ExoS Rho GTPase-activating Protein Activity Stimulates Reorganization of the Actin Cytoskeleton through Rho GTPase Guanine Nucleotide Disassociation Inhibitor*

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ExoS is a bifunctional Type III cytotoxin of Pseudomonas aeruginosa with N-terminal Rho GTPase-activating protein (RhoGAP) and C-terminal ADP-ribosyltransferase domains. Although the ExoS RhoGAP inactivates