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Phosphorylation of Platelet Actin Binding Protein Protects Against Proteolysis by Calcium Dependent Sulfhydryl Protease *

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When Actin Binding Protein (ABP) isolated from human blood platelets is phosphorylated in vitro with a cyclic AMP dependent kinase it becomes resistant to proteolysis by the Calcium Dependent Sulfhydryl Protease (CDSP). This protection against proteolytic cleavage is specific for CDSP since phosphorylation of ABP does not protect against proteolysis by trypsin, papain and thermolysin. Thus, there appears to be a distinct phosphorylation site on the ABP molecule which is essential for regulating the initial proteolytic degradation of ABP by CDSP.

The interaction of actin binding protein (ABP) and F-actin is thought to be a critical factor in the organization of the cytoskeleton of human blood platelets (1). Because the cytoskeleton is intimately associated with the platelet plasma membrane the regulation of this interaction, as well, may be essential in determining the variety of shape changes and secretory processes observed upon stimulation of platelets with a number of different mitogenic agents. One such regulatory factor, phosphorylation, had previously been

ABBREVIATIONS

CDSP, Calcium Dependent Sulfhydryl Protease; ABP, Actin Binding Protein; EGTA, Ethylene Glycol bis (2-Amino ethyl ether) N,N'-tetraacetic acid; PEG-6000, Polyethylene glycol; TCA, Trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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reported from this laboratory to regulate the interaction of ABP with F-actin, *in vitro*, and the suggestion was made that the phosphorylation-dephosphorylation of ABP may control platelet shape changes *in situ* (2).

Another factor to be considered in the regulation of cytoskeletal formation and deformation is the susceptibility of ABP to proteolysis by a Ca^{++}-dependent sulfhydryl protease (CDSP). This protease has been described in platelets (3,4) as well as other tissues although its function remains obscure. Fox and Phillips (5) have reported that ABP is proteolyzed *in situ* upon platelet stimulation with thrombin presumably through the activation of CDSP. The amount of breakdown, detected in their study, however, was minimal and may be related to the difficulty in maintaining ABP completely free of its breakdown products.

During the course of our investigations on the state of ABP phosphorylation we observed an increased stability of ABP to proteolysis by CDSP subsequent to its phosphorylation by a cyclic-AMP dependent kinase. In this report we present the results of this study.

MATERIALS AND METHODS

Platelet preparation and cytoskeleton isolation were carried out as described by Rosenberg et. al (1). ABP was purified as described previously (1) and stored in assembly buffer consisting of 40 mKCl, 10mM PIPES, pH 6.8, 1 mM EGTA and 1mM NaN_3.

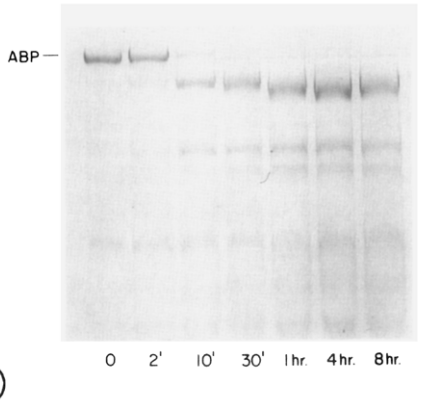
The purification of platelet CDSP to homogeneity in our laboratory involved several steps in the procedure (4). However, it was found sufficient for the purposes of this study to use the protease obtained after step III as described in reference 4. The protease was stored frozen in 25 mM Tris pH 7.4 and 1mM EGTA. Determination of CDSP activity using either ABP or ^{14}C-casein as substrate was carried out by the standard procedures of this laboratory (4).

Phosphorylation of ABP was carried out using approximately 0.5 mg cAMP-dependent kinase (bovine heart, Sigma, St. Louis) 1mM cAMP, 1 mM $MgCl_2$ 2mM ATP. The ATP used was either as the [^{32}P] labelled form or unlabelled. The incubation was allowed to proceed for 2 hours at 37°C after it had been previously determined that maximum incorporation of ^{32}P into ABP occurred after this period of time.

