Glucosylation and ADP Ribosylation of Rho Proteins: Effects on Nucleotide Binding, GTPase Activity, and Effector Coupling†

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ABSTRACT: We studied the effects of glucosylation of RhoA, Rac1, and Cdc42 at threonine-35 and -37 by *Clostridium difficile* toxin B on nucleotide binding, GTPase activity, and effector coupling and compared these results with the ADP ribosylation of RhoA at asparagine-41 catalyzed by *Clostridium botulinum* C3 transferase. Whereas glucosylation and ADP ribosylation had no major effects on GDP release from RhoA, Rac1, and Cdc42, the rate of GTPγS release from Rho proteins was increased 3−6-fold by glucosylation. ADP ribosylation decreased the rate of GTPγS release by about 50%. Glucosylation reduced the intrinsic activities of the GTPases by 3−7-fold and completely blocked GTPase stimulation by RhoGAP. In contrast, ADP ribosylation slightly increased GTPase activity (∼2-fold) and had no major effect on GAP stimulation of GTPase. Whereas ADP ribosylation did not affect the interaction of RhoA with the binding domain of protein kinase N, glucosylation inhibited this interaction. Glucosylation of Rac1 markedly diminished its ability to support the activation of the superoxide-generating NADPH oxidase of phagocytes. Glucosylated Rac1 did not interfere with NADPH oxidase activation by unmodified Rac1, even when present in marked molar excess, indicating that it was incapable of competing for a common effector. The data indicate that the functional inactivation of small GTPases by glucosylation is mainly caused by inhibition of GTPase−effector protein interaction.

Rho subfamily proteins (Rho, Rac, and Cdc42) are low-molecular mass GTPases that regulate the actin cytoskeleton (*1*, *2*) and several signaling pathways (*3*−*6*). The Rho subtype proteins RhoA, -B, and -C control formation of stress fibers and adhesion complexes. They are involved in regulation of phosphoinositide-3-kinase (*7*), phosphatidylinositol-4-phosphate-5-kinase (*8*), phospholipase D (*9*), smooth muscle contraction (*10*), cell−cell contact (*11*), endocytosis (*12*), and transcriptional activation (*13*, *14*). The Rac proteins (Rac1 and Rac2) participate in membrane ruffling and formation of lamellipodia induced by growth factors (*1*, *15*) and may play a role in transcriptional activation (*14*, *16*). Moreover, Rac proteins were shown to regulate NADPH oxidase in some cell types (*17*, *18*). A third member of the Rho subfamily, Cdc42 (at least two mammalian isoforms exist), participates in receptor-induced formation of microspikes and filopodia (*19*, *20*). In addition to participating in the cortical actin filament system, Cdc42 may be also involved in various signaling processes, including mast cell activation via the antigen receptor (*21*) and activation of transcription factors (*14*, *16*).

Several potential effectors of Rho subfamily proteins have been reported (*3*−*6*). Some of these effectors are lipid kinases as described above. Others are protein kinases such as the Rho kinase (*22*, *23*), protein kinase N (PKN)[1] (*24*, *25*), and p21-activated kinases (PAK) (*26*). Another group of effectors appear to be function as adapter proteins such as WASP (*27*) and p67^{phox} (*28*). The latter adapter protein interacts with Rac to activate the multicomponent system of the NADPH oxidase.

Like other GTP-binding proteins, Rho subfamily proteins are regulated by a GTPase cycle. They are active in the GTP-bound form and inactive when GDP is bound. The active state is terminated by hydrolysis of bound GTP caused by stimulation of the intrinsic GTPase activity. Basal GTPase activity is rather low, but several GTPase-activating proteins (GAP proteins) (*29*) were shown to be involved in turning off the GTP-binding proteins by causing severalfold stimulation of GTP hydrolysis. The prototype of the family of GTPase-activating proteins is RhoGAP, a 50 kDa protein (*30*). Other members of this GAP family are p190, myr5, bcr, chimaerin, and others (for review, see ref *29*).

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[1] Abbreviations: HPLC, high-performance liquid chromatography; ECL, enhanced chemiluminescence; GTPγS, guanosine 5′-(γ-thiotriphosphate); mant-GDP, 2′(3′)-*O*-(*N*-methylanthraniloyl)guanosine 5′-diphosphate; GAP, GTPase-activating protein; PKN, protein kinase N.

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Rho subfamily proteins are selective substrates for various groups of bacterial toxins (*31*). C3-like toxins [e.g. *Clostridium botulinum* exoenzyme C3 (*32*), *Clostridium limosum* transferase (*33*), and EDIN (*34*)] ADP ribosylate RhoA, -B, and -C at asparagine-41 (*35*), thereby inactivating the GTP-binding proteins. Rac and Cdc42 are poorly or not at all modified by these transferases (*33*). Recently, it has been reported that Rho, Rac, and Cdc42 are the eukaryotic targets for *Clostridium difficile* toxins A and B (*36*, *37*). These toxins, which are major virulence factors for induction of antibiotic-associated diarrhea and pseudomembranous colitis (*38*−*41*), belong to a family of "large" clostridial toxins having molecular masses of 270 (toxin B) and 308 kDa (toxin A), respectively. These toxins possess glucosyltransferase activity and modify the GTPases by using UDPglucose as a cosubstrate. RhoA is glucosylated at threonine-37, and Rac1 and Cdc42 are glucosylated at the homologous amino acid threonine-35, thereby inactivating the GTP-binding protein (*36*). The threonine residue at position 37 of Rho (position 35 of Rac or Cdc42) is located in the so-called effector region and extremely conserved among all small GTPases. This amino acid residue participates in coordination of the bound magnesium ion and thereby plays a pivotal role in nucleotide binding (*42*). Therefore, we studied the consequences of glucosylation of Rho subfamily proteins on nucleotide binding, GTPase activity, and their interaction with effector proteins and compared these results with the functional consequences of toxin-induced ADP ribosylation.

