Vol. 172, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1990 Pages 256-261

ENHANCEMENT OF GTP γS-BINDING ACTIVITY BY cAMP-DEPENDENT PHOSPHORYLATION OF A FILAMIN-LIKE 250 KDa MEMBRANE PROTEIN IN HUMAN PLATELETS

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Received September 3, 1990

Summary: The treatment of human platelets with the dibutyryl cyclic AMP (dbcAMP) revealed the presence of a 250 kDa protein which enhanced its GTP-binding activity. This protein was purified from platelet membranes by successive chromatographies on DEAE-cellulose, Ultrogel AcA34, Mono Q, HCA-hydroxyapatite, and TSK-3000SW columns. The positive cross-reaction of the 250 kDa protein with the anti-filamin antibody indicated that this protein is filamin or very close to it. The GTP γS-binding activity of this protein, when phosphorylated with cyclic AMP-dependent protein kinase (A-kinase), showed an over tenfold increase, with the specific activity being 3.6 nmol/mg protein. Dephosphorylation of the phosphorylated protein with alkaline phosphatase reduced the GTP γS-binding activity to the control untreated level.

It has been known that the treatment of human platelets with dbcAMP suppress platelet functions mediated by agonists such as thrombin (1,2). The mechanism underlying this suppression is poorly understood, but it is suggested that phosphorylation of the receptor by A-kinase activated by the cAMP-treatment decreases the affinity for thrombin, thereby inhibiting its coupling to GTP-binding protein (G-protein) associated-phospholipase C (PLC) (3).

We have previously demonstrated that the PLC activity of human platelet membranes was activated by GTP γS and also that such activation was diminished in either membranes prepared from dbcAMP-pretreated platelets (membranes A) or membranes prepared from untreated platelets and subsequently incubated with cAMP/ATP (membranes B) (4). It was then assumed that the inhibitory effect of cAMP was due to phosphorylation by A-kinase of G-protein, PLC, or other component(s). There were no changes in the basal PLC activity and the ADP-ribosylation by pertussis toxin in either membranes A or B. In the course of the study, we have observed a protein band with M.W. 250 kDa on SDS-PAGE whose

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Abbreviations: dbcAMP, dibutyryl cyclic AMP; ABP, actin-binding protein; A-kinase, cyclic AMP-dependent protein kinase; G-protein, GTP-binding protein; 8-N_3-[γ-^{32}P]GTP, 8-azido-[γ-^{32}P]GTP; App(NH)p, adenosine 5'-(β,γ-imido)-triphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; GP, glycoprotein.

0006-291X/90 $1.50

GTP-binding activity was remarkably enhanced by phosphorylation through activation of A-kinase.

In this communication, we have extended the previous work and purified a 250 kDa protein from the human platelet membrane for characterization, which was identified to be an actin-binding protein (ABP) filamin or very close to it.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was purchased from New England Nuclear, 8-N_3-[γ-^{32}P]GTP from ICN, purified bovine heart A-kinase, chicken gizzard filamin and alkaline phosphatase attached to beaded agarose from Sigma. App(NH)p and GTPγS from Boehringer Mannheim. Anti-filamin antibody was obtained from Seikagaku Kogyo. Anti-spectrin antibody was kindly supplied by Dr. Y. Yahata of Kawasaki Medical College. All other chemicals were of reagent grade.

Preparation of cytoskeletal proteins from the human platelet membrane fraction

Preparation of membrane fraction from human platelets was carried out as described previously (4-6). Outdated human platelet concentrate was centrifuged at 200 $\times$g for 5 min to remove contaminating red blood cells. Platelets were then collected and washed twice with Tris-bicarbonate buffer, pH 6.8, containing 5 mM EGTA and 10 mM EDTA by centrifugation at 2,000 $\times$g for 15 min. The washed cells were resuspended in 25 mM Tris-HCl buffer, pH 7.5, containing 5 mM EGTA, 5 mM EDTA and 1 mM phenylmethylsulfonylfluoride (PMSF) and were homogenized in a teflon-glass homogenizer. After centrifugation at 20,000 $\times$g for 25 min at 4 ℃, the pellet was suspended in Buffer A (2 mM Tris-HCl, 5 mM EGTA, 0.5 mM PMSF, pH 9.0) at room temperature. The suspension was incubated to extract cytoskeleton-rich fraction by continuous stirring for 30 min at 37 ℃. The suspension was centrifuged at 15,000 $\times$ g for 15 min and the final supernatant was stored at -80 ℃.

