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IN VITRO PHOSPHORYLATION OF THE RED BLOOD CELL CYTOSKELETON COMPLEX BY CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM ERYTHROCYTE MEMBRANE

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Hydrosoluble proteins extracted from human erythrocyte ghosts by dialysis at low ionic strength and alkaline pH contain a cyclic AMP-dependent protein kinase which phosphorylates in vitro the cytoskeleton components in crude extracts. Spectrin components 1 and 2, actin and protein band 4.1 undergo this cyclic AMP-dependent endogenous phosphorylation together with low molecular weight peptides solubilized with the cytoskeleton in hydrosoluble extract. However, pure spectrin and purified erythrocyte G-actin were not phosphorylated by purified cyclic AMP-dependent protein kinase from erythrocyte membrane. Purified G-actin when added free to crude extract does not undergo phosphorylation by the cyclic AMP-dependent protein kinase present in this extract. In contrast, purified cyclic AMP-dependent protein kinase added either to crude extract or to the purified cytoskeleton complex (spectrin, actin and protein band 4.1), phosphorylates spectrin, actin and protein band 4.1. We can conclude that (1) cyclic AMP-dependent phosphorylation of red cell cytoskeleton occurs in vitro only when the cytoskeleton components are in a complexed form; (2) red cell actin, like other cellular actins, may be phosphorylated by cyclic AMP-dependent protein kinase but only in the oligomeric form and not in the G form.

Introduction

Several protein kinase activities are present in the red cell membrane: activities of cyclic AMP-dependent histone kinase [1–4] and cyclic nucleotides-independent casein kinases [5]have been identified in human erythrocyte. These activities catalyse endogenous phosphorylation of membrane proteins. A cyclic AMP-independent casein kinase has been extracted from membranes and purified [6–8]: it specifically phosphorylates the spectrin component 2, one or several components of the complex band 3 and some other minor peptides; it uses ATP as a phosphoryl donor and differs from the cytosol casein kinase which is also able to act on the same membrane substrate [9–12]. The cyclic AMP-dependent protein kinase which is a type I histone kinase [13, 14] has a very high potential activity but seems to play an unimportant role in phosphorylation of intact red cells and isolated ghosts: in endogenous phosphorylation experiments, the only apparent effect of the kinase stimulation by cyclic AMP is a slight increase of the overall phosphorylation [4,15]; analysis of phosphorylated peptides by SDS-polyacrylamide gel electrophoresis shows limited increase of spectrin phosphorylation, no noticeable change in phosphorylation of component 3 but important phosphorylation of ankyrin (band 2.1) and components 4.5 and 4.8. Indeed, the part of the cyclic AMP-dependent protein phosphorylation is generally considered not prominent owing to the fact that red cell membranes contain only weak adenylcyclase activity.

As previously reported [13], cyclic AMP-dependent protein kinase is present in the membrane frac-

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

tion extracted at pH 9 in a medium of low ionic strength; furthermore, such a fraction of hydrosoluble proteins contains the main components of the membrane cytoskeleton.

We demonstrate herein that spectrin, actin and protein band 4.1 undergo endogenous cyclic AMP-dependent phosphorylation in the medium of hydrosoluble membrane proteins. This phosphorylation only occurs when spectrin, actin and band 4.1 are complexed; isolated spectrin and actin are insensitive to the purified cyclic AMP-dependent protein kinase activity.

Material and Methods

Blood samples were drawn on heparin from volunteers, physicians and workers of the laboratory and used immediately. Erythrocytes were washed thrice in buffered saline (phosphate buffer, pH 8, 0.005 M) and ghosts were prepared according to Dodge et al. [16].

Endogenous phosphorylation of ghosts and isolated protein fractions was assayed according to Guthrow et al. [2] using in a total volume of 200 μl, 100–120 μg proteins, 0.5 nM [γ-^{32}P]ATP (Amersham Center, specific activity 2–4 Ci/nmol) in 0.05 M acetate buffer, pH 6.5/0.3 mM EGTA, 10mM magnesium acetate and when indicated 1 nM cyclic AMP. Assay mixture was incubated at 30°C for 10 min, unless otherwise indicated. Reaction was stopped by trichloracetic acid precipitation (4 ml at 7.5%); precipitate was dissolved in 0.2 ml 1 M NaOH then precipitated again with trichloroacetic acid (2 ml at 5%). Three cycles of precipitation-dissolution were carried out. Finally, the dissolved precipitate was added to 10 ml scintillation fluid (Dimilume Packard) and radioactivity was determined by liquid-scintillation spectrometry (Intertechnique ABAC SL 40). For SDS-polyacrylamide gel electrophoresis, (performed according to Fairbanks et al. [17]), reaction was stopped by 1% SDS in final concentration in the presence of 1 mM EDTA/3% β-mercaptoethanol (v/v). After Coomassie blue staining, peptide bands were cut up, dissolved in H_2O_2 for 2 h at 80°C and the radioactivity included in each slice was counted.

