PHOSPHOPROTEIN PHOSPHATASE OF THE HUMAN ERYTHROCYTE

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Summary. Human erythrocytes contain phosphoprotein phosphatase activity that can be assayed by measurement of ^{32}Pi release from partially purified [^{32}P]spectrin. The activity is entirely cytoplasmic and is readily detected at very high dilution (e.g., 10^{-4} relative to packed cells). Because the reaction in vitro is highly inhibited by Pi, ATP and 2,3-DPG at physiologic concentrations, turnover of membrane protein-bound phosphate in intact erythrocytes may be modulated by the availability of these ligands.

Among the major components of the human erythrocyte membrane are two polypeptides, referred to as "spectrin" (1), that are distinctive in their high molecular weights and susceptibility to elution at very low ionic strength (1-3). The spectrin chains are localized on the cytoplasmic face of the membrane (3-5), where they are believed to interact with ATP, divalent cations and actin in determining erythrocyte shape and membrane elasticity (3,5-10). Several laboratories have demonstrated that polypeptide 2 (MW 215,000--ref. 3) of spectrin is phosphorylated, both in isolated membranes exposed to [γ-^{32}P]ATP (11-15) and in intact cells incubated with ^{32}Pi (15,16). These observations suggest that the organization of the erythrocyte membrane may be modulated by regulatory phosphorylations of spectrin. Because such a regulatory pathway should be reversible, we have examined the erythrocyte for the presence of a phosphoprotein phosphatase with activity against spectrin. In this report we describe the detection of such an enzyme and present a preliminary description of its properties.

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MATERIALS AND METHODS

Preparation of ^{32}P-labeled spectrin. Erythrocyte membranes were prepared as described previously (2). Fifty to 60 ml of packed red cell ghosts were incubated at 37° for 30 minutes with 2.3 μM [γ-^{32}P]ATP (New England Nuclear NEG-002X; 10-20 Ci/mmole) in 10 mM Mg^{++}--0.2 M KCl--0.25 mM ouabain. The reaction was terminated by addition of nine volumes of cold 5 mM Tris-chloride (pH 8) containing 2 mM ATP. The membranes were collected by centrifugation was washed eight times. After the third wash, ATP was omitted; between the fifth and sixth wash, the membranes were frozen and thawed. To elute spectrin, the washed ^{32}P-labeled membranes were diluted into nine volumes of warm 1 mM Tris-chloride (pH 8)--1 mM DTT† and incubated for one hour at 37° (14). The suspension was then ejected twice through a 27 guage needle and centrifuged at 25,000 rpm for one hour. The supernatant was brought to 20 mM in sodium-HEPES (pH 7) and concentrated by ultrafiltration to a protein concentration of 1 to 2 mg/ml. The material was stored at -20° in 0.25 M sucrose--20 mM HEPES (pH 7)--1 mM DTT.

Characterization of ^{32}P-labeled spectrin. Polyacrylamide gel electrophoresis in 1% SDS revealed that the preparation of labeled spectrin contained two major polypeptides (1 and 2--refs. 2,3), together comprising over 90% of the material stained with Coomassie blue, plus numerous trace peptides. The ^{32}P-labeled components were characterized in several ways. Only 0.5-3% of the ^{32}P in the extract was not adsorbed on activated charcoal and was extracted into isobutanol in the presence of H_2SO_4 and ammonium molybdate. Fixed, stained gels contained a single peak of ^{32}P coincident with band 2 in the staining profile. Gels analyzed for ^{32}P without prior fixation and revealed one major and two minor peaks of ^{32}P (Figure 1). The major peak was identical to that detected in band 2 in replicate fixed, stained gels. A second peak contained 2-5% of the total ^{32}P in the extract and had an electrophoretic mobility identical to that of [γ-^{32}P]ATP (Figure 1). If non-radioactive ATP was omitted during the washing of the ^{32}P-labeled membranes, the ^{32}P content of the second peak increased to over 70% of the total ^{32}P in the spectrin extract. This material appears to be tightly, but not covalently, bound to protein, as it was appreciably removed by ultrafiltration or dialysis only after the addition of SDS. Thus, 2-5% of the radioactivity associated with [^{32}P]spectrin prepared as phosphatase substrate is probably tightly bound [^{32}P]ATP.