EXPERIMENTAL PROCEDURES

Materials. Mant-GDP [2′(3′)-*O*-(*N*-methylanthraniloyl)-guanosine 5′-diphosphate] was synthesized, purified, and characterized as previously described (*43*). Mant-GDP was analyzed on a Nucleosil-NH_2 HPLC column from Macherey & Nagel (D-52313 Düren, Germany) by an isocratic elution with 85% sodium phosphate (0.4 M, pH 5.1)/15% methanol. *C. difficile* toxin B and *C. botulinum* C3 transferase were purified as described (*44*, *45*). All other proteins used were expressed as a glutathione transferase fusion protein in pGEX2T in *Escherichia coli* (TG1) and purified by affinity chromatography on glutathione−Sepharose. The Cdc42 plasmid was a gift from M. Aepfelbacher (Munich, Germany); the RhoA ΔCAAX plasmid was from G. Rex (Freiburg, Germany), and Rac1, RhoA, and p30-GAP plasmids were from A. Hall (London, U.K.). From the C-terminal deleted RhoA ΔCAAX, we produced much more protein in the preparations, so all experiments described for RhoA were performed with RhoA ΔCAAX. We proved that the investigated biochemical properties of RhoA are not affected by the deletion of the last four C-terminal amino acids. From the protein kinase N (PKN), only the NH_2 terminus (amino acids 7−155) was expressed, which is responsible for binding RhoA (*46*). The plasmid was a gift from K. Kaibuchi (Ikoma, Japan). Baculoviruses carrying cDNA for $p47^{phox}$ and $p67^{phox}$ were gifts from T. L. Leto (NIH, Bethesda, MD). The GST-Rho subfamily proteins were cleaved with thrombin to separate the GTPase from the glutathione transferase and finally purified by gel filtration on a Superdex 75 10/30 column from Pharmacia with buffer C [10 mM triethanolamine/HCl (pH 7.5), 150 mM NaCl, and 2.5 mM $MgCl_2$]. The purity of the proteins (>95%) was checked by SDS−PAGE, and concentrations of the proteins were determined according to Bradford (*47*) using serum albumin as the standard.

Glucosylation of the Rho Proteins. Glucosylation of the Rho subfamily proteins (∼10 μM) was performed for 45 min at 37 °C in a buffer containing 2.5 mM EGTA, 1 mM $MnCl_2$, 2.5 mM $MgCl_2$, 100 mM KCl, 100 μM UDPglucose, and 16 nM toxin B essentially as described (*36*). Thereafter, the reaction mixture was applied to a Superdex 75 10/30 column to separate the GTPases from toxin B and to bring the protein into buffer C. Complete glucosylation of GTPases was checked by a second glucosylation reaction in the presence of UDP[^{14}C]glucose followed by SDS−PAGE and phosphorimaging.

ADP Ribosylation of RhoA. RhoA was ADP ribosylated for 45 min at 37 °C in buffer C supplemented with 100 μM NAD^+ and 20 nM C3 toxin. Modification of RhoA was checked by the shift of the RhoA band in nondenaturating gel electrophoresis caused by ADP ribosylation (*48*).

Assay of GTPase Activity. For RhoA, the GTPase activity was determined by a HPLC assay. RhoA was loaded with nonradioactive GTP as described above. To separate free nucleotides, the reaction mixture was gel filtered with buffer C on a Superdex 75 10/30 column at 4 °C. The hydrolysis of RhoA-bound GTP was determined at 30 °C. Two hundred microliters of the RhoA solution was added to 25 μL of denaturing buffer (1 M sodium phosphate at pH 1.66) and the mixture incubated for 3 min on ice. The denatured Rho protein was sedimented by centrifugation (3 min at 14000*g*) and the supernatant applied to a Nucleosil-NH_2 column. The nucleotides were eluted isocratically with 0.4 mM sodium phosphate at pH 5.1 and detected by UV absorption at 252 nm. GTP was quantified as the relation of the peak area of GTP to the sum of GTP and GDP areas (both nucleotides have the same absorption coefficient at 252 nm of 13 700 M^{-1} cm^{-1}).

The GTPase activity of Rac1 and Cdc42 was determined in a [γ-^{32}P]GTP hydrolysis assay. [γ-^{32}P]GTP−Rac1 or [γ-^{32}P]GTP−Cdc42 was formed by incubation of 10 μM Rac1 and Cdc42 with 100 μM [γ-^{32}P]GTP and 10 mM EDTA in buffer C for 10 min at 4 °C. Then the free Mg^{2+} concentration was adjusted to 2.5 mM, and free nucleotides were removed by gel filtration (with buffer C) on a NAP5 column (Pharmacia, Freiburg, Germany). The removal of free nucleotide is essential because glucosylation increased the dissociation rate of GTP by about 3−7-fold (see Results). Thus, in the presence of free GTP, nucleotide exchange interferes with GTP hydrolysis. The GTPase activity was measured in buffer C in the presence of 1 mg/mL BSA at 30 °C. At the indicated times, samples were taken and filtered on nitrocellulose and protein-bound [γ-^{32}P]GTP was quantified by liquid scintillation counting.

Binding and Release of [^{35}S]GTPγS. The GTPases (1 μM) were incubated with 50 μM [^{35}S]GTPγS in buffer C (and 1 mg/mL BSA) at 30 °C. Samples were filtered on nitrocellulose at the indicated times, and the bound radioactivity was quantified by liquid scintillation counting. After maximal incorporation of [^{35}S]GTPγS, 2.5 mM GTPγS was added to displace the [^{35}S]GTPγS from the GTPases.

Charcoal Assay for GTP Hydrolysis. Control and modified GTPases (about 15 μM) were loaded with [γ-^{32}P]GTP, and the free nucleotide was removed by gel filtration with buffer C on a Superdex 75 10/30 column at 4 °C. Then the

GTPases were incubated for up to 360 min in buffer C at 30 °C. Ten aliquots (50 μL) were taken at the appropriate times to give the exact course of GTP hydrolysis. For determination of released [γ-^{32}P]orthophosphate, duplicates of 50 μL aliquots were added to 450 μL of ice-cold charcoal buffer [50 mM H_3PO_4 (pH 1.76) and 5% (w/v) charcoal]. After mixing for 20 s and centrifugation for 2 min (14000*g*), the radioactivity was measured in 200 μL of the supernatant.