The results of overall phosphorylation were expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP per mg protein in 10 min incubation time. Radioactivity included in peptide bands after SDS-polyacrylamide gel electrophoresis was expressed as counts per min, a reliable specific activity measurement being not possible.

Unless otherwise indicated, hydrosoluble proteins were extracted from ghosts by overnight dialysis at 4°C against water alkalinized at pH 9 by NH_4OH/1 mM EDTA/1 mM β-mercaptoethanol. Stripped ghosts were eliminated by centrifugation at 100 000 × *g* for 1 h in a Beckman preparative ultracentrifuge and the supernatant was collected. In some experiments, hydrosoluble proteins were solubilized from ghosts at 37°C for 30 min in 0.001 M phosphate buffer, pH 7.6/5 mM β-mercaptoethanol, then concentrated in vacuum and dialyzed against 0.025 Tris-HCl buffer, pH 8/25 mM NaCl/5 mM β-mercaptoethanol.

Spectrin was further purified from hydrosoluble proteins by $SO_4(NH_4)_2$ precipitation (50% saturation), acidic precipitation and finally gel filtration on a AcA34 column (LKB).

Fractionation of hydrosoluble proteins was carried out by ultracentrifugaiton through a 10–30% sucrose gradient, in a Beckman Ultracentrifuge rotor SW 40 for 14 h at 40 000 rev/min. Collected proteins were submitted to SDS-polyacrylamide gel electrophoresis. For fractionation of phosphorylated proteins, hydrosoluble extracts were phosphorylated in large quantities by endogenous phosphorylation, using the same conditions as in the standard assay, except that incubation time was 1 h.

Pure protein kinases used in some experiments were prepared as previously reported [6,13].

Results

Cyclic AMP-dependent phosphorylation of hydrosoluble proteins (Table I)

In our experimental conditions, the overall autophosphorylation of membranes (16 controls) was 107 pmol/mg protein per 10 min (σ = 24.2, extremes 64–141) without cyclic AMP and 148 pmol/mg protein per 10 min (σ = 34.1 Extremes 95–214) with cyclic AMP. Therefore, cyclic AMP increases phosphorylation by about 38%. Differences between phosphorylation with and without cyclic AMP were statistically significant ($t = 3.94, P < 0.001$).

Autophosphorylation of membrane proteins ex-

TABLE I

Autophosphorylation was expressed as pmol of ^{32}P transferred from [γ-^{32}P]ATP in experimental conditions (see text) per mg proteins for 10 min incubation time.

Autophosphorylation	Whole ghosts (16 controls)	Hydrosoluble proteins (8 controls)
– cAMP	m = 107 (64–141)	m = 94 (83–109)
+ cAMP	m = 148 (95–214)	m = 450 (350–540)
$R = \frac{+ \text{cAMP}}{- \text{cAMP}}$	1.38	4.50

trated as reported in Material and Methods (eight controls) was between 83 and 109 pmol without cyclic AMP and 350–540 pmol with cyclic AMP. The ratio of phosphorylation + cyclic AMP/phosphorylation – cyclic AMP was not seriously modified when incubation time was prolonged as long as 1 h.

Identification of substrates: cyclic AMP-dependent phosphorylation of cytoskeleton components

(1) Preparative ultracentrifugation of phosphorylated hydrosoluble proteins (Fig. 1). Our purpose was to look for the possible in vitro cyclic AMP-dependent phosphorylation of cytoskeleton; we studied radioactivity of the various molecular forms of spectrin and other cytoskeleton components, separated by preparative ultracentrifugation from endogenous phosphorylated crude hydrosoluble proteins. 8 mg hydrosoluble proteins prepared according to Marchesi et al. [18] were phosphorylated with cyclic AMP for 30 min as indicated above, then dialysed for 18 h against the phosphorylating buffer (0.05 M acetate buffer, pH 6.5/10 mM magnesium acetate/0.3 mM EGTA) to eliminate excess ATP, and separated by preparative ultracentrifugation. Radioactivity incorporated in the protein peaks corresponding to the dimeric and tetrameric forms of spectrin was proportional to the protein concentration, and specific activity of both the spectrin forms was similar. Furthermore, radioactivity measurements showed: (1) a protein component present in very small amounts but highly phosphorylated, eluted from the gradient just before the dimeric form of spectrin, corresponding possibly to ankyrin which,

