Monoglucosylation of low molecular mass GTPases is an important post-translational modification by which microbes interfere with eukaryotic cell signaling. Ha-Ras is monoglucosylated at effector domain amino acid threonine 35 by Clostridium sordellii lethal toxin, resulting in a blockade of the downstream mitogen-activated protein kinase cascade. To understand the molecular consequences of this modification, effects of glucosylation on each step of the GTPase cycle of Ras were analyzed. Whereas nucleotide binding was not significantly altered, intrinsic GTPase activity was markedly decreased, and GTPase stimulation by the GTPase-activating protein p120GAP and neurofibromin NF-1 was completely blocked, caused by failure to bind to glucosylated Ras. Guanine nucleotide exchange factor (Cdc25)-catalyzed GTP loading was decreased, but not completely inhibited. A dominant-negative property of modified Ras to sequester exchange factor was not detectable. However, the crucial step in downstream signaling, Ras-effector coupling, was completely blocked. The $K_d$ for the interaction between Ras-GTP and the Ras-binding domain of Raf was 15 nM, whereas glucosylation increased the $K_d$ to >1 mM. Because the affinity of Ras-GDP for Raf ($K_d = 22 \mu M$) is too low to allow functional interaction, a glucose moiety at threonine 35 of Ras seems to block completely the interaction with Raf. The net effect of lethal toxin-catalyzed glucosylation of Ras is the complete blockade of Ras downstream signaling.

* Clostridium difficile toxins A and B are protein toxins with molecular masses of 308 and 270 kDa, respectively, and are the causative agents of antibiotic-associated pseudomembranous colitis (1–3). In cell monolayers, the toxins are cytotoxic, causing preferential destruction of the microfilament system (1, 4). Both toxins have been recently identified as glucosyltransferases that selectively modify the Rho subfamily proteins Rho, Rac, and Cdc42, but not prototypes of other Ras subfamilies such as Ras, Rab, and Ran (5–7). Glucosylation occurs at threonine 37 in Rho and threonine 35 in Rac and Cdc42, which are located in the effector region of the GTPases. Microinjection of glucosylated Rho freed of toxin induces morphological and cytoskeletal alterations that are identical to those induced by the toxin itself, indicating that the glucose moiety in the effector region grossly changes the properties of Rho to act in a dominant-negative manner (5). Thus, glucosylation of Rho seems to be the molecular mechanism through which at least toxin B acts cytotoxically on cells.

The lethal toxin from Clostridium sordellii, which belongs to the same family of large clostridial cytotoxins as do toxins A and B, is causally involved in diarrhea and enterotoxemia in domestic animals and in gas gangrene in man (8). Lethal toxin has also been identified as a glucosyltransferase that, however, exhibits a different protein substrate specificity. Whereas the substrate specificity of toxin A/B is confined to the Rho subfamily proteins, isoforms of lethal toxin glucosylate both Rho and Ras subfamily proteins; Rac from the Rho subfamily and Ras, Raf, and Rap from the Ras subfamily are substrates of lethal toxin (9–11). Ras is glucosylated at threonine 35 (10), which is equivalent to threonine 37 in Rho. Glucosylation renders cellular Ras inactive, resulting in a blockade of growth factor-stimulated p42/44 mitogen-activated protein kinase (9, 10).

Ras proteins are essential for the regulation of cell differentiation and proliferation (12–14). They are involved in the transduction of extracellular signals to the nucleus. The active GTP-bound state is promoted by guanine nucleotide exchange factors such as SOS and Cdc25 that allow interaction with their effectors. The best studied effector is the Raf kinase, which is translocated to the membrane by interaction with Ras-GTP. Activated Raf initiates a linear downstream kinase cascade in which Erk kinase (p42/44 kinase) eventually leads to activation of transcription factors. In addition to Raf kinase, phosphatidylinositol 3-kinase, p120GAP, neurofibromin, MEKK-1, protein kinase Cζ, and the Ras GEFs are putative effectors (15). The active state of Ras is terminated by GTPase-activating proteins (GAPs) that interact with the GTP-bound form of Ras to stimulate severalfold the intrinsic GTP-hydrolyzing activity (16, 17). p120GAP, neurofibromin and GAP1 are have been reported to exhibit GAP activity for Ras (18).

We report here the functional consequences of glucosylation of the effector domain of Ras on guanine nucleotide release, on the GAP-induced stimulation of Ras GTPase activity, and on the coupling to the effector Raf kinase. The study of each step of the GTPase cycle gives evidence that glucosylation blocks Ras-effector coupling, resulting in complete inhibition of Ras downstream signaling. This inhibition seems to be the basis of the molecular mode of action of lethal toxin from C. sordellii.

