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Erythrocyte thiol status regulates band 3 phosphotyrosine level via oxidation/reduction of band 3-associated phosphotyrosine phosphatase

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Abstract Oxidative stress-induced tyrosine phosphorylation has been ascribed to activation of phosphotyrosine kinase or to inhibition of phosphotyrosine phosphatase (PTP). We have previously identified a PTP associated with band 3 in the human erythrocyte membrane, a PTP that is normally highly active and prevents the appearance of band 3 phosphotyrosine. Here we show that treatment of erythrocytes with the thiol-oxidizing agent diamide leads to the formation of PTP disulfides (PTP-band 3 mixed disulfides) and inhibition of dephosphorylation, allowing the accumulation of band 3 phosphotyrosine. Upon reduction of the disulfides, the band 3 phosphotyrosine is dephosphorylated. Erythrocyte thiol alkylation by *N*-ethylmaleimide results in irreversible PTP inhibition and irreversible phosphorylation. The results are consistent with the notion that alterations in cellular thiol status affect the cell phosphotyrosine status and that oxidative stress-induced tyrosine phosphorylation involves inhibition of PTP.

Key words: Phosphotyrosine phosphatase; Band 3 phosphorylation; Thiol status; Thiol oxidation; Diamide

1. Introduction

Phosphorylation of protein tyrosine residues plays an essential role in the regulation of diverse cellular functions. The level of any particular protein phosphotyrosine is controlled by members of two opposing enzyme families, phosphotyrosine kinases (PTKs) and phosphotyrosine phosphatases (PTPs), acting at that specific protein site [1–3]. Normally, the activity of PTP is very high relative to that of PTK, so that a very low basal level of phosphotyrosine is maintained in the cell [1–3]. Tyrosine phosphorylation of receptors and of certain cellular proteins is promoted by the binding of ligands (e.g. EGF, insulin) to their receptors [4,5]. Protein tyrosine phosphorylation in the intact cell can also be promoted by a variety of agents, notably agents which affect the system via oxidative stress [5–16]. These include hydrogen peroxide (usually added to the cells together with the PTP inhibitor vanadate, or generated within the cell) [5,8–13], the thiol oxidizing agent diamide [6–8], thiol alkylating agents [11,13], nitric oxide [14,15], as well as UV irradiation [13]. In some cases, the enhanced tyrosine phosphorylation was suggested to result from direct oxidation and activation of PTK [7,10,12]. However, since all PTPs contain at their active center a highly reactive cysteine residue that is essential for phosphatase activity [1,17], inhibitory effects of the oxidants on PTP activity have been considered to be the more likely mechanism, indirectly responsible for the tyrosine phosphorylation observed [5,11–16]. The PTPs involved and the alterations induced by the agents in the PTPs and in the substrates have not been identified.

We have previously identified a PTP associated with band 3 in the human erythrocyte membrane [18]. The PTP seems to be related to PTP1B (identified using an antibody to an epitope in its catalytic domain and by molecular mass), with the major fraction tightly bound to the cell membrane, along with the major part of band 3. The system described allows the study of the interaction of a PTP with a known, endogenous substrate under normal conditions and the identification of agent-induced alterations in the system. We show here that alterations in erythrocyte thiol status lead to alterations in PTP and result in band 3 tyrosine phosphorylation. Treatment with the thiol-oxidizing agent diamide [19] leads to the formation of PTP disulfides (which appear to be mixed disulfides involving band 3) and results in inhibition of PTP activity and in band 3 tyrosine phosphorylation. Both the disulfide formation and the phosphorylation are reversible. Irreversible erythrocyte thiol alkylation by *N*-ethylmaleimide (NEM) results in irreversible PTP inhibition and irreversible phosphorylation. The results are consistent with the notion that alterations in cellular thiol status may, as part of the perturbations in cellular functions, affect the cell phosphotyrosine status.

