Entrapment of Rho ADP-ribosylated by Clostridium botulinum C3 Exoenzyme in the Rho-Guanine Nucleotide Dissociation Inhibitor-1 Complex*

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The Rho subfamily of low molecular mass GTPases comprises RhoAB/C, Rac1/2/3, Cdc42, RhoD, RhoE/Rnd, RhoG, TC10, and TTF. The best characterized of them, Rho, Rac, and Cdc42, have been shown to be involved in the formation of the actin cytoskeleton. Whereas Rho is involved in the regulation of focal adhesions and stress fibers, Rac participates in the control of lamellipodia and Cdc42 in the formation of filopodia. To their function as regulators of the microfilament system, the Rho proteins are involved in the management of several cellular functions such as membrane trafficking, smooth muscle contraction, phospholipid metabolism, cell cycle progression, cell transformation, apoptosis, and transcriptional activation (1, 2).

In the GTP-bound form, Rho translocates to plasma membranes where it interacts with effectors to transduce the signal downstream. In the inactive GDP-bound form, Rho is complexed by the guanine nucleotide dissociation inhibitor (GDI), thereby localizing it to the cytosolic fraction. Three isoforms of GDI have been described: Rho-GDI-1 that interacts with Rho, Rac, and Cdc42 and the D4-GDI or Ly-GDI that is selectively expressed in hematopoetic cell lines (3–5). Rho-GDI1 and the murine form Rho-GDI3 possess a hydrophobic N-terminal elongation to be associated with membranous compartments (6, 7). GDI1 seems to function with RhoG but not with Rac. It is poorly understood how Rho is released from the GDI complex. Phospholipids, especially PIP2, are reported to liberate Rho from the GDI complex (8, 9). Recently, moesin, a member of the ERM (ezrin, radixin, moesin) family, that binds to CD44 or intercellular adhesion molecules (ICAMs) has been reported to bind GDI thereby releasing Rho (10–13).

Because Clostridium botulinum exoenzyme C3 turns off the Rho-dependent signal pathway by selective ADP-ribosylation of RhoA, -B, and -C but not of other low molecular mass GTPases, C3 has been classified as an indispensable tool in cell biology. The disadvantage of poor cell accessibility because of failure of receptor binding and translocation domain has been overcome by different approaches: 1) microinjection of C3 (14); 2) electroporation of cells in the presence of C3 (15); 3) permeabilization by digitonin (16) or streptolysin O (17); 4) intracellular expression of C3 (18–20); 5) using chimeric toxins that recruit the cell entry machinery of other toxins, C3 is fused to enzyme-deficient diptheria toxin or C. botulinum C2 toxin (21–23). The latter possibility, a chimera with the binary C. botulinum C2 toxin, was applied in this study.

In intact cells, ADP-ribosylation of Rho is accompanied by disaggregation of actin filaments leading to the notion that ADP-ribosylation turns Rho inactive. It has been reported that the typical feature of the disrupted actin filament system caused by C3-catalyzed ADP-ribosylation is reversed by the cytotoxic necrotizing factor (CNF) (22, 24) that is known to transform Rho constitutively active by deamidation of glutamine 63 (25, 26). Because deamidation merely blocks GTP hydrolysis, ADP-ribosylated Rho is able to interact functionally with its effectors. Thus, inhibition of Rho-effector coupling seems not to be the mode of how ADP-ribosylation inactivates Rho functions.

Therefore, we studied the influence of ADP-ribosylation on the Rho-GDI interaction and on the subcellular distribution of

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1 The abbreviations used are: GDI-1, guanine nucleotide dissociation inhibitor; GTPγS, guanosine 5′-O-(thiotriphosphate); C21, Rho binding domain of rhoetkin; C3, the chimeric C2IN-C3 transferase; RBD, Rho binding domain; ROK, rhokinase; PIPγ, phosphatidylinositol 4,5-bisphosphate; CNF, cytotoxic necrotizing factor; GST, glutathione S-transferase; GEF, guanine nucleotide exchange factor.
Rho and report here that ADP-ribosylation prevents membrane binding and traps Rho in the GDI complex thereby blocking Rho cycling between cytosol and membranes.