Purification of a 250 kDa protein from cytoskeletal fraction

All procedures were carried out at 0-4 ℃ unless otherwise indicated. The supernatant (250 ml) obtained above was applied to a column of DEAE-cellulose (1.6 $\times$8.0 cm) pre-equilibrated with 20 mM Tris-acetate buffer, pH 7.6, containing 10 mM NaCl, 0.1 mM EDTA, and 0.1 % 2-mercaptoethanol (Buffer B). The column was first washed with 250 ml of Buffer B and then eluted with 50 ml of a linear NaCl gradient (10-500 mM) in the same buffer. After five chromatographic steps using DEAE-cellulose, Ultrogel AcA34, Mono Q (I), HCA-hydroxyapatite, TSK-3000SW columns applied to a column of Mono Q (II) (HR 5/5) which had been equilibrated with Buffer A. The column was washed and eluted at a flow rate of 1 ml/min with the following series of NaCl gradients using a Pharmacia FPLC system; 10 mM for 2 min; 10-250 mM for 1 min; 250-350 mM for 8 min; 350-1000 mM for 1 min; 1000 mM for 2 min. The eluate was collected in fractions of 0.2 ml. In the purification steps, each fraction was treated with exogeneous A-kinase and then subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7) and autoradiographed.

Phosphorylation and dephosphorylation of 250 kDa protein

Phosphorylation of a 250 kDa protein by A-kinase was assayed essentially as described previously (8). The reaction mixture (final volume, 200 μl) contained 25 mM Mops buffer, pH 7.5, 5 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP, 1 μM cAMP and 10 μg of A-kinase purified from bovine heart. Dephosphorylation of phosphorylated 250 kDa protein by A-kinase was performed by mixing with alkaline phosphatase attached to beaded agarose (Sigma). The reaction mixture (final volume, 1 ml) contained 50 mM Mops buffer, pH 9.5, 1 mM $MgCl_2$, phosphorylated 250 kDa protein (50 μg) and alkaline phosphatase attached to beaded agarose (0.2 ml). The reactions were run at 37℃ for 30 min and stopped by centrifuging the mixture at 4℃ for 5 min after neutralization. The phosphorylated or dephosphorylated proteins were analyzed by SDS-PAGE.

Assays of GTPγS-binding activity and photoaffinity labelling with 8-azido-[γ-^{32}P]guanosine 5'-triphosphate

[^{35}S]GTPγS-binding activity was assayed according to the method described earlier (9). Photoaffinity labelling was carried out essentially as described previously (4,10). The standard reaction mixture contained 20 mM Mops buffer,

pH 7.5, 1 mM EDTA, 100 mM NaCl, 25 mM $MgCl_2$, 1 μM 8-N_3-[γ-^{32}P]GTP and protein (5 μg) in the presence or absence of 1 mM unlabelled GTP. The mixture was incubated in the dark for 10 min at 4°C and then irradiated for 10 min with a UV lamp (254 nm) from a distance of 1 cm. The labelled protein was analyzed by SDS-PAGE and visualized by autoradiography.

Immunoblot analysis

Immunoblot analysis was performed essentially as described previously (11). The purified 250 kDa protein (approximately 7 μg of protein each) was analyzed by 8-16 % SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. Protein transfered to the membrane was immunostained.

Other methods

Protein was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

In the present investigation, we have extended the previous study describing the presence of a 240-250 kDa protein whose GTP-binding activity was increased by cAMP, probably via A-kinase (4), and have attempted to purify and identify the platelet membrane protein. Since the 250 kDa protein was suggested to be a cytoskeletal protein based on molecular weight, abundant presence in cytoskeletal fraction and phosphorylation by A-kinase. A modified method of O'Halloran et al (5, 6) was employed for extraction of the cytoskeletal fraction. Its crude extract was purified by successive chromatographies on DEAE-cellulose, Ultrogel AcA34, Mono Q (I), HCA-hydroxyapatite, TSK-3000SW, and Mono Q (II) columns. The fractions obtained at each purification step were incubated with A-kinase (bovine heart; Sigma) in the presence of [γ-^{32}P]ATP and subjected to SDS-PAGE and autoradiographed. Fig. 1 shows an elution profile of the Mono Q (II) at the final purification step. The peak fraction gave a

