The small GTPases Rho, Rac, and Cdc42 are monoglucosylated at effector domain amino acid threonine 37/35 by Clostridium difficile toxins A and B. Glucosylation renders the Rho proteins inactive by inhibiting effector coupling. To understand the functional consequences, effects of glucosylation on subcellular distribution and cycling of Rho GTPases between cytosol and membranes were analyzed. In intact cells and in cell lysates, glucosylation leads to a translocation of the majority of RhoA GTPase to the membranes whereas a minor fraction is monomeric in the cytosol without being complexed with the guanine nucleotide dissociation inhibitor (GDI-1). Rho complexed with GDI-1 is not substrate for glucosylation, and modified Rho does not bind to GDI-1. However, a membranous factor inducing release of Rho from the GDI complex makes cytosolic Rho available as a substrate for glucosylation. The binding of glucosylated RhoA to the plasma membrane is saturable, capable of unmodified Rho-GTPγS guanosine 5′-O-(3-thiotriphosphate), and takes place at a membrane protein with a molecular mass of about 70 kDa. Membrane-bound glucosylated Rho is not extractable by GDI-1 as unmodified Rho is, leading to accumulation of modified Rho at membranous binding sites. Thus, in addition to effector coupling inhibition, glucosylation also inhibits Rho cycling between cytosol and membranes, a prerequisite for Rho activation.

The Rho subfamily (Rho, Rac, and Cdc42) of low molecular mass GTP-binding proteins are important regulators of the actin cytoskeleton, phospholipid metabolism, membrane trafficking, smooth muscle contraction, cell cycle progression, cell transformation, apoptosis, and transcriptional activation (1–9). The Rho proteins are molecular switches in intracellular signaling. In the GDP-bound form they are switched off, and in the GTP-bound form they are switched on. The on-off state is tightly governed by regulatory proteins. The activation is catalyzed by guanine nucleotide exchange factors (GEFs), which promote the exchange with GTP. In the active GTP state, the Rho protein couples to effector proteins, e.g. Ser/Thr kinases such as Rho kinase or PAK for downstream signaling. The active state is terminated by the GTPase-activating proteins (GAPs), which supply an arginine finger to increase the GTPase activity, resulting in the formation of inactive GDP-bound Rho (10). The cycling between the two nucleotide-bound states is accompanied by cycling between the cytosolic fraction and the membranes (11–17). The guanine nucleotide dissociation inhibitors (GDIs) are involved in this subcellular cycling. GDIs are unique for the Rho and Rab subfamilies but not found in the other subfamilies of the Ras superfamily. The GDIs bind to postranslationally modified (isoprenylated) Rho proteins, keep them in the GDP-bound form in the cytosolic fraction, and inhibit nucleotide exchange (5, 18–22). The GDP form is preferentially bound to GDI-1, but in the GTP-bound form GDI-1 interacts with GAP (23–26).

There are several reports that phosphatidylinositol bisphosphate (PIP₂) liberates Rho from the GDI complex but the physiological significance of this finding is still unclear (27, 28). Recently, the ezrin, moesin, and radixin (ERM) proteins have been reported to release Rho from the GDI complex. The ERM proteins are composed of a N-terminal membrane anchorage domain (binding to CD44 or ICAM) and a C-terminal F-actin binding domain allowing them to link the cytoskeleton to the membrane (29, 30). The ERMs are inactive in the cytosol through head to tail interaction (31). A so far unidentified signal induces opening of the ERMs allowing the N-terminal part to bind to GDI-1. ERM-GDI interaction results in the release of Rho, which subsequently interacts with the cognate effectors to be activated for downstream signaling (15, 32, 33). In this regard, the ERM family functions as a displacement factor for Rho from the GDI complex (8).

GD1-1 is exclusively localized in the cytosol and interacts with Rho, Rac, and Cdc42 (12, 18). Ly-GDI (D4-GDI) exhibits the same properties but is preferentially expressed in hematopoietic cell lines (34). In addition to the cytosolic GDIs, GDIα and GDI-3 are membrane-bound through a N-terminal extension. The latter GDIs are expressed in a more tissue-specific manner, and they show more selective interaction with Rho subfamily proteins (21, 22).

