Phosphotyrosine phosphatase associated with band 3 protein in the human erythrocyte membrane

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The anion-exchange band 3 protein is the main erythrocyte protein that is phosphorylated by tyrosine kinase. To study the regulation of band 3 phosphorylation, we examined phosphotyrosine phosphatase (PTP) activity in the human erythrocyte. We show that the human erythrocyte membrane contains a band 3-associated neutral PTP which is activated by Mg^{2+} and inhibited by Mn^{2+} and vanadate. The PTP is active in the intact cell and in the isolated membrane. A major fraction of the PTP is tightly bound to the membrane and can be extracted from it by Triton X-100; a minor part is associated with the Triton X-100-insoluble cytoskeleton. The behaviour of the PTP parallels that of band 3, the major fraction of which is extractable by detergents with a

minor fraction being anchored to the cytoskeleton. Moreover, band 3 is co-precipitated when the PTP is immunoprecipitated from solubilized membranes, and PTP is co-precipitated when band 3 is immunoprecipitated. The PTP appears to be related to PTP1B (identified using an antibody to an epitope in its catalytic domain and by molecular mass). The system described here has a unique advantage for PTP research, since it allows the study of the interaction of a PTP with an endogenous physiological substrate that is present in substantial amounts in the cell membrane. The membrane-bound, band 3-associated, PTP may play a role in band 3 function in the erythrocyte and in other cells which have proteins analogous to band 3.

INTRODUCTION

Phosphorylation of protein tyrosine residues is important in the regulation of cell proliferation, differentiation and metabolism. Very little phosphotyrosine is usually detected in intact cells and cell fractions. A significant increase in tyrosine phosphorylation can, however, be achieved by the use of compounds known to inhibit phosphotyrosine phosphatase (PTP) [1–3]. It is generally agreed that the level of protein phosphotyrosine is determined by the activity of protein tyrosine kinases (PTKs) and the opposing activity of protein PTPs [1–4]. It has been proposed that the PTPs normally act to maintain a very low level of phosphorylated tyrosine [1]. Erythrocytes contain PTK activity, with the band 3 protein being the major substrate for this kinase [5–8]. As is the case for other cells and proteins, band 3 phosphorylation is markedly enhanced when reagents such as vanadate are used [5–8]. PTP inhibitors were required to prevent loss of phosphorylation during purification of band 3 from phosphorylated membranes, indicating that PTP activity was co-purified with band 3 [7]. Several PTPs have been detected in erythrocytes [9–11]. The band 3 tyrosine residues which can be phosphorylated have been identified and the properties of the PTKs have been studied [6–8], but relatively little is known about the PTPs in the erythrocyte.

We show here that the human erythrocyte membrane contains a band 3-associated neutral PTP which is activated by Mg^{2+} and inhibited by Mn^{2+} and vanadate. The major fraction of the PTP is extractable by Triton X-100, and a minor part is associated with the cytoskeleton. This behaviour parallels the behaviour of band 3, the major fraction of which is extractable by detergents whereas a minor fraction is anchored to the cytoskeleton [12,13]. The membrane-bound PTP appears to be PTP1B or a related PTP. The physiological role of band 3 tyrosine phosphorylation

and the significance of dephosphorylation are not known. Band 3 is the anion-exchange protein, and it also binds various cytoskeletal proteins as well as haemoglobin and cytoplasmic glycolytic enzymes. Phosphorylation of band 3 has been proposed to regulate glycolysis; band 3 phosphorylation and dephosphorylation may also influence anion exchange [7,14,15]. Band 3-associated PTP may thus be important for band 3 function in the erythrocyte and in other cells which have proteins analogous to band 3.

MATERIALS AND METHODS

Erythrocytes, erythrocyte membranes and membrane fractions

Fresh blood was obtained from healthy humans using EDTA as an anticoagulant. Blood was centrifuged, plasma and buffy coat removed and the erythrocytes washed three times with 150 mM NaCl. Erythrocyte membranes were obtained by haemolysing cells in 5 mM sodium phosphate buffer, pH 8.0, containing 1.0 mM EDTA and 0.1 mM PMSF [7]. Membranes were washed with the same buffer, then further washed with 10 mM NaCl/0.1 mM PMSF to obtain haemoglobin-free membranes (white membranes). To obtain membrane vesicles, white membranes were freed of band 6, then depleted of most spectrin and actin according to established methods [16]. The spectrin- and actin-depleted membrane vesicles were incubated with 20 vol. of 1.0 M KCl/1.0 mM dithiothreitol/1.0 mM EDTA/0.1 mM PMSF/5.0 mM sodium phosphate, pH 8.0, at 37 °C for 30 min [17] to yield vesicles essentially free of spectrin and containing little of bands 4.1 and 4.2. The N-terminal cytoplasmic fragment (43/41 kDa) was isolated by treating membrane vesicles with chymotrypsin, followed by adsorption on to DE52 cellulose and

elution of the soluble 43/41 kDa fragment by KCl, according to published procedures [7].