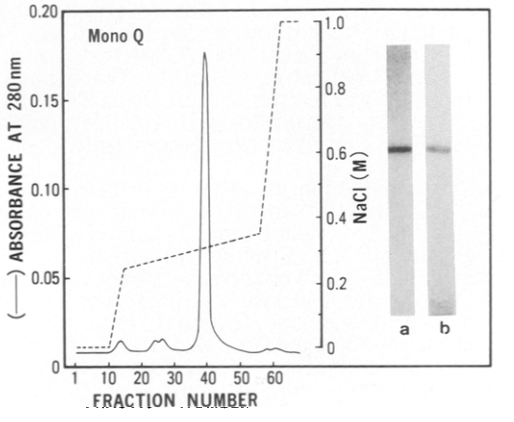

Fig. 1. Mono Q (II) chromatography and SDS-polyacrylamide gel electrophoresis of 250 kDa protein purified from human platelet membranes
250 kDa protein-rich fraction was applied to Mono Q (II) column and eluted with NaCl linear gradients. Purified 250 kDa protein was phosphorylated by A-kinase in the presence of cAMP, [γ-^{32}P]ATP and $MgCl_2$. Samples (6 μg protein) were subjected to gradient (8-16 %) SDS-PAGE. Lane a, Coomassie blue staining; lane b, autoradiograph at -80°C for 24 h using X-Omat film.

single band with an apparent molecular mass of 250 kDa as examined by SDS-PAGE. The same band was phosphorylated by incubation with exogenous A-kinase in the presence of [γ -^{32}P]ATP.

For further characterization, the purified 250 kDa protein was analyzed by immunoblotting and was found to cross-react with the anti-filamin antibody but not with the anti-spectrin antibody, as shown in Fig. 2. It was furthermore observed that this protein formed a complex with actin purified from rabbit muscle (data not shown). These results strongly suggested that the 250 kDa protein was filamin or filamin-like actin-binding protein (ABP) (12).

We have then examined using a photosensitive GTP analogue whether or not the GTP-binding activity of the purified protein was changed by phosphorylation with A-kinase (Fig. 3). The control unphosphorylated 250 kDa protein showed little or no significant binding activity for 8-N_3-[γ -^{32}P]GTP (lane 1), while the phosphorylated protein revealed a profound increase in the binding activity (lane 3). However, dephosphorylation of the phosphorylated protein with alkaline phosphatase resulted in a loss of the GTP-binding activity (lane 4). These results demonstrated that the GTP-binding activity of this protein was

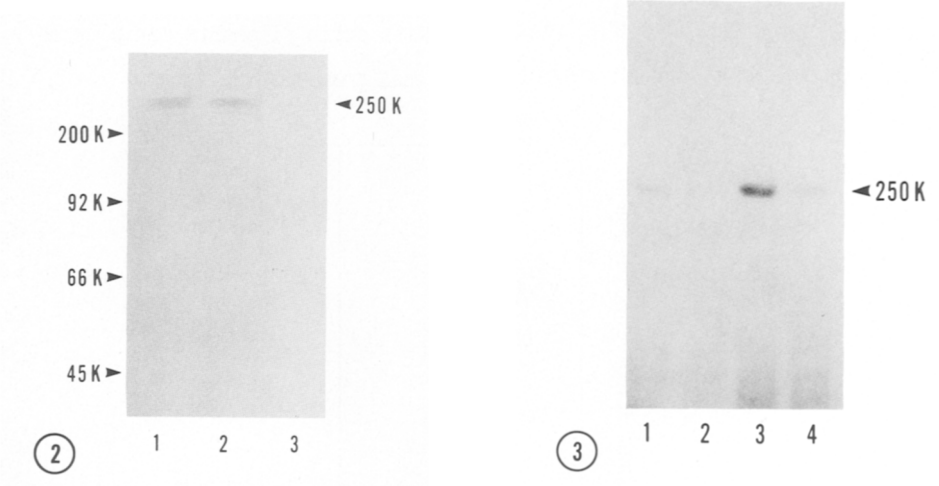

Fig. 2. Immunoblot analysis of 250 kDa protein
Samples (7 μg of protein) were analyzed by 8-16 % SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. Lane 1, commercially available filamin transfered to the membrane and immunostained with anti-filamin antibody;lane 2, purified 250 kDa protein transfered to the membrane and immunostained with anti-filamin antibody; lane 3, purified 250 kDa protein transfered to the membrane and immunostained with anti-spectrin antibody.

