A rho Gene Product in Human Blood Platelets

II. EFFECTS OF THE ADP-RIBOSYLATION BY BOTULINUM C3 ADP-RIBOSYLTRANSFERASE ON PLATELET AGGREGATION*

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Narito Morii, Toshiyuki Teru-uchi, Tomoko Tominaga, Naokazu Kumagai, Shunji Kozaki†, Fumitaka Ushikubi, and Shuh Narumiya

From the Department of Pharmacology, Kyoto University Faculty of Medicine, Sakyoku, Kyoto 606 and the †Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan

In the accompanying paper (Nemoto, Y., Namba, T., Teru-uchi, T., Ushikubi, F., Morii, N., and Narumiya, S. (1992) J. Biol. Chem. 267, 20916–20920), we have identified rhoA protein as the sole substrate protein for botulinum C3 ADP-ribosyltransferase (C3 exoenzyme) in human blood platelets. Here we examined the role of rhoA protein in platelet functions. C3 exoenzyme added to washed platelets dose- and time-dependently ADP-ribosylated rhoA protein in situ in the cells. Concomitant with this modification, inhibition of thrombin-induced platelet aggregation was observed. This inhibition was not reversed by washing the treated platelets, but was not found when C3 exoenzyme was pretreated with mouse monoclonal anti-C3 exoenzyme antibody. C3 exoenzyme treatment did not affect thrombin-induced inositol 1,4,5-trisphosphate production. Secretion of preloaded [14C]serotonin was not influenced. In addition, the enzyme treatment also suppressed platelet aggregation induced by phorbol myristate acetate. These results suggest that rhoA protein plays a role mainly in the aggregation process downstream from receptor-phospholipase C coupling. This, together with the previous finding that rhoA protein modulates stress fiber formation in cultured fibroblasts (Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) J. Cell Biol. 111, 1001–1007), suggests that rhoA protein regulates the assembly of actin filaments and the avidity of the platelet integrin (glycoprotein IIb-IIIa) complex on the platelet surface. The enzyme treatment also suppressed platelet aggregation induced by phorbol myristate acetate. These results suggest that rhoA protein plays a role mainly in the aggregation process downstream from receptor-phospholipase C coupling. This, together with the previous finding that rhoA protein modulates stress fiber formation in cultured fibroblasts (Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) J. Cell Biol. 111, 1001–1007), suggests that rhoA protein regulates the assembly of actin filaments and the avidity of the platelet integrin (glycoprotein IIb-IIIa) complex in the aggregating process.

rhot gene products (rho proteins) are low molecular weight GTP-binding proteins sharing 30–40% homology with ras p21 and constitute a subfamily in the ras-related superfamily of proteins (1, 2). They are distinguished from other low molecular weight GTP-binding proteins in that they can serve as substrates for botulinum C3 ADP-ribosyltransferase (C3 exoenzyme) (3–5). C3 exoenzyme is a 24-kDa exoenzyme produced by Clostridium botulinum types C and D (6, 7) and ADP-ribosylates a specific asparagine residue located in the putative effector domain of these proteins (8). This ADP-ribosylation, which neither inhibits the activities of GTP binding and hydrolysis nor affects their interaction with rho-specific GTPase-activating protein (8, 9), interferes with their biological activity possibly by affecting their interaction with unknown effector molecules. C3 exoenzyme, when incubated with or microinjected into cultured cells, ADP-ribosylates a rho protein in the cells and causes morphological changes. A typical change is rounding up of cell bodies with disassembly of actin filaments (10, 11). On the other hand, microinjection of activated rhoA mutant protein (Val14-rhoA) into fibroblasts enhances actin filament organization and induces finger-like cell processes (12). Because actin filaments are linked to adhesion plaques as stress fibers and change in cell shape is dependent on the organization of stress fiber and cell adhesion to the substratum (13), we have hypothesized that rho proteins are involved in the organization of an adhesion plaque and actin filament attachment to the plaque. Platelets appear to be a good system to test this hypothesis because they respond to various stimuli to undergo shape change, secretion, and adhesion. These processes involve various cytoskeletal organizations, and adhesion in these cells is elicited as aggregation, which is caused by the assembly of actin filaments and the activation of the platelet integrin (GPIIb-IIIa) (14–17). As reported in the accompanying paper (18), platelets contain high levels of rhoA protein as the ADP-ribosylation substrate for C3 exoenzyme. In this study, we examined the effects of its ADP-ribosylation by C3 exoenzyme on the secretion and aggregation of platelets and the expression of GPIIb-IIIa at the cell surface.