Preparation of crude phosphoprotein phosphatase. Washed, packed human erythrocytes were hemolyzed by dilution into thirty volumes of 5 mM sodium phosphate (pH 8) at 0° (2). After centrifugation at 18,000 rpm for 20 minutes, the supernatant was removed and used as the source of protein phosphatase; HEPES buffer was used for further dilutions of the hemolysate. Fresh supernatant was prepared for each experiment, although storage for one week at -20° in the presence of 5 mM DTT resulted in a decrease of only 10% in phosphatase activity relative to the fresh hemolysate.

Assay of phosphoprotein phosphatase activity. The crude enzyme was incubated at 37° with 35-50 μg of [^{32}P]spectrin in a volume of 0.05 ml containing 20 mM HEPES (pH 7)--0.1 M KCl--4 mM DTT. The release of ^{32}Pi from the labeled substrate was assessed in two ways. Method 1: The incubation was terminated by addition of 3 ml of a suspension of Norit A (40 mg/ml) in 0.1 M HCl--0.02% bovine serum albumin--1 mM sodium phosphate--1 mM sodium pyrophosphate at 0°. After standing for 10-15 minutes at 0°, the suspension

†Abbreviations: DTT, dithiothreitol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

was filtered (0.45 μm Millipore filter) and the residue was washed twice with 2 ml of 0.01 N HCl--1 mM sodium phosphate. The ^{32}Pi not adsorbed to the charcoal was determined by counting Cerenkov radiation produced by ^{32}P in the filtrate (17). Method 2: The reaction was terminated by addition of 0.3 ml of cold 25% trichloroacetic acid, followed by 200 μg of bovine serum albumin in 0.05 ml. The suspension was allowed to stand for 10 minutes at 0° and was then centrifuged for 10 minutes at 2500 rpm. A 0.3 ml portion of the resulting supernatant was mixed with 0.2 ml of 4% ammonium molybdate in 2 M H_2SO_4 and extracted with 1.0 ml of isobutanol. Following centrifugation to promote phase separation, 0.5 ml of the isobutanol phase was withdrawn for liquid scintillation counting. All data were corrected by subtraction of blank values obtained by simultaneous incubation of [^{32}P]spectrin in the absence of enzyme; blanks were 0.5-3% of the total radioactivity in incubation mixtures.

Other methods. Protein was determined by the method of Lowry et al. (18). Polyacrylamide gel electrophoresis in 1% SDS at pH 2.4; gel fixation, staining and destaining; and analysis of ^{32}P distribution in gels by autoradiography or counting of transverse slices were performed as described previously (14,19).

RESULTS

Release of ^{32}Pi from ^{32}P-labeled erythrocyte membranes. In previous studies, erythrocyte membranes phosphorylated in the presence of Mg^{++} and [γ-^{32}P]ATP showed no loss of ^{32}P from band 2 in incubations at 37° for up to 10 minutes (14). However, incubation of washed, ^{32}P-labeled erythrocyte membranes at 25° in the presence of a 1:30 dilution of erythrocyte cytoplasm (supernatant of the initial hemolysis) increased the rate of ^{32}Pi release fifteen-fold relative to that measured in control incubations of ghosts in hemolysis buffer alone (data now shown). Boiling the erythrocyte cytoplasm for five minutes abolished its stimulatory effect. Polyacrylamide gel electrophoresis in SDS of the membranes recovered after incubation revealed that 70% of the decrease in the ^{32}P content of the hemolysate-treated membranes was due to loss of ^{32}P from the region of band 2; no evidence of proteolysis was observed on these gels. These findings suggested that erythrocyte cytoplasm contained a phosphatase activity capable of acting on band 2.

Demonstration of spectrin phosphatase. When [^{32}P]spectrin was incubated under the conditions of the enzyme assay, but in the absence of added erythrocyte cytoplasm, or in the presence of boiled cytoplasm, no