White membranes were extracted with Triton X-100 according to published methods [12], with some modifications. Aliquots of membranes were suspended in cold 25 mM Hepes buffer, pH 7.3, containing 1.0 mM dithiothreitol, 0.1 mM PMSF (buffer A) and 0.2 % or 2.0 % Triton X-100, or 0.8 M NaCl/0.2 % Triton X-100 [10] (NaCl/Triton). The membrane suspensions were incubated on ice for 30 min and centrifuged at 40000 *g*. The detergent-insoluble pellets were washed twice in buffer A. The supernatants were dialysed against buffer A. For the identification of the proteins in the membranes and membrane fractions, aliquots were solubilized and boiled in Laemmli SDS buffer (sample buffer) [18], resolved by SDS/10 %-PAGE and stained with Coomassie Blue. Protein bands were quantified by densitometric scanning of the negatives of the gel photographs.

Phosphorylation of erythrocytes and of erythrocyte membranes

Erythrocytes were suspended to a 10 % (v/v) haematocrit in 25 mM Hepes buffer, pH 7.3/125 mM NaCl and incubated in the presence or absence of 5.0 mM Mg^{2+} or Mn^{2+} , with or without 0.1 mM sodium orthovanadate (vanadate). Erythrocyte suspensions were incubated at 37 °C for 1 h and membranes prepared, as described above, using 0.1 mM orthovanadate in the haemolysing and washing solutions. For membrane phosphorylation, white membranes were suspended at 1.0 mg of protein/ml in buffer A containing 5 μ M ATP and incubated at 30 °C for 15 min in the presence or absence of 10 mM Mg^{2+} or 5.0 mM Mn^{2+} , with or without 0.1 mM vanadate. Membranes were solubilized and boiled in sample buffer.

Analysis of phosphotyrosine and of band 3 by immunoblotting

Proteins from the solubilized membranes were resolved by SDS/10 %-PAGE, followed by transfer to nitrocellulose membranes. The nitrocellulose membranes were blocked for 15 h at room temperature in a solution of 50 mM Tris, pH 7.4/150 mM NaCl/0.1 % Tween-20 (TNT), containing 0.5 % BSA, 1.0 % low-fat dried milk (blocking solution) and 0.05 % NaN_3 , then incubated for an additional 2 h at room temperature with a monoclonal anti-phosphotyrosine antibody (BioMakor, Rehovot, Israel; antibody diluted 1:2000). After washing with the blocking solution, the membranes were incubated for 1 h with goat anti-mouse peroxidase-conjugated antibodies (Amersham), washed in TNT and analysed using the ECL detection system (Amersham). A similar procedure was carried out for band 3 immunoblotting, using monoclonal anti-(band 3) antibody (BioMakor; antibody diluted 1:20000).

Determination of PTP activity and PTP analysis by immunoblotting

PTP activity was determined by using *p*-nitrophenyl phosphate (p-NPP) as substrate according to published procedures [10], with some modifications. Erythrocyte white membranes, membrane vesicles, membrane pellets and soluble fractions were suspended in buffer A containing 15 mM p-NPP and incubated at 37 °C for 30 min with or without 20 mM Mg^{2+} , in the presence or absence of 0.1 mM vanadate. The reaction was terminated by addition of 0.1 M NaOH, the samples were centrifuged (9000 *g*), and the release of *p*-nitrophenol from p-NPP was measured spectroscopically at 410 nm.

PTP activity was also tested by carrying out autodephosphorylation of phosphorylated membranes. Phosphorylated membranes were obtained by incubation of

membrane suspensions with ATP in the presence of vanadate and Mn^{2+} , as described above. The membrane suspensions were then placed on ice and washed with buffer A, and NaCl/Triton extracts were prepared as described above. Phosphorylated membranes, NaCl/Triton extracts and pellets were then suspended in buffer A and incubated at 37 °C with 10 mM Mg^{2+} or 5.0 mM Mn^{2+} , in the presence or absence of 0.1 mM vanadate. Aliquots were removed at various intervals, solubilized and boiled in sample buffer; proteins were resolved by SDS/10 %-PAGE, followed by anti-phosphotyrosine immunoblotting, as described above.