EXPERIMENTAL PROCEDURES

Materials—C3 exoenzyme was purified from the culture filtrate of C. botulinum strain 003, and mouse monoclonal anti-C3 exoenzyme antibody (IgG1 subclass) was prepared as previously reported (19). [1-14C]NAD (800 Ci/mmol) was obtained from Du Pont-New England Nuclear. 5-[side chain-2-14C]Hydroxy-tryptamine ([14C]serotonin) (55 mCi/mmol) was obtained from Amersham Corp. Thrombin and FMA were from Sigma, and mouse IgG was from ICN Immunobiologicals. Mouse monoclonal anti-human GPIIb-IIIa antibody P2 (IgG1 subclass) was from Immunotech S. A. (Marseille, France). The fluorescein isothiocyanate-conjugated F(ab')2 fragment of goat anti-mouse IgG antibody was from Cappel Research Products. Other chemicals used were of reagent grade.

Preparation of Washed Platelets—Washed human platelets were obtained from healthy volunteers and prepared as described (20). In

1 The abbreviations used are: GP, glycoprotein; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP, inositol 1,4,5-trisphosphate; FMA, phorbol myristate acetate; TBS, Tris-buffered saline.
standard procedures, 27 ml of venous blood was mixed with 0.1 volume of citrate/glucose buffer (100 mM sodium citrate, pH 6.5, and 140 mM glucose) and centrifuged at 165 × g for 15 min at room temperature. Platelet-rich plasma (8 ml) was then mixed with 0.5 volume of the same buffer and centrifuged at 750 × g for 10 min at 4°C. The platelet pellet was suspended by gentle pipetting in 6 ml of citrate-buffered saline (136 mM sodium chloride, pH 6.5, 10 mM sodium citrate, and 30 mM glucose) and left on ice for 1 h. The platelet suspension was then centrifuged and washed once with 2 ml of the above saline. Platelets were finally suspended in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and kept on ice until use.

C3 Exoenzyme Treatment and Aggregation—Washed platelets (2 × 10^9/200 µl) were supplemented with 5 mM MgCl₂ and 5 mM CaCl₂ and incubated with citrate-buffered saline containing 10 mM EGTA, and centrifuged at 4°C for 10,000 rpm for 1 h at 16°C. With or without various concentrations of C3 exoenzyme. At the indicated times, either thrombin or PMA was added, and aggregation was measured by light transmission.

Serotonin Secretion—Platelets were preloaded with [³⁵S]serotonin according to the method of Weiss et al. (21). Four ml of platelet-rich plasma was mixed with 0.46 µCi of [³⁵S]serotonin and incubated for 30 min at 37°C. After incubation, washed platelets were prepared as described above. By this procedure, 80% of the total [³⁵S]serotonin added was recovered in the washed platelets. Platelets (4.5 × 10^9/300 µl) were incubated with or without 15 µg of C3 exoenzyme at 37°C for 1.5 h as described above. Leakage or spontaneous release of serotonin during this incubation was <6% of the total [³⁵S]serotonin in the platelets. Thrombin (0.5 unit/ml) was then added, and aggregation was monitored on the Hematracer. Thirty-µl aliquots of the suspension were taken at the indicated times, immediately mixed with 30 µl of citrate-buffered saline containing 10 mM EGTA, and centrifuged at 4°C for 10,000 × g for 9 s. Thirty-µl supernatants were taken, and the [³⁵S]serotonin released was measured in a Triton/toluene scintillator by a Packard Minaxi Model 4530 liquid scintillation system.

IP₃ Production—Platelets (3 × 10^9/500 µl) were incubated with or without 50 µg/ml C3 exoenzyme for 1 h as described above, and 50-µl aliquots of these suspensions were then taken and used in each measurement. These aliquots were incubated with 2 units/ml thrombin at 37°C. At the indicated times, the reaction was terminated by the addition of 16 µl of 10% perchloric acid. IP₃ content in the acid supernatants was measured by the IP₃ assay system (Du Pont-New England Nuclear) according to the manufacturer's protocol using half of the recommended volumes. In our hands, 0.1–10 pmol of IP₃/tube was detected with reproducible results.

