Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 508-513

ROLE OF ACTIN BINDING PROTEIN PHOSPHORYLATION IN PLATELET CYTOSKELETON ASSEMBLY

Zhuang, Qing-Qi*, Rosenberg, S.+, Lawrence, J. and Stracher, A.o

Department of Biochemistry, State University of New York, Downstate Medical Center, Brooklyn, NY 11203

Received December 12, 1983

Actin binding protein from human blood platelets is shown to exist in the resting platelet as a phosphorylated protein and contains two residues of phosphate per 260,000 kd. Removal of one-half of these residues with E. coli alkaline phosphatase results in the loss of its ability to crosslink F-actin into a low speed sedimentable complex (its cytoskeleton) and to bind to an F-actin affinity column. Thus, phosphorylation-dephosphorylation of ABP may be an important regulatory mechanism by which the platelet regulates its shape via its cytoskeletal structure.

The isolation of a platelet cytoskeleton composed of three proteins, actin binding protein (ABP), α-actinin and F-actin (1) and their subsequent purification (2,3) has made it possible to study their interactions and the regulation of these interactions in vitro. We have already reported that it is possible to recombine ABP and F-actin in vitro to form a structural complex that resembles the triton insoluble cytoskeleton (1) and which is sedimentable at low speed (<3000 rpm). Although it has been reported that both spectrin (4,5) and filamin (6) could exist as phosphoproteins, no correlation could be found between the state of phosphorylation of these proteins and their ability to interact with and/or cross link F-actin filaments. We had previously reported (1) that reformation of the ABP-F-actin complex in vitro, required that ABP be in the phosphorylated state and that actin be in the F-form. This requirement dictated that if the cytoskeleton is a pre-existing structure then ABP must exist as a phosphoprotein and F-actin must be the predominant form in the "resting platelet." In this study we present evidence that indeed, ABP does exist as a phosphoprotein and that removal of

* Visiting Assoc. Professor from Shanghai First Medical College, People's Republic of China
+ Present Address: Sidney Farber Cancer Inst., Boston, MA.
o To whom all correspondence should be addressed.

ABBREVIATIONS: ABP, actin binding protein; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide, gel electrophoresis; EDTA, Ethylene diamine tetraacetic acid; EGTA, Ethylene Glycol bis (2-Amino ethyl ether) N,N'-tetraacetic acid; PIPES, piperazine-N,N'-bis (2-ethanesulfonic acid).

0006-291X/84 $1.50

at least one-half of its phosphate results in its inability to cross link F-actin into a low speed sedimentable complex.

Materials and Methods

Platelet preparation and cytoskeleton isolation were carried out as described by Rosenberg, et. al. (1). ABP and actin were purified as described previously (1,3). The staining of phosphoproteins directly on SDS-gels was carried out by the procedure of Cutting and Roth (8) and the determination of the P_i content of ABP was by the method of Itaya and Ui, (9) as modified by Stull and Buss, (10).

The phosphoserine content of ABP was estimated by automatic amino acid analysis of the hydrolyzed protein using a Beckman Model 120C Analyzer.

Removal of P_i was carried out using E. coli Alkaline phosphatase (Sigma). ABP was incubated with or without the enzyme in 0.7M tris-HCl pH 8.0 for two hours at 37°C and then dialyzed overnight against Buffer A which consisted of 40 mM KCl, 10 mM PIPES, 1 mM EGTA and 1 mM NaN_3. Control or phosphatase-treated ABP was then incubated with actin for 15 minutes at room temperature and centrifuged at 5,000 x g. The resulting pellets and supernatants were separated and processed for SDS-polyacrylamide gel electrophoresis. The ABP:phosphatase ratio used in this experiment was 1:1 (w:w) and the phosphatase had 38 units of activity/mg. The control contained the same ratio of ABP:enzyme except that 10 mM EDTA was added to completely inactivate the phosphatase.

