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PHOSPHORYLATION AND DEPHOSPHORYLATION REACTIONS BY ERYTHROCYTE PLASMA MEMBRANE ENZYMES *

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Summary

Human erythrocyte membranes contain a phosphoprotein phosphatase able to dephosphorylate membrane protein previously phosphorylated by the endogenous protein kinase.

The level of dephosphorylation obtained after prolonged incubation is about one half of the phosphorylated residues.

The characteristics of this enzyme are similar to those described for the cytoplasmic phosphoprotein phosphatase.

In a membrane preparation the phosphorylation and dephosphorylation reactions can be repeated, at least twice, achieving similar levels of phosphate esterified or hydrolyzed.

The coordination of these two enzyme systems might play a role in some of the functions attributed to the protein kinase system.

Introduction

It has become evident that several hormonal-induced cell responses are expressed through the phosphorylation of specific proteins, which in part is cyclic AMP dependent [1]. In the human erythrocyte, as well as in other eukaryotic cells, this system seems to be part of the plasma membrane, and its components have been partially identified and purified [2]. The phosphorylation of at least one of the membrane proteins, spectrin complex, has been

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Abbreviation: SDS, sodium dodecyl sulphate.

related to changes in the shape of the erythrocyte [3], and may therefore provide the basis for the known ATP-dependent control of red cell shape [4].

If the phosphorylation of some specific membrane proteins indeed plays a role in functional responses of the cell, it is mandatory that phosphoprotein phosphatase(s) should exist in close proximity to the phosphorylated residues. Such enzymes have been demonstrated in the cytoplasm of several cells, including erythrocytes [5] and there have been reports of membrane-bound protein phosphatases [6,7]. It is certain that most of the proteins phosphorylated on the erythrocyte membrane are located on its internal face in close contact with the cytoplasm. But the presence of a membrane-bound phosphoprotein phosphatase would seem to be advantageous, especially if this system were coordinated with the phosphorylation by protein kinases.

The present study demonstrates the presence in the human erythrocyte plasma membrane of a phosphoprotein phosphatase and examines its relation to phosphorylation reactions.

Materials and Methods

Heparinized human blood was obtained following informed consent from healthy donors, and erythrocyte membranes, prepared according to Dodge et al. [8], were studied on the same day.

Membrane protein phosphorylation and dephosphorylation. For the phosphorylation experiments the membranes were washed once with 5 mM sodium acetate (pH 6.6) and incubated at 30° C for 5—10 min in a solution containing 50 mM sodium acetate (pH 6.5); 2 mM magnesium acetate; 0.06 mM EGTA; 0.06 mM ATP [γ-^{32}P]ATP (The Radiochemical Center, Amersham: 3.0 Ci/mM) and where indicated, 0.0025 mM cyclic AMP. At the end of the incubation time, aliquots were added to 20 mM Tris buffer (pH 7.4) containing 2% SDS.

The protein phosphatase reaction was performed with the remaining membranes. They were washed once in 10 mM Tris buffer (pH 7.4) containing 50 mM ATP, twice in the 10 mM Tris buffer (pH 7.4) and incubated in: 50 mM Tris buffer (pH 7.4); 100 mM KCl, and 10 mM Mg^{2+}. The reaction was stopped by addition of 20 mM Tris buffer (pH 7.4) containing 2% SDS.

Aliquots from both types of experiments were examined by gradient acrylamide gel electrophoresis (between 3.5 and 10.5%) as described by Fairbanks et al. [9]. The gels were either stained and sliced and the radioactivity measured in a liquid scintillation counter, or dried and used for radioautography. The ^{32}P-labelled proteins were estimated by weighing the main areas of the ^{32}P-labelled proteins from the scannings of the radioautograms.

Cytoplasmic fractions were obtained from washed erythrocytes lysed in 2 vols. of ice-cold water. After centrifugation at 20 000 × *g* for 20 min, aliquots of the supernatant were kept at −80° C and thawed only once.

Spectrin was isolated from freshly prepared ^{32}P-labelled membranes incubated in 0.3 mM phosphate buffer (pH 7.6) at 37° C for 30 min [10]. Membrane proteins were estimated by the method of Lowry et al. [11] using bovine serum albumin as a standard.