Fluorescence Assay for Nucleotide Exchange. All measurements were carried out on a LS50B spectrofluorometer from Perkin-Elmer at 30 °C. We used the increase in fluorescence intensity at 444 nm of mant-nucleotides upon binding to GTP-binding proteins to measure the nucleotide exchange of the Rho subfamily proteins. To use a 100-fold molar excess of mant-GDP over protein-bound nucleotide, we did not excite the mant-fluorescence directly at 344 nm, which yields a poor signal-to-basal ratio. Instead, we excited the fluorescence of tryptophan residues of the GTPases at 295 nm (the mant-fluorescence is hardly excited at this wavelength) and used the transfer of fluorescence resonance energy from the tryptophan fluorophore (emission at ~340 nm) to the mant-moiety to detect the binding of mant-GDP (*49*). Mant-GDP (50 μM) was added to GTPase (0.5 μM) in buffer C, and the fluorescence intensity was measured at 444 nm with excitation at 295 nm. To avoid photobleaching, the samples were exited in a cycle of 1 min for 1 s.

Calculation of the Rate Constants for the Nucleotide Dissociation and GTPase Activity. The rate constants were obtained by fitting a single-exponential curve to the experimentally obtained data with a nonlinear regression using the program SigmaPlot. The coefficients of variation never exceeded 2% in the fluorescence assay, 5% in the GTPase assay, and 10% in the [^{35}S]GTPγS filter binding assay.

Coprecipitation Assay. The Rho subfamily proteins (10 μM) were preloaded with 2.5 mM GTPγS or GDP in buffer C for 90 min at 30 °C in a volume of 100 μL. Then 50 μL of glutathione−Sepharose suspension (50%, v/v) with Rho-binding proteins immobilized as GST fusion proteins was added and the mixture incubated for 30 min at 30 °C. After three washes with 500 μL of buffer C, the remaining protein was eluted from the glutathione beads with 50 μL of SDS buffer and applied for SDS−PAGE. RhoA was identified with monoclonal mouse anti-RhoA IgG1 (26C4, Santa Cruz) by Western blot analysis. Primary antibodies were detected by horseradish peroxidase-conjugated secondary antibody and ECL reaction.

Rac1-Dependent Activation of NADPH Oxidase. Native and glucosylated Rac1 were subjected to exchange to GTPγS by incubation for 30 min at 30 °C with 300 μM nucleotide in the presence of 9 mM EDTA, followed by the addition of $MgCl_2$ to a final concentration of 25 mM. The ability of the two forms of Rac1 to support the activation of NADPH oxidase was tested in a lithium dodecyl sulfate (LiDS)−stimulated cell-free superoxide (O_2^-) generating system (*17*). This consisted of solubilized guinea pig macrophage membrane (corresponding to 5 nM cytochrome b_{559}) (*50*), 100 nM baculovirus-derived p47^{phox} and p67^{phox} (*51*), and either control or glucosylated Rac1, at concentrations ranging from 0 to 200 nM. The components were diluted in assay buffer (*50*), containing 200 μM cytochrome *c* and 130 μM LiDS, to a total volume of 200 μL in a 96-well microplate and incubated for 90 s at room temperature, with gentle shaking.

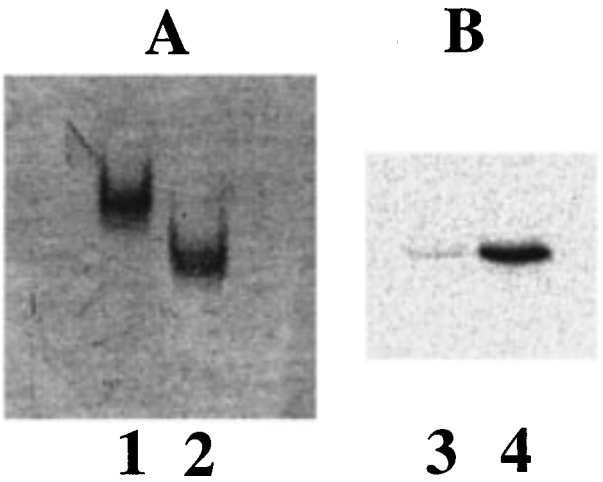

FIGURE 1: ADP ribosylation and glucosylation of Rho subfamily proteins. (A) Coomassie blue-stained native gel of ADP-ribosylated and control RhoA. RhoA was ADP ribosylated for 45 min at 37 °C in buffer C supplemented with 100 μM NAD^+ and 20 nM C3 toxin. After gel filtration on a Superdex 75 column, the modification of RhoA was checked by nondenaturing gel electrophoresis: lane 1, control RhoA; and lane 2, ADP-ribosylated RhoA. (B) Autoradiography of SDS−PAGE of ^{14}C-glucosylated RhoA. Glucosylation of the GTPase was performed as described in Experimental Procedures. Thereafter, the GTPase was separated from toxin B and brought into buffer C by gel filtration on a Superdex 75 column. Complete glucosylation of the GTPase was proved by a second glucosylation reaction with glucosylated (lane 3) and control RhoA (lane 4) protein in the presence of UDP[^{14}C]glucose followed by SDS−PAGE and phosphorimaging. Glucosylation of Cdc42 and Rac1 was performed and checked in the same way (not shown).

Upon addition of 294 μM NADPH, the rate of O_2^- production was determined by measuring the kinetics of cytochrome *c* reduction, at 550 nm, in a Spectramax 340 microplate reader (Molecular Devices). The results are expressed as turnover values (moles of O_2^- per second per mole of cytochrome b_{559}), and blank values, represented by O_2^- production rates in the absence of Rac1-GTPγS, were deducted. In each experiment, each measurement was performed in triplicate and results were averaged.