Preparation of Platelets: Human platelet concentrates, fresh from the Greater New York Blood Center, were freed from contaminating erythrocytes and leukocytes and sequentially washed according to the procedures described by Rosenberg et. al. (1,3).

Preparation of rabbit skeletal muscle actin: The muscle actin was isolated by the method as described by Spudich & Watt (11).

Preparation of F-actin-Sepharose 4B - The activation of Sepharose by cyanogen bromide was carried out under the conditions given by Blackburn et al. with the modification that 1 M Na_2CO_3 solution was used (12). The coupling of skeletal muscle F-actin (1.5 mg per g (wet weight) to the activated gel was carried out in borate buffer (0.8M, pH 9) for 24 hours with gentle mixing by inversion at 4°C. The resulting F-actin gel was further treated with 1 M ethanolamine in a similar manner for an additional 24 hrs.

SDS-polyacrylamide gel electrophoresis was carried out by the procedure used routinely in this laboratory and described by Rosenberg, et. al. (1,3). All reagents are the highest grade commercially available.

Results

A number of criteria were used to ascertain the state of phosphorylation in actin binding protein. As shown in Fig. 1 when the purified ABP is stained with malachite green, a band is obtained in the high molecular weight region corresponding to ABP. Treatment with E-coli alkaline phosphatase results in the loss of this band.

Further confirmation for the presence of phosphate in ABP was determined as seen in Table 1. By both amino acid analysis for phosphoserine and by quantitative micro analysis for P_i, approximately 4 residues of phosphate per mole of ABP can be demonstrated.

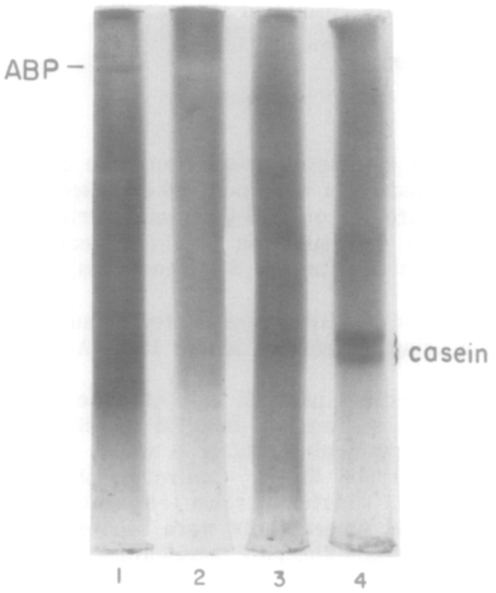

Figure 1. SDS-polyacrylamide gels stained with malachite green to detect phosphoproteins. Procedure as in Materials and Methods. Lane 1, ABP; Lane 2, ABP treated with E. Coli Alk. phosphatase; Lane 3, Bovine serum albumin control; Lane 4, casein as phosphoprotein control.

Interaction of Phosphorylated ABP with F-Actin.

Two criteria were used to ascertain the role of the phosphate residue on ABP in the interaction with F-actin. As seen in Fig. 2 when ABP isolated from unstimulated platelets was mixed with platelet F-actin a turbidity immediately appears and centrifugation at low speed (< 3000 rpm) results in a sedimentable pellet (Fig. 2, C) in which over 90% of the ABP-actin complex can be found in the pellet. After treatment of ABP with E-coli Alkaline Phosphatase (Fig. 2, A.P.) no low speed sedimentable complex is observed and most of the ABP and F-actin are found in the supernatant (see Fig. 2, A.P.). If this supernatant is spun at 100,000 x g for 3 hours, however, both ABP and F-actin are found in the pellet. ABP alone will not sediment under these conditions.