Immunoblot analysis of PTP protein was carried out on membranes and membrane fractions using a monoclonal anti-PTP1B antibody, FG6-1G (Oncogene Science), at a concentration of 2 μ g/ml [4]. Following SDS/PAGE (10 % gels) and transfer to nitrocellulose membranes, the blots were processed as described above for anti-phosphotyrosine immunoblotting, but with 0.3 % gelatin in the blocking solution instead of BSA and dried milk.

Immunoprecipitation of PTP and of band 3

White membranes were extracted with NaCl/Triton in buffer A and centrifuged (40000 *g*). The membrane extracts were immunoprecipitated with anti-PTP antibody or with anti-(band 3) antibody and the immunoprecipitates were analysed for PTP and for band 3. For immunoprecipitation of PTP, an aliquot of 200 μ l of the membrane extract, containing 100 μ g of protein, was mixed with 1.0 μ g of FG6-1G. After gentle agitation at 4 °C for 1 h, 30 μ l of goat anti-mouse IgG/Protein A-agarose complex (Oncogene Science) was added and gentle agitation continued for 15 h. The mixture was then centrifuged at 2500 *g*, and the pellet was washed four times and suspended in buffer A containing 0.2 % Triton X-100. An aliquot was solubilized in sample buffer and electrophoresed. Identification of co-precipitated band 3 was carried out by immunoblotting using the anti-(band 3) antibody at a dilution of 1:5000. Another aliquot was used for the analysis of PTP activity (see below).

For immunoprecipitation of band 3, aliquots of the membrane extracts were mixed with anti-(band 3) antibody at a dilution of 1:1000 and further treated as described above for PTP immunoprecipitation. The identification of co-precipitated PTP by anti-PTP immunoblotting was not possible, due to interference by the IgG present in the precipitates, since the IgG heavy chain and PTP migrate at similar mobilities on SDS/PAGE. Therefore phosphorylated membranes were used for band 3 immunoprecipitation, and PTP activity was analysed in the band 3 immunoprecipitate by following dephosphorylation of band 3 in the precipitates. Aliquots of the resuspended band 3 immunoprecipitate pellets were incubated at 37 °C with 0.1 mM vanadate, or 10 mM Mg^{2+} or with a combination of 10 mM Mg^{2+} and PTP immunoprecipitate. Reactions were terminated by solubilization and boiling in sample buffer. Samples were electrophoresed and band 3 phosphotyrosine was analysed by anti-phosphotyrosine immunoblotting, as described above, using the anti-phosphotyrosine antibody at a dilution of 1:500.

RESULTS

Band 3 tyrosine phosphorylation in intact erythrocytes and in isolated erythrocyte membranes

Erythrocytes were incubated with and without Mg^{2+} or Mn^{2+} , in the absence and presence of vanadate. Cell membranes were then prepared and solubilized, and proteins were resolved by SDS/PAGE and analysed for phosphotyrosine content by anti-

Figure 1 Band 3 tyrosine phosphorylation in intact erythrocytes and in erythrocyte white membranes

Left panel: erythrocytes were incubated at 37 °C for 60 min with 5.0 mM Mg²⁺ (lane 1), 5.0 mM Mn²⁺ (lane 2), 0.1 mM vanadate (lane 3), 5.0 mM Mg²⁺ + 0.1 mM vanadate (lane 4) or 5.0 mM Mn²⁺ + 0.1 mM vanadate (lane 5). A Coomassie Blue-stained protein profile is shown in lane 6. Middle panel: densitometric scan of samples from lanes 3, 4 and 5. Right panel: erythrocyte white membranes were suspended in buffer A containing 5 μ M ATP and incubated at 30 °C for 15 min with 10 mM Mg²⁺ (lane 1), 0.1 mM vanadate (lane 2), 10 mM Mg²⁺ + 0.1 mM vanadate (lane 3), 5.0 mM Mn²⁺ (lane 4) or 5.0 mM Mn²⁺ + 0.1 mM vanadate (lane 5). For further details, see the Materials and methods section.