EXPERIMENTAL PROCEDURES

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GST, glutathione S-transferase; RBD, Ras-binding domain; GTPγS, guanosine 5′-O-(3-thiotriphosphate); HPLC, high pressure liquid chromatography; Gpp(NH)p, guanosine 5′-β,γ-imidotriphosphate; mant, N-methylanthraniloyl; GDI, guanine nucleotide dissociation inhibitor.

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Monoglucosylation of Ras

6018 as described (33). Preparation of recombinant proteins was from Escherichia coli expression systems. Full-length Ha-Ras (34), the catalytic domain of p120Gal (GAP-334) covering residues 714–1047 (16), the GST fusion protein with the catalytic domain of neurofibromin NF1-333 (GST-NF1-333) covering residues 1198–1530 (35), the Ras-binding domain (RBD) of Raf covering residues 51–131 (20), the catalytic domain of the mammalian exchange factor Cdc25 (Cdc25-GEFcat) covering residues 976–1260 (36), and the RBD of the Raf GEF (22) were prepared as described.

Glucosylation Reaction—For the time course, Ha-Ras (2 μM) previously loaded with GDP or GTPyS was incubated with lethal toxin (3 nM) in a buffer containing 30 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 2 mM dithioerythritol at 37 °C for 3 h. The control was treated in the same atmosphere for 10 min. The samples were then immobilized on glutathione-Sepharose beads. Protein concentration was determined and then adjusted. GDP release from Ha-Ras (200 nM) was removed by gel filtration using elution buffer. Protein concentration was determined and then adjusted. Dissociation rate constants (k off) were obtained by fitting single exponentials to the data. The constants were fitted according to Ref. 21 to yield k off. The same experiments were performed with the Raf GEF (16 μM) instead of the Raf RBD and with NF1-333 (5 μM). To test competition between glucosylated and control Ras, Ras-mant-Gpp(NH)p (50 nM) binding to the Raf RBD (100 nM) was measured in the presence of 850 nM control Ras or 2500 nM glucosylated Ras, each loaded with unlabeled Gpp(NH)p.

RESULTS

C. sordellii lethal toxin O-glucosylated Ha-Ras in the presence of the cosubstrate UDP-glucose. In the GDP-bound form, incorporation was 0.94 mol of glucose/mol of Ras, consistent with a monoglucosylation type of reaction (Fig. 1). Loading of Ras with the nonhydrolyzable GTP analogue GTPyS abolished modification almost completely, indicating that the hydroxyl group of the acceptor amino acid threonine 35 is involved in binding GTP. Therefore, we tested whether the glucose moiety at threonine 35 alters nucleotide binding, exchange, hydrolysis, and interaction with Ras regulatory proteins.

Guanine Nucleotide Exchange—N-Methylanthraniloyl (mant)-labeled guanine nucleotides were used to determine GDP and GTP dissociation from Ras. Dissociation of mant-GDP/GTP from Ras caused a decrease in mant fluorescence, which was monitored in a Perkin-Elmer LS50 fluorescence spectrometer; emission was monitored at 442 nm with excitation at 366 nm. Guanine nucleotide exchange was determined according to Lenzen et al. (38).

Ha-Ras (control and glucosylated) was incubated with 2.5 mM mant-GDP in the presence of 10 mM EDTA on ice for 2 h. Unbound mant-GDP was removed by gel filtration using elution buffer. Protein concentration was determined and then adjusted. GDP release from Ha-Ras (200 nM) was induced by addition of 100 μM unlabeled GDP in the absence and presence of Cdc25-GEFcat (50 and 100 nM) at 20 °C. The time course of the decrease in fluorescence was monitored. Dissociation rate constants (k off, min⁻¹) were calculated by single exponential fit. To test whether glucosylated Ras sequesters Cdc25, Cdc25-GEFcat (50 nM)–stimulated mant-GDP release from control Ras (100 nM) was determined in the presence of 10 μM control Ras or 10 μM glucosylated Ras, each loaded with unlabeled GDP.

Fig. 1. Time course and nucleotide dependence of the glucosylation reaction of Ha-Ras. Ha-Ras (2 μM) was loaded with either GDP (○) or GTPyS (△), followed by ³⁵C-glucosylation reaction as described under “Experimental Procedures.” At the indicated time points, samples were taken and run on 12.5% SDS-polyacrylamide gel. The amount of glucose incorporated (mol/mol of Ras) was calculated from PhosphorImager (Molecular Dynamics, Inc.).