2. Materials and methods

2.1. Thiol modification of erythrocytes

Blood (obtained from healthy humans using EDTA as an anticoagulant) was centrifuged, plasma and buffy coat removed and the erythrocytes washed three times with 150 mM NaCl. Erythrocytes were suspended to 10% hematocrit in 25 mM HEPES buffer, pH 7.3/125 mM NaCl and incubated for 30 min at 37°C in the presence or absence of 1.0 mM diamide [19] or 2.0 mM NEM. Aliquots of the treated erythrocytes were washed and reincubated for 30 min in buffer containing 5.0 mM dithiothreitol (DTT).

2.2. Erythrocyte membranes and membrane extracts

Erythrocyte membranes were prepared by hemolyzing the cells in 5 mM sodium phosphate buffer, pH 8.0, containing 1.0 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Membranes were washed with the same buffer, then further washed with 10 mM NaCl/0.1 mM PMSF to obtain hemoglobin-free membranes. For the identification of the membrane proteins, aliquots were solubilized and boiled in Laemmli's SDS sample buffer under nonreducing and reducing conditions (i.e. in the absence and presence of 10 mM DTT), resolved by 10% SDS/PAGE and stained by Coomassie blue. Membrane extracts were prepared by suspending membranes in cold 25 mM HEPES buffer, pH 7.3, containing 1.0 mM DTT, 0.1 mM PMSF and 0.2% Triton X-100 (HEPES/Triton), as previously described [18]. The Triton-treated membrane suspensions were kept on ice for 30 min, then centrifuged at $40000 \times g$. The supernatants were used as the membrane extracts.

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2.3. Analysis of phosphotyrosine, of band 3 and of PTP by immunoblotting

Proteins of the solubilized membranes and extracts were resolved by 10% SDS/PAGE, followed by transfer to nitrocellulose membranes and immunoblotting analysis, as previously described [18]. For the analysis of phosphotyrosine, 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 3.0% bovine serum albumin (BSA) and 0.05% NaN_3, then incubated for additional 2 h at room temperature with monoclonal anti-phosphotyrosine antibody (BioMakor, Rehovot, Israel; antibody diluted 1:2000). After washing with the blocking solution (without NaN_3), the membranes were incubated for 1 hr with goat anti-mouse peroxidase-conjugated antibodies (Amersham), washed in TNT and analyzed, using the ECL detection system (Amersham). Similar procedure was carried out for band 3 immunoblotting, using monoclonal anti-band 3 antibody (BioMakor; antibody diluted 1:20000). For the immunoblot analysis of PTP, the nitrocellulose membranes were processed as described above for anti-phosphotyrosine immunoblotting, but with 0.3% gelatin in the blocking solution instead of BSA, using monoclonal anti-PTP1B antibody FG6-1G (Oncogene Science) at a concentration of 2 μg/ml [18].

3. Results

3.1. Tyrosine phosphorylation in diamide-treated erythrocytes

Erythrocytes were incubated in the presence and absence of diamide, membranes prepared, solubilized in nonreducing and reducing sample buffer and analyzed by SDS-PAGE and by immunoblotting for phosphotyrosine. Coomassie blue staining of membrane proteins obtained from diamide-treated erythrocytes and electrophoresed under nonreducing conditions showed high molecular weight (HMW) fractions at the origin of the gel (Fig. 1, lane 1). Phosphotyrosine was observed in the diamide-treated erythrocytes, in the band corresponding to band 3 protein and in the HMW bands (Fig. 1, lane 4). When diamide-treated cells were reincubated with DTT, the HMW fractions were reduced and the protein profile reverted to the original, control protein profile (Fig. 1, lane 2). The diamide-treated erythrocytes which were reincubated with DTT did not show any tyrosine phosphorylation (Fig. 1, lane 3). When membranes isolated from diamide-treated erythrocytes were solubilized in sample buffer containing DTT, the HMW phosphorylated fractions diminished significantly, with band 3 remaining strongly phosphorylated (Fig. 1, lane 5). No phosphotyrosine was observed in erythrocytes incubated in the absence of diamide (Fig. 1, lane 6). Thus, oxidation of erythrocyte thiols resulted in band 3 tyrosine phosphorylation, which was reversible upon reduction of the disulfides in the intact cells by DTT.