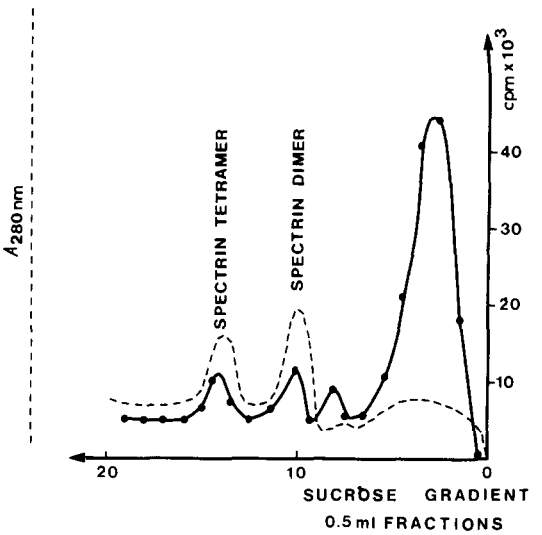

Fig. 1. Preparative ultracentrifugation in a sucrose gradient 10–30 g/100 (w/v) of hydrosoluble proteins extracted by overnight dialysis at 4°C, low ionic strength and pH 9. After extraction, proteins were phosphorylated with cyclic AMP, then dialyzed to eliminate excess radioactive ATP. (- - - - -) 280 nm absorbance; (———) ^{32}P radioactivity.

as well known, is phosphorylated by cyclic AMP-dependent protein kinase; (2) a major peak of radioactivity at the top of the gradient in a zone of low molecular weight components, only detectable by a slight shift of the 280 nm absorbance. About 70% of the total radioactivity was included in this peak. Actin was among these components which were important substrates for cyclic AMP-dependent protein kinase. We have previously observed such peptides after SDS-polyacrylamide gel electrophoresis of hydrosoluble proteins [19] but we do not know their nature and role.

Whatever might be these peptides, preparative ultracentrifugation demonstrated cyclic AMP-dependent phosphorylation of di- and tetrameric forms of spectrin.

(2) SDS-polyacrylamide gel electrophoresis analysis of phosphorylated hydrosoluble proteins. We will consider only the radioactivity incorporated in cytoskeleton components after SDS-polyacrylamide gel electrophoresis. After whole ghosts phosphorylation without cyclic AMP, radioactivity of the cytoskeleton complex was divided as follow: 80–85% in spectrin component 2; 9–12% in spectrin band 1; about 4% in

the peptide band 4.1, and 3% in band 5. When phosphorylation of whole ghosts was performed with cyclic AMP, the radioactivity incorporated in spectrin bands 1 and 2 was increased by about 20%; radioactivity included in actin (band 5) was increased 2-times and was not significantly modified in band 4.1.

Analysis of the same cytoskeleton components after endogenous phosphorylation of hydrosoluble proteins without cyclic AMP, using identical conditions and after electrophoresis separation, shows the following distribution of radioactivity: about 5% of radioactivity were incorporated in high molecular weight complexes which scarcely penetrate the gel; about 25% were incorporated in spectrin component 1; 50% in spectrin component 2; 5% in peptide band 4.1, and 7% in band 5. When cyclic AMP was present, phosphorylations of high molecular weight complexes and spectrin components 1 and 2 were increased by about 2-times. Peptide band 4.1 and actin radioactivity were, respectively, multiplied 5–10-times and 10–18-times in comparison with corresponding radioactivities obtained after phosphorylation without cyclic AMP.

When [2,8-^{3}H]ATP was used instead of [γ-^{32}P]-ATP, no radioactivity was found, neither in spectrin bands 1 and 2 nor in band 4.1 and actin. These results confirm that the radioactivity incorporated in cytoskeleton components after incubation of hydrosoluble proteins with [γ-^{32}P]ATP was due to a real transfer of ^{32}P from ATP and not to the possible binding of whole [γ-^{32}P]ATP to proteins.