RESULTS

ADP Ribosylation and Glucosylation of Rho Proteins. Before the effects of ADP ribosylation and glucosylation on biochemical properties of the Rho subfamily proteins were measured, complete modification was checked. ADP ribosylation of RhoA was checked by the shift of the RhoA band in nondenaturing gel electrophoresis (Figure 1A) caused by the incorporation of the charged ADP ribose (*48*). Complete glucosylation of GTPases was checked by a second glucosylation reaction in the presence of UDP[^{14}C]glucose followed by SDS−PAGE and phosphorimaging (Figure 1B). If the first glucosylation is complete, no further radioactive [^{14}C]glucose can be bound to Thr-35 and -37.

Effects of ADP Ribosylation and Glucosylation on Nucleotide Binding. Nucleotide binding was studied by monitoring the increase in fluorescence intensity of mant-GDP. We used a 100-fold molar excess of the labeled nucleotide over the primary protein-bound GDP, and we checked by HPLC that after purification the wild-type Rho proteins (RhoA, Cdc42, and Rac1) have bound GDP. Figure 2 shows the influence

Table 1: Nucleotide-Binding of Control and Modified Rho Proteins

	k_{-GDP} (min^{-1}) from fluorescence assay	k_{-GDP} (min^{-1}) from filter binding assay	$k_{-GTP\gamma S}$ (min^{-1}) from filter binding assay	$t_{1/2}$ (min)	$t_{1/2}$ (min)	$t_{1/2}$ (min)
con-RhoA	0.047 ± 0.002	0.048 ± 0.002	0.013 ± 0.001	14.8	14.4	53.3
glu-RhoA	0.056 ± 0.002	0.055 ± 0.001	0.033 ± 0.001	12.4	12.6	21.0
ADPr-RhoA	0.066 ± 0.008	0.063 ± 0.003	0.007 ± 0.001	10.5	11.0	99.0
con-Cdc42	0.027 ± 0.001	0.029 ± 0.001	0.006 ± 0.001	25.7	23.9	115.0
glu-Cdc42	0.038 ± 0.002	0.035 ± 0.001	0.025 ± 0.002	18.2	19.8	27.7
con-Rac1	0.031 ± 0.002	0.028 ± 0.001	0.008 ± 0.001	22.4	24.8	86.6
glu-Rac1	0.031 ± 0.002	0.029 ± 0.001	0.054 ± 0.002	22.4	23.9	12.8

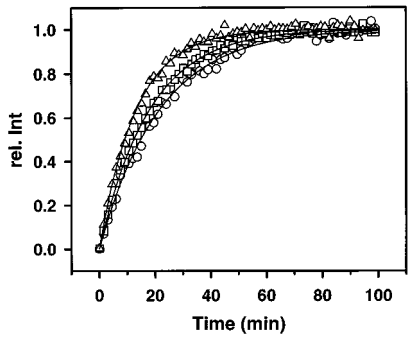

FIGURE 2: Influence of glucosylation and ADP ribosylation on binding of mant-GDP to RhoA. RhoA was glucosylated by *C. difficile* toxin and ADP ribosylated by *C. botulinum* C3 transferase as described in Experimental Procedures. The exchange reaction was started by adding mant-GDP (50 μM, final concentration) to 0.5 μM RhoA with bound GDP at 30 °C. Nucleotide exchange was monitored by the increase in the fluorescence of mant-GDP at 444 nm upon binding to RhoA. We excited the tryptophan fluorescence of RhoA at 295 nm and used the transfer of fluorescence energy to the mant-fluorophore to detect the binding of mant-GDP: (○) control RhoA, (□) RhoA glucosylated by toxin B, and (△) RhoA ADP ribosylated by C3 toxin. The curves were fitted by a single-exponential function (line), and the increase in fluorescence was normalized to 1.

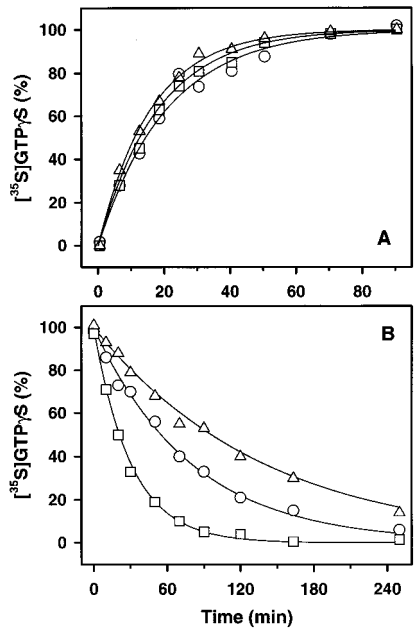

FIGURE 3: Influence of glucosylation and ADP ribosylation of Rho on binding and release of [^{35}S]GTPγS. (A) Binding of [^{35}S]GTPγS to 1 μM RhoA (GDP form) after addition of 50 μM [^{35}S]GTPγS. At the indicated times, samples were taken and filtered on nitrocellulose and bound radioactivity was determined. (B) Release of bound [^{35}S]GTPγS from RhoA was initiated by addition of a 50-fold molar excess (2.5 mM) of unlabeled GTPγS after maximal incorporation of radioactivity: (○) control RhoA, (□) RhoA glucosylated by toxin B, and (△) RhoA ADP ribosylated by C3 toxin.

of ADP ribosylation and glucosylation on the binding of mant-GDP to RhoA in the GDP-bound form. As the dissociation of bound nucleotide is the rate-limiting step, the increase in mant-fluorescence upon binding to the GTPase reflects the dissociation of the original bound GDP (*52*). All data could be fitted with a single-exponential function from which the dissociation rate was determined. ADP ribosylation and glucosylation of RhoA increased the dissociation rate of GDP from 0.047 to 0.066 min^{-1} and to 0.056 min^{-1}, respectively (Table 1). The same assay was applied for unmodified and glucosylated Cdc42 and Rac1. The glucosylation of Cdc42 increased the dissociation rate of GDP from 0.027 min^{-1} for control Cdc42 to 0.038 min^{-1} for glucosylated Cdc42. For Rac1, we could not find a significant difference after glucosylation. The dissociation rates of GDP for control and glucosylated Rac1 were 0.031 min^{-1}.