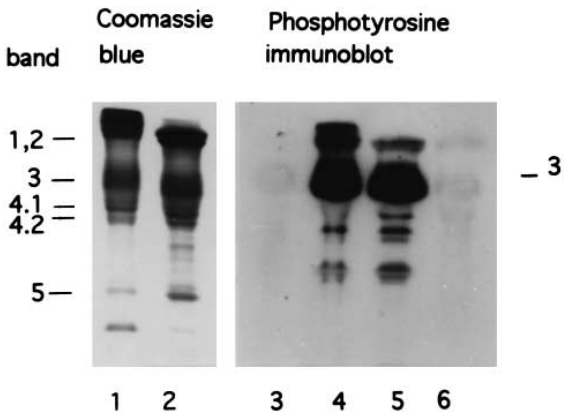

Fig. 1. Tyrosine phosphorylation in diamide-treated erythrocytes. Erythrocyte suspensions (10%) were incubated for 30 min at 37°C in the presence and absence of 1.0 mM diamide. One part was then washed and incubated with 5.0 mM DTT for 30 min. Membranes were prepared, solubilized in sample buffer in the absence or presence of DTT and analyzed by SDS-PAGE and phosphotyrosine immunoblotting, as described in Section 2. Lanes 1 and 4, membranes of diamide-treated erythrocytes solubilized in the absence of DTT; lanes 2 and 3, diamide-treated erythrocytes reincubated with DTT; lane 5, membranes of diamide-treated erythrocytes solubilized in the presence of DTT; lane 6, control erythrocytes.

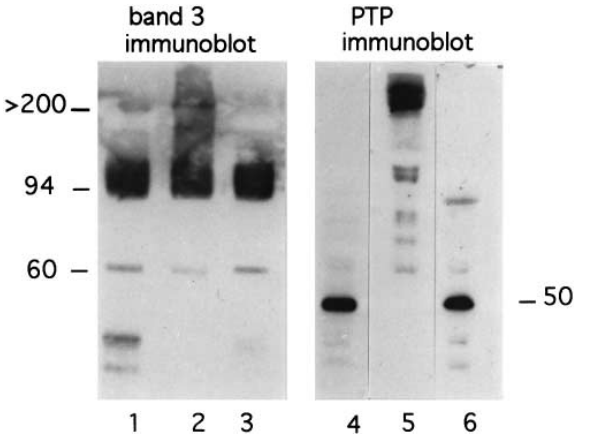

Fig. 2. Band 3 and PTP in diamide-treated erythrocytes. Experimental conditions were the same as in Fig. 1. Membranes were solubilized in sample buffer in the absence of DTT and analyzed for band 3 and PTP by immunoblotting, as described in Section 2. Lanes 1 and 4, control erythrocytes; lanes 2 and 5, diamide-treated erythrocytes; lanes 3 and 6, diamide-treated erythrocytes incubated with DTT, then solubilized.

3.2. Band 3 and PTP oxidation in diamide-treated erythrocytes

Erythrocytes were treated as described above, membranes solubilized and analyzed by immunoblotting for band 3 and PTP. In the control cells, band 3 appeared as approximately 95 kDa band (Fig. 2, lane 1). Part of band 3 was oxidized to HMW fractions in diamide-treated cells (Fig. 2, lane 2) and was reduced back to the 95 kDa band in erythrocytes reincubated with DTT after the diamide treatment (Fig. 2, lane 3). PTP, which appeared as a 50 kDa band in the control erythrocytes (Fig. 2, lane 4), was oxidized to HMW fractions (Fig. 2, lane 5) and was reduced back to the 50 kDa band in cells reincubated with DTT after diamide (Fig. 2, lane 6). The position of the oxidized PTP appeared to be similar to that of the oxidized band 3 (Fig. 2, lanes 2 and 5).

A two-dimensional SDS-PAGE and immunoblotting showed that the oxidized PTP and band 3, present in the same HMW fraction in the non-reduced gels, were resolved on the second dimension reducing gel, to PTP and band 3 (data not shown).