The Rho subfamily proteins are cellular targets of various bacterial toxins to be covalently modified. CNF1 catalyzes deamidation of glutamine 63 of Rho to generate constitutively active GTPase (35, 36). Clostridium botulinum C3 exoenzyme inactivates RhoA, -B, and -C but not Rac and Cdc42 by ADP-ribosylation at asparagine 41 (37, 38). Clostridium difficile toxins A and B, which are cytotoxins to cause disaggregation of the actin filament system, catalyze monogluicosylation of Rho, Rac, and Cdc42, thereby inactivating the GTPases (39, 40). The modification occurs at threonine 37 (Rho) and threonine 35 (Rac, Cdc42), which is located in the effector loop. Glucosylation does not significantly alter binding of GDP and GTP but decreases the intrinsic GTPase activity by a factor of 5 and
Membrane Binding of Glucosylated Rho

**EXPERIMENTAL PROCEDURES**

**Materials and Chemicals**—RhoA and Rac1 were expressed in *S. frugiperda* cells (Sf9 cells) and purified from a Triton X-100-soluble fraction of cells as glutathione S-transferase fusion protein. GDI-1 was purified as glutathione S-transferase fusion protein from *Escherichia coli*. The glutathione fusion proteins were isolated by affinity purification with glutathione-Sepharose beads (Amersham Pharmacia Biotech) previously equilibrated with separation buffer. Glutathione fusion proteins were isolated by affinity purification with glutathione-Sepharose beads (Amersham Pharmacia Biotech).

*C. difficile* toxin B (42), *C. botulinum* C2 toxin (43), and the chimeric C3 toxin (ADP-ribosyltransferase C3 is fused to the catalytically deficient *C. botulinum* C2 toxin) (44) were purified as described. UDP-[14C]glucose was purchased from Bio Trend (Cologne, Germany).

**Cell Culture**—NIH3T3 fibroblasts were grown in Dulbecco’s medium supplemented with 10% fetal calf serum, 4 mM glutamine/penicillin/streptomycin.

**Toxin Treatment and Lysis of Cells**—Cell cultures of 10-cm dishes were treated with *C. difficile* toxin B (0.1 μg/ml), *C. botulinum* C2 toxin (400 ng/ml C2II plus 200 ng/ml C2I) or the chimeric C3 toxin (400 ng/ml C2 toxin (43) and the chimeric C3 toxin (400 ng/ml C2 toxin (43)] for different times as noted in the legends. The cells were rinsed with 5 ml of ice-cold phosphate-buffered saline and scraped off in 300 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM MgCl2, 40 μg/ml apotinin, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 80 μg/ml benzamidine) per dish. The cells were disrupted mechanically by sonification (five times on ice), followed by centrifugation for 10 min at 1,000 × g to remove the nuclear fraction and intact cells. The supernatant (1 mg/ml protein) was used as cell lysate.

**Fractionation of Cell Lysates**—Lysates 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 with lysis buffer and resuspended in the original volume of lysis buffer.

**ADP-ribosylation Reaction**—Lysates from NIH3T3 fibroblasts (1 mg/mL) were subjected to ADP-ribosylation by C3 toxin (0.1 μg/ml) in the presence of 10 μM NAD and 10 μM thymidine for 30 min at 37°C. The reaction was determined by addition of Laemmli sample buffer.

**Glucosylation Reaction**—Lysates from NIH3T3 fibroblasts (1 mg/mL) were incubated with toxin B (1 μg/ml) in the presence of 30 μM UDP-[14C]glucose and 1 mM MnCl2 at 37°C for 1 h. Recombinant RhoB (2 μg) dissolved in glucosylation buffer (50 mM HEPES, pH 7.4, 2 mM MgCl2, 5 mM GTPγS, 1 mM dithiothreitol) was incubated with toxin B (1 μg/ml) and UDP-[14C]glucose (30 μM) at 37°C for 30 min. Cytosols from NIH3T3 fibroblasts (0.8 mg/ml) were incubated with 0.2 mg/ml PIP2 or 50 μg/ml GDI-1 at 37°C for 30 min followed by [14C]glucosylation reaction. The reaction was determined by addition of Laemmli sample buffer.

**SDS-Polyacrylamide Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed with 12.5% polyacrylamide gels. The gels were visualized by PhosphoImage SI from Molecular Dynamics.

**Immunoblot Analysis**—Proteins were separated on 12.5% polyacrylamide gels and transferred onto nitrocellulose for 2 h at 250 mA, followed by blocking with 5% (w/v) nonfat dried milk for 1 h. Blots were incubated with 2 h with the appropriate primary antibody (diluted 1:3,000) in buffer B (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM KCl, 0.05% (w/v) Tween 20) and then for 45 min with a horseradish peroxidase-conjugated secondary antibody.

