A) shows the amounts of band 3 recovered in the various subfractions after extraction with Triton X-100 (lanes 2–5) or n-octyl glucoside (lanes 6–9). Even-numbered lanes correspond to detergent-soluble fractions, while odd-numbered lanes correspond to detergent-insoluble material (membrane skeleton). Lane 1 contains a whole-cell lysate. It was confirmed that the two detergents display different solubilizing properties: Triton X-100 extracted more band 3 (Figure 6A, lanes 2 and 4) than n-octyl glucoside (lanes 6 and 8).

Irrespective of the amount of band 3 extracted, the native (50 kDa) form of PTP1B was always found in the detergent-

Figure 6 Partitioning of band 3 and PTP1B in detergent-soluble and detergent-insoluble fractions from control and Ca^{2+} /A23187-treated erythrocytes

Cells were incubated at 10% Ht in the absence and in the presence of 0.3 mM Ca^{2+} and $5\text{ }\mu\text{M}$ A23187 at 37°C . Aliquots were sampled before addition of Ca^{2+} /A23187 and after a 20 min incubation with Ca^{2+} /A23187. Whole erythrocytes were extracted with 2% (w/v) Triton X-100 (lanes 1–5) or 2% (w/v) n-octyl glucoside (lanes 6–9) and the insoluble fractions sedimented as described in the Experimental section. (A) Immunodetection of band 3; (B) immunodetection of PTP1B. Lane 1, whole-cell lysate; lanes 2 and 6, control cells (soluble fraction); lanes 3 and 7, control cells (membrane skeletons); lanes 4 and 8, Ca^{2+} /A23187-treated cells (soluble fraction); lanes 5 and 9, Ca^{2+} /A23187-treated cells (membrane skeletons). Samples of each subfraction were loaded in amounts equivalent to 10^7 erythrocytes. Results are representative of three similar experiments.

soluble fraction, both before and after Ca^{2+} treatment (Figure 6B), thus ruling out selective recruitment of PTP1B to the membrane skeleton in response to the treatment. A major portion of the 42 kDa form of PTP1B was found in the insoluble fraction. We

have no explanation for this finding, which may indicate the presence of a subpopulation of 42 kDa PTP1B tightly associated with the membrane skeleton even under resting conditions. This portion, however, did not increase in response to elevations in the intracellular Ca^{2+} concentration.

DISCUSSION

Ca^{2+} -dependent vesiculation of human erythrocytes is accompanied by a net loss of KCl (Gardos effect) and, consequently, of cell water, which is followed by cell shrinkage. The decrease in cell volume induces an increase in tyrosine phosphorylation of band 3 that is also detected in vesicles [11,12]. In the present work we have shown that tyrosine phosphorylation of band 3 in microvesicles released by Ca^{2+} /A23187-treated erythrocytes is stable under conditions where complete dephosphorylation of band 3 can be observed in the remnant erythrocytes. An explanation for the relatively stable phosphorylation of band 3 in vesicles was provided by the very low levels of cytosolic phosphatases and, surprisingly, by the complete absence of the major membrane-bound PTP of human erythrocytes from this compartment. This finding prompted us to investigate how the principal components of the molecular machinery that controls the tyrosine phosphorylation/dephosphorylation of band 3 partition between the two compartments during vesiculation.

The PTKs p72^{syk} and $\text{p56/53}^{\text{lyn}}$ were both present in the vesicle compartment. Persistent phosphorylation in vesicles due to the continuous action of PTKs was excluded, since the released vesicles, incubated separately from the cells in the presence of staurosporine (to inhibit kinase activity) for 2 h were still fully phosphorylated on band 3, whereas complete dephosphorylation occurred in parent cells incubated with staurosporine over the same time span (Figure 1). p72^{syk} appeared to be retained in the cells, during both vesiculation and erythrocyte aging (Figures 3 and 5C), to a greater extent than $\text{p56/53}^{\text{lyn}}$ (Figures 3 and 5D). This may be related to the modality of interaction with the membrane of the latter kinase, which is probably acylated, like other members of the Src family of PTKs, and therefore can interact directly with the lipid bilayer.