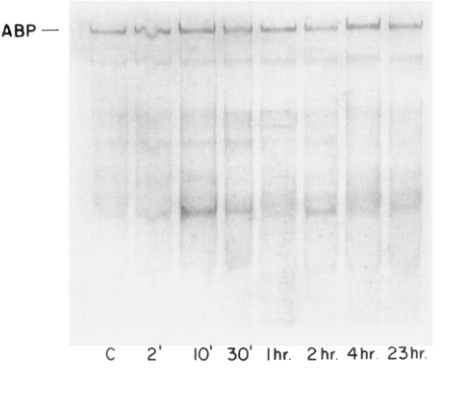
SDS-PAGE was carried out as described in previous publications (4).

RESULTS AND DISCUSSION

In a previous study (2) we had described that ABP as isolated is present as a phosphoprotein and that after removal of these phosphate groups with E.



(1)



(2)

Figure 1. Proteolysis of non phosphorylated ABP with CDSP. Sample of ABP (approx. 100 ug) in assembly buffer with the addition of 4 mM $CaCl_2$(final conc.). CDSP (approx. 10-20 ug) was added and proteolysis was allowed to proceed for the specified periods of time. Aliquots were removed, added to TCA first to stop the reaction and prepared for SDS-PAGE. Stained with Coomassie Blue.

Figure 2. Proteolysis of ABP which had been previously phosphorylated with cAMP dependent kinase. Procedure as in legend to Figure 1.

coli alkaline phosphatase ABP would no longer interact with actin. In this study we describe that further phosphorylation of ABP with a cAMP-dependent kinase, at a site presumably different from that previously described, protects against proteolysis by CDSP.

As seen in Fig. 1, ABP, as isolated from resting platelets, is ordinarily very susceptible to proteolysis by CDSP, whereas it can be seen in Fig. 2 that further phosphorylation of this ABP with c-AMP dependent kinase protects the molecule almost completely against subsequent degradation by CDSP for up to 23 hours. Addition of fresh CDSP at 4 and 8 hrs., as well, produced no additional breakdown of ABP (data not shown). In some experiments partial breakdown up to 30-40% could be achieved if the ratio of CDSP to ABP was increased. By determining the specific activity of the phosphorylated ABP it was possible to estimate that 2-4 P_i groups had been incorporated per 500 kDa. This is in addition to the 4 residues/mole found to be present in ABP as isolated (2). The quantitation of the data shown in Figs. 1 and 2 is depicted in a typical experiment as shown in Fig. 3. That this protection is specific to CDSP is shown in Fig. 4 where it can be seen that phosphorylation of ABP does not protect against other proteases such as trypsin, papain and thermolysin. This specificity of protection against CDSP has a number of

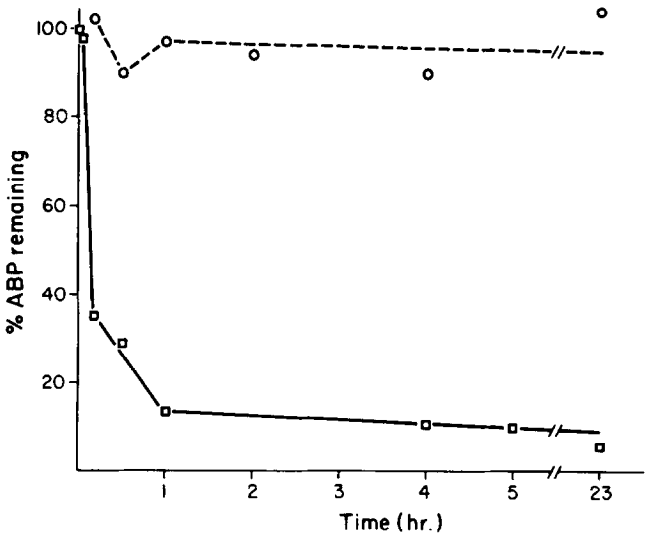


Figure 3. Comparison of rates of proteolysis of non-phosphorylated vs. phosphorylated ABP. Data from Figs. 1 and 2 were quantified by densitometric scans of the SDS gels.

important physiological implications not the least of which might be the stabilization of the cytoskeletal structure to degradation in situ. Since ABP has been reported to interact with glycoprotein Ib (6-8) in the plasma membrane as well as being associated with the cytoskeleton this stabilization of ABP to degradation may be important in maintaining cytoskeleton membrane interactions.