Fig. 3. Photoaffinity labelling with 8-N_3-[γ -^{32}P]GTP
Photoaffinity labelling was performed as described in Meterials and Methods. Samples are as follows: lane 1, purified 250 kDa protein incubated with 8-N_3-[γ -^{32}P]GTP in the dark and irradiated with UV; lane 2, A-kinase-phosphorylated 250 kDa protein incubated with 8-N_3-[γ -^{32}P]GTP in the dark; lane 3, A-kinase-phosphorylated 250 kDa protein incubated with 8-N_3-[γ -^{32}P]GTP in the dark and irradiated with UV; lane 4, dephosphorylated 250 kDa protein with alkaline phosphatase, incubated with 8-N_3-[γ -^{32}P]GTP in the dark and irradiated with UV.

increased when phosphorylated with A-kinase and was reduced to the initial level before phosphorylation when dephosphorylated. As indicated in Fig. 4, the specific [^{35}S]GTPγS-binding activities for the control and the phosphorylated 250 kDa protein were 0.32 nmol/mg of protein and 3.4 nmol/mg of protein, respectively. Upon dephosphorylation with alkaline phosphatase, its binding activity was decreased nearly to the control level (0.45 nmol/mg of protein). The binding of [^{35}S]GTPγS was specifically inhibited with 0.1-1.0 mM of GTP or GDP, but not with ATP nor App(NH)p.

Although we are unable to correlate directly the modification in GTPγS-binding by phosphorylation-dephosphorylation with the regulation of PLC activity, some recent reports suggest that ABP and other cytoskeletal proteins are involved not merely in the maintenance of cellular structure but in intracellular signal transduction system. The ABPs such as profilin (13), gelsolin (14) and α-actinin (15) have been demonstrated to interact with phosphoinositides. Recently, Goldschmidt-Clermont *et al.* (16) have shown evidence that profilin competes with platelet cytosolic PLC for interaction with phosphatidylinositol 4,5-bisphosphate (PIP_2) and thereby inhibits PIP_2 hydrolysis by this enzyme. Furthermore, some glycoproteins (GPs) of platelet membranes have been reported to link to actin filaments (17,18). Nakano *et al.* (19) have suggested that the linkage of GPIa with actin filaments may play an important role in collagen receptor-mediated signal transduction system.

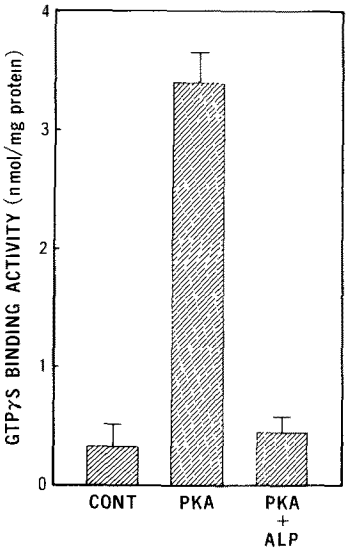

Fig. 4. Effect of phosphorylation or dephosphorylation on the [^{35}S]GTPγS-binding of the purified 250 kDa protein
Column 1, purified intact 250 kDa protein; column 2, phosphorylated 250 kDa protein by A-kinase; column 3, dephosphorylated 250 kDa protein by alkaline phosphatase. The results shown are representatives of three independent experiments.

Although it has been reported that increased levels of cAMP in platelets are associated with its reduced ability to bind and respond to thrombin (1-3), our findings suggested that some other site(s) in the signal transduction may be affected by cAMP. It is tempting to speculate that binding of GTP to G-protein may be competitively suppressed by the increased GTP-binding activity of the 250 kDa protein upon phosphorylation by A-kinase. It is also conceivable that the phosphorylated 250 kDa protein directly would interact with one or more of three principal components; receptor, G-protein and PLC or some other as-yet unidentified component(s) present in membranes, or that the phosphorylated 250 kDa protein would somehow inhibit coupling of G-protein to PLC. It is presumed that a group of cytoskeletal proteins form a direct or indirect network with the cytoplasmic components or integral components involved in signal transduction system.

Further experiments including the reconstitution system should be performed to elucidate the function of the 250 kDa protein in platelet activation.

ACKNOWLEDGMENT

This study was supported in part by the grant from Ministry of Education, Science and Culture, Japan.

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