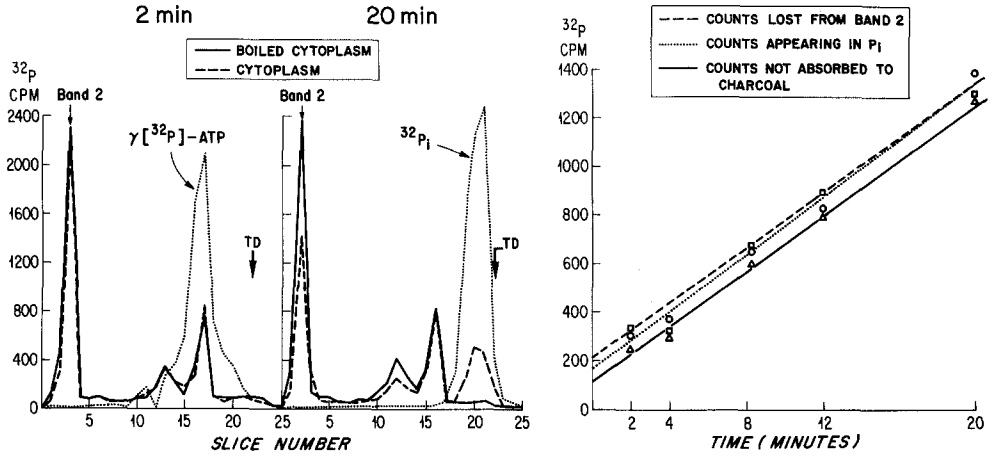

Figure 1. Characterization of [^{32}P]spectrin and identification of ^{32}Pi released in reactions with cytoplasmic phosphatase. Spectrin was prepared from membranes phosphorylated with [γ-^{32}P]ATP as described in Materials and Methods, except that nonradioactive ATP was included only in the medium used to terminate the reaction. The final preparation of [^{32}P]spectrin thus retained sufficient [γ-^{32}P]ATP to allow an examination of its fate in the subsequent incubations. Aliquots of [^{32}P]spectrin (0.75 mg protein, approximately 150,000 cpm) were incubated at 37° with erythrocyte cytoplasm (overall dilution, 1/600) or boiled cytoplasm in 1.0 ml volumes containing 20 mM Tris-chloride (pH 8.0)--60 mM NaCl--4 mM DTT. At 2, 4, 8, 12 and 20 minutes, 0.2 ml portions were removed, and the reaction terminated by mixing with 0.05 ml of 0.6 M sucrose--5% SDS--0.2 M DTT--50 µg/ml pyronin Y, with further incubation for 30 minutes at 37°. From each 0.25 ml solution, two 0.04 ml aliquots were removed for assay of ^{32}Pi release (Method 1) and three 0.05 ml aliquots were subjected to SDS-polyacrylamide gel electrophoresis at pH 2.4 (19).

(a) Distribution of ^{32}P in electrophograms of samples removed after two or 20 minutes incubation with cytoplasm (dashed lines) or boiled cytoplasm (solid lines). The distributions of [γ-^{32}P]ATP and ^{32}Pi subjected to electrophoresis under identical conditions are indicated by dotted lines at the left and right, respectively. The gels were frozen and transected without fixation and staining. Radioactivity in 2 mm slices was determined by counting Cerenkov radiation in water (19); recovery of ^{32}P in the gels was 100 $\pm$ 5%.

(b) Rate of ^{32}Pi release from [^{32}P]spectrin measured by depletion of ^{32}P in band 2 (dashed line), increase in counts in the ^{32}Pi zone (dotted line; average of total counts in slices 19-22 from duplicate gels), and appearance of counts not adsorbed to charcoal (solid line; average of duplicate determinations).

release of ^{32}Pi or loss of ^{32}P from band 2 occurred (Fig. 1). The addition of erythrocyte cytoplasm promoted the release of ^{32}P selectively from band 2 without change in the magnitude of bound [γ-^{32}P]ATP. The ^{32}P released had the electrophoretic mobility of ^{32}Pi, was not adsorbed by charcoal (Fig. 1),

and yielded a molybdate complex that was extracted into isobutanol (data not shown). Furthermore, at a time when 35% of the ^{32}P in band 2 had been released, the electrophoretic mobility and Coomassie blue staining intensity of band 2 was unaltered (Fig. 1), clearly excluding proteolysis as the mechanism of ^{32}P release.