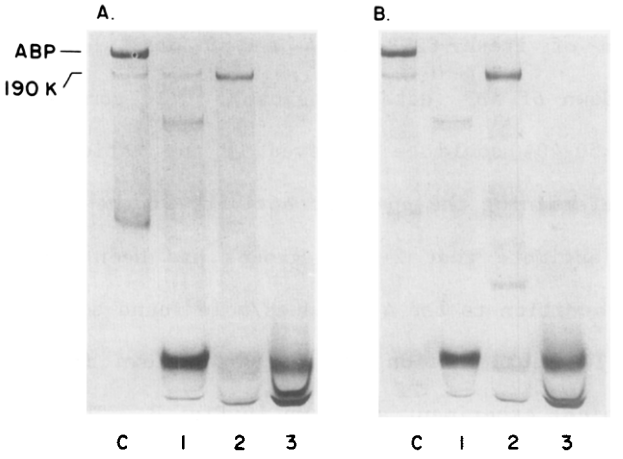


Figure 4. Proteolysis of phosphorylated and non-phosphorylated ABP with 1) papain 2) thermolysin and 3) trypsin. A) phosphorylated ABP B) non-phosphorylated ABP. Proteolysis was allowed to proceed at pH 7.5 for 30 min. at 25°C. Ratio of substrate to enzyme approximately 50:1 on a weight basis.

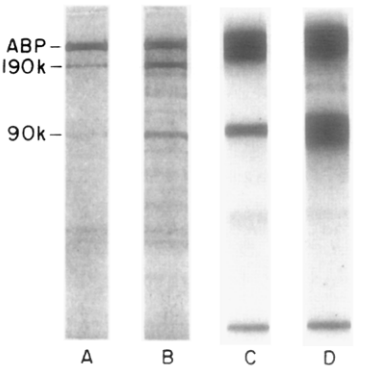


Figure 5. Proteolysis of ^{32}P-ABP with CDSP. A) control ^{32}P-ABP B) ^{32}P-ABP proteolyzed with CDSP. (A) and (B) were stained with Coomassie Blue. (C) autoradiogram of (A). (D) autoradiogram of (B). See legend to Fig. 1 for procedure.

Thus, from these and our previous studies there appear to be 2 regions of the ABP molecule which when fully phosphorylated manifest two potentially important characteristics, namely, interaction with F-actin and stabilization against proteolytic degradation.

When native ABP of 270 kDa is proteolyzed by CDSP two major fragments appear, one of approximately 190 kDa and a second of approximately 90 kDa (see Fig. 1). Because of the stabiliation effect produced by phosphorylation it was difficult to produce these proteolytic fragments of phosphorylated ABP using CDSP. Partial cleavage, however, could be achieved with several additions of CDSP and the results of such an experiment suggest that the phosphorylation site resulting in stabilization to proteolysis appears to be on the 90 kDa region of the molecule since the ^{32}P label after phosphorylation of ABP with cAMP dependent protein kinase and subsequent proteolysis remains exclusively with this fragment. This can be seen in Fig. 5 where all of the ^{32}P which has been incorporated into ABP by the use of cAMP kinase was accounted for in the 90 kDa fragment produced after partial proteolysis by CDSP. This could be further confirmed if native ABP were first completely proteolyzed to its 2 major fragments and the fragments treated with cAMP kinase. In this instance only the 90 kDa fragment became labelled with ^{32}P (data not shown). These results point to the phosphorylation occurring in a defined region of the molecule. Degradation of the 90 kDa fragment with

papain further places the phosphorylated residues in a 30 kDa area containing all of the original ^{32}P counts (unpublished observations). Studies on the sequence of the ^{32}P-containing site are continuing. It is tempting to speculate that it may be this fragment which is associated with glycoprotein Ib in the plasma membrane. At the present time the locale of the phosphorylated residues occurring in native ABP which appear to regulate its interaction with F-actin is not known. However, our previous study (4) had suggested that F-actin bound to the 190 kDa fragment after proteolysis suggesting these residues to be on this fragment. Further studies to localize the F-actin binding region of ABP are in progress.

Thus, phosphorylation-dephosphorylation of ABP in platelets may be a unique mechanism by which ABP can maintain its attachment to F-actin at one phosphorylation site and be stabilized to proteolysis by phosphorylation at a separate site on the molecule. ABP, therefore may play a central role in maintaining the organization of the cytoskeletion and its attachment to the membrane and may also act as a transducer of information from the extracellular receptor to the intracellular cytoskeleton.

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