With a filter binding assay, we verified the data for the GTPases obtained with the fluorescence assay. Binding of [^{35}S]GTPγS was performed with a 50-fold molar excess of [^{35}S]GTPγS over protein-bound GDP. Figure 3A shows the incorporation of [^{35}S]GTPγS in RhoA and the fitting of the data by a single-exponential function. Data from the filter binding assay were in good agreement with those obtained by the fluorescence assay (Table 1). After maximal incorporation of [^{35}S]GTPγS, a 50-fold molar excess of unlabeled GTPγS was added to displace the radioactive [^{35}S]GTPγS from RhoA. As shown in Figure 3B, ADP ribosylation and glucosylation of RhoA exhibited more distinct effects on the dissociation rate of [^{35}S]GTPγS than on the dissociation rate of GDP. The ADP ribosylation of RhoA decreased the dissociation rate of [^{35}S]GTPγS from 0.013 min^{-1} for control RhoA to 0.007 min^{-1}. On the contrary, glucosylation increased the rate to 0.033 min^{-1}. For glucosylated Cdc42 and Rac1, a similar increase in the dissociation rates of bound GTPγS was detected. Glucosylation of Rac1 and of Cdc42 increased the dissociation rate of [^{35}S]GTPγS from 0.008 to 0.054 min^{-1} and from 0.006 to 0.025 min^{-1}, respectively. The data of the nucleotide exchange of the investigated Rho proteins are summarized in Table 1.

Effects of ADP Ribosylation and Glucosylation on GTPase Activity. The GTPase activity of RhoA was measured with a HPLC assay (Figure 4). Because free nucleotide was separated after loading and the amounts of both nucleotides (GDP and GTP) remaining were determined by HPLC, this assay is not affected by nucleotide exchange. ADP ribosylation and glucosylation of RhoA exhibited opposite effects on the intrinsic GTPase activity of RhoA. The ADP ribosylation enhanced the GTPase activity of RhoA from 0.027 to 0.060 min^{-1}. In contrast, glucosylation decreased

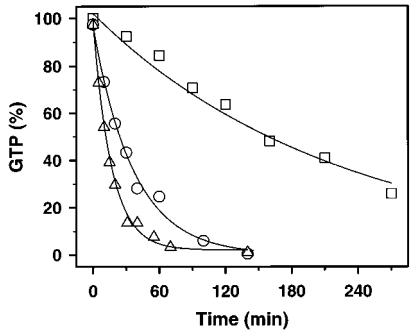

FIGURE 4: Influence of glucosylation and ADP ribosylation of RhoA on GTPase activity. RhoA was loaded with GTP, free nucleotide removed by gel filtration on a Superdex 75 10/30 column at 4 °C, and the hydrolysis of RhoA-bound GTP determined. At the indicated times, samples (200 μL) were taken and the protein was denatured by addition of 25 μL of 1 M sodium phosphate buffer (pH 1.66) and sedimented by centrifugation for 3 min at 14000*g*. Then 200 μL of the supernatant was applied to a Nucleosil-NH_2 column and analyzed for nucleotide by HPLC: (○) control RhoA, (□) RhoA glucosylated by toxin B, and (△) RhoA ADP ribosylated by C3 toxin. Data are means of three independent experiments.

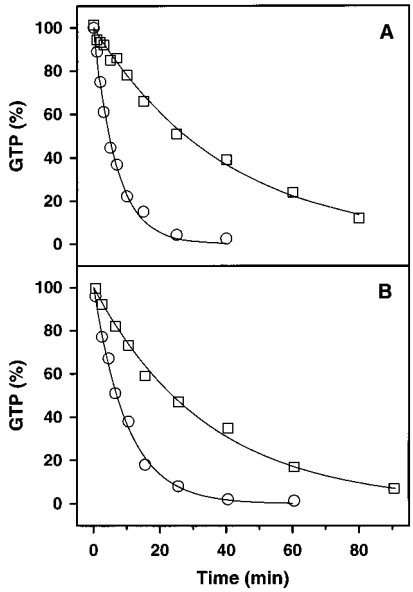

FIGURE 5: Influence of glucosylation of Cdc42 (A) and Rac1 (B) on GTPase activity. Proteins were loaded with [γ-^{32}P]GTP, and free nucleotide was removed by gel filtration on a NAP5 column as described in Experimental Procedures. The hydrolysis of protein-bound [γ-^{32}P]GTP was measured by a nitrocellulose filter binding assay: (○) control Cdc42 and Rac1 and (□) glucosylated GTPases. Data were fitted by a single-exponential decay (line).

GTPase activity to a rate of 0.0045 min^{-1}. A prerequisite for the HPLC assay is the availability of large amounts of protein of the GTPase. Because preparations of Cdc42 and Rac1 yielded low amounts of protein, we determined their GTPase activities by using [γ-^{32}P]GTP and a filter binding assay. To exclude that exchange of [γ-^{32}P]GTP for nonradioactive GTP interferes with determination of GTPase activity, free radioactive GTP was separated by gel filtration. As shown in Figure 5, glucosylation decreased the intrinsic GTPase activity of Cdc42 and Rac1 from 0.15 to 0.025 min^{-1} and from 0.10 to 0.03 min^{-1} , respectively. In addition, glucosylation-induced inhibition of GTPase activity was confirmed by measuring the release of [^{32}P]orthophosphate with a charcoal assay (not shown).