When studying erythrocyte senescence, a decline with cell age was measured in the content per cell of the two PTKs examined. The average decreases were 64% for $\text{p56/53}^{\text{lyn}}$ and 47% for p72^{syk} , when comparing the F6 subpopulation with the total population (Figures 5C and 5D). The 17% decrease in membrane surface extension reported previously in old erythrocytes relative to the total population of cells, and the corresponding decrease in cell volume (25%) [18], therefore do not appear to be sufficient to explain the decreases in $\text{p56/53}^{\text{lyn}}$ and p72^{syk} . However, our results are likely to reflect two superimposed phenomena: a true decline in the amount of PTKs per cell, and a decline due to decreasing contamination of the cell subpopulations by kinase-rich reticulocytes. Thus contamination by reticulocytes is higher in the F1, F2 and F3 (Figure 4) subpopulations, which could therefore significantly increase the level of PTKs detected in these fractions. On the other hand, in subpopulations F4–F6, where the reticulocyte count was similar and very low (Figure 4), the amounts of the PTKs continued to decrease (Figures 5C and 5D). Since both kinases can be recovered in the vesicular compartment, these results are compatible with the view that at least one component of the cell-age-dependent decline in kinase content is due to loss by vesiculation, although the possibility cannot be excluded that proteolytic processes might also be involved.

Behaviour similar to that described for the PTKs was observed for the cytosolic phosphatase SHPTP-2, which was detected in

vesicles produced *in vitro*, and appeared to decline throughout erythrocyte life in the circulation (Figures 3 and 5B), although at a lower rate than the kinases examined.

A different situation was observed for PTP1B. Three lines of evidence support the conclusion that, in the human erythrocyte, this PTP is localized in a subcellular membranous compartment that is restricted in its ability to migrate to regions destined for vesiculation. On the one hand, two substantially different models of *in vitro*-induced membrane vesiculation (one dependent on the elevation of intracellular Ca^{2+} associated with increased levels of band 3 tyrosine phosphorylation, and the other based on depletion of ATP stores in the absence of external Ca^{2+} and of tyrosine phosphorylation of band 3) confirmed that PTP1B was absent from the vesicles. On the other hand, the examination of *in vivo* aged erythrocytes indicated that the cells retain the original endowment of PTP1B as they age in the circulation, decrease progressively in volume and lose membrane surface extension [18,20]. Constant levels of PTP1B in equal numbers of cells of progressively smaller size (Figure 5A) also imply enrichment of PTP1B relative to band 3 (its substrate), the density of which appears comparable in the membranes of cells and vesicles [14–16]. This substantiates the hypothesis that the decrease in cell size that occurs during aging occurs via a mechanism of vesiculation that selects regions of the membrane to be released in the vesicles.

The main membrane-associated PTP in human erythrocytes has been classified as PTP1B, although possible identification as the closely related T-cell PTP has not been excluded [8]. Our observation that the PTP in the erythrocyte is subjected to the same Ca^{2+} -dependent processing that occurs to PTP1B in activated platelets [29,30] provides additional support to the original classification.

Previous work aimed at characterizing the subcellular localization of PTP1B revealed a tight association between this phosphatase and bulk band 3 in the membrane [8]. In a recent study, it was proposed that a rise in the Ca^{2+} concentration in the erythrocyte promotes the dissociation of PTP1B from band 3, while both proteins remain in the membrane [33]. The results of the present study do not contradict this model, but suggest a more complex situation. A highly selective sorting of PTP1B was observed during vesiculation, resulting in the complete absence of this PTP from vesicles. The observation, reported previously [17,34], that vesicles may contain less band 3 per unit surface area than the parent cells does not help to explain the absence of PTP1B from vesicles. In fact, if we admit a tight association of PTP1B with band 3, then it could be hypothesized that, during vesiculation, either the PTP dissociates (by a still unknown mechanism) from the band 3 molecules targeted to the vesicles, or only the proportion of band 3 that is originally not engaged with the phosphatase localizes to the vesicles.