Results

The pattern of membrane protein phosphorylation was similar to that previously reported [12] although some quantitative differences were found in the proportions of the phosphorylated proteins. From 14 experiments, the mean value of the ^{32}P-labelled protein bands, computed from acrylamide gels sliced and counted (see Materials and Methods for details), revealed the following: in the absence of cyclic AMP, protein bands II, III and IV_5 contained 20, 25 and 4 pmol ^{32}P . mg total membrane proteins^{-1} · 5 min^{-1}, respectively, and in the presence of the cyclic nucleotide the corresponding values were 43, 27 and 43 pmol.

We have observed that membrane ^{32}P-labelled proteins showed no loss of radioactivity if reincubated at pH 6.6 for up to 30 min (the pH value at which the protein kinase reaction was performed) (data not shown). By exploring other pH values, we found that phosphorylated membranes washed free of ATP and reincubated in 20 mM Tris buffer (pH 7.4) showed the presence of phosphoprotein phosphatase activity. Aliquots of ^{32}P-labelled membranes were reincubated for various times up to 180 min, and examined on acrylamide gel electrophoresis. The gels were stained, dried and the radioautograms scanned. As shown on Fig. 1, when membranes were phosphorylated in the presence and absence of cyclic AMP, the radioactive phosphate was progressively lost. This loss of radioactivity was not due to degradation of the proteins (i.e. activation of proteolytic enzymes) since their patterns on Coomassie blue-stained gels (see insert Fig. 1) remained unchanged on several examinations throughout the reincubation period. In two experiments 72% of the ^{32}P-labelled proteins were recovered as $^{32}P_i$ after filtration (Millipore filters type EA) at the end of the phosphoprotein phosphatase reaction.

In order to study the behaviour of the phosphoprotein phosphatase(s) in somewhat more details, two separate membrane preparations were phosphorylated for 10 min, washed, and reincubated for various lengths of time. Aliquots were resolved on gels, the radioautograms scanned (Fig. 2), and the percentage of radioactivity in the main protein peaks was calculated (using as the 100% value the extent of phosphorylation of the ^{32}P-labelled membranes which were washed but not reincubated). The membranes phosphorylated in the presence of cyclic AMP showed close to 50% dephosphorylation after 120 min. Membranes initially phosphorylated only by the cyclic AMP-independent kinase showed a different pattern of dephosphorylation. Both bands II and III appeared dephosphorylated to a larger extent. The low level of phosphorylation of protein band IV_5 (in the absence of cyclic AMP) did not allow an accurate determination of this value.

From Fig. 2 we calculated the initial rates for both phosphorylation and dephosphorylation reactions (Table I). For protein bands II and IV_5, slightly less than 10% of the phosphate esterified in the presence of cyclic AMP was hydrolyzed, while somewhat higher values were obtained for bands II and III when phosphorylation was done in the absence of the cyclic nucleotide.

The dependence of the extent of dephosphorylation on Mg^{2+} concentration was studied. The dephosphorylation pattern of the membrane ^{32}P-labelled proteins changed somewhat with the Mg^{2+} concentration. In the absence of Mg^{2+}

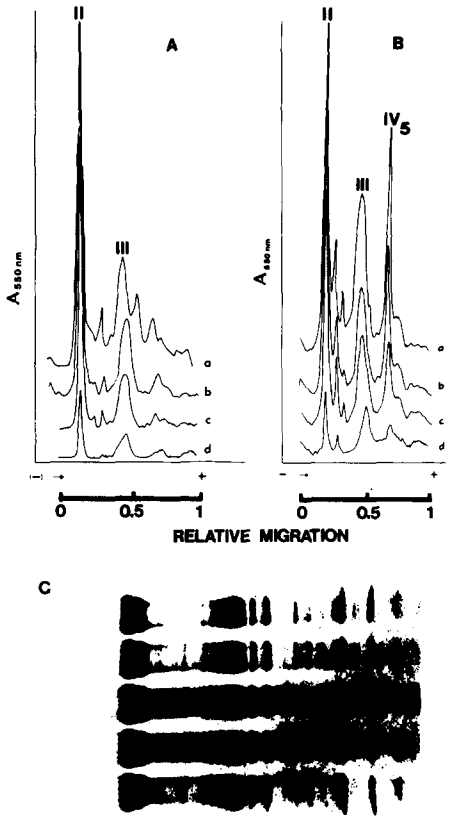

Fig. 1. Scanning of radioautogram after dephosphorylation of ^{32}P-labelled membrane proteins by membrane protein phosphatase(s). Erythrocyte proteins were phosphorylated for 10 min with (B) or without (A) cyclic AMP, washed free of ATP and resuspended for one phosphatase reaction. Aliquots were removed at various times, resolved on polyacrylamide gel electrophoresis and the radioautograms scanned. (a) Zero time phosphatase reaction; (b—d) 30, 60 and 180 min, respectively. (C) Coomassie blue-stained gels from aliquots taken at different times of the protein phosphatase reaction (0, 30, 60 and 180 min, respectively).