Table 1 Influence of various agents on PTP activity in erythrocyte membranes

PTP activity in erythrocyte membranes was measured using p-NPP as substrate, as described in the Materials and methods section. Activities are expressed as a percentage of the activity measured in control samples. The control samples contained erythrocyte membranes (40–80 μ g of protein) suspended in 0.1 ml of 25 mM Hepes buffer, pH 7.3, 1.0 mM dithiothreitol, 0.1 mM PMSF (buffer A), 15 mM p-NPP and 20 mM MgCl₂. The results represent the means of duplicate samples from a representative experiment which was repeated three times.

Agent	Concentration (mM)	PTP activity (% of control)
MgCl ₂	0	10
	1.0	58
	10	85
Orthovanadate	0.1	0
Dithiothreitol	0	86
	10	106
ZnCl ₂	0.05	92
	0.25	62

phosphotyrosine immunoblotting (Figure 1, left panel). No tyrosine phosphorylation was observed in cells incubated with Mg²⁺ or Mn²⁺ alone (Figure 1, left panel, lanes 1 and 2). A marked phosphorylation of band 3 tyrosine was achieved in the presence of vanadate, with or without added Mg²⁺. Tyrosine phosphorylation was further enhanced when both Mn²⁺ and vanadate were present (Figure 1, left panel, lanes 3–5, and Figure 1, middle panel). Under these conditions little or no phosphotyrosine was observed in other membrane proteins (Figure 1, left panel, lanes 5 and 6). These results show that a marked phosphorylation of band 3 tyrosine can be achieved in the intact cells, but only when the phosphatase inhibitor vanadate is used. The results suggest that PTP activity is very high in the intact erythrocyte.

Band 3 tyrosine phosphorylation occurred also in isolated haemoglobin-free ('white') membranes. Membrane suspensions were incubated with ATP, with and without Mg²⁺ or Mn²⁺, in the absence and presence of vanadate. Membranes were then solubilized and analysed for phosphotyrosine as described above.

Band 3 phosphotyrosine was not observed in membranes incubated with Mg²⁺ alone or with vanadate alone (Figure 1, right panel, lanes 1 and 2), but was observed in membranes incubated with both Mg²⁺ and vanadate (lane 3). Band 3 tyrosine phosphorylation was observed in membranes incubated with Mn²⁺ alone, and was especially marked when both Mn²⁺ and vanadate were used (lanes 4 and 5). These results show that Mg²⁺-dependent tyrosine phosphorylation can be achieved in the isolated membranes when the phosphatase inhibitor vanadate is used. Mn²⁺-dependent phosphorylation can be achieved in the presence of Mn²⁺ alone, and is enhanced upon the addition of vanadate. The results indicate the presence of a membrane-associated PTP activity which is inhibited by Mn²⁺ and vanadate.

PTP activity of erythrocyte membranes and membrane fractions

Membrane PTP activity was estimated using p-NPP as substrate. As shown in Table 1, membrane PTP activity was significantly enhanced by Mg²⁺, partially inhibited by Zn²⁺ and completely inhibited by vanadate. The optimal activity was found at pH 7.0–7.5, with 80 % of that activity observed at pH 5.5 or 10.5.

In order to define the nature of the PTP–membrane association, erythrocyte membranes were extracted by several means (as described in the Materials and methods section) and the fractions obtained were analysed for PTP activity, using p-NPP as substrate. Membrane vesicles retained all the PTP activity of the original membrane suspension (Figure 2, top panel, fraction 1); the membrane vesicles were freed of most of the spectrin and actin and were depleted of band 6 (Figure 2, middle panel, lane a). KCl-treated membrane vesicles retained about 80 % of the original membrane PTP activity (Figure 2, top panel, fraction 2); they also retained all of the band 3 as well as band 2.1, were freed of most band 4.1 and some band 4.2, and were depleted of spectrin and band 5 (Figure 2, middle panel, lane b). Based on these results it appeared that the PTP activity was not associated with the cytoskeletal proteins spectrin, band 4.1, band 4.2 and actin.