(3) Phosphorylation of pure spectrin and actin by membrane cyclic AMP-dependent protein kinase. We tried to obtain phosphorylation of pure spectrin and pure actin by the cyclic AMP-dependent membrane protein kinase purified from hydrosoluble proteins. Spectrin, purified as reported in Material and Methods, was able to undergo a weak autophosphorylation when incubated in the standard assay mixture with [γ-^{32}P]ATP: phosphoryl transfer was about 15–20 pmoles of ^{32}P per mg spectrin in 30 min and was not stimulated by cyclic AMP. To 200 μg pure spectrin, we added in the phosphorylation medium 0.1 μg pure cyclic AMP-dependent protein kinase and 1 nM cyclic AMP; no complementary phosphorylation of spectrin was observed. On the contrary, pure spectrin can be highly phosphorylated by purified cyclic AMP-independent casein kinase extracted from human erythrocyte membrane.

50 μg erythrocyte G-actin, purified according to Spudich and Watt [20] were incubated in the phosphorylating mixture with 0.1 μg cyclic AMP-dependent membrane protein kinase: no phosphorylation was observed.

From these experiments, we could conclude that (1) the two spectrin chains (specially the light chain), actin and peptide band 4.1 were phosphorylation by cyclic AMP-dependent endogenous protein kinase in the crude hydrosoluble proteins solutions; (2) in contrast, neither the purified spectrin nor the pure erythrocyte G-actin was phosphorylated by purified cyclic AMP-dependent protein kinase from human erythrocyte membrane.

Influence of the physical state of spectrin-actin-band 4.1 complex on the cytoskelton component phosphorylation

The above-mentioned discrepancies could be due either to the physical state of the cytoskeleton components in the hydrosoluble protein medium or to differences in enzyme activity when assayed in crude extract or after purification.

To verify the first hypothesis, we chose actin which was highly phosphorylated by endogenous cyclic AMP-dependent protein kinase in the crude membrane extract. We added free G-actin purified from erythrocyte membrane to the crude hydrosoluble proteins; then we performed endogenous phosphorylation and we compared phosphorylation assay results of samples with and without free actin added. The results are given in Table II.

Other samples of the mixtures were subjected to SDS-polyacrylamide gel electrophoresis; after gel staining, band 5 was cut up and radioactivity included in the disc was counted. The results are given in Table III.

Our experimental conditions being unfavourable to the actin binding to cytoskeleton elements, we can assume that actin added to hydrosoluble proteins remained free. Indeed, free actin appeared non-phosphorylatable in a medium in which actin complexed with other cytoskeleton components was highly phosphorylated.

With the aim on one hand to study the relationship between the various possible conformations of the cytoskeleton components and on the other hand

TABLE II

60 μl of a 0.7 mg/ml red cell actin solution (in 2 mM Tris-HCl buffer, pH 9/0.5 mM β-mercaptoethanol 0.2 mM ATP/0.5 mM $CaCl_2$ were added to 200 μl of standard phosphorylation mixture containing 480 μg of hydrosoluble proteins. The results are expressed as pmol of ^{32}P transferred per 10 min and per mg protein in experimental conditions. Difference between phosphorylation without and with Actin added was not significant.

	Overall phosphorylation of hydrosoluble proteins	
	– Cyclic AMP	+ Cyclic AMP
Without actin	145	502
With actin	145	535

their phosphorylability, we fractionated hydrosoluble proteins by gel filtration and performed the cyclic AMP-dependent phosphorylation of each fraction with and without pure cyclic AMP-dependent protein kinase added.

We then separated the phosphorylated peptides by SDS-polyacrylamide gel electrophoresis and counted the radioactivity of each peptide after gel staining.

Addition of kinase to crude extract increased by about 20% the radioactivity of spectrin, actin, and band 4.1; in the complex obtained from the first peak of gel filtration, phosphorylation of spectrin component 1 and component 2 were, respectively, increased by about 50 and 35%, and phosphorylation of actin by 7-fold when cyclic AMP-dependent protein kinase was added. Phosphorylations of spectrin chain I and spectrin chain II were increased by 3.5- and 2-times, respectively, in the spectrin dimer preparation which was devoid of actin.

TABLE III

Samples of mixtures described in Table II were subjected to SDS-polyacrylamide gel electrophoresis. After gel staining, band 5 was cut up, the gel disc was dissolved in H_2O_2 and radioactivity included in the disc was counted. Differences between preparations without and with red cell actin were not significant.