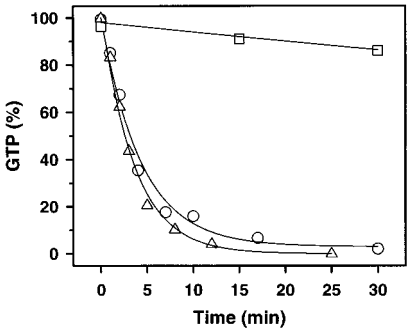

FIGURE 6: Influence of ADP ribosylation and glucosylation of RhoA on GAP-stimulated GTPase activity. RhoA GTPase (4 μM) was stimulated by addition of the 30 kDa active fragment of p50Rho-GAP (10 nM) at 30 °C. Hydrolysis of protein-bound GTP was determined by the HPLC assay as described in Figure 5: (○) control RhoA, (□) RhoA glucosylated by toxin B, and (△) RhoA ADP ribosylated by C3 toxin. GTPase activity of glucosylated RhoA determined in the presence of 100 nM Rho-GAP fragment was the same as that for 10 nM GAP.

Table 2: GTPase Activity of Control and Modified Rho-Proteins

	k (min^{-1})	$t_{1/2}$ (min)	stimulation by p30-GAP
con-RhoA	0.027 ± 0.001	25.7	+
glu-RhoA	0.0045 ± 0.0003	154	−
ADPr-RhoA	0.060 ± 0.003	11.6	+
con-Cdc42	0.150 ± 0.003	4.6	+
glu-Cdc42	0.025 ± 0.001	27.8	−
con-Rac1	0.10 ± 0.01	6.9	+
glu-Rac1	0.030 ± 0.001	23.1	−

Next, we investigated the effects of ADP ribosylation and glucosylation on the stimulation of the GTPase activity by p30-GAP, an active fragment of p50 Rho-GAP, which is a GTPase-activating protein for RhoA, Cdc42, and Rac1 (*30*). p30-GAP (10 nM) increased the GTPase activity of control RhoA and of ADP ribosylated RhoA by about 8- and 5-fold, respectively (Figure 6). In contrast, stimulation of the GTPase of RhoA was completely blocked by glucosylation even at a concentration of 100 nM p30-GAP. Similarly, toxin-induced glucosylation inhibited GTPase stimulation of Cdc42 and Rac1 by p30-GAP (data are summarized in Table 2).

To study the influence of ADP ribosylation and glucosylation on binding of RhoA to GAP, we employed coprecipitation experiments. To this end, p30-GAP, immobilized as a GST fusion protein to glutathione−Sepharose, was incubated with control and modified RhoA. After the Sepharose beads were washed, bound proteins were eluted and subjected to SDS−PAGE and RhoA was detected with immunoblotting (Figure 7A). RhoA was loaded with GTPγS or GDP prior to coprecipitation. In contrast to a recent report (*30*), only the triphosphate (GTPγS) form of RhoA bound to p30-GAP, indicating that the affinity of Rho for GAP may depend on the nucleotide bound (Figure 7A). ADP ribosylation at Asn-41 caused no inhibition of binding to p30-GAP compared to that of control RhoA. In contrast, after glucosylation at Thr-37, no binding of RhoA to p30-GAP was detected.

Effects of ADP Ribosylation and Glucosylation of Rho Proteins on Interaction with Effectors. The amino acid residues which are modified by ADP ribosylation (in RhoA at Asn-41) or by glucosylation (in RhoA at Thr-37 and in Cdc42 and Rac1 at Thr-35) are localized in loop 2, which is part of the effector-binding region of the GTPases. To

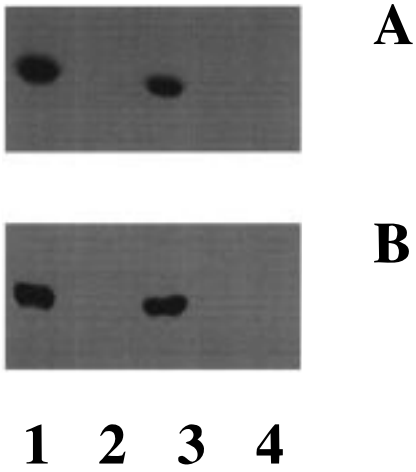

FIGURE 7: Influence of glucosylation and ADP ribosylation of RhoA on GAP and PKN binding. (A) Coprecipitation of RhoA with the 30 kDa fragment of p50-GAP. The binding of control, ADP-ribosylated, or glucosylated RhoA to p30-GAP immobilized as a GST fusion protein to glutathione−Sepharose was determined as described in the Experimental Procedures: lane 1, GST−p30-GAP incubated with ADP-ribosylated RhoA (GTPγS-bound form); lane 2, glucosylated RhoA (GTPγS-bound form); lane 3, control RhoA (GTPγS-bound form); and lane 4, control RhoA (GDP-bound form). RhoA was detected with monoclonal mouse anti-RhoA IgG followed by an ECL reaction with a horseradish peroxidase-conjugated secondary antibody. (B) Western blot analysis of the coprecipitation of RhoA with the Rho-binding domain of PKN. The coprecipitation assay with PKN and Rho was the same as that in panel A: lane 1, PKN with ADP-ribosylated RhoA (GTPγS-bound form); lane 2, glucosylated RhoA (GTPγS-bound form); lane 3, control RhoA (GTPγS-bound form); and lane 4, control RhoA (GDP-bound form).

examine the interaction of RhoA with protein kinase N (PKN), a putative effector for RhoA (*24*, *25*), precipitation studies were performed. As observed with p30-GAP, ADP ribosylation of RhoA had no effect on the binding to PKN, whereas glucosylation completely blocked this interaction (Figure 7B).

Rac1 was found to be required for the activation of the O_2^--generating NADPH oxidase complex of phagocytes (*17*). We examined the effect of glucosylation of Rac1 at Thr35 on this function. In Figure 8, the level of activation of NADPH oxidase, in a semirecombinant cell-free system, was plotted against the concentrations of control and glucosylated Rac1. It is apparent that glucosylated Rac1 is much less efficient as an activator; its V_{max} was 6.88 mol of O_2^- s^{-1} (mol of cytochrome b_{559})$^{-1}$, compared to 36.12 mol of O_2^- s^{-1} (mol of cytochrome b_{559})$^{-1}$ exhibited by the unmodified Rac1. Next, we investigated the ability of glucosylated Rac1 to compete with control Rac1 for a target molecule participating in the activation of the oxidase. As is apparent from Figure 9, NADPH oxidase activation supported by Rac1 at 5, 10, 20, and 100 nM was not significantly reduced by the simultaneous presence of glucosylated Rac1, at either identical or 10-fold higher concentrations.