Some properties of the cytoplasmic phosphatase. The rate of ^{32}Pi release increased with increasing [^{32}P]spectrin concentration up to 0.8 mg protein/ml and began to plateau thereafter; saturating concentrations were > 1.2 mg/ml. The rate of ^{32}P release was strictly linear with time up to approximately 25% release and gradually declined thereafter; the maximal

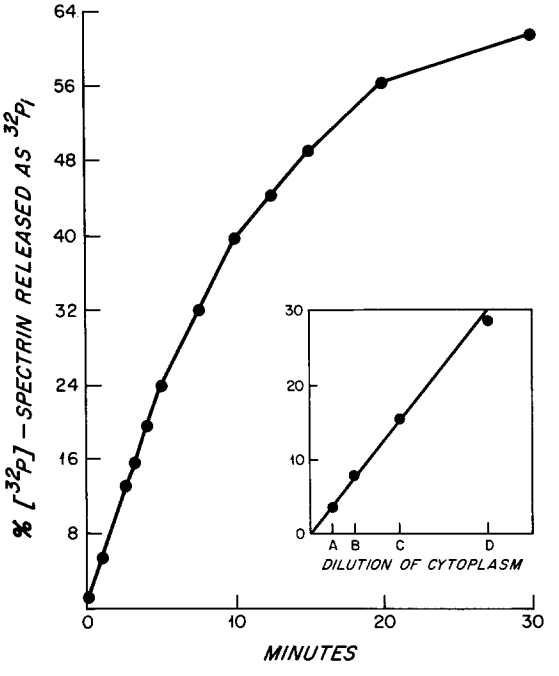

Figure 2. Kinetics and concentration dependence of spectrin phosphatase reaction. Erythrocyte cytoplasm (overall dilution, 1/600) was incubated with [^{32}P]spectrin (0.84 mg/ml) in the assay medium described in Materials and Methods. At the times indicated, replicate aliquots were withdrawn and assayed for ^{32}Pi by Method 1. Activity is expressed as percent release of ^{32}Pi relative to total ^{32}P in the sample. The insert shows activity (percent ^{32}Pi released in 20 minutes at 37°) as a function of overall dilution of the cytoplasm: A = 1/12000, B = 1/6000, C = 1/3000, D = 1/1500.

release of ^{32}Pi was 60-65% with all preparations (Fig. 2). The pH optimum was near 7. Spectrin phosphatase was essentially entirely cytoplasmic in localization: 96% of the activity was recovered in the initial hemolysate supernatant and the relative specific activity in the purified ghost membranes was less than 2% of that of the cytoplasm, whether assayed in the presence or absence of Triton X-100 (0.2% or 1%, w/v).

Regulation of spectrin phosphatase (Table I). Sodium and potassium ions were mildly stimulatory, with an optimum near 0.1 M, whereas the divalent cations tested were only inhibitory when present at 10 mM and above. Fluoride was completely inhibitory above 10 mM and compounds containing phosphate and pyrophosphate were, in general, strongly inhibitory. Especially striking was the inhibition exerted by Pi, ATP and 2,3-DPG at concentrations found in erythrocyte cytoplasm (20). Cyclic AMP and cyclic GMP were without effect (10^{-6} to 10^{-2} M).

Table I. Effect of various agents on dephosphorylation of [^{32}P]spectrin by cytoplasmic phosphoprotein phosphatase

Agent	Conc.	Relative Activity	Agent	Conc.	Relative Activity	Agent	Conc.	Relative Activity
	mM	%		mM	%		mM	%
Mg^{++}	10	58	F^-	100	0	ATP or GTP	1	2
"	1	95	"	10	10	" "	0.1	22
Ca^{++}	10	68	"	1	93	ADP or GDP	1	12
"	1	97	Pi	10	2	AMP or GMP	1	71
Mn^{++}	10	28	"	1	39	Adenosine	1	110
"	1	86	"	0.1	95	Ribose-5-P	1	80
EGTA	1	97	PPi	10	0	2,3-DPG	5	12
EDTA	1	106	"	1	0	"	1	52
			"	0.1	10	"	0.5	85

Phosphoprotein phosphatase was assayed by Method 1. Erythrocyte cytoplasm (overall dilution, 1:12000) was incubated with 42 µg [^{32}P]spectrin in 0.05 ml mixtures containing 20 mM HEPES (pH 7.0)--0.1 M KCl--4 mM DTT--1 mM EDTA. Cation chlorides, sodium salts of anions, or other agents were added at the concentrations indicated. In examining effects of EGTA and divalent cations, EDTA was omitted from the incubation mixtures. Release of ^{32}Pi did not exceed 25% of the total radioactivity in the assay mixture. Activity is expressed as percent release of ^{32}Pi relative to controls incubated without additions.