	Radioactivity incorporated in band 5 (cpm)	
	– Cyclic AMP	+ Cyclic AMP
Without actin	231	1250
With actin	278	1456

From these experiments, we conclude that (1) cyclic AMP-dependent protein kinase added to crude hydrosoluble proteins was active towards endogenous substrates of the cytoskeleton components eventhough the same enzyme was inactive towards pure spectrin and actin; (2) the substrate which was the most sensitive to the enzyme activity was actin, but only when it was included in the spectrin-actin-band 4.1 complex.

Discussion

The present work demonstrates that spectrin, actin and protein band 4.1 extracted as a complex from human erythrocyte membrane are phosphorylated by the cyclic AMP-dependent protein kinase which elutes from the membrane together with these cytoskeleton components. In contrast, as previously noticed [8,13], purified spectrin and G-actin are not phosphorylatable by the pure cyclic AMP-dependent protein kinase from erythrocyte membrane. Such a discrepancy is not clearly explained and needs further work. Three hypothesis might be discussed.

(1) The loss of phosphorylatable sites of spectrin and actin in the course of purification of these proteins. Spectrin is very sensitive to endogenous proteolysis. Tryptic digestion may rapidly remove all the phosphorylable sites sensitive to spectrin kinase [21] and we cannot exclude the possibility that the highly phosphorylated small peptides found in the crude hydrosoluble extract are proteolytic fragments with phosphorylatable sites. Arguments against this hypothesis are the following: spectrin phosphorylated by endogenous cyclic AMP-dependent protein kinase in crude hydrosoluble extract can be purified later without loss of radioactivity, therefore without loss of its phosphorylated sites; in another way pure spectrin which cannot be phosphorylated by cyclic AMP-dependent protein kinase is highly phosphorylated by the membrane casein kinase; however, the sites for both enzymes may be different.

(2) The presence in the hydrosoluble extract of an intermediate agent between the cyclic AMP-dependent protein kinase and the protein substrates. Such

an hypothetical intermediate could be a casein kinase, the activity of which would be stimulated by phosphorylation by the cyclic AMP-dependent protein kinase. In fact, we have eliminated this hypothesis: a low casein kinase activity is present in hydrosoluble proteins due to small amounts of spectrin kinase extracted at low ionic strength, but complete inhibition of this activity by heparin (unpublished data) does not abolish the cyclic AMP-dependent phosphorylation of the cytoskeleton components.

Calcium ions do not intervene, since the preparations and the phosphorylation assays are performed in the presence of EDTA or EGTA.

(3) The presence of the complexed form of spectrin, actin and band 4.1 is obligatory to the action of cyclic AMP-dependent protein kinase. A strong argument for this hypothesis is the non-phosphorylation of free actin added to the hydrosoluble extract. Furthermore, when pure cyclic AMP-dependent protein kinase is added to the crude hydrosoluble extract, its activity increases phosphorylation of spectrin slightly and phosphorylation of actin and protein 4.1 greatly. The same effect is observed when kinase is added to the spectrin-actin-band 4.1 complex obtained from crude extract by gel filtration; a weak phosphorylation is observed with the 'spectrin-dimer' obtained from the gel filtration peak, in which spectrin is not really pure, and finally no phosphorylation is present when purified spectrin or actin is used.

At the present day, we do not know why such a complexed form of substrates is necessary for the cyclic AMP-dependent protein kinase activity. We do not know also if the cyclic AMP-dependent phosphorylation of spectrin, actin and protein 4.1 observed in vitro in a protein extract of red cell membrane is of physiological significance in vivo. However, the most important result of this work is the demonstration of a cyclic AMP-dependent phosphorylation of red cell actin. Phosphorylation of a non-muscle actin by a cyclic AMP-dependent protein kinase has been observed in a culture of mouse lymphoma cells [22]; however, only a small amount of the cell actin was phosphorylated, probably the 'nascent' chains and perhaps the oligomeric forms occurring during the filament synthesis. Phosphorylation of red cell actin has been previously reported. It is generally accepted that red cell actin is under an oligomeric form [23]. Our results show that G-actin is not phosphorylated, in contrast to the native form of actin in the cytoskeleton complex. These results could be in agreement with those of Steinberg [22] if the phosphorylation of only oligomeric forms of actin is confirmed. Nevertheless, the significance of this phosphorylation with respect to the actin function in the red cell cytoskeleton, remains to be determined.

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