**Nucleotide Exchange Reaction**—Isoprenylated RhoA and Rac1 were incubated in buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2.5 mM MgCl2) in the presence of 100 μM GTPγS or 100 μM GDP on ice for 1 h.

**Sucrose Density Centrifugation**—100 μl of reaction mixture were 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 at 4°C (1 h at 30,000 × g). The supernatant was precipitated and resolved in 30 μl of Laemmli sample buffer. The pellet was dissolved in the same volume of sample buffer.

**Binding of Isoprenylated RhoA**—RhoA was incubated with toxin B (0.1 μg/ml) and loaded with GDP in *C. difficile* (data not shown). All steps were carried out at 4°C. Washed NIH3T3 membranes (0.35 mg/ml) were incubated in binding buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2.5 mM MgCl2, 0.1 mM bovine serum albumin, 100 μM GTPγS, 100 μM phenylmethylsulfonyl fluoride) with [14C]glucosylated RhoA as indicated for 30 min on ice. The mixture was fractionated by sucrose density centrifugation (as described above), and the pellet was counted for radioactivity. The amount found in the total absence of membranes was set blank (about 0.1%).

**RhoA Overlay**—RhoA (200 μg/ml) was glucosylated in the presence of 100 μM non-radioactive UDP-glucose as described above, followed by nucleotide exchange with 100 μM GDP or GTPγS on ice for 1 h. Complete glucosylation was checked by [32P]ADP-ribosylation (data not shown). After transfer of NIH3T3 membranes, the nitrocellulose was blocked with 5% nonfat dried milk, followed by renaturation of proteins in buffer B overnight at 4°C. Binding was performed at 24°C for 2 h with 2 μg/ml glucosylated or unmodified RhoA (loaded either with GTPγS or GDP) dissolved in buffer B supplemented with 2.5 mM MgCl2. After washing four times with buffer B for 10 min, the membranes were probed for RhoA as described above.

**Gel Permeation Chromatography of the Rho-GDI-1 Complex**—Cytochrome c was prepared as described above. 0.5 mg of protein dissolved in 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA was loaded onto a Superdex 25 column (Amersham Pharmacia Biotech) previously equilibrated with separation buffer (10 mM imidazole, pH 6.8, 400 mM NaCl, 250 mM sucrose).

**Separation of Monomeric Rho from Rho-GDI Complex by 30-kDa Cut-off Membrane Filters**—Cytopsins (250 μg of protein dissolved in 500 μl of separation buffer) were applied onto a 30-kDa cut-off membrane (Microcon 20, Amicon, Beverly, MA) at 7,000 × g for 30 min at room temperature. Supernatant and filtrate were brought to the same protein concentration, followed by immunoblot analysis.

**RESULTS**

**Influence of the Glucosylation on the Subcellular Distribution of RhoA**—The majority of RhoA (>90%) was localized in the cytosolic fraction when lysates from NIH3T3 cells were fractionated by ultracentrifugation (Fig. 1A). After treatment of NIH3T3 cells with toxin B, the subcellular distribution changed and about 50% of RhoA was localized to the membranous fraction (Fig. 1A). To exclude that this redistribution was merely based on disaggregation of the actin cytoskeleton, *C. botulinum* C2 toxin was applied which directly ADP-ribosylates monomeric actin thereby turning it incapable of polymerization. As shown in Fig. 1A, disruption of the actin filaments did not induce translocation of Rho to the membranes. Furthermore, neither toxin B nor C2 toxin treatment of the cells led to a change in GDI-1 localization (Fig. 1A). To prove whether translocation of Rho to the membranes was due to the bound glucose moiety, membrane binding of Rho ADP-ribosylated by C3 toxin was studied. In contrast to toxin B, C3 selectively modifies Rho but not Rac and Cdc42. ADP-ribosylated Rho was found in the cytosol (Fig. 1A) but not at the membranes, indicating that redistribution of Rho was based on the glucose moiety and not on mere changes in structure by posttranslational modification.

The translocation to the membranes was a time-dependent process proceeding with the increase in Rho glucosylation (Fig. 1B). Glucosylation induced a shift of the majority of Rho to the membranes, but a fraction (about 40%) remained in the cytosol. Under these conditions, all cellular Rho was glucosylated in vivo as was shown by a second [14C]glucosylation of the lysates (Fig. 1C). That only a fraction of the completely glucosylated...
cellular Rho bound to the membranes may be an indication for limited binding capacity of the membranes.