DISCUSSION

Erythrocyte cytoplasm clearly contains a protein phosphatase capable of acting on phosphorylated band 2, whether in aqueous suspension or on the erythrocyte ghost membrane. Under optimal conditions *in vitro*, [^{32}P]spectrin is rapidly dephosphorylated in the presence of minute amounts of erythrocyte cytoplasm. However, the limited data available indicate that turnover of ^{32}P in band 2 in intact red cells is quite slow (G. Fairbanks and J. Avruch, unpublished observations). This suggests that the activity of the phosphoprotein phosphatase is inhibited in the intact erythrocyte. The present data point up several possible sites of regulation. First, our preliminary experiments indicated that the rate of ^{32}P release from membrane-bound [^{32}P]spectrin is much slower than release from [^{32}P]spectrin in aqueous suspension (at comparable concentrations of polypeptide 2 and phosphatase). This difference might reflect a critical structural feature of membrane-bound spectrin or might be due to more trivial factors, such as artifactual permeability barriers between enzyme and substrate or mild denaturation of the eluted spectrin. Similarly, the factors controlling the binding of the cytoplasmic phosphatase to its membrane-bound substrate are unknown. In addition to these topologic considerations, it is apparent that the sodium salts of ATP, 2,3-DPG and Pi are strongly inhibitory at concentrations of these anions in erythrocyte cytoplasm. A number of protein phosphatases from other sources are similarly inhibited by compounds containing phosphate and pyrophosphate groups (21). However, the effects of these compounds may vary depending on the phosphoprotein substrate employed and in some cases are altered greatly by the presence of a divalent cation. In view of these complexities, further study will be required to clarify both the site of action and regulatory role of ATP, Pi, and 2,3-DPG in spectrin dephosphorylation. The present studies serve to identify spectrin phosphatase and suggest that the regulation of its activity may be subject to several types of control.

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REFERENCES

1. Marchesi, V.T., and Steers, E., Jr. (1968) Science 159, 203-204.
2. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
3. Steck, T.L. (1974) J. Cell Biol. 62, 1-19.
4. Nicolson, G.L., Marchesi, V.T., and Singer, S.J. (1971) J. Cell Biol. 51, 265-272.
5. Tilney, L.G., and Detmers, P. (1975) J. Cell Biol. 66, 508-520.
6. Rosenthal, A., Kregenow, F.M., and Moses, H.L. (1970) Biochim. Biophys. Acta 196, 254-262.
7. Pinder, J.C., Bray, D., and Gratzer, W.B. (1975) Nature 258, 765-766.
8. Painter, R.G., Sheetz, M., and Singer, S.J. (1975) Proc. Natl. Acad. Sci. 72, 1359-1363.
9. LaCelle, P.L., and Kirkpatrick, F.H. (1975) in Erythrocyte Structure and Function (Brewer, G.J., ed.) Alan R. Liss, New York, pp. 535-553.
10. Kirkpatrick, F.H. (1976) Life Sciences 19, 1-18.
11. Guthrow, C.E., Jr., Allen, J.E., and Rasmussen, H. (1972) J. Biol. Chem. 247, 8145-8153.
12. Rubin, C.S., and Rosen, O.M. (1973) Biochem. Biophys. Res. Commun. 50, 421-429.
13. Roses, A.D., and Appel, S.H. (1973) J. Biol. Chem. 248, 1408-1411.
14. Avruch, J., and Fairbanks, G. (1974) Biochemistry 13, 5507-5514.
15. Greenquist, A.C., and Shohet, S.B. (1975) in Erythrocyte Structure and Function (Brewer, G.J., ed.) Alan R. Liss, New York, pp. 515-531.
16. Palmer, F.B.St.C., and Verpoorte, J.A. (1971) Can. J. Biochem. 49, 337-347.
17. Avruch, J., and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334-347.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Avruch, J., and Fairbanks, G. (1972) Proc. Natl. Acad. Sci. 69, 1216-1220.
20. Beutler, E., and Srivastava, S.K. (1972) in Hematology (Williams, W.J., Beutler, E., Erslev, A.J., and Rundles, R.W., eds.) McGraw-Hill, New York, pp. 94-100.
21. Nakai, C., and Thomas, J.A. (1974) J. Biol. Chem. 249, 6459-6467.