Membranes extracted with 0.2 % Triton X-100 retained about 35 % of the original PTP activity (Figure 2, top panel, fraction 3). Under these conditions about 30–35 % of band 3 remained with the pellet, along with most spectrin, band 4.2, band 4.1 and actin (results not shown). Membranes extracted with 2 % Triton X-100 retained in the pellet about 15 % of the PTP activity and

Figure 2 PTP activity in erythrocyte membrane fractions

Membrane fractions were prepared and PTP activity was determined, using p-NPP as substrate, as described in the Materials and methods section. Final volumes of the membrane fractions were adjusted to the same volume as that of the whole (white) membrane suspensions. Top: PTP activity and band 3 content in the various fractions are expressed as percentages of those in the white membranes. Fractions: 1, membrane vesicles; 2, membrane vesicles treated with 1.0 M KCl; 3, membranes treated with 0.2% Triton X-100; 4, membranes treated with 2% Triton X-100; 5 and 6, membranes treated with 0.8 M NaCl and 0.2% Triton X-100 (NaCl/Triton) (5, pellet; 6, supernatant). Middle: SDS/PAGE profiles and densitometric scans of proteins from membrane vesicles (lane a) and from KCl-treated membrane vesicles (lane b). Numbers indicate bands detected. Bottom: SDS/PAGE profiles and densitometric scans of proteins from whole membranes (lane a) and from the supernatant (lane b) and pellet (lane c) of NaCl/Triton-treated membranes.

about 15% of band 3 (fraction 4). Treatment of membranes with a combination of 0.8 M NaCl and 0.2% Triton X-100 resulted in solubilization of all of the membrane PTP activity, whereas the pellet exhibited only a trace of PTP activity (fractions 5 and 6). Under these conditions about 70% of band 3, along with 50% of band 4.2 and a major part of band 2.1, was solubilized; the pellet contained the majority of spectrin, band 4.1 and actin, along with the remainder of band 3 and associated proteins (Figure 2, bottom panel, lanes a–c). These results indicate that, as analysed by hydrolysis of p-NPP, a major part of the membrane PTP activity is tightly associated with the membrane and can be removed by Triton X-100; a small part appears to be retained in the cytoskeleton and can be dissociated from the membrane by salt at a high ionic strength. A combination of high salt and detergent solubilizes all of the PTP activity.

Autodephosphorylation of membranes

Erythrocyte membranes were phosphorylated, washed and re-incubated in the absence and presence of vanadate, Mg^{2+} or Mn^{2+} . Immunoblotting of the membranes with the anti-(band 3) antibody identified the 90 kDa band 3 and two minor band 3 protein fragments of 60 kDa and 42 kDa, known to exist in the erythrocyte membrane [8]. As shown in Figure 3 (top and middle panels), dephosphorylation of band 3 occurred in membranes incubated in the absence of vanadate, but not in its presence. Mg^{2+} enhanced the rate of band 3 dephosphorylation. These results indicate that Mg^{2+} enhances PTP activity towards its natural substrate. This enhancement occurs to a lesser degree than the stimulation by Mg^{2+} of PTP activity towards the artificial substrate p-NPP (Table 1). In contrast to the effect of Mg^{2+} , Mn^{2+} was found to inhibit band 3 dephosphorylation; at a concentration of 5 mM, Mn^{2+} inhibited membrane band 3 dephosphorylation to an extent similar to that caused by 0.1 mM vanadate. Following extraction with NaCl/Triton, dephosphorylation of band 3 was observed in the soluble extract, but not in the pellet (Figure 3, bottom panel). These results show that the membrane PTP is active towards its endogenous substrate in a manner similar to its activity towards the artificial substrate, i.e. it is activated by Mg^{2+} and dephosphorylates band 3 in the NaCl/Triton extract, but activity is not present in the NaCl/Triton pellet. The results also show that the membrane PTP is inhibited by Mn^{2+} [the effect of Mn^{2+} on p-NPP hydrolysis cannot be studied, since $Mn(OH)_2$ appears as a coloured product upon termination of the reaction by NaOH].

Immunoblotting and immunoprecipitation of PTP and of band 3

Erythrocyte white membranes, the soluble fraction and the insoluble pellet obtained by extraction with NaCl/Triton were analysed by immunoblotting, using the monoclonal anti-PTP1B antibody FG6-1G, as described in the Materials and methods section. As shown in Figure 4(A), a band of 50 kDa was observed in the white membranes. The NaCl/Triton-soluble extract contained a PTP band, whereas the insoluble pellet did not. Thus the presence of PTP protein corresponded to the presence of PTP activity in the NaCl/Triton extract, whereas no PTP protein was detected in the pellet, which lacked PTP activity. Immunoprecipitation of the NaCl/Triton-soluble extract with FG6-1G was carried out as described in the Materials and methods section. The PTP immunoprecipitate contained band 3, as identified by immunoblotting with anti-(band 3) antibody (Figure 4B, lane 1). When the NaCl/Triton extracts were treated with anti-tubulin antibody as an unrelated primary antibody, or with the secondary antibody/Protein A-agarose complex only, no