**Membrane Binding of Glucosylated Rho**—The effect of glucosylation on the translocation of Rho was not only observed in intact cells; but also when Rho was glucosylated in cell lysates (Fig. 2A). The failure of ADP-ribosylated Rho to bind to membranes was also found in the in vitro system. This finding indicated that the machinery of the intact cell was not needed for translocation but merely the modification with glucose.

To prove this hypothesis binding of \[^{14}C\]glucosylated RhoA in the GTP-γS form to membranes from NIH3T3 cells freed from endogenous Rho was performed. Bound Rho was separated from non-bound by sucrose density centrifugation. Fig. 2B shows that glucosylated RhoA-GTP-γS bound in a saturable manner to the membranes. Maximal binding was about 2.5 μg of Rho/mg of membranes. Binding of glucosylated RhoA was competed by unmodified RhoA-GTP-γS (Fig. 2C) but not by RhoA-GDP (data not shown), suggesting comparable affinities to the membrane binding site. Glucosylated Rho bound to GDP also showed binding but less compared with the GTP-γS bound Fig. 2D.

Saturable binding of glucosylated and unmodified Rho indicated that the binding was not merely mediated through lipid-lipid interaction by the geranyl-geranyl moiety but rather through a protein-protein interaction. To obtain more information on the binding site, we performed an overlay assay. Membranes from NIH3T3 cells were electroblotted and then overlaid with unmodified and glucosylated RhoA either bound to GDP or GTP-γS. Bound RhoA was detected with anti-RhoA. Fig. 2D shows that unmodified RhoA bound when loaded with GTP-γS, whereas in the GDP-bound form there was only a very faint binding. The membrane protein to which RhoA bound exhibited an apparent molecular mass of about 70 kDa (p70). Glucosylated RhoA bound to the same protein; however, it bound in an almost nucleotide-independent manner. Addition of RhoA-GTP-γS to the overlay with glucosylated RhoA-GTP-γS clearly decreased the binding of glucosylated RhoA (data not shown), corroborating the results of binding to native membranes and indicating competition at the same binding site.

**Glucosylation of the Rho-GDI Complex**—The majority of Rho is localized in the cytosol, and this cytosolic Rho is not monomeric but bound to GDI-1 in a high affinity complex. The cytosolic Rho-GDI complex is reported to be cleaved by PIP2 (27, 28).

Treatment of intact cells as well as cell lysates with toxin B resulted in complete glucosylation of cellular Rho (Fig. 1C). This observation implicated that also Rho from the GDI complex was modified. Therefore, we tested the influence of GDI-1 on the glucosylation of Rho. Toxin B-catalyzed glucosylation of Rho/Rac/Cdc42 from cytosolic fractions was increased after addition of PIP2; conversely, addition of recombinant GDI-1 to the cytosol completely abolished glucosylation, an effect that was completely reversed by PIP2 (Fig. 3A). These data indicated that the Rho GTPases complexed with GDI-1 were not substrates for toxin B. To test this assumption directly, we studied glucosylation with recombinant proteins, using isoprenylated RhoA and Rac1 from S9 cells because only isoprenylated Rho is capable of binding to GDI-1. RhoA as well as Rac1 bound to GDI-1 were not glucosylated by toxin B. However, addition of PIP2 turned RhoA and Rac1, respectively, glucosylatable (Fig. 3B). To exclude that PIP2 had no direct stimulating effect on the enzyme reaction, increasing concentrations of PIP2 were added to the glucosylation reaction of RhoA. As shown in Fig. 3C, the phosphoinositide did not change the glucosylation of recombinant RhoA, indicating that the effect of PIP2 on the glucosylation of cellular Rho/Rac/Cdc42 was indeed due to the cleavage of the Rho-GDI complex.

The finding that cellular Rho was completely glucosylated in intact cells and cell lysates was apparently inconsistent with the observation that the Rho-GDI complex was resistant to glucosylation. To solve this contradiction, we tested whether membranes contained factors that released Rho from the GDI complex. To this end cytosolic fractions were incubated with increasing concentrations of membranes, followed by toxin B-catalyzed glucosylation (Fig. 3D). The very low glucosylation rate in the cytosol was enhanced by membranes that stimulated glucosylation comparable to the addition of PIP2. Thus, membranes contained a factor or factors that make Rho available for glucosylation. PIP2 or the ERM proteins both have been reported to be releasing factors for Rho (8, 32). In intact cells and also in cell lysates, Rho cycles between the GDI-bound and the membrane-bound form; the intermediary free, i.e. monomeric Rho, became substrate for glucosylation.