there was virtually no dephosphorylation after 60 min of incubation. When the concentration of Mg^{2+} was increased to 20 mM, band II was maximally dephosphorylated, but with the other two proteins, these higher concentrations of Mg^{2+} were slightly inhibitory. With protein band III, the protein phosphatase seemed to be most active at 3 mM Mg^{2+}.

It seemed probable that dephosphorylation in situ might occur at higher rate since some enzymes might be lost during membrane preparation, or cytoplasmic phosphatase [5] might play a role in these reactions. Therefore several agents known to modify the activity of soluble phosphatase(s) were used. In Table II, it is shown that some of the agents capable to inhibit the cytoplasmic phosphatase like P_i, PP_i, ATP and ADP [5] are also strong inhibitors for the membrane-bound enzyme, whereas for some other inhibitors of the soluble enzyme, like 2,3-diphosphoglycerol, 10 mM Mg^{2+} and 100 mM KCl, there are some differences. The addition of 10 mM Mg^{2+} promoted dephosphorylation of band II without affecting the other protein bands. 2,3-Diphosphoglycerol

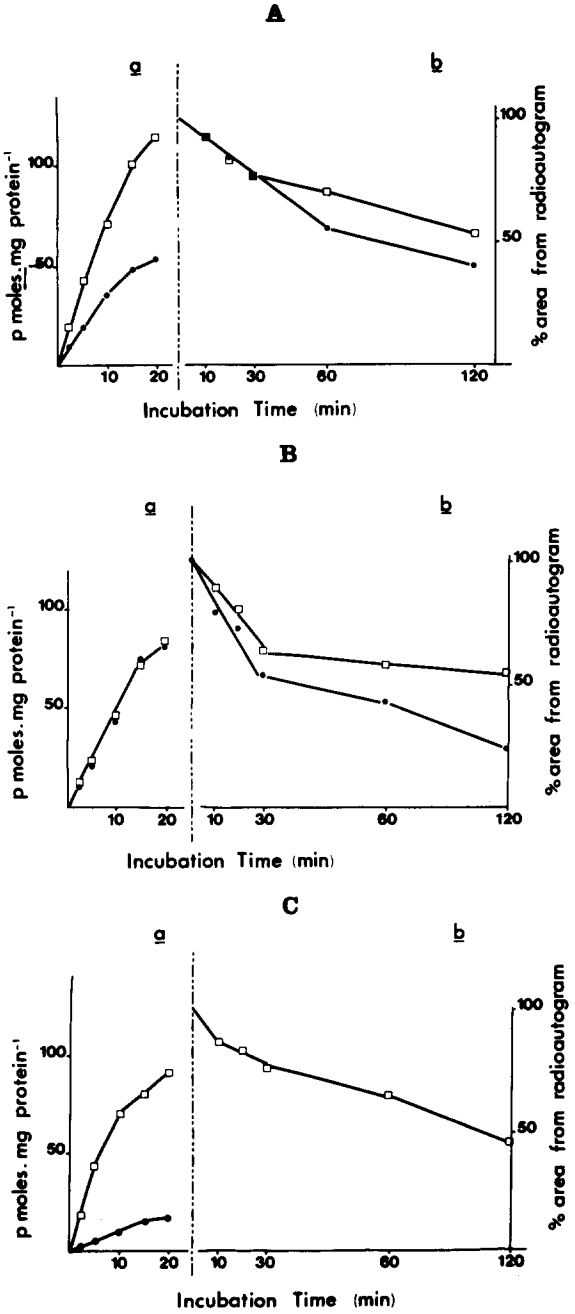

Fig. 2. The rate of phosphorylation and dephosphorylation of erythrocyte membrane proteins by endogenous enzymes. (a) Membranes were phosphorylated in the presence and absence of cyclic AMP. The ^{32}P-labelled proteins were obtained by slicing the gels and counting the radioactivity. (b) ^{32}P-labelled membranes were reincubated for dephosphorylation. Aliquots were resolved on polyacrylamide gel electrophoresis and the areas obtained from the radioautograms. (A) Protein II; (B) protein III; (C) protein IV$_5$. □, + cyclic AMP; ●, — cyclic AMP.