Since we had already shown that ABP and F-actin interact at low ionic strength and dissociate at high ionic strength (2,3) we studied the binding of ABP to an F-actin affinity column. As seen in Fig. 3A if ABP in 0.1 M KCl is added to the column it binds and can be eluted with a high salt buffer. The peak eluted after the application of the high salt buffer could be shown by SDS-PAGE to be ABP (see gel inset). The

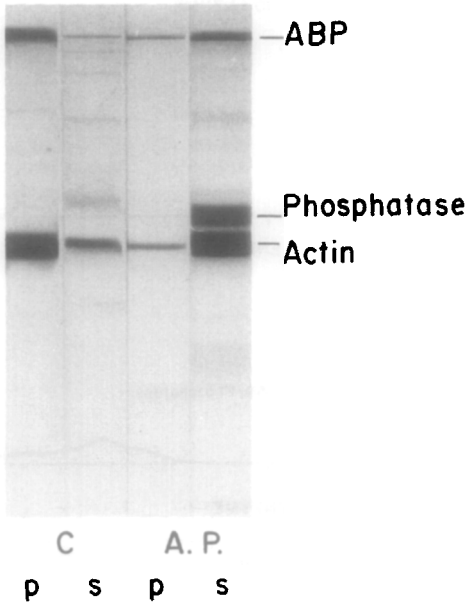

Figure 2. 5.5% SDS-polyacrylamide gels showing the effects of E. coli alkaline phosphatase on ABP binding to actin. ABP was incubated with (A. P.) or without (C) E. coli alkaline phosphatase as described in Methods. Control (C) or phosphatase-treated (A.P) ABP was then incubated with actin for 15 minutes at room temperature and centrifuged at 5,000 x g. The resulting pellets (P) and supernatants (S) were separated and processed for SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue.

peak at the void volume contains residual polyethylene glycol used in the initial purification of ABP from platelets (1). If, however, ABP treated witn E. coli alkaline phosphatase was added to the affinity column, it could be seen that ABP was not bound and eluted in the void peak, suggesting no interaction with F-actin occurred (see Fig. 3c). A control with inactive alkaline phosphatase (Fig. 3B) gave the same results as seen in Fig. 3A.

Discussion

It is clear from the results presented here that ABP exists in the resting platelet as a phosphoprotein and the stability of the platelet cytoskeletal structure is dependent upon the state of phosphorylation of ABP. Removal of at least one-half of the phosphate groups from ABP (see Table 1) results in its inability to organize F-actin into a highly cross-linked structure as evidenced from its lack of binding to an F-actin affinity column as well as the loss of its ability to form a low speed sedimentable complex.

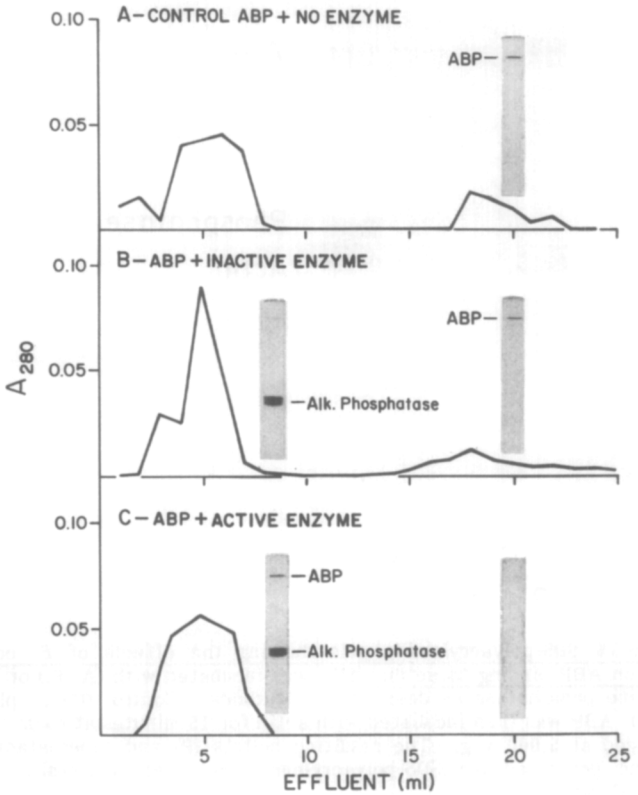

Figure 3. Chromatography of alkaline phosphatase treated and untreated ABP on F-actin-Sepharose 4B affinity column. The column was initially equilibrated and eluted with 0.1M KCl. At 12 ml the eluting buffer was changed to 0.6M KCl, 10 mM PIPES, pH 6.8. The inset gels represent the protein present in the peak tubes. Over 90% of the applied ABP could be recovered in either peak 1 or peak 2.