ADP-ribosylation of Platelet Homogenates—Washed platelets were incubated with or without C3 exoenzyme as described above. At the indicated times, 50-µl aliquots containing 1.5 × 10^8 platelets were taken and chilled on ice. An equivalent amount of C3 exoenzyme was then added to the aliquots of control platelets, and platelets were homogenized on ice. The presence of 0.4 mM phenylmethylsulfonyl fluoride, 5 mM EGTA, 5 mM leupeptin, 5 µM pepstatin, and 2 mM benzamidine on ice by sonication for 5 s, three times with 5-s intervals in a Tomy Model UR-20P microsonicator. The homogenates were immediately subjected to the in vitro ADP-ribosylation reaction using [³²P]NAD (specific activity of 2500 cpm/pmol) as described previously (19). The protein amount in the homogenate was determined by the method of Bradford (22) using bovine serum albumin as a standard.

Flow Cytometry—Washed platelets (1 × 10^9/100 µl of TBS) were incubated with or without 5 µg of C3 exoenzyme for 1 h as described above. Twenty-five µl of the suspension was then mixed with 3 volumes of phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.2) containing 4 µg of mouse monoclonal anti-OPHB-III antibody and 0.45% bovine serum albumin. The mixture was incubated for 1 h at 4°C. The platelets were then washed twice with 400 µl of ice-cold Hanks' balanced salt solution containing 0.75% bovine serum albumin and 0.05% sodium azide (Hanks' balanced salt solution/bovine serum albumin), resuspended in 100 µl of the same solution, and incubated with 1 µg of fluorescein isothiocyanate-conjugated anti-mouse IgG F(ab′)₂ fragment for 30 min on ice. The platelets were washed again three times with 160 µl of Hanks' balanced salt solution/bovine serum albumin, suspended in 1 ml of the same solution, and subjected to flow cytometric analysis on Cytoror (Ortho Diagnostic System Co., Tokyo).

ADP-ribosylation of rhoA Protein in Platelets and Inhibition of Aggregation by C3 Exoenzyme—Platelets were incubated with or without various concentrations of C3 exoenzyme for various lengths of time. Homogenates were made after incubation and then were incubated with [³²P]NAD and C3 exoenzyme to determine the amount of the ADP-ribosylation substrate remaining in these platelets. As shown in Fig. 1A, the ADP-ribosylation substrate in control platelets did not change significantly in amount during 3 h of incubation. On the other hand, that in platelets treated with C3 exoenzyme decreased significantly with incubation time; the decreases were 7, 27, and 42% with 1, 2, and 3 h of incubation, respectively. As shown in Fig. 1B, this decrease in the amount of the ADP-ribosylation substrate was dependent on the amount of C3 exoenzyme used in the treatment; the decreases were 8, 12, and 21% in platelets treated with 50, 75, and 150 µg/ml C3 exoenzyme for 1 h, respectively. The observed decrease was not due to ADP-ribosylation during homogenate prepa-
ration because we added, before homogenization, the same amount of C3 exoenzyme to control platelets and did not see any decrease in the substrate amount in these platelets. These results suggest that rhoA protein in platelets had undergone ADP-ribosylation in situ during the C3 exoenzyme treatment and became unable to be modified further in vitro.

Concomitant with this ADP-ribosylation, thrombin-induced platelet aggregation was inhibited by the C3 exoenzyme treatment. This inhibition occurred in a time- and dose-dependent manner (Fig. 2, A and B). When platelets were incubated with 10 μg of C3 exoenzyme, inhibition became apparent after 1 h and was nearly complete at 2 h. When incubation was carried out with various amounts of C3 exoenzyme for 2 h, inhibition was observed on treatment with 2.5 μg of enzyme and was nearly complete at 10 μg. This inhibition caused by C3 exoenzyme was not reversed by washing treated platelets free of the enzyme, as shown in Fig. 2C. Inhibition by C3 exoenzyme was consistently observed, although the extent of inhibition varied with various batches of platelet suspensions. This depends largely on reactivity of incubated platelets to aggregation stimuli. When treated platelets responded well to thrombin, inhibition by C3 enzyme treatment was overcome by the addition of higher doses of thrombin and on prolonged incubation (Fig. 2D).