TABLE I

PHOSPHORYLATION AND DEPHOSPHORYLATION IN ERYTHROCYTE MEMBRANE PROTEINS

From the linear portion of Fig. 3 the initial rates for both kinases and phosphatases were calculated. The values for the protein kinase reactions represent phosphate esterified on proteins, whereas the values of protein phosphatases reflect the amount of phosphate hydrolyzed · min^{-1}. The results are expressed as pmol · mg^{-1} protein · min^{-1}.

Proteins	Protein kinases		Phosphoprotein phosphatase(s) *	
	—cyclic AMP	+cyclic AMP	—cyclic AMP	+cyclic AMP
Band				
II	3.6	7.2	0.6	0.6
III	4.5	4.5	0.7	0.7
IV_5	—	7.0	—	0.6

inhibited dephosphorylation of bands II and III (— cyclic AMP) without affecting band IV_5 (+ cyclic AMP) and 100 mM KCl inhibited dephosphorylation of band IV_5. The addition of hemolysate increased to some extent dephosphorylation of bands II and IV_5 without affecting band III, thus suggesting that part of the enzyme is membrane bound. In one experiment ^{32}P-labelled spectrin was incubated to examine the dephosphorylation reaction with the

TABLE II

DEPHOSPHORYLATION OF MEMBRANE PROTEINS BY PROTEIN PHOSPHATASE(S)

Membranes phosphorylated with and without cyclic AMP were washed, and reincubated for 120 min. The ^{32}P-proteins were resolved on gradient slab electrophoresis, and the radioautograms were scanned. The areas of the main protein bands obtained from the samples prior to dephosphorylation are considered as 100%. The areas obtained after dephosphorylation are expressed as percentage of these initial values.

	Protein band (percentage remaining radioactivity)				
	II		III		IV_5
	—cyclic AMP	+cyclic AMP	—cyclic AMP	+cyclic AMP	+cyclic AMP
Buffer A *	54	70	36	54	46
+ 10 mM Mg^{2+}	40	30	41	53	41
+ 100 mM KCl	48	57	27	54	62
+ 4 mM dithiothreitol	41	55	29	51	56
+ 10 mM NaF	67	82	48	76	87
+ 10 mM P_i	67	77	52	82	100
+ 1 mM PP_i	80	100	53	78	100
+ 1 mM ATP	77	100	45	73	91
+ 1 mM ADP	77	88	66	78	98
+ 1 mM cyclic AMP	36	100	41	100	—
+ 5 mM 2,3-diphosphoglycerol	61	67	60	64	77
+ cytosol Diluted 1:10	32	37	41	52	32
Diluted 1:5	30	28	36	47	27

* Buffer A 50 mM Tris buffer (pH 7.4); 2 mM $MgCl_2$.

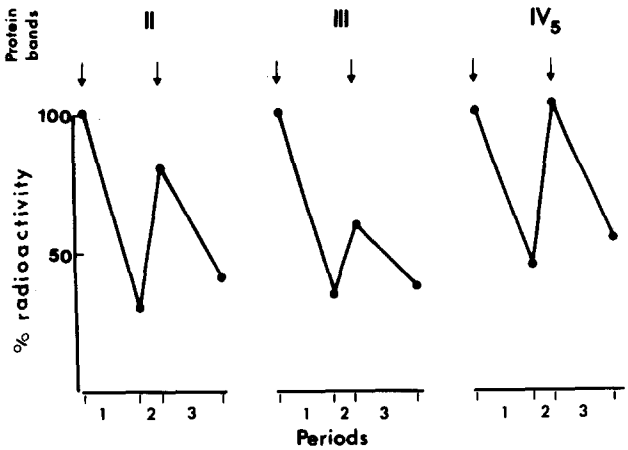

Fig. 3. Cycles of phosphorylation-dephosphorylation reactions in erythrocyte membranes. Membranes were phosphorylated for 10 min in the presence of cyclic AMP, followed by a dephosphorylation of 120 min (period 1). They were then phosphorylated (period 2) and dephosphorylated (period 3) under the same conditions. Aliquots obtained from each step were resolved on polyacrylamide gel electrophoresis and the radioautograms scanned.

membrane-bound enzyme, revealing only a small amount of dephosphorylation.