Whether the phosphorylation sites on ABP are equivalent, i.e. two per chain and whether both are essential remains to be determined. It would appear from these results that only one site per chain is essential.

TABLE 1
INORGANIC PHOSPHATE AND PHOSPHOSERINE CONTENT OF NATIVE AND TREATED ABP

Determination	NATIVE ABP	TREATED ABP*
	moles /mole ABP	
P_i (n=4)^o	3.96 ± 0.3	2.09 ± 0.2
Phosphoserine$^+$ (n=3)	4.21 ± 0.4	1.78 ± 0.2

* Treated for 3 hr. with E. Coli alk. phosphatase
\+ Determined by amino acid analysis
o Determined by the method of Stull and Buss (10)

The results reported here are in contrast to those reported by Brenner and Korn (4) and Pinder et. al. (5) who reported that phosphorylation of purified spectrin did not alter its ability to induce actin polymerization. Davies et al. (6) reported that extracts of fibroblasts incubated with ^{32}P-ATP showed an incorporation of ^{32}P into filamin. Furthermore, they were able to phosphorylate chicken gizzard filamin in vitro with skeletal muscle protein kinase and cAMP. There have been no reports, however, on the function of filamins' phosphorylation or on what effect, if any, phosphorylation has on filamin ability to bind to and cross link F-actin. At the present, no kinase has been isolated in which platelet ABP, once dephosphorylated could be re-phosphorylated nor has an endogenous phosphatase been found to dephosphorylate ABP. Carroll and Bernard (13) have shown that rapid dephosphorylation of ABP occurs in aggregating platelets in a 3-5 minute time course. These same authors have suggested that thrombin stimulation of platelets results in a 2-3 fold increase in ^{32}P incorporation into ABP although the significance of the levels of incorporation achieved remains to be determined. Our contention that ABP already exists as a phosphoprotein could explain the low levels of ^{32}P-incorporation in these studies.

References

1. Rosenberg, S., Stracher, A. and Lucas, R.C. (1981) J. Cell. Biol. 91: 201-211.
2. Rosenberg, S., Stracher, A. and Burridge, K. (1981) J. Biol. Chem. 256: 12986-12991.
3. Rosenberg, S., Lawrence, J. and Stracher, A. (1982) Cell Motility 4: 317-322.
4. Brenner, S.L. and Korn, E.D. (1979) J. Biol. Chem. 251: 8620-8627.
5. Pinder, J.C., Bray, D. and Gratzer, W.B. (1977) Nature, 270: 752-754.
6. Davies, P., Shizuta, Y., Olden, K., Gallo, M. and Pastan, I. (1977) Biochem, Biophys. Res. Comm. 74: 300-07.
7. Rosenberg, S., Stracher, A., Detwiler, T.C. and Lucas, R.C. (1978) Fed. Proc. 37: 1790.
8. Cutting, J.A. and Roth, T.F. (1973) Analyt. Biochem. 54: 386-394.
9. Itaya, K. and U.M. (1966) Clin. Chim. Acta 14: 361-366.
10. Stull, J.T. and Buss, J.E. (1977). J. Biol. Chem. 252: 851 857.
11. Spudich, J.A. and Watt, S. (1971). J. Biol. Chem. 246: 4866-4871.
12. Blackburn, P., Wilson, G. and Moore, S. (1977) J. Biol. Chem. 252: 5904-5910.
13. Carroll, R.C. and Gerrard, J.M. (1982) Blood 59: 466-471.