To confirm that this inhibition is due to C3 exoenzyme itself and not to any contaminants in the enzyme preparation, we incubated the enzyme with mouse monoclonal anti-C3 exoenzyme antibody and added the mixture to washed platelets. A control experiment was carried out using commercially available mouse IgG. As shown in Fig. 3, C3 exoenzyme elicited the inhibition of platelet aggregation in the presence of mouse IgG, whereas the addition of anti-C3 exoenzyme antibody abolished the inhibition of platelet aggregation by C3 exoenzyme.

Effects of C3 Exoenzyme on Thrombin-induced IP₃ Production, [³⁹⁵C]Serotonin Secretion, and PMA-stimulated Platelet

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**Fig. 2.** Inhibition of platelet aggregation by C3 exoenzyme treatment. **A,** time course. Platelets were incubated with 10 μg of C3 exoenzyme for 30, 60, and 120 min, and platelet aggregation induced by thrombin was determined as described under "Experimental Procedures." Typical traces are shown, and the addition of thrombin (0.5 unit/ml) is indicated (V). The second addition of thrombin (2.5 unit/ml) is indicated (V) in platelets treated for 2 h. **B,** dose dependence. Washed platelets were incubated with various amounts of C3 exoenzyme for 2 h, and aggregation induced by 0.5 unit/ml thrombin was determined as described under "Experimental Procedures." Typical traces are shown, and the added amounts of C3 exoenzyme are indicated. **C,** effect of washing. Platelets were incubated with or without 10 μg of C3 exoenzyme in 200 μl of TBS for 2 h. After incubation, the platelets were mixed with 400 μl of ice-cold TBS containing 10 mM EGTA and centrifuged at 750 x g for 10 min at 4°C. The platelet pellets were washed again with 400 μl of the same buffer and resuspended in 200 μl of TBS containing 5 mM MgCl₂ and 5 mM CaCl₂. Platelets treated with C3 exoenzyme at the same time and without washing were used as a positive control. Aggregation was induced with 0.5 unit/ml thrombin as indicated (V). The upper, middle, and lower traces show aggregation of control platelets and of platelets treated with C3 exoenzyme before and after washing, respectively. **D,** antagonism by higher doses of thrombin. Platelets were incubated with and without 5 μg of C3 exoenzyme in 200 μl of TBS for 2 h, and aggregation was induced by the successive addition of 0.2 (V) and 1.0 (V) unit/ml thrombin. In this experiment, control platelets showed full aggregation. C3 exoenzyme-treated platelets, which showed ~40% inhibition on the first challenge, aggregated to the same extent as control platelets on prolonged incubation after the second addition.
approximately 80% of the total [14C]serotonin was released of released [14C]serotonin in the medium gradually decreased
Aggregation—To locate the signaling step affected by C3 exoenzyme treatment, we first examined its effect on thrombin-induced IP$_3$ production. Washed platelets were incubated with or without C3 exoenzyme for 1 h. Aliquots were examined for aggregation by 0.5 unit/ml thrombin (A) and for IP$_3$ production by 2 unit/ml thrombin (B). All procedures were carried out as described under “Experimental Procedures.” Typical traces are shown in A, and the addition of thrombin is indicated (V). Time courses of IP$_3$ production in control (O) and C3 exoenzyme-treated (●) platelets are shown.
Aggregation—To locate the signaling step affected by C3 exoenzyme treatment, we first examined its effect on thrombin-induced IP$_3$ production. Thrombin induced a rapid and transient increase in IP$_3$ levels in both control and C3 exoenzyme-treated platelets (Fig. 4). Whereas platelet aggregation examined at the same time was significantly inhibited in the C3 exoenzyme-treated platelets (Fig. 4A), there were no appreciable differences between the control and C3 exoenzyme-treated platelets in the basal and peak amounts of IP$_3$ as well as the time course of its production (Fig. 4B); the maximal values were 5.1 and 4.4 pmol/3 × 10$^8$ platelets, respectively, which were attained 10 s after thrombin stimulation in both platelet preparations. The IP$_3$ level decreased rapidly thereafter; and after 30 s, the levels returned to those before stimulation.
We next examined the effects of C3 exoenzyme treatment on platelet secretion (Fig. 5). This was evaluated by thrombin-induced secretion of [14C]serotonin preloaded into platelets. [14C]Serotonin secretion was rapid in control platelets. Approximately 80% of the total [14C]serotonin was released within 2 min after the addition of thrombin, and the amount of released [14C]serotonin in the medium gradually decreased thereafter presumably by re-uptake of released serotonin (Fig. 5B). In C3 exoenzyme-treated platelets, the secretion of [14C] serotonin was slower; the half-maximal release was observed at 1 min in the C3 exoenzyme-treated platelets, whereas it occurred within 30 s in the control platelets. The amount of released [14C] serotonin increased until 7.5 min, and >70% of the total [14C] serotonin was released as the maximal response in C3 exoenzyme-treated platelets. This value was comparable to that of the control platelets.
The above results suggest that C3 exoenzyme treatment preferentially affects the aggregation mechanism downstream from agonist-mediated phosphatidylinositol turnover. To confirm this, we examined the effect of C3 exoenzyme on PMA-induced platelet aggregation. As shown in Fig. 6, the aggregation of control platelets induced by 1 μM PMA proceeded much slower, but eventually led to almost the same extent as aggregation caused by 0.5 unit/ml thrombin. Incubation with C3 exoenzyme significantly suppressed this aggregation, as observed in thrombin-induced aggregation.
Effect of C3 Exoenzyme Treatment on Expression of GPIIb-IIIa Complex on Platelets—To examine the possibility that C3 exoenzyme treatment alters expression of the GPIIb-IIIa on the platelet surface, we carried out flow cytometry using a specific anti-GPIIb-IIIa antibody. As shown in Fig. 7, control and C3 exoenzyme-treated platelets yielded essentially identical histograms of specifically fluorescent labeled cells. This suggests that C3 exoenzyme treatment did not alter the amount of GPIIb-IIIa expressed on the platelet surface.
conjugated goat anti-mouse IgG as described under "Experimental Procedures." Fluorescence intensity is expressed on a log scale. A, control platelets treated without anti-GPIIb-IIIa antibody; B, control platelets; C, C3 exoenzyme-treated platelets. Mean fluorescence intensities from control and C3 exoenzyme-treated platelets were 154.8 and 146.6, respectively.