For all other experiments, Ha-Ras (400 μM) was incubated with lethal toxin (60 nM) in a buffer containing 30 mM Tris-HCl (pH 7.4), 4 mM UDP-glucose, 0.5 mM MnCl₂, 1.5 mM MgCl₂, 100 mM KCl, and 0.5 mM dithioerythritol at 37 °C for 3 h. The control was treated in the same way, but in the absence of lethal toxin. To test the amount of glucosylation, samples were diluted and then glucosylated in the presence of UDP-[¹⁴C]glucose as described above. Previously glucosylated Ras showed no incorporation of [¹⁴C]glucose, indicating complete modification in the first reaction. In addition, samples were analyzed by electrospray mass spectrometry (Finnigan) to prove complete glucosylation (162-Da increase in mass).

Intrinsic GTPase Activity—Ha-Ras (control and glucosylated) was incubated with 50 mM GTP in the presence of 10 mM EDTA on ice for 2 h. Unbound GTP was removed by gel filtration using elution buffer containing 30 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 2 mM dithioerythritol. Protein concentration was determined and then adjusted. Ha-Ras (100 μM) was incubated at 37 °C. At the indicated time points (see Fig. 3), samples were taken and subjected to HPLC (C₁₈ column) to separate GDP and GTP (37). The ratio of the integrated areas of GTP to those of GTP plus GDP gives the extent of GTP hydrolysis. For determination of the GAP-stimulated GTPase activity, GAP-334 or NF1-333 was added to the assay described above. For control Ras, 3.8 nM GAP or 9 nM NF1-333 was used, and for glucosylated Ras, 15 μM GAP or 9.1 μM NF1-333 was used.

Binding of Glucosylated Ras to the Catalytic Domain of Neurofibromin NF-1 (GST-NF1-333)—Control Ras and glucosylated Ras (2.7 μM; both loaded with Gpp(NH)p) were incubated with 1.3 μM GST and GST-NF1-333, respectively, in binding buffer containing 30 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, and 2 mM dithioerythritol at room temperature for 10 min. The samples were then immobilized on glutathione-Sepharose beads. After washing the beads four times with binding buffer, the proteins were eluted by incubation with 20 mM glutathione dissolved in binding buffer on ice for 5 min. Eluted proteins were separated by 15% SDS-polyacrylamide gel electrophoresis.

Guanine Nucleotide Exchange—N-Methylanthraniloyl (mant)-labeled guanine nucleotides were used to determine GDP and GTP dissociation from Ras. Dissociation of mant-GDP/GTP from Ras caused a decrease in mant fluorescence, which was monitored in a Perkin-Elmer LS50 fluorescence spectrometer; emission was monitored at 442 nm with excitation at 366 nm. Guanine nucleotide exchange was determined according to Lenzen et al. (38).

Ha-Ras (control and glucosylated) was incubated with 2.5 mM mant-GDP in the presence of 10 mM EDTA on ice for 2 h. Unbound mant-GDP was removed by gel filtration using elution buffer. Protein concentration was determined and then adjusted. GDP release from Ha-Ras (200 nM) was induced by addition of 100 μM unlabeled GDP in the absence and presence of Cdc25-GEFcat (50 and 100 nM) at 20 °C. The time course of the decrease in fluorescence was monitored. Dissociation rate constants (h off, min⁻¹) were calculated by single exponential fit. To test whether glucosylated Ras sequesters Cdc25, Cdc25-GEFcat (50 nM)—stimulated mant-GDP release from control Ras (100 nM) was determined in the presence of 10 μM control Ras or 10 μM glucosylated Ras, each loaded with unlabeled GDP.

Measurement of Raf RBD Inhibition of Nucleotide Exchange—Ha-Ras (control and glucosylated; 55 μM) was incubated with 2 mM mant-Gpp(NH)p in the presence of 2 units/ml alkaline phosphatase, 200 mM (NH₄)₂SO₄, and 100 mM ZnCl₂ on ice for 2 h. Unbound mant-Gpp(NH)p was removed by gel filtration using elution buffer. Protein concentration was determined and then adjusted. Dissociation rate constants (h off, μM⁻¹ min⁻¹) were calculated by single exponential fit. To test whether glucosylated Ras inhibited binding, exchange, and interaction with Ras regulatory proteins.