Figure 3 Dephosphorylation of band 3 in erythrocyte membranes and membrane fractions

White membranes were phosphorylated, then extracted with NaCl/Triton, as described in the Materials and methods section. Phosphorylated membranes, and the supernatant and pellet of the NaCl/Triton-treated phosphorylated membranes, were suspended in buffer A and incubated with and without 10 mM Mg^{2+} or 5.0 mM Mn^{2+} , in the presence and absence of 0.1 mM vanadate. Aliquots were removed at various intervals, solubilized, analysed by SDS/PAGE and immunoblotting, further processed and quantified as described in the Materials and methods section. Top: rate of dephosphorylation of band 3 in membranes. A representative experiment is shown; the experiment was repeated three times. Middle: immunoblot of phosphorylated membranes incubated for 90 min. Bottom: immunoblot of supernatant and pellet of NaCl/Triton-treated membranes incubated with 10 mM Mg^{2+} in the absence of vanadate. Lanes 1–3, supernatant; lanes 4–6, pellet.

band 3 was detected in the precipitates (results not shown). The co-precipitation of band 3 protein in the PTP immunoprecipitate indicates that PTP is associated with band 3 protein. The identification of PTP in the immunoprecipitates by immunoblotting is precluded, due to the presence in the precipitates of IgG heavy chain, which migrates on SDS/PAGE at a mobility similar to that of PTP (Figure 4B, lane 2). Therefore, in order to

Figure 4 Identification of membrane PTP by immunoblotting and immunoprecipitation of PTP and band 3

(A) White membranes and the soluble extract and pellet of NaCl/Triton-treated membranes were electrophoresed and immunoblotted with FG6-1G, as described in the Materials and methods section. Lane 1, white membranes; lane 2, NaCl/Triton-insoluble pellet; lane 3, NaCl/Triton-soluble extract. (B) Lane 1, the NaCl/Triton extract was immunoprecipitated with FG6-1G. The immunoprecipitate was electrophoresed and immunoblotted with anti-(band 3) antibody. Lane 2, immunoblot of mouse IgG electrophoresed under the same conditions. (C) The NaCl/Triton extract of phosphorylated membranes was immunoprecipitated with anti-(band 3) antibody. Aliquots of the immunoprecipitate were incubated as described in the Materials and methods section with: lane 1, vanadate; lane 2, Mg^{2+} ; lane 3, Mg^{2+} and an aliquot of the PTP precipitate. Samples were electrophoresed and immunoblotted with an anti-tyrosine antibody. Numbers on the right are molecular masses (kDa) of protein standards.

demonstrate the presence of PTP in the PTP immunoprecipitate and to find out whether PTP co-precipitates with band 3, the following procedure was carried out. Band 3 was immunoprecipitated by anti-(band 3) antibody from phosphorylated membranes. Aliquots of this immunoprecipitate were incubated with vanadate or Mg^{2+} (Figure 4C, lanes 1 and 2) and analysed for phosphotyrosine by anti-phosphotyrosine immunoblotting, as described in the Materials and methods section. Dephosphorylation of band 3 occurred in the samples incubated in the absence of vanadate (Figure 4C, lane 2), indicating the presence of PTP activity in the band 3 immunoprecipitate. The addition of an aliquot of PTP immunoprecipitate to the phosphorylated band 3 immunoprecipitate led to further band 3 dephosphorylation (Figure 4C, lane 3). The finding of PTP activity confirmed the presence of this enzyme in the PTP immunoprecipitate and showed PTP co-precipitation with band 3 in the band 3 immunoprecipitate.