**DISCUSSION**

In this study, we showed that C3 exoenzyme added to a platelet suspension caused in situ ADP-ribosylation of the substrate in the cells and inhibited platelet aggregation stimulated by thrombin and PMA. The two events correlated well in their time course and dependence on the amount of C3 exoenzyme. Inhibition of platelet aggregation took >30 min to occur and was not reversed by washing the treated platelets. The inhibitory effect of the enzyme was abolished by incubation of C3 exoenzyme with monoclonal anti-C3 exoenzyme antibody. The platelet substrate for this ADP-ribosylation is most probably rhoA protein because we identified it as the sole C3 exoenzyme substrate in human platelets (18). These results suggest that rhoA protein is involved in the aggregation reaction and that C3 exoenzyme affects aggregation via its ADP-ribosylation.

On treatment with 50 μg/ml C3 exoenzyme for 2 h, in situ ADP-ribosylation composed only ~25% of the total platelet substrate and was relatively low when compared to other systems (11, 23). Nonetheless, this treatment blocked aggregation almost completely. One question is how this small percentage of ADP-ribosylated protein exerts such inhibition. Low molecular weight GTP-binding proteins such as rho proteins are presumed to serve as molecular switches in various cellular responses. Upon cell stimulation, they are converted to the active GTP-bound form and convey the respective signal. Not all of the molecules are activated by this signal. Not all of the molecules are activated by this signal. Nonetheless, treatment more marked by synergistic inhibitory actions. Although C3 exoenzyme treatment suppressed platelet aggregation almost completely, it did not affect thrombin-induced IP₃ production. [¹⁴C]Serotonin secretion was delayed by the treatment, but reached almost the same extent as the control platelets. C3 exoenzyme treatment also inhibited PMA-induced platelet aggregation. These results suggest that the ADP-ribosylation did not affect coupling of the receptor and phospholipase C and that rho protein is involved in the aggregation process downstream from receptor-phospholipase C coupling. Aggregation and secretion are two major events derived from phospholipase C activation. Under some experimental conditions, the two events can be dissociated. Carroll et al. (15) found that treatment of platelets with cytochalasin B delays full aggregation without a change in the secretion process. On the other hand, treatment of PMA itself induced full aggregation with reduced serotonin secretion. Electron microscope observations demonstrated that the aggregation process is preceded by the formation of numerous pseudopods by bundling of actin filaments, which is secretion induced by the formation and constriction of a microfilament network termed "contractile gel" (28). Thus, the two events are different, but are similarly dependent on the organization of actin filaments and their localization at specific sites in the cell. Our results indicate that the ADP-ribosylation of rho protein affects some mechanism common to both processes that is essential to aggregation, but is less important in secretion, leading to the suppression of aggregation and delay in secretion.