DISCUSSION

We studied the functional consequences of glucosylation and ADP ribosylation of Rho subfamily proteins on nucleotide binding, GTPase activity, and effector coupling. Whereas ADP ribosylation by C3-like transferases is restricted to RhoA, -B, and -C (*33*), *C. difficile* toxins A and

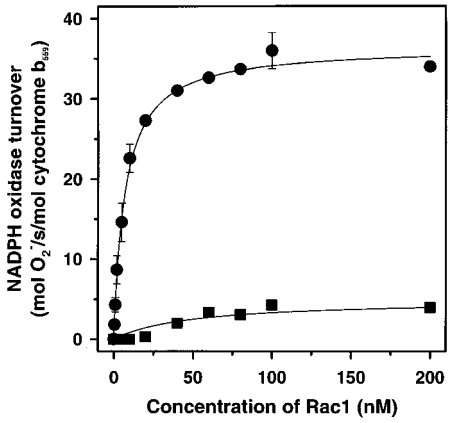

FIGURE 8: Activation of NADPH oxidase in a cell-free system by control and glucosylated Rac1. Rac1-dependent O_2^- production was assayed in a LiDS-activated cell-free system, consisting of solubilized macrophage membrane (5 nM cytochrome b_{559}), p47^{phox} (100 nM), p67^{phox} (100 nM), and various concentrations of control (●) or glucosylated (■) Rac1, as detailed in Experimental Procedures. Data plotted in the figure represent means ± SE of three experiments.

B glucosylate Rho, Rac, and Cdc42 (*36*, *37*). The acceptor amino acids for glucosylation by *C. difficile* toxins are Thr-37 in RhoA and Thr-35 in Cdc42 and Rac1. This threonine residue, which is conserved in all small GTP-binding proteins, is located in loop 2 of the GTPase and is involved in coordinating the γ-phosphate of bound GTP (*42*). Only small changes in binding of GDP are observed after glucosylation of Cdc42, RhoA, and Rac1. The dissociation rates of GDP for Cdc42 and RhoA were increased by about 1.4-fold after glucosylation, and we did not detect any change in dissociation of GDP with glucosylated Rac1. In agreement with the specific role of Thr-37 and -35 in GTP binding (*42*), dissociation of GTPγS was much more affected by glucosylation, resulting in a 3−6-fold increase in the rate of [^{35}S]GTPγS dissociation.

ADP ribosylation of RhoA occurs at Asn-41 (*35*). After ADP ribosylation, the release of bound GDP was accelerated by about 1.4-fold; a similar finding was reported recently (*53*). In contrast, the exchange of bound GTPγS was about 2-fold slower. Asn-41 is four amino acid residues apart from the acceptor amino acid Thr-37 for glucosylation and is probably also part of the effector-binding region (*35*). This amino acid residue is not directly involved in nucleotide binding (*54*). Therefore, the effect of ADP ribosylation on release of bound nucleotides might be explained by conformational changes induced by attachment of ADP ribose or by charge effects of the phosphate groups.

As observed for the binding of GTPγS to RhoA, ADP ribosylation and glucosylation exerted opposite effects on the intrinsic GTPase activity of RhoA. While ADP ribosylation doubled the GTPase activity, glucosylation decreased the GTPase activity by about 85% compared to that of the control protein. Similarly, glucosylation decreased the GTPase activity of Rac and Cdc42 by about 80 and 85%, respectively, indicating the pivotal role of this residue for GTPase activity.

Major differences between ADP ribosylation and glucosylation were observed also in GAP stimulation of GTPase activity. As reported recently (*55*, *56*), we also observed that GTPase stimulation by the Rho-GAP (*30*) did not change

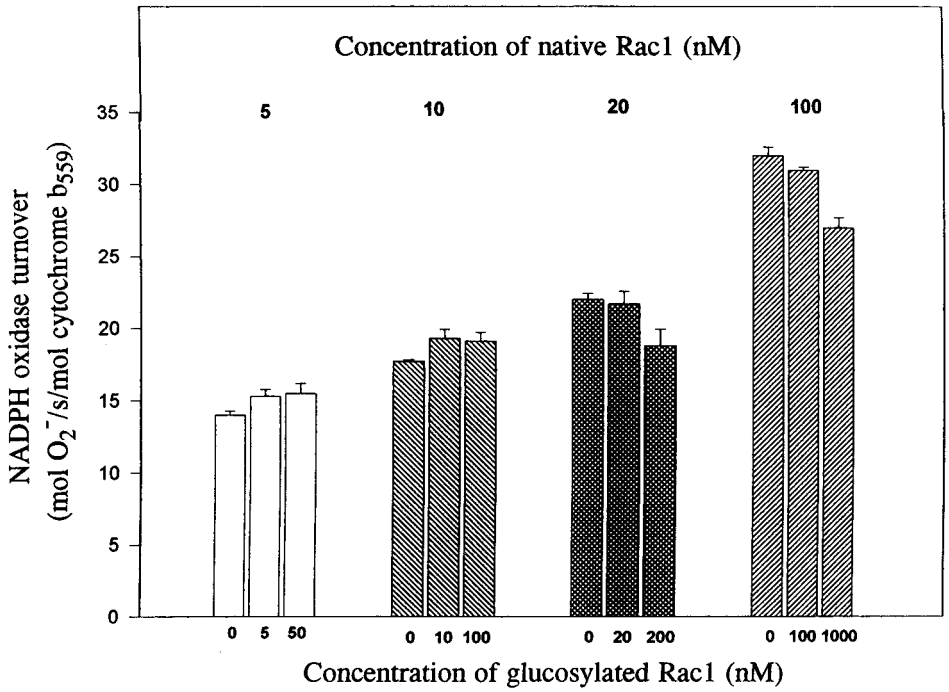

FIGURE 9: Effect of glucosylated Rac1 on activation of NADPH oxidase by control Rac1. Rac1-dependent O_2^- production was assayed in a cell-free system, as described in the legend to Figure 8. Native Rac1 was present at concentrations of 5, 10, 20, and 100 nM, and for each concentration of control Rac1, O_2^- production was measured in the absence or presence of an equimolar or 10-fold molar excess of glucosylated Rac1. Results represent means ± SE of three experiments.