EXPERIMENTAL PROCEDURES

Materials—[3H]NAD was purchased from PerkinElmer Life Sciences. Phosphatidylinositol 4,5-bisphosphate and GTP-S were obtained from Roche Molecular Biochemicals. The antibody against RhoA and GDI-1 were purchased from Santa Cruz Biotechnology, Inc.

Recombinant Proteins—RhoA was expressed in S/F9 insect cells using a baculovirus expression system (Pharmgene, S/F9 cells (1 × 10³ cells/ml) were cultured at 25°C in TMF-H insect medium containing 10% fetal calf serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin. Infected with pAE9H7L containing RhoA baculovirus transfer vectors (multiplicity of infection of 5–10) was performed at 25°C for 48 h. Thereafter, the cells were lysed by sonication in buffer A (50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1% (v/v) Triton X-100, 10 µM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.5). The beads were removed by centrifugation, and thrombin was added to 20,000 × g for 1 h. GST-RhoA from the supernatant was precipitated with glutathione-Sepharose beads at 4°C for 30 min followed by intensive washing with buffer A to remove unbound proteins. RhoA was released from the parent GST fusion proteins by incubation with thrombin (Pharmgene, 10 units) at 4°C overnight in buffer (150 mM NaCl, 5 mM MgCl₂, 2.5 mM CaCl₂, 1 mM dithiothreitol, and 50 mM Tris-HCl, pH 8.0). The beads were removed by centrifugation, and thrombin was precipitated using p-aminobenzamidine beads. The homogeneity of the recombinant RhoA was proven by SDS-PAGE.

GDI-1 was purified as glutathione S-transferase fusion proteins from Escherichia coli followed by thrombin cleavage (100 µg/ml for 30 min at 22°C). Thrombin was removed by precipitation with benzamidine-Sepharose beads (Amersham Biosciences). The chimeric C2-IN-C3 toxin (exoenzyme C3 is fused to the catalytically deficient C2I toxin) (23) and C. botulinum C2 toxin were purified as described (27). In this study C3 was synonymous with C2IN-C3.

Toxin Treatment—NIH3T3 fibroblasts were grown in Dulbecco's medium supplemented with 10% fetal calf serum, 4 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Fibroblasts on 10-cm dishes were treated with C2-IN-C3 fusion toxin or C2 toxin (C2I plus C2II) (each 1 µg/ml) for 3 h. The medium was removed and the cells were washed with 5 ml of ice-cold phosphate-buffered saline and scraped in 300 µl/l dish lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 40 µg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 80 µg/ml benzamidine, 1 mM dithiothreitol, 1 mM EDTA). The cells were disrupted mechanically by sonication (five times on ice), followed by centrifugation for 10 min at 2000 × g to remove the nuclear fraction. The supernatant was used as cell lysate.

Lysates from NIH3T3 cells were centrifuged at 100,000 × g for 1 h to prepare cytosolic and total particulate fractions. The high speed pellet, which consists of the heavy and the light membrane fractions, was washed and resuspended in lysis buffer (28). Cytosolic and membranous fractions were brought to the same concentration of protein measured by the method of Bradford (51).

ADP-ribosylation—RhoA (50 µg/ml) was subjected to ADP-ribosylation by C3 exoenzyme (1 µg/ml) and 10 µM [3H]NAD for 30 min at 37°C. Nucleotide exchange reaction: isoprenylated RhoA (200 µg/ml) dissolved in lysis buffer was incubated in the presence of 100 µM GTP-S or 100 µM GDP for 1 h on ice.

Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis was performed on 12.5% acrylamide gels. The gels were analyzed by PhosphorImager SI from Molecular Dynamics. Proteins were separated on ice, transferred onto nitrocellulose for 2 h at 250 mA. The membranes were blocked for 1 h with 5% (w/v) nonfat dried milk. Blots were incubated for 2 h with anti-RhoA monoclonal antibody (Santa Cruz), anti-GDI-I polyclonal antibody (both diluted 1:2,000) and anti-PLD1 (1:500) in phosphate-buffered saline containing 0.05% Tween 20. Then, for 45 min with a horseradish peroxidase-conjugated secondary antibody. The proteins were visualized by enhanced chemiluminescence. Western blotting of purified recombinant proteins confirmed that the antibodies were specific (results not shown).