3.3. Autodephosphorylation of erythrocyte membranes

We previously found that following erythrocyte band 3 tyrosine phosphorylation in erythrocyte membranes in the presence of vanadate, dephosphorylation of band 3 was achieved by removing the vanadate and incubating the membranes with Mg^{2+} and DTT [18]. In order to examine whether the presence of band 3 phosphotyrosine in the oxidized cells was due to inhibition of PTP, membranes were isolated from diamide-treated erythrocytes and incubated with Mg^{2+}, in the presence of either diamide (to ensure that the membranes remained in

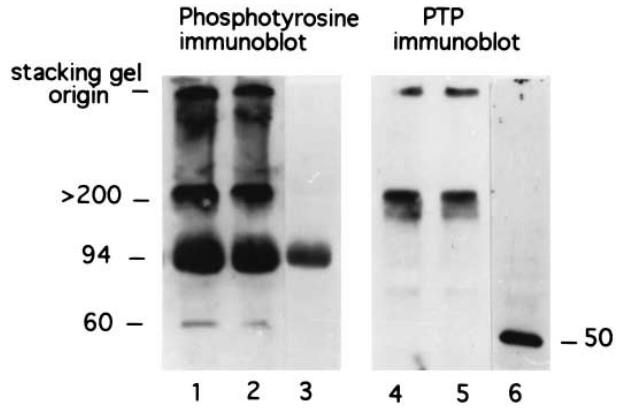

Fig. 3. Phosphotyrosine dephosphorylation in erythrocyte membranes. Membranes were prepared from diamide-treated erythrocytes and suspended at 1.0 mg protein/ml in HEPES/PMSF buffer containing 10 mM Mg^{2+}. Membrane suspensions were incubated for 30 min at 37°C in the presence of either 0.5 mM diamide or 5.0 mM DTT, solubilized and analyzed for phosphotyrosine and for PTP. Lanes 1 and 4, unincubated membranes; lanes 2 and 5, membranes incubated with diamide; lanes 3 and 6, membranes incubated with DTT.

the oxidized state) or DTT. When the membranes, isolated from the oxidized cells, were incubated in the presence of diamide, very little, if any, band 3 dephosphorylation occurred, with the phosphorylated HMW fractions remaining (Fig. 3, lanes 1 and 2). Under these conditions, PTP was present in the HMW fractions, including those at the origin of the stacking gel and at the origin of the separating gel (Fig. 3, lanes 4 and 5). In the presence of DTT, the HMW phosphorylated fractions disappeared and band 3 protein underwent dephosphorylation (Fig. 3, lane 3). Immunoblotting for PTP showed that in the presence of DTT, PTP disappeared from the HMW fractions and reappeared as a 50 kDa fraction (Fig. 3, lane 6). The results indicate that PTP thiol oxidation causes inhibition of phosphotyrosine dephosphorylation and that such an inhibition can be reversed and dephosphorylation achieved by reduction of the diamide-induced disulfides.

3.4. Effects of NEM on band 3 tyrosine phosphorylation

Erythrocytes were treated with NEM, washed and reincubated with DTT. Band 3 phosphotyrosine was found in erythrocytes incubated with NEM (Fig. 4, lane 1). No dephosphorylation was observed in the erythrocytes reincubated with DTT (Fig. 4, lane 2). Membranes which were prepared from NEM-treated erythrocytes and incubated with Mg^{2+} and DTT did not show any dephosphorylation (Fig. 4, lane 3). In contrast, band 3 dephosphorylation did occur in membranes which were prepared from vanadate-treated cells and incubated in the absence of vanadate (Fig. 4, lanes 4 and 5). The results indicate that, whereas vanadate-induced PTP inhibition and band 3 tyrosine phosphorylation is reversible, the phosphorylation following the irreversible thiol alkylation by NEM is not reversible. Immunoblot analysis of PTP from NEM-treated erythrocytes showed the same 50 kDa band as the control PTP (not shown).