Guanine Nucleotide Exchange of Ha-Ras—Guanine nucleotide exchange of Ras was followed by changes in fluorescence resulting from the release of bound fluorophore-labeled nucleotide mant-GDP. To this end, glucosylated Ras was loaded with mant-GDP, followed by removal of unbound mant-GDP; exchange was started by addition of a surplus of unlabeled GDP. Fig. 2A shows the nucleotide exchange of glucosylated versus unmodified Ras. The calculated k off values are 0.00010 s⁻¹ for glucosylated Ras and 0.00002 s⁻¹ for control Ras. Thus, glucosylation caused only an ~5-fold enhancement of GDP release. GEF-stimulated GDP release was studied with the catalytic domain of the exchange factor Cdc25 (Cdc25-GEFcat). Compared with unmodified Ras, the exchange rate of glucosylated Ras was stimulated 10 times less (Fig. 2A). The same decrease was found when the concentration of Cdc25-GEFcat was doubled (100 nM) at a constant Ras concentration (200 nM). Whereas Cdc25-GEFcat–stimulated exchange of control Ras by a factor of 144, modified Ras was stimulated only by a factor of 12. Thus, glucosylation of Ras resulted in an increase in unstimulated GDP release, but a decrease in GEF-stimulated exchange. To test the possibility of Cdc25 sequestration by modified Ras, we studied whether glucosylated Ras was able to block Cdc25-stimulated exchange of control Ras. To this end, Cdc25-GEFcat (50 nM)–stimulated exchange of control Ras–mant-GDP (100 nM) was determined in the presence of a
employed the catalytic domain of p120GAP (GAP-334) and that GTPase activity of Ras was potently stimulated by GAPs. We half-time up to 128 min (200 nM) loaded with mant-GDP. Release of mant-GDP from control (A, not exhibit this dominant-negative property. Glucosylated Ras does ever, sequestration of Cdc25 resulting in blocked exchange higher affinity than unmodified Ras to sequester Cdc25. How- illustrates that glucosylated Ras acted like unmodified Ras, of the affinity (Kd) is based on the so-called GDI effect of Raf. Raf binding to Ras blocks GTP release from Ras. The dissociation constant (Kd) of the Ras-Raf complex can be calculated from the correlation of Raf concentration to inhibition of GTP release from Ras (20, 21). Control and glucosylated Ras were loaded with the mant-labeled nonhydrolyzable GTP analogue Gpp(NH)p to prevent nucleotide hydrolysis. Release of mant-Gpp(NH)p in the absence and presence of the Raf RBD was induced by addition of unlabeled GTP, and the time course of of neurofibromin NF-1 (NF1-333). At a concentration of 3.8 nM GAP-334, the Ras GTPase was enhanced by a factor of 50,000 as depicted in Fig. 3 and Table I; NF1-333 (9.2 nM) was less stimulatory. However, both GAP-334 and NF1-333 were not able to enhance glucosylated Ras GTPase (Fig. 3); even 1000-fold higher concentrations than used for control Ras (15 µM GAP-334 and 9.2 µM NF1-333) did not enhance GTPase activity. Mere reduction, but not full inhibition, of the intrinsic GTPase is actually not caused by residual unmodified Ras; the complete inhibition of GAP stimulation argues against remnant effects, and furthermore, differential glucosylation and mass spectrometric analyses clearly showed complete modification. Thus, glycosylation of Thr-35 resulted in a decrease in GTPase activity to 20% and in unresponsiveness to GAP stimulation. To test whether this unresponsiveness is caused by inhibition of Ras-GAP interaction by the glucose moiety, coprecipitation assays were performed. Because NF1-333 exhibited higher affinity for Ras than GAP-334, GST-NF1-333 fusion protein was employed. Control and glucosylated Ras both loaded with Gpp(NH)p were incubated with GST-NF1-333 immobilized on Sepharose beads, followed by release of NF-1 and NF-1-Ras complexes, respectively, with glutathione. Fig. 4 shows the SDS-polyacrylamide gel electrophoresis of eluted proteins. Whereas NF-1 bound to Ras, glucosylated Ras had lost this property. This finding was corroborated by a different approach, namely the GDI effect of NF-1 as described below. The glucose moiety in Thr-35 blocks NF-1-Ras interaction, thus preventing the stimulation of the GTPase activity.