In order to probe further the interaction of PTP with band 3, the N-terminal cytoplasmic fragment was isolated from the membrane vesicles, as described in the Materials and methods section. The solution containing the 43/41 kDa cytoplasmic fragment was analysed using antibodies and immunoprecipitated with FG6-1G. Both PTP and the 43/41 kDa fragment were present in the solution, as detected by the anti-PTP and anti-(band 3) antibodies (Figure 5, lanes 1 and 2). Under the experimental conditions used, 23–26% of the PTP originally present in the membrane vesicles was recovered in this solution, along with 20–23% of the 43/41 kDa domain of the original band 3. These results indicate that PTP was not selected for or excluded during the isolation of the band 3 cytoplasmic fragment. The immunoblot of PTP showed, in addition to the 50 kDa band, a band of 35 kDa (Figure 5, lane 1); this fraction may have resulted from partial cleavage of PTP by chymotrypsin that was used to cleave the 43/41 kDa fragment from the membrane. Immunoprecipitation of PTP with FG6-1G was accompanied by co-precipitation of the 43/41 kDa fragment (Figure 5, lane 3), indicating an association of PTP with the band 3 cytoplasmic domain.

Figure 5 Identification of PTP and of the 43/41 kDa band 3 fragment by immunoblotting and immunoprecipitation of PTP

An aliquot of a solution of 41/43 kDa band 3 fragment was electrophoresed and immunoblotted with FG6-1G (lane 1) and with anti-(band 3) antibody (lane 2). Another aliquot of this solution was immunoprecipitated with FG6-1G; the immunoprecipitate was then electrophoresed and immunoblotted with anti-(band 3) antibody (lane 3). For further details, see the Materials and methods section.

DISCUSSION

An extremely low level of tyrosine phosphorylation is observed in a variety of nucleated cells under normal conditions, but a marked increase in phosphorylated tyrosine residues is observed when compounds known to inhibit PTP (e.g. vanadate, H_2O_2 /vanadate) are added to the cells [1–3,19]. Similarly, tyrosine phosphorylation in intact erythrocytes and isolated erythrocyte membranes is markedly increased when vanadate or H_2O_2 /vanadate is used ([5–8] and the present paper). Assuming that the effect of the inhibitors used is through the inhibition of PTP, the results in the various studies imply that the action of PTP is responsible for the low level of tyrosine phosphorylation normally present in cells and in isolated membranes [1–3,5–8,19].

Several PTPs have been described in erythrocytes, namely cytoplasmic, low-molecular-mass acid phosphatases and a membrane-associated neutral PTP activity [9–11]. These phosphatase activities have been found to be inhibited by Mn^{2+} and Mg^{2+} [9,10]. The PTP identified in the present work is a membrane-associated PTP. Its activity is enhanced by Mg^{2+} and inhibited by Mn^{2+} and vanadate, as shown by their effects on phosphorylation and dephosphorylation of band 3 in intact cells and membranes. That the activity of this PTP is enhanced by Mg^{2+} is shown by its effects on p-NPP hydrolysis and band 3 dephosphorylation. The fact that band 3 tyrosine phosphorylation in the intact erythrocytes required vanadate alone, whereas phosphorylation in isolated membranes required both vanadate and added Mg^{2+} , indicates that Mg^{2+} is required for both phosphorylation and dephosphorylation reactions, i.e. it induces tyrosine phosphorylation by membrane PTK (via an effect on Mg^{2+} -ATPase) and also enhances dephosphorylation by PTP. Since the erythrocyte contains an appreciable amount of Mg^{2+} (about 2.5 mM [20]), the vanadate added to the intact cell overcomes the Mg^{2+} -induced enhancement of PTP activity and allows the Mg^{2+} effect on tyrosine phosphorylation to become apparent. In the case of isolated membranes, however, the addition of both agents is required to achieve tyrosine phosphorylation. In contrast, Mn^{2+} is shown here to inhibit dephosphorylation of band 3 and, since it can substitute for Mg^{2+} in the phosphorylation reaction, the addition of Mn^{2+} alone to the erythrocyte membranes suffices to achieve both PTP inhibition and PTK activity (Mn^{2+} penetrates the erythrocyte at

a low rate, so that it cannot by itself overcome the Mg^{2+} -induced enhancement of PTP or inhibit dephosphorylation in the intact cell).

The fact that a major part of the PTP activity could be extracted from the erythrocyte membrane by Triton X-100 indicates that the interaction between this PTP and the membrane is mostly hydrophobic. The small fraction of the total PTP activity extracted from the membrane by a high salt concentration represents a fraction that is associated with membrane component(s) through electrostatic interactions; a combination of Triton X-100 and high salt allows the total solubilization of PTP activity. Using antibodies to a conserved epitope in the catalytic domain of PTP1B [4], we identified a protein band of 50 kDa in the erythrocyte membrane which was solubilized by Triton X-100/high salt along with the solubilization of the PTP activity.