3.5. Effects of band 3 thiol alterations on dephosphorylation of band 3 phosphotyrosine

We previously found that Triton X-100 extracts of erythrocyte membranes contain band 3 and associated PTP, and that band 3 dephosphorylation can be achieved in the Triton-extracts of phosphorylated membranes by the addition of Mg^{2+} and DTT [18]. In order to find out whether thiol-altered band 3 is a substrate for PTP, we carried out the following experiment. Membranes isolated from control and from NEM-treated erythrocytes were extracted with Triton X-100. Extracts of the alkylated samples were mixed with extracts of control samples (Fig. 5, lanes 1 and 2), or with extract buffer alone (Fig. 5, lanes 3 and 4). Aliquots of the mixtures were kept in ice (Fig. 5, lanes 1 and 3) or incubated in the presence of Mg^{2+} and DTT (Fig. 5, lanes 2 and 4) and phosphotyrosine analyzed by immunoblotting. Incubation of the alkylated sample that was mixed with control extract led to dephosphorylation of the phosphotyrosine in the thiol-altered band 3 (Fig. 5, lane 2 versus lane 1). In contrast, no dephosphorylation was observed in the sample lacking the control extract (Fig. 5, lane 4 versus lane 3). These results indicate that thiol-altered band 3 is accessible to PTP (the suitability of diamide-oxidized band 3 as a substrate for PTP cannot be ascertained under these experimental conditions, since the disulfides are reduced by the added DTT).

4. Discussion

Perturbation in the cellular and membrane thiol status may occur as a result of certain physiological processes or pathological conditions and can be achieved by the use of oxidizing and alkylating agents [20]. Alterations in the redox status may be of importance in the regulation of cell protein tyrosine phosphorylation. The promotion of tyrosine phosphorylation by physiological or agent-induced oxidative stress, shown to occur in a variety of systems, has been ascribed to activation of PTK or to inhibition of PTP. The diamide-induced autophosphorylation of the endoplasmic reticulum tyrosine kinase Ltk was considered to be due to the oxidation and activation

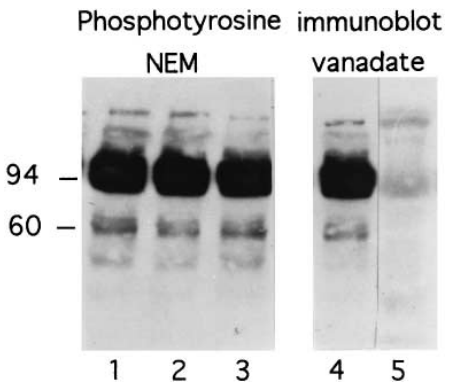

Fig. 4. Effects of NEM on band 3 phosphotyrosine as compared to the effects of vanadate. Erythrocyte suspensions were incubated for 30 min at 37°C with 2.0 mM NEM and washed. An aliquot of the NEM-treated cells was then incubated with 5.0 mM DTT for 30 min. Other erythrocyte suspensions were incubated with 0.1 mM sodium orthovanadate (vanadate), as previously described [18]. Membranes, prepared from NEM or from vanadate-treated erythrocytes, were suspended in HEPES/PMSF buffer containing 10 mM Mg^{2+} and 1.0 mM DTT, incubated for 30 min at 37°C, solubilized and analyzed for phosphotyrosine. Lane 1, NEM-treated erythrocytes; lane 2, NEM-treated erythrocytes incubated with DTT; lane 3, membranes (prepared from NEM-treated erythrocytes) incubated with Mg^{2+} and DTT. Lane 4, vanadate-treated erythrocytes; lane 5, membranes (prepared from vanadate-treated erythrocytes) incubated with Mg^{2+} and DTT.