after ADP ribosylation at Asn-41. In contrast, glucosylation at Thr-37 completely blocked the stimulation of the GTPase activity by Rho-GAP. Similarly, glucosylation inhibited GAP stimulation of Rac and Cdc42 GTPase activity. These findings reflect a central role of this threonine residue in GAP stimulation of the GTPases. Accordingly, it has been shown that the Thr-37 → Ala mutation of Rho is insensitive toward p50 Rho-Gap (*57*). The inhibition could be due to a decreased affinity of the Rho proteins to GAP after glucosylation. Another possibility may be that the covalently bound glucose blocks the GAP-induced mechanism for stimulation of the RhoA GTPase activity. In a coprecipitation assay, we found no change in the affinity of RhoA to p30-GAP after ADP ribosylation, which correlates with the unaffected stimulation of the GTPase activity. After glucosylation, however, binding of RhoA to p30-GAP was no longer detectable. Thus, decreased affinity is one explanation for the inhibition of GTPase stimulation by GAP observed after glucosylation of RhoA. However, it is noteworthy that other mutations in the effector region of Rho (even a chimeric protein with residues 23−48 of Ras) did not affect GAP stimulation of Rho (*57*), indicating that selectively Thr-37 but not the region in the vicinity of this threonine residue is important for GTPase activity and GAP stimulation.

We studied Rho effector coupling by using protein kinase N (PKN), which selectively interacts with this GTPase (*46*). Surprisingly, ADP ribosylation did not interfere with the Rho binding to PKN, while glucosylation completely blocked the interaction between Rho and PKN. A Thr-37 → Ala mutation of RhoA was also shown to be incapable of binding PKN (*46*). From these findings, we conclude that both modifications exert their effects on Rho-mediated signaling by completely different mechanisms. Glucosylation blocks Rho−effector coupling, while ADP-ribosylated RhoA was still capable of interacting with PKN. Thus, the hypothesis that inactivation of RhoA by C3-induced ADP ribosylation is simply caused by steric hindrance of RhoA−effector interactions appears not to be true (*48*). However, one has to consider that we used a fragment of PKN (amino acids 7−155) harboring the Rho-binding domain. One cannot entirely exclude the possibility that the binding of full length PKN proteins is somehow affected by ADP ribosylation. Furthermore, it remains to be clarified whether the attachment of ADP ribose in the vicinity of the effector region of RhoA affects the binding of other Rho-interacting effectors or whether the activation of Rho protein itself by guanine nucleotide exchange factors is affected (e.g. by sequestration of GEFs).

For Rac1, we were able to test the direct influence of glucosylation on its ability to participate in the activation of NADPH oxidase. Our finding that glucosylation at Thr-35 markedly reduced the NADPH oxidase activating ability of Rac1 is in good agreement with earlier work. Thus, it was reported that a domain homologous to the canonical effector region of Ras (residues 26−48) was essential for the oxidase activating function of Rac1. This was based on the finding that several point mutations within this sequence, including Thr-35 → Ala, resulted in an impaired ability to support NADPH oxidase activation (*28, 58−60*). A similar conclusion was reached by testing of chimeric molecules consisting of segments of Rac1 and either Cdc42Hs (*60*) or RhoA (*61*). It was proposed that the molecular target of Rac1, among the members of the NADPH oxidase complex, is the cytosolic component $p67^{phox}$ (*58*). Indeed, a Thr-35 → Ala mutation also resulted in the loss of the ability of Rac1 to bind to $p67^{phox}$ (*58*). Our results can be interpreted as corroborating the role of Thr35, in particular, and of the effector domain, in general, in this interaction. However, more recent work, utilizing "peptide walking" (*62*), chimerization (*61*), and point mutations (*63*), has highlighted the

involvement of additional domains in oxidase activation by Rac1, including residues 103−107, 124−135, and 163−169. It was also proposed that some of these domains might be involved in interaction with oxidase components, other than $p67^{phox}$ (*63*). It is, therefore, likely that the Thr35-containing effector domain is required but not sufficient for oxidase activation. Our finding that glucosylated Rac1 is unable to compete with unmodified Rac1 is compatible with a model based on a single-domain interaction between Rac1 and its oxidase target protein but is difficult to reconcile with the evidence for the involvement of multiple Rac1 domains. A possible solution to this discrepancy could be that binding of Rac1 to oxidase target(s) is dependent on the simultaneous engagement of several Rac1 domains, and the dysfunction of any single domain prevents binding. Alternatively, the effector domain might be responsible for the primary binding of Rac1 to its target molecule, whereas the other domains could be required for activation-related events, secondary to binding, which might involve other components of the NADPH oxidase complex. Moreover, our findings on Rho and Rac support the notion that the mechanism of inactivation of Rho subfamily proteins by glucosylation is the inhibition of binding of the GTPases to their effectors. However, this mechanism is insufficient to explain the dominant negative effects of glucosylated RhoA observed previously after microinjection of modified proteins (*36*).

In summary, although both ADP ribosylation and glucosylation by the clostridial toxins occur in the effector binding domain of Rho subfamily proteins and cause similar morphological changes, e.g. rounding up of cells and redistribution of the actin cytoskeleton, the data presented show that the principles underlying these functional consequences are largely different for both types of covalent modification.

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