It was of interest to determine whether the ^{32}P-labelled proteins, once dephosphorylated by membrane-bound phosphoprotein phosphatase, could still serve as substrate for a second cycle of phosphorylation (Fig. 3). After the phosphorylation reaction, the membranes washed free of ATP were reincubated under conditions of the phosphatase reaction for 120 min and slightly over 60% of their initial radioactivity was hydrolyzed. The membranes were then washed in the acetate buffer for the kinase reaction, reincubated for 10 min for a second cycle of phosphorylation, and the ^{32}P-labelled membranes examined again on acrylamide gels. The autoradiograms revealed that proteins of bands II and IV_5 reached levels of ^{32}P-labelled proteins similar to those found after the first cycle of phosphorylation, whereas protein band III showed only slight rephosphorylation. A second dephosphorylation was performed, and levels similar to those seen in the first cycle were obtained. Therefore it seemed that isolated membranes might undergo several cycles of these reactions, and that the amounts of protein phosphorylated and protein phosphates hydrolyzed in each cycle are similar for protein bands II and III.

Discussion

Our findings indicate the presence in isolated human erythrocyte membranes of phosphoprotein phosphatase(s) capable of dephosphorylating endogenous phosphorylated membrane proteins. This enzyme although with similar properties to the soluble erythrocyte one remains bound to the membrane after extensive washing. This suggests that it might form part of the membrane and have a local function.

The two cycles of phosphorylation-dephosphorylation experiments each

showing similar levels of phosphate esterification and hydrolysis for protein bands II and IV_5 indicate that the same number of protein sites can be rephosphorylated after one cycle of dephosphorylation and that probably, these same sites can be hydrolyzed again by the phosphatase(s). Thus the system remains relatively stable throughout these experimental steps. Similar results have been obtained after three cycles of phosphorylation-dephosphorylation.

A striking finding was the relatively low levels of dephosphorylation observed after prolonged incubation, i.e. after 120 min, less than one half of the ^{32}P-labelled proteins had disappeared. And secondly, that at short incubation times, similarly to those utilized for the protein kinase reactions, the extent of hydrolysis of the ^{32}P label was quite small.

By calculating both the phosphorylation and the dephosphorylation during the initial linear part of the reactions, we observed that in the presence of cyclic AMP, bands II—III and IV_5 had a phosphorylation rate of 7.3, 4.6 and 7.1 pmol, respectively, for the first 10 min. At the end of this period, only 0.6 pmol $\cdot$ min^{-1} of the ^{32}P had been removed by the phosphoprotein phosphatase(s). Several interpretations are possible. Either an activator of the phosphoprotein phosphatase exists in vivo which is not present in the membrane preparation, or cytoplasmic enzyme(s) might be involved in these reactions, and both enzymes, the soluble and the membrane-bound one, might act concomitantly. It is also possible that the membrane preparation contains inhibitors such as P_i, PP_i or nucleoside phosphates [5] which might remain bound on the isolated membrane.

Graham et al. [5] have shown that the cytoplasm of human erythrocytes even at high dilution, contains enzyme(s) capable of dephosphorylating purified ^{32}P-labelled spectrin. In our experiments, the addition of rather larger amounts of cytoplasm did not significantly increase the extent of dephosphorylation of the membrane-bound ^{32}P-labelled proteins. It is possible that limited accessibility of the cytoplasmic enzyme to the phosphorylated sites on the membrane might be partly responsible for this effect, but it is also possible that membrane-bound phosphoproteins are partially dephosphorylated by membrane-associated enzymes in close proximity to the phosphorylated proteins. These membrane-bound enzymes differ in some of their properties from the soluble ones.

The advantage of a possible coordinated multienzyme system forming part of the plasma membrane seems evident. Our group and others [13—15] have shown the presence in the membrane of adenyl cyclase, which in some mammals is modulated by hormones. The cyclic AMP generated can be utilized in situ for the phosphorylation of specific membrane proteins, thereby probably changing some responses of the cell [16]. The phosphatase(s), also part of the membrane, may dephosphorylate some specific site(s) on the protein which again can serve as substrate for the kinase reaction. This might be part of a general modulating system capable of modifying cellular responses.

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