Binding of RhoA to Membranes—Recombinant RhoA from S/F9 cells was ADP-ribosylated as described above followed by pre-binding of GDP or GTP-S. RhoA (2 µg) was incubated with NIH3T3 membranes (50 µg) on ice. The reaction mixture was layered onto 200 µl of sucrose (20% (w/v)) sucrose supplemented with 0.1 mg/ml bovine serum albumin and 0.02% (w/v) sodium azide) using microcentrifugation tips and centrifuged for 1 h at 30,000 × g at 4°C. The supernatant was precipitated and resolved in 30 µl of Laemmli sample buffer. The pellet was dissolved in the same volume of sample buffer. Supernatants and pellets were analyzed for RhoA content by immunoblot.

Separation of RhoA from the GDI Complex—Cytosol was prepared as described above. 1 mg of protein dissolved in 500 µl of separation buffer was loaded onto a Superdex 75 column (Amersham Biosciences) previously equilibrated with separation buffer (10 mM imidazole, pH 6.8, 400 mM NaCl, 250 mM sucrose). The flow rate was 0.2 ml/min, and the fraction size was 500 µl. The fractions were precipitated with chloroform/methanol and resolved in 30 µl of Laemmli buffer. Fractions were analyzed by immunoblot for RhoA and GDI-1. Calibration of the column was performed with bovine serum albumin (66 kDa, fractions 4 and 5), chicken albumin (45 kDa, fractions 7 and 8), and chymotrypsin (25 kDa, fractions 11 and 12). Cytosols (0.5 mg/ml) prepared in lysis buffer supplemented with 250 mM sucrose were incubated in the presence of 0.2 mg/ml PIP₂ or buffer at 37°C for 10 min and applied to a 30-kDa cut-off membrane (Microcon 30, Amicon, Beverly) at 7000 × g for 30 min at room temperature. Supernatant and filtrate were brought to the same protein concentration followed by immunoblot analysis for RhoA and GDI-1.

Pull-down Experiments with GST-C21—Pull-down experiments were performed as described by Reid et al. (29). Ice-cold lysis buffer (500 µl; 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 1% Nonidet P-40, 0.25% Triton X-100, 10 mM dithiothreitol, 100 µM phenylmethylsulfonyl fluoride) was added to cell lysates containing 1 mg of protein in total. For recombinant assay, 1 µg of GDP- or GTP-S-loaded RhoA was added to 500 µl of lysis buffer. Samples were centrifuged for 10 min at 15,000 × g, and the supernatant was used for pull-down assay. To this end, 20 µl of beads slurry of the Rho binding domain GST-C21 from rhotein bearing ~30 µg of fusion protein were added to each sample and rotated at 4°C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer. To each sample 20 µl of Laemmli buffer were added to the beads, and 12% SDS-PAGE with subsequent transfer of proteins onto nitrocellulose was performed. RhoA was detected by Western blot using specific antibody.

RESULTS

Activation of ROK by ADP-ribosylated RhoA—The consequences of ADP-ribosylation on the Rho-dependent activation of the effector protein ROK were tested in a recombinant system. The activity of ROK was determined in terms of phosphorylation of the myosin binding subunit in the presence or absence of isoprenylated RhoA. As shown in Fig. 1, unmodified RhoA-GTP/S as well as ADP-ribosylated RhoA-GTP/S stimulated ROK activity, the latter to a lesser extent. Thus, ADP-ribosylation does not interfere with Rho-dependent ROK activation per se.

Subcellular Distribution of RhoA in C3-treated NIH3T3 Fibroblasts—In terms of the cytosol membrane model of the
NIH3T3 fibroblasts were treated with the indicated toxins (each 1 µg/ml for 4 h) until the cells were completely rounded; the lysates were fractionated by ultracentrifugation (60 min at 100,000 × g). Cytosol (cyt) and membranes (mem) were adjusted to the same protein concentration. Proteins were separated by SDS-PAGE and analyzed for RhoA and GDI-1 by immunoblot analysis. A, cellular Rho from C3-treated cells is inactive. Recombinant RhoA as well as Rho from lysates were subjected to C21 pull-down as described under “Experimental Procedures.” Precipitated Rho was detected using Western blot analysis. Complete ADP-ribosylation of Rho is indicated by gel shift of RhoA to a higher apparent molecular weight.