Interaction of Glucosylated Ha-Ras with the Raf Kinase—Downstream signaling of Ras is based on the physical interaction of GTP-bound Ras with the Raf kinase to translocate Raf to the plasma membrane. This Ras-effector coupling can be characterized by the affinity of the Ras-Raf complex. Determination of the affinity (Kd) is based on the so-called GDI effect of Raf. Raf binding to Ras blocks GTP release from Ras. The dissociation constant (Kd) of the Ras-Raf complex can be calculated from the correlation of Raf concentration to inhibition of GTP release from Ras (20, 21). Control and glucosylated Ras were loaded with the mant-labeled nonhydrolyzable GTP analogue Gpp(NH)p to prevent nucleotide hydrolysis. Release of mant-Gpp(NH)p in the absence and presence of the Raf RBD was induced by addition of unlabeled GTP, and the time course of

FIG. 2. Influence of glucosylation on guanine nucleotide exchange. A, basal and Cdc25-GEFcat-stimulated exchange of Ha-Ras (200 nM) loaded with mant-GDP. Release of mant-GDP from control (●, □) and glucosylated (●, ○) Ras was induced by addition of 100 µM unlabelled GDP (C, □) and by addition of GDP plus exchange factor Cdc25-GEFcat (50 nM) (●, ●), respectively. Decrease in fluorescence (arbitrary units (a. u.)) was temporally monitored at 20 °C. B, competition of glucosylated Ras with control Ras. Cdc25 (50 nM)-induced GDP exchange of Ras (100 nM) loaded with mant-GDP was performed in the absence (□) or presence of either 10 µM control Ras (●) or 10 µM glucosylated Ras (○), both loaded with unlabelled GDP. Basal GDP exchange of Ras (100 nM) in the absence of Cdc25 (○) is also shown.

FIG. 3. Intrinsic and GAP-stimulated GTPase activities. Control (●, ○) and glucosylated (□, ○) Ras were loaded with GTP and then incubated at 37 °C. At the indicated time points, samples were subjected to reversed-phase HPLC to determine the amounts of GTP and GDP. ● and □, intrinsic GTPase. For determination of the GAP-stimulated GTPase activity, the catalytic domain of p120GAP (GAP-334) or NF-1 (NF1-333) was added. 3.8 nM GAP-334 (○) or 9.2 nM NF1-333 (○) to 100 µM control Ras and 15 µM GAP-334 (□) or 9.2 µM NF1-333 (□) to 100 µM glucosylated Ras.
TABLE I

Parameters measured for unmodified and glycosylated Ras

| Affinity for Raf RBD, Kd | Nucleotide dissociation | GTPase |
|--------------------------|-------------------------|--------|
|                          | k<sub>d</sub> mant-GTP | mant-GDP | Cdc25-stimulated | Intrinsic, k<sub>d</sub> | GAP-stimulated |
| Ras                      | 15 nM                   | 0.00071 | 0.00002 | 144 | 0.028 | 50,000 |
| Glucosylated Ras         | >1 mM                   | 0.00170 | 0.00010 | 12  | 0.0054 | No stimulation |

FIG. 4. Binding of glucosylated Ras to the catalytic domain of NF-1. Control (cont-Ras) and glucosylated (gluc-Ras) Ras, both loaded with Gpp(NH)p, were incubated with the catalytic domain of NF-1 (GST-NF1-333) or with GST as a control, both immobilized on glutathione-Sepharose beads, at room temperature for 10 min. After washing, the proteins were eluted by glutathione and analyzed by 15% SDS-polyacrylamide gel electrophoresis (Coomassie Blue-stained gel shown). M, molecular mass markers. Ras (second lane) was used as a control. Binding of control Ras to GST-NF1-333 was ~80%.

The K<sub>d</sub> for the Ras complex with the Raf RBD and the k<sub>d</sub> value for mant-GTP were calculated from the measurement of the Ras-Raf interaction; the k<sub>d</sub> value for mant-GDP was calculated from the data of the exchange experiments. Calculation was performed as described under “Experimental Procedures.”

The k<sub>d</sub> value of 15 nM reflects complete abstraction of mant-Gpp(NH)p from Ras.

Nucleotide exchange of 0.1 μM control Ras (k<sub>d</sub> = 0.00075 min<sup>-1</sup>) was decreased by 5 μM NF1-333 (k<sub>d</sub> = 0.00016 min<sup>-1</sup>), whereas glucosylated Ras (k<sub>d</sub> = 0.00147 min<sup>-1</sup>) showed no significant decrease.
change in the $k_{off}$ in the presence of NF1-333 (0.00153 min$^{-1}$).