To test whether glucosylated RhoA was capable of binding to GDI-1, coimmobilization experiments with GST-GDI-1 immobilized to Sepharose were performed. As shown in Fig. 3E, RhoA-GDP binding to GDI-1 was superior to binding of RhoA-GTP-γS corroborating reported data (25). Glucosylated RhoA, however,
showed no binding to GDI-1 independently of the nucleotide bound. The same results were found for Rac1 with the exception that also Rac1-GTP bound to GDI-1. Thus, glucosylation inhibited binding to GDI-1 or significantly decreases the affinity to GDI-1. From the finding that glucosylation prevented binding to GDI-1, it can be deduced that glucosylated Rho, in contrast to nonmodified Rho, should be monomeric in the cytosol from toxin B-treated cells. Indeed, gel permeation chromatography of cytosol prepared from control and toxin B-treated cells revealed that Rho from control cells was eluted with an apparent molecular mass of about 50 kDa (22 kDa of Rho plus 23 kDa of GDI-1) and that glucosylated Rho was eluted with a mass of 22 kDa consistent with the monomeric property of modified Rho (Fig. 4A).

Extraction of Rho from Membranes—One property of GDI-1 is to extract Rho bound to membranes. Because glucosylated Rho lost its property to bind to GDI-1, GDI-1 should not be able to extract modified RhoA from membranes. Membranes from control and toxin B-intoxicated cells were loaded with GDP, followed by extraction with GDI-1 or buffer as control. Thereafter, the membranes were separated from the soluble fraction by sucrose density centrifugation, and Rho was detected by immunoblot, GDI-1 completely extracted unmodified Rho from the membranes but was incapable of releasing glucosylated Rho (Fig. 5). Thus, glucosylation of Rho led to trapping of modified Rho at the Rho binding site of the membranes.

DISCUSSION

The Rho subfamily proteins Rho, Rac, and Cdc42 are monoglucosylated at effector domain amino acid threonine 37 (Rho) and threonine 35 (Rac/Cdc42) by C. difficile toxins A and B. The glucose moiety located at switch I loop of Rho alters the GTPase cycle: Activation of Rho by GEFs is reduced but not completely inhibited when the properties of glucosylated Ras are transferred to that of glucosylated Rho (45). The intrinsic GTPase activity of Rho is reduced but the GAP-stimulated activity is completely inhibited (41). The pivotal step for signaling, the effector coupling, is completely blocked (41). From these biochemical data, it can be concluded that glucosylated Rho should be trapped in the GTP-bound state but is incapable of downstream signaling. In contrast to the Ras subfamily, the Rho subfamily proteins are additionally regulated by GDIs, which keep the Rho proteins in the GDP-bound form. The high affinity Rho-GDI complex reflects the cytosolic pool of inactive Rho. Furthermore, GDI-1 extracts membrane-bound Rho and delivers it to the cytosolic pool. Thus, GDI-1 is the key player of the cytosol-membrane cycling, which in conjunction with GTP binding is important for downstream signaling (8, 46).

GDI-1 consists of a well structured C-terminal part with a pocket to bind the isoprenyl moiety of the Rho proteins (47). The N-terminal part is very flexible and is proposed to interact.
with the effector loop of the Rho proteins (47). Rho in the GDI-1 complex is not substrate for toxin B. This may be due to the inaccessibility of the toxin recognition site on the Rho surface and/or the inaccessibility of the acceptor amino acid threonine 37. The fact that glucosylated Rho does not form a complex with GDI-1 strongly argues for the proposed interaction of GDI-1 with the effector loop of Rho GTPases.

In intact cells and cell lysates, cellular Rho is completely glucosylated and no unmodified is left. This finding is surprising because the majority of Rho is bound in the GDI-1 complex in which Rho is not substrate for the toxins. The Rho-GDI complex, the recombinant or cytosolic one, is cleaved by PIP2. Whether PIP2 is the physiological release factor is unclear but PIP2 is involved in the displacement of Rho from the GDI complex by the ERM proteins (48). These proteins mediate the membrane association of the actin filaments (30, 31, 49), and they displace Rho from the Rho-GDI complex initiating the activation of Rho (32, 33). We showed that membranes contain displacement activity to release Rho from the GDI complex; released, monomeric Rho is then glucosylated by toxin B. This finding explains why the complete quantity of cellular Rho is glucosylated in intact cells as well as in lysates.