Using C3 exoenzyme, several groups already studied the role of rho proteins in various types of cultured cells. Chardin et al. (11) found that the addition of C3 exoenzyme to cultured Vero cells causes the disappearance of microfilaments and rounding of cells. Paterson et al. (12) reported that microinjection of ADP-ribosylated rhoA protein into fibroblasts induces similar rounding of cells. They further reported that microinjection of the Val¹⁴-rhoA mutant, a constitutively active form of the protein, induces an extensive microfilament network in the cells and yields characteristic parallel finger-like cytoplasmic processes. They also found that microinjection of this active mutant into contact-inhibited cells induces the organization of a dense microfilament network. Thus, ADP-ribosylation of a rho Protein and Platelet Aggregation

**FIG. 7. Flow cytometric analysis of expression of GPIIb-IIIa in control and C3 exoenzyme-treated platelets.** Platelets were treated with or without C3 exoenzyme and stained with monoclonal anti-GPIIb-IIIa antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG as described under "Experimental Procedures." Fluorescence intensity is expressed on a log scale. A, control platelets treated without anti-GPIIb-IIIa antibody; B, control platelets; C, C3 exoenzyme-treated platelets. Mean fluorescence intensities from control and C3 exoenzyme-treated platelets were 154.8 and 146.6, respectively.

**FIG. 8. Current hypothesis on role of rho protein and effect of its ADP-ribosylation by C3 exoenzyme.** In this scheme, the GTP-bound form of rho protein and its ADP-ribosylated GDP-bound form act on the same effector molecule as an agonist and an antagonist, respectively, to modulate actin filament assembly and avidity of cell adhesion. The ADP-ribosylated GTP-bound form of rho protein has been shown to be biologically inactive (12), but this form will be converted to the GDP-bound form by the action of GTPase-activating protein in the cell (9). rho (GTP)-ADP, GTP-bound form of ADP-ribosylated rho protein; rho (GDP)-ADP, GDP-bound form of ADP-ribosylated rho protein; GAP, GTPase-activating protein for rho protein.
events caused by the ADP-ribosylation and activation of rho protein appear opposite in terms of cell morphology and actin filament organization. On the other hand, injection of a GDP-bound form of rhoA did not cause any change. From those observations and from the findings that the microfilament in the cell is attached at its ends to adhesion plaques composed of integrins and other cytoskeletal elements and that loss of adhesion leads to cell rounding (13), we hypothesize the action of rho protein and the effect of its ADP-ribosylation as shown in Fig. 8. In this scheme, rho protein is activated upon some stimuli to the GTP-bound form, which works on the putative effector molecule and promotes linkage of the actin filament to adhesion receptors to organize stress fiber and enhance cell attachment. On the other hand, this protein, when ADP-ribosylated, binds to the same effector molecule, but prevents the signal of the active form, which leads to loss of cell adhesion and dissolution of microfilaments. Thus, we propose that ADP-ribosylated rho protein acts as an antagonist for the effector. This may explain why some biological effects of the ADP-ribosylation are observed with modification of only that ADP-ribosylated rhoA protein and its ligands such as fibrinogen and fibronectin. The GPIIIa complex is constitutively expressed on the platelet surface, but does not bind adhesion molecules unless it is activated. The detailed mechanism of this activation is not known, but association of the actin filament with the integrin receptor during activation has been reported (14, 29, 30). By analogy to the proposed role of rho protein in cultured cells, we suggest that rhoA protein in platelets is activated by receiving a signal of platelet activation and promotes organization of the actin filament and its binding to the integrin receptor complex to cause activation of the integrin receptor and aggregation. On the other hand, it could be argued that rhoA is not necessarily involved in aggregation signaling and that the inhibition of platelet aggregation observed here is caused nonspecifically by disruption of the cytoskeleton with ADP-ribosylated rhoA. To exclude such a possibility, we should show the association of rho protein activation with aggregation stimuli. Experiments in this line and analysis of the activation mechanism are in progress in our laboratory.

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