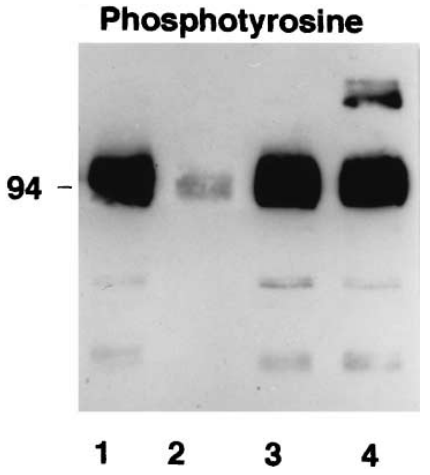

Fig. 5. Band 3 dephosphorylation in membrane extracts. Triton extracts were prepared from membranes of NEM-treated erythrocytes and from control membranes, as described in Section 2. Extract prepared from the NEM-treated erythrocytes was mixed with control extract or with extract buffer (one volume of control extract or extract buffer was added to one volume of extract of NEM-treated cells). Aliquots were incubated for 30 min at 37°C in HEPES/PMSF buffer containing 10 mM Mg^{2+} and 1.0 mM DTT. Unincubated and incubated aliquots were solubilized and analyzed for phosphotyrosine. Lanes 1 and 2, extract of NEM-treated cells mixed with control extract; lanes 3 and 4, extract of NEM-treated cells mixed with extract buffer; lanes 1 and 3, unincubated samples; lanes 2 and 4, incubated samples.

of the kinase [7]. However, based on the finding of an endoplasmic reticulum-associated PTP1B, it was subsequently proposed (but not proved) that the diamide-induced activation of Ltk might be explained by oxidation and inhibition of PTP [21]. The insulinomimetic agents H_2O_2/vanadate have been shown to augment the autophosphorylation of the insulin receptor kinase and its cellular target proteins, concomitant with inhibition of PTP activity in the cytosolic fraction prepared from the treated cells [5,22]. Diamide has been shown to inhibit PTP activity in fibroblast cytoplasm without altering the kinase activity associated with the EGF receptor [6]. The H_2O_2-induced phosphorylation of EGF receptor was considered to be due to activation of a tyrosine kinase [12]. However, since dephosphorylation of receptor tyrosine kinases was shown to be inhibited by oxidative stress-inducing agents [23], it has been proposed that the phosphorylation of the receptors promoted by these agents is due to inhibition of PTP [23].

The studies quoted above indicate that oxidative stress-induced tyrosine phosphorylation of certain receptors and substrates may be due not to direct effect on PTK but to loss of PTP activity. The data presented here provide direct evidence for PTP being a target for oxidative stress and for a role of the thiol-altered PTP in promoting protein tyrosine phosphorylation. We have previously shown that treatment of erythrocytes with the thiol oxidizing agent diamide leads to the formation of disulfide-linked, HMW membrane protein fractions that include some band 3 disulfides [24]. Of the five cysteine residues in human band 3, the two present in the N-terminal cytoplasmic domain (residues 201 and 317) are considered to be reactive and susceptible to oxidation and alkylation [25,26]. Here we show that diamide induces the oxidation of PTP, in addition to oxidation of some of the band 3 protein. Based on the molecular masses of the oxidized fractions and on the presence of both PTP and band 3 in the same HMW fractions, i.e. fractions which are reduced to PTP and band 3 upon treatment by DTT, it appears that the PTP forms mixed disulfides with oxidized band 3 (HMW band 3 that is composed of two or more monomers). The formation of mixed disulfides is consistent with the association we found previously between PTP and band 3 [18]. The formation of mixed disulfides between proteins and small thiols (e.g. glutathione) is considered to be an early cellular response to oxidative stress [27]. The presence of mixed disulfides between associated proteins such as band 3 and PTP would indicate that in addition to the protein-small thiol mixed disulfides, protein-protein mixed disulfides may be formed in response to oxidative stress. Such alterations may directly affect the function of the associated proteins.

We show here that the diamide-induced band 3 phosphorylation is reversed upon the reduction of the disulfides in the intact cells and that band 3 dephosphorylation in membranes isolated from the diamide-treated cells does not occur unless the disulfides are reduced back to thiols. These results indicate that the oxidation-induced phosphorylation is due to inhibition of PTP. The fact that the dephosphorylation of band 3 in NEM-treated erythrocytes or in membranes isolated from such cells is irreversibly inhibited, whereas the alkylated band 3 is a substrate for native PTP, lends further support to the conclusion that thiol-alteration and inhibition of PTP is responsible for the appearance of phosphorylated band 3.

Since PTPs are important for the control of cell protein phosphotyrosine level, the regulation of PTP activity is in turn important for the prevention of uncontrollable dephosphorylation. Modulation of cellular thiol status would provide one of the means for modulation of PTP activity, with target specificity possible for both PTP activity and inhibition, via an association of an active PTP with the substrate and inhibition via the formation of PTP-substrate mixed disulfides.

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