The incapability of glucosylated Rho to bind to GDI-1, shown by gel filtration of intoxicated cytosol as well as by coprecipitation assay using recombinant Rho and GDI-1, has a functional implication. It is generally accepted that Rho is translocated to the membranes during the activation process and that inactive Rho is extracted by GDI-1 to allow new Rho to translocate. Glucosylated Rho accumulates at the membranes for two reasons. (i) Rho is kept in the GTP-bound form. (ii) Rho cannot be extracted by GDI-1 thereby inhibiting the translocation of unmodified Rho to the membrane. The inhibition of the cytosol-membrane cycling of Rho contributes to its functional inactivation.

It is very surprising that Rho with a glucose moiety at threonine 37 shows increased binding to the membranes. The binding of glucosylated Rho to membranes is saturable and competeable by unmodified Rho, as shown in the membrane binding assay. The subfractionation experiments showed that the majority of cellular Rho but not the complete amount of glucosylated Rho translocated to the membranes. This finding also argues for the existence of limited binding sites at the cytosolic face of the membranes. These binding sites may be also shared with Rac, an additional reason for the partial membrane binding of glucosylated Rho. Furthermore, the binding takes place at a membrane protein with a molecular mass of 70 kDa as shown in the overlay experiments. The binding to p70 is also competed by unmodified Rho. Finally, the native
structure of glucosylated Rho is essential because denaturation by removal of the Mg\(^{2+}\) ion completely abolishes membrane interaction. These data argue for a specific binding to a membrane protein, which is mediated rather by a protein-protein interaction than by a nonspecific lipid-lipid interaction through the isoprenyl moiety. Glucosylated Rho is unresponsive to GAP and should therefore be trapped in the GTP-bound form, and GTP-bound glucosylated Rho should bind to membranes as unmodified does. However, the experimental data, i.e. the membrane binding and the overlay assay, clearly show that the binding of modified Rho is almost independent of the nucleotide-bound state. Thus, it is conceivable that glucosylated Rho does bind through a mechanism involving the glucose moiety to p70, a mechanism that is different from the binding of unmodified Rho.

Because recombinant glucosylated RhoA in its native form binds to p70, the glucose seems to change properties of RhoA. A direct involvement of the glucose moiety at threonine 37 in binding is very unlikely. The effector loop seems not to be involved in membrane binding because membrane-bound Rho interacts with its effector proteins without being released from the membranes.

The membranous p70 is not a selective binding site for toxin-modified Rho. Normal cellular Rho in its GTP-bound form also binds to p70. However, p70 seems to be not an effector protein for Rho because glucosylated Rho does bind. Recently, saturable high affinity binding of GTP-bound Rho to membrane preparations from erythrocytes has been reported (50). It is tempting to speculate that the reported saturable binding is mediated through p70. p70 may be a multifunctional adapter protein.

The acceptor amino acid of glucosylation, threonine 37, resides in the effector domain. However, it becomes more and more clear that there are several domains that participate in the communication with the effector proteins. Recently, it has been reported that Rho possesses accessory domains that determine the effector specificity of Rho (51, 52). One class (citrkon) solely interacts with the classical effector domain covering amino acids 23–40, whereas the class of ROCK (Rho-associated coiled-coiled-containing protein kinase) and the class of rhophilin bind to two distinct regions, amino acids 23–40 (switch I) and amino acids 75–92. Switch I seems to be indispensable for Rho effector coupling, and therefore glucosylation at Thr-37 blocks communication with every Rho effector.

Based on the data of the present study, we present a refined model on the functional consequences of glucosylation of the Rho GTPases by C. difficile toxins A and B (Fig. 6). Glucosylation at threonine 37/35 slows down activation by GEFs but completely inhibits downstream signaling of GTP-bound Rho. Glucosylated Rho binds to a membranous binding site but cannot be released from the membranes by GDI-1 as unmodified Rho is, because of failure to interact with GAP and to bind to GDI-1. Glucosylated Rho then accumulates at the membranes and prevents translocation of unmodified Rho to the membranes for signaling. Thus, glucosylation results in two functional consequences, inhibition of effector coupling and of the cycling of the Rho GTPases from cytosol to membranes and vice versa. The inhibition of the subcellular cycling may explain why glucosylated Rho is dominant negative in intact cells (39). Glucosylated Rho occupies the membrane binding sites and inhibits membrane translocation of unmodified cellular Rho.

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REFERENCES
1. Machesky, L. M., and Hall, A. (1996) Trends Cell Biol. 6, 304–310
2. Narumiya, S. (1996) J. Biochem. (Tokyo) 120, 215–228