We tested whether Ca^{2+} /A23187 treatment could induce the re-location of PTP1B molecules, alone or in association with band 3, from the membrane to the membrane skeleton. Cells were treated with two different detergents to characterize the population of detergent-resistant, skeleton-associated band 3, as well as the localization of PTP1B before and after Ca^{2+} treatment. PTP1B could be stably associated only with higher oligomers of band 3, which have been reported to be the preferential form of band 3 bound to the membrane skeleton [25]. The detergent n-octyl glucoside, which has been shown to preserve the oligomeric state and the molecular interactions of band 3 better than Triton X-100 [26], was used in parallel with the latter detergent. It was confirmed that Triton X-100 extracted more band 3 than did n-octyl glucoside from whole, resting erythrocytes, and that Ca^{2+} treatment did not change this characteristic partitioning markedly. Most importantly, PTP1B was solubilized completely by either

detergent, except for a portion of the 42 kDa fragment, which remained stably associated with the membrane skeleton, but did not increase upon Ca^{2+} treatment.

The fact that a large population of band 3 molecules remained associated with the membrane skeletal component after extraction of cells with *n*-octyl glucoside, while the same detergent solubilized PTP1B completely, suggests that the association between the two proteins involves mostly hydrophobic (as opposed to electrostatic) interactions, which may be direct or mediated by a lipid component. A prevalent hydrophobic association with the membrane seems more probable for PTP1B, which was originally found to be inserted, via its highly hydrophobic C-terminus, within the lipid bilayer of the endoplasmic reticulum in placental cells [35]. In erythrocytes, which lack an endoplasmic reticulum, PTP1B can interact only with the lipid bilayer of the plasma membrane, probably in close proximity, or via a direct interaction with the integral protein band 3.

The data presented here are compatible with dissociation of PTP1B from band 3 in cells that undergo vesiculation [33]. Such a dissociation is likely to be related, by a mechanism that deserves future study, to a rearrangement of the composition of the lipid moiety of the membrane. Several events are triggered by increased intracellular Ca^{2+} levels during Ca^{2+} -induced vesiculation (and some also occur in vesiculation induced by ATP depletion or storage): loss of the asymmetrical distribution of phospholipids between the two membrane leaflets, hydrolysis of phosphoinositides to diacylglycerol and phosphoinositol, and an increase in the amount of phosphatidic acid from phospholipase D-catalysed phosphatidylcholine hydrolysis. As we have shown in the present work (Figure 1), the dissociation of PTP1B from band 3 would not compromise dephosphorylating activity towards band 3 within the cell, and it would explain the absence of PTP1B from vesicles, in association with band 3. It is not, however, sufficient to explain how the PTP is sequestered entirely within the cell compartment.

It would be of interest to examine whether a lipid-raft-based mechanism of sorting exists in erythrocytes, which is responsible for the exclusion of PTP1B from the budding vesicle. The presence of membrane subdomains as lipid rafts has been described recently in erythrocytes [34]. The organization of signalling components in macromolecular complexes within lipid rafts seems to be a general feature in cells with active signalling transducing pathways. Interestingly, in lymphocytes, one of the few membrane proteins that appears to be excluded from the rafts is a PTP (CD45) [36].

The regulation of PTPs appears to be complex, and to depend largely on their subcellular localization. The observation that PTP1B is selectively retained during erythrocyte aging leads us to speculate that the maintenance of stable PTP activity is under selective pressure in erythrocytes, for the fine regulation of band 3 tyrosine phosphorylation throughout the life of the cell in the circulation. The present results may contribute to ongoing efforts in the characterization of the role of band 3 tyrosine phosphorylation in human erythrocyte pathophysiology.

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