Rho-GTPase cycle, active Rho is translocated to the membranes where effector proteins are also present (30). To study whether ADP-ribosylated Rho does in fact bind to membranes, the subcellular distribution of Rho after treatment of NIH3T3 cells with the chimeric C3 toxin was determined (Fig. 2A). When cytosolic and membrane fractions of control NIH3T3 cells were adjusted to the same protein concentration, about one-half of the cellular Rho was localized in the cytosolic fraction and the other half in the membranes. ADP-ribosylation, however, resulted in a complete shift to the cytosol, and no Rho was detected anywhere in the membrane fractions (Fig. 2A). To exclude that the observed translocation of Rho was based on the reorganization of the actin cytoskeleton, that is the consequence of ADP-ribosylation of Rho, the actin filaments were depolymerized by C. botulinum C2 toxin, that directly ADP-ribosylates actin thereby rendering it incapable of polymerization (31, 32). Rho from these cells showed almost the same subcellular distribution as control cells (Fig. 2A). Thus, ADP-ribosylation rendered Rho incapable of binding to the membranes and led to its accumulation in the cytosol. The total ADP-ribosylation rendered Rho incapable of binding to the membranes and led to its accumulation in the cytosol. The total subcellular distribution of Rho after treatment of NIH3T3 fibroblasts with the chimeric C3 toxin was determined (Fig. 2A).

Rho Activity State—To check the activity state of Rho within the C3-treated cell, the amount of active ADP-ribosylated Rho was determined by pull-down experiments using the Rho binding domain of rhotekin (C21) (29). To exclude that ADP-ribosylation hampers the Rho-C21 interaction, this experiment was first carried out with recombinant proteins. As shown in Fig. 2B, recombinant GTPγS-bound RhoA was pulled down with GST-C21 independently of the ADP-ribosylation. Subsequently, Rho from lysates of C3-intoxicated cells was subjected to the pull-down assay with C21. As expected, some active Rho was detected anywhere in the membrane fractions (Fig. 2B). Thus, ADP-ribosylated Rho was completely cytosolic (Fig. 2B).

Binding of ADP-ribosylated RhoA to GDI-1—Because cellular ADP-ribosylated Rho was completely cytotoxic, we addressed the question whether the modified Rho was monomeric or complexed with GDI-1 (33, 34). To separate monomeric RhoA from the Rho-GDI complex, cytosolic fractions were passed through an ultracentrifugation membrane with a 30-kDa cut-off. As shown in Fig. 3A, both RhoA (molecular mass 20–30 kDa) and GDI-1 (molecular mass 40–50 kDa) were detected in the cytosol. Fractionation of cytosol using a 30-kDa cut-off membrane filter. Filtrate and supernatant were analyzed for RhoA and GDI-1 by immunoblot analysis. B, gel permeation chromatography. Cytosols from control and C3-treated cells were chromatographed on Superdex 75 column. Fractions were analyzed for RhoA and GDI-1 by immunoblotting. RhoA and GDI-1 from control cytosol eluted in fractions 6–9 corresponding to an apparent molecular mass of 50–40 kDa. A minor amount of monomeric RhoA eluted in fraction 11 (molecular mass 20–30 kDa). Cytosolic Rho from C3-treated cells eluted also in fractions 6–9, corresponding to an apparent molecular mass of 40–50 kDa. C, nucleotide-dependent binding of ADP-ribosylated RhoA to GDI-1. RhoA was ADP-ribosylated and preloaded with GDP or GTPγS as described under “Experimental Procedures.” GST-GDI-1 and GST as control immobilized to glutathione agarose were incubated with RhoA as indicated at 4 °C for 1 h. Eluted proteins were probed for RhoA by immunoblot analysis.