PTP1B is known to be distributed in both the cytosolic and particulate cell fractions [21]. The PTP1B in the particulate fraction in human placenta has been shown to be in part in the membrane (extracted from the particulate fraction by Triton X-100), with the other part associated with the cytoskeleton [21]. About 85 % of PTP activity (presumably PTP1B, as identified by antibodies) has been found to be associated with the particulate fraction in rat myoblast cells [4]. As for the protein mass, it appears that the PTP1B originally purified from human placenta as a protein of 37 kDa is the N-terminal portion derived from a full-length PTP1B of 48–50 kDa [21,22]. Based on localization, solubilization properties, the antibody used and molecular mass, the PTP described here appears to be PTP1B or related to it. It may also be related to TC-PTP, which exhibits marked sequence similarity to PTP1B. When expressed in hamster kidney cells TC-PTP was associated with the particulate fraction, from which it could be released by a combination of Triton X-100 and high salt [23]. Additional studies are necessary to further refine the classification of the band 3 PTP described here.

The pattern of extraction of the PTP described here is similar to that of band 3 protein. The major part of band 3 is extractable by Triton X-100, whereas a minor population (about 10–20 % of total band 3) is linked to the cytoskeleton via band 2.1 (ankyrin) [12,13]. The fact that most of the membrane PTP is extracted by Triton X-100 and that the 15–20 % of the PTP that remains with the cytoskeleton is not associated with spectrin, band 4.1, 4.2 or actin suggests an association with band 3. It has been shown that, if vanadate was omitted during purification of phosphorylated band 3, phosphorylation of Tyr-8 of band 3 was lost, indicating that PTP activity was co-purified with band 3 [7]. The co-precipitation of band 3 with PTP and of PTP activity with band 3 shown here indicates an association between PTP and band 3. The observations that the 43/41 kDa fragment solution contained PTP and that, upon immunoprecipitation of PTP from this solution, this band 3 fragment was co-precipitated further support such an association and indicate that PTP is associated with the band 3 N-terminal cytoplasmic domain. An additional association of PTP with ankyrin is not excluded and should be further studied.

In contrast to the behaviour of the PTP described here, most of the erythrocyte membrane PTK activity has been shown to be retained in the cytoskeleton when the major part of band 3 is removed by Triton X-100; most of the PTK is dissociated from the membrane by high ionic strength [6]. The PTK (recently identified as a p72^{syk} PTK [8]) may thus be preferentially bound by electrostatic interactions to the band 3 molecules which are anchored to the membrane cytoskeleton [6]. The physiological significance of the difference between the behaviour of band 3 PTK and PTP is not clear. The preferential localization of PTK near the cytoskeleton-linked band 3 molecules may allow for the

differential regulation of phosphorylation and dephosphorylation in the two band 3 populations. Tyr-8 of band 3 (near to the N-terminal cytoplasmic domain) is the major site of phosphorylation [7,8,24]. Phosphorylation of this residue prevents the binding of glycolytic enzymes to the N-terminal region of the protein. Since these enzymes are inhibited when bound to band 3, glycolysis in the erythrocyte is enhanced by band 3 phosphorylation [14,25]. Thus the locations of PTP and PTK and the balance of PTK and PTP activities may play a role in erythrocyte metabolism.

The association of a PTP with a substrate protein located in a certain cellular compartment creates substrate specificity and ensures controlled access to phosphorylated sites, since the associated protein may determine the localization and directs the action of the PTP [1,2,26]. Little information is available on this aspect, since many of the studies on PTPs have been carried out on non-physiological substrates [2]. In a study employing co-expression of several different PTPs, cellular compartmentalization seemed to play a major role in PTP activity [27]. It has been predicted that intracellular PTPs will be found to be associated with individual protein anchors in membranes [1]. The association between the PTP and band 3 protein shown here demonstrates an interaction of a phosphatase with an endogenous substrate located in the membrane, providing support for this prediction.

The physiological substrates for PTPs have not been clearly identified. The system described here provides a useful model, since it allows the study of an interaction of a PTP with an endogenous substrate that is present in substantial amounts in the cell membrane. Proteins related to band 3 are found in other cell membranes [28–31]. Studies on the phosphorylation and dephosphorylation of band 3 in the erythrocyte may, therefore, help us to understand these processes and their relationship to normal and abnormal cell functions in other cells and tissues [32].

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