Nucleotide Release—From the exchange experiments based on GDP loading and the experiments of Ras-Raf interaction based on GTP release, no major difference in nucleotide release was detected between modified and unmodified Ras (Table I). These findings reflect the integrity of the protein structure of Ras and clearly argue against degradation as a basis for the effects of glucosylation on the GTPase cycle.

**DISCUSSION**

Low molecular mass GTPases of the Ras superfamily are important regulators of intracellular signal transduction, and these cellular functions seem to predispose them to be preferred targets for bacterial protein toxins. Whereas the C3-like exoenzymes catalyze ADP-ribosylation of RhoA, RhoB, and RhoC at Asn-41, C. difficile toxins A and B monoglucosylate the members of the Rho subfamily, Rho, Rac, and Cdc42, at threonine 37/35, respectively. These GT-Pases are involved in the regulation of different aspects of the actin cytoskeleton, and modification renders them inactive (5, 6, 24, 25). C. sordellii lethal toxin, which belongs to the same family as C. difficile toxins, monoglucosylates Rac, Ras, Rap, and Rap proteins, thus covering two subfamilies of small GTPases as targets (9, 10).

The glucosylated amino acid in Ras is threonine 35 (10). As can be deduced from the crystal structure of Ha-Ras, the hydroxyl moiety of threonine 35, together with serine 17, is involved in the coordination of Mg$^{2+}$, which ligates the $\gamma$- and $\beta$-phosphates of GTP. Thus, the hydroxyl group of Thr-35 is not assessable for modification (26, 27). In the GDP-bound form, loop-2 moves and threonine 35 is exposed to the surface of the molecule. Furthermore, threonine 35 is important for coordination of the Mg$^{2+}$ ion and the positioning of the nucleophilic water molecule in the GTP conformation (28). These structural considerations imply that Ras-GDP rather than Ras-GTP should be the preferred substrate for glucosylation. This notion is supported by experimental data: inactive GDP-bound Ras is monoglucosylated, whereas active Ras stabilized by binding to nonhydrolyzable GTP$\gamma$S prevents glucosylation. Thus, this type of toxin-catalyzed modification is strictly dependent on the conformation of the target protein Ras. Although the bound phosphate significantly affects the glucosylation reaction, the glucosylation of Ras does not grossly alter binding of GDP of GTP (Table I). Nucleotide dissociation is only slightly increased by glucosylation; the $k_{off}$ for GTP dissociation is doubled, and that for GDP is increased $\sim$5-fold. In contrast to the intrinsic nucleotide dissociation, GEF-stimulated GDP exchange is decreased by glucosylation. This effect has been proven not to be based on GEF sequestration by modified Ras, thus excluding any increase in binding affinity of modified Ras for Cdc25. Mediation of the nucleotide exchange by GEFs (Cdc25) is not fully understood. Most likely, the GEFs induce a conformational change through binding to Ras, which then facilitates nucleotide release. The decreased exchange activity toward glucosylated Ras may be caused by the inability of modified Ras to perform the GEF-induced conformational change or, alternatively, by reduced binding affinity for Cdc25. However, the latter possibility cannot be easily tested because of the low affinity interaction of Cdc25 with Ras. Nevertheless, GEF-Ras interaction is qualitatively different from that of GAP and effector proteins with Ras because glucosylation modulates Cdc25 activity, but completely blocks GAP activity and the interaction with effectors.

Glucosylation reduces the intrinsic GTPase activity of Ras to 20%, but obviously does not cause full blockade. Complete inhibition, however, is observed for stimulation of GTP hydrol-

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2 R. Cool, personal communication.
The observed effects of glucosylation on divers Ras functions have been also found for glucosylated Rho and Rac GTPases that are modified by \textit{C. difficile} toxins A and B (39). Thus, the functional consequences of a glucose group in the effector region seem to be identical for the homologous low molecular mass GTPases.

In conclusion, glucosylation of the Ras effector region by bacterial exotoxins causes several effects on the Ras GTPase. Whereas nucleotide binding is not grossly altered, intrinsic GTPase activity is markedly decreased, and GAP stimulation is completely blocked, findings that indicate activation of Ras rather than inactivation. Guanine nucleotide exchange factor-catalyzed GTP loading is decreased, but not inhibited. However, the crucial step in downstream signaling, Ras-effector coupling, is completely blocked, and this functional consequence finally determines the net effect of glucosylation on the GTPase cycle, i.e. the blockade of Ras signal transduction.

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