![Fig. 2. A, effect of chimeric C3 and C. botulinum C2 toxin on the subcellular distribution of RhoA and GDI-1 in NIH3T3 fibroblasts. NIH3T3 fibroblasts were treated with the indicated toxins (each 1 µg/ml for 4 h) until the cells were completely rounded; the lysates were fractionated by ultracentrifugation (60 min at 100,000 × g). Cytosol (cyt) and membranes (mem) were adjusted to the same protein concentration. Proteins were separated by SDS-PAGE and analyzed for RhoA and GDI-1 by immunoblot analysis. B, cellular Rho from C3-treated cells is inactive. Recombinant RhoA as well as Rho from lysates were subjected to C21 pull-down as described under “Experimental Procedures.” Precipitated Rho was detected using Western blot analysis. Complete ADP-ribosylation of Rho is indicated by gel shift of RhoA to a higher apparent molecular weight.](http://www.jbc.org/)

![Fig. 3. ADP-ribosylated RhoA is complexed with GDI-1. A, fractionation of cytosol using a 30-kDa cut-off membrane filter. Cytosols from control and C3-treated cells were incubated without or with 0.2 mg/ml PIP2, followed by separation through a 30-kDa cut-off membrane filter. Filtrate and supernatant were analyzed for RhoA and GDI-1 by immunoblot analysis. B, gel permeation chromatography. Cytosols from control and C3-treated cells were chromatographed on Superdex 75 column. Fractions were analyzed for RhoA and GDI-1 by immunoblotting. RhoA and GDI-1 from control cytosol eluted in fractions 6–9 corresponding to an apparent molecular mass of 50–40 kDa. A minor amount of monomeric RhoA eluted in fraction 11 (molecular mass 20–30 kDa). Cytosolic Rho from C3-treated cells eluted also in fractions 6–9, corresponding to an apparent molecular mass of 40–50 kDa. C, nucleotide-dependent binding of ADP-ribosylated RhoA to GDI-1. RhoA was ADP-ribosylated and preloaded with GDP or GTPγS as described under “Experimental Procedures.” GST-GDI-1 and GST as control immobilized to glutathione agarose were incubated with RhoA as indicated at 4 °C for 1 h. Eluted proteins were probed for RhoA by immunoblot analysis.](http://www.jbc.org/)
The latter condition is not to disturb the stability of the Rho-GDI complex in the absence of any detergents. The reason for the pull-down was the addition of PIP$_2$ (Fig. 2B). Active Rho was determined by rhotekin pull-down. In contrast to the condition in Fig. 2A, lysates from C3-treated cells (data not shown). Thus, the ADP-ribosylated RhoA was complexed with GDI-1 or other proteins but was not monomeric.

Binding of ADP-ribosylated Rho to GDI-1 was also tested in a recombinant system by nucleotide-dependent co-precipitation of Rho with GST-GDI-1 immobilized to Sepharose. As shown in Fig. 3C, unmodified as well as modified RhoA bound to GDI-1 when loaded with GDP but only faintly when bound to GTP$\gamma$S. The faint binding of GTP$\gamma$S-loaded Rho reflects a low affinity binding of RhoA-GTP$\gamma$S to GDI-1. ADP-ribosylated Rho bound to GDI-1 with higher affinity in comparison to non-modified Rho (Fig. 3C). A further indication for higher affinity came from a precipitation experiment of RhoA from cytosolic fractions using immobilized GST-GDI-1. Added GST-GDI-1 competed with endogenous GDI-1 for RhoA binding by complete precipitation of cytosolic Rho. However, GST-GDI-1 was incapable of precipitating modified RhoA from cytosols prepared from C3-treated cells (data not shown). Thus, the ADP-ribosylated RhoA-GDI-1 complex seemed to be more stable than the complex with non-modified Rho.

**Activation of ADP-ribosylated Rho from Lysates**—To confirm that the increased stability of the ADP-ribosylated Rho-GDI complex in fact hampers Rho activation, lysates were treated with and without C3 and NAD followed by GTP$\gamma$S incubation. Active Rho was determined by rhotekin pull-down. In contrast to the condition in Fig. 2B, the pull-down was carried out in the absence of any detergents. The reason for the latter condition is not to disturb the stability of the Rho-GDI complex. As shown in Fig. 4, unmodified Rho-GTP$\gamma$S was active whereas Rho from C3-treated lysates turned out to be inactive. This finding underlines that GTP$\gamma$S is capable of releasing unmodified Rho but not ADP-ribosylated Rho from the GDI complex. As a control the sequence of reaction was reversed: lysates were pretreated with GTP$\gamma$S followed by ADP-ribosylation. This reaction of reaction guaranteed the presence of monomeric Rho and GDI-1. Monomeric Rho-GTP$\gamma$S was then ADP-ribosylated and pulled down by rhotekin (Fig. 4). Thus, entrapment of ADP-ribosylated Rho in the GDI complex effectively prevented Rho activation by GTP$\gamma$S.

**DISCUSSION**

Because of the widespread application of C3 as a tool in cell biology, the knowledge of the cellular mode of action is essential for the understanding of C3 effects on signal transduction. However, it is still unclear how ADP-ribosylation causes inactivation of the Rho GTPases. Studies on the functional consequences of ADP-ribosylation on the GTPase cycle of Rho do not reveal remarkable changes in nucleotide binding, direct inhibition of the Rho binding domain (RBD)-deficient ROK is constitutively active and that an antibody toward the RBD of ROK is sufficient for activating ROK, thus mimicking Rho (44). Based on the latter finding, mere binding of ADP-ribosylated RhoA should be sufficient for ROK activation as presented above in a recombinant system. However, direct inhibition of Rho-effector coupling is obviously not the mode of action for how Rho is inactivated by ADP-ribosylation in intact cells. The notion that ADP-ribosylated Rho is biologically active is further supported by the observation that sequential treatment of cells with C3 followed by CNF1 results in typical features of the CNF1 morphology (22, 24). ADP-ribosylated RhoAQ63E, likely loaded with cellular GTP, is obviously active, i.e. capable of downstream signaling. The ADP-ribose appears to be inert.

The prominent finding after treatment of cells with C3 was the altered subcellular distribution of Rho, so that ADP-ribosylated cellular Rho was completely localized to the cytosolic fraction. From this finding arises the question whether cytosolic Rho is monomeric or bound to GDI-1. From the gel filtration and the ultrafiltration experiments, it became clear that ADP-ribosylated Rho is bound to GDI. Comparable data, the complexation of ADP-ribosylated Rho with GDI, have been shown in smooth muscle strips using immunoprecipitation techniques (45). We confirmed this finding directly, by the binding of recombinant ADP-ribosylated RhoA to recombinant GDI-1 thereby excluding the involvement of any additional factors. From the structure of the RhoA-GDI-1 complex can be deduced that the ADP-ribose at Asn-41 is directed to the solvent and thereby excluding the involvement of any additional factors. The ADP-ribosylated recombinant GDI can compete with cellular GDI for Rho binding. This finding underlines that GTP$\gamma$S-loaded recombinant RhoA to recombinant GST-GDI-1 immobilized to Sepharose. As shown in Fig. 3C, unmodified as well as modified RhoA bound to GDI-1 when loaded with GDP but only faintly when bound to GTP$\gamma$S. The faint binding of GTP$\gamma$S-loaded Rho reflects a low affinity binding of RhoA-GTP$\gamma$S to GDI-1. ADP-ribosylated Rho bound to GDI-1 with higher affinity in comparison to non-modified Rho (Fig. 3C). A further indication for higher affinity came from a precipitation experiment of RhoA from cytosolic fractions using immobilized GST-GDI-1. Added GST-GDI-1 competed with endogenous GDI-1 for RhoA binding by complete precipitation of cytosolic Rho. However, GST-GDI-1 was incapable of precipitating modified RhoA from cytosols prepared from C3-treated cells (data not shown). Thus, the ADP-ribosylated RhoA-GDI-1 complex seemed to be more stable than the complex with non-modified Rho.
through prevention of its membrane binding. In a recombinant system, ADP-ribosylation reduced Lbc-catalyzed GTP loading of Rho (22). Both effects together may prevent GTP loading of ADP-ribosylated Rho. This notion is supported by our finding on the absence of any active ADP-ribosylated Rho from C3-treated cells as determined by rhoeatin pull-down.

In terms of the cytosol-membrane model of Rho GTPase cycle a functional effector protein is characterized by membrane association. Because ADP-ribosylation entraps Rho in the cytosolic GDI complex, ADP-ribosylation indirectly blocks coupling of Rho to a membrane-bound effector protein. This notion meets the former hypothesis of blocked Rho-effector coupling by ADP-ribosylation; however, under special conditions such as the sequential intoxication with C3 and CNF1, ADP-ribosylated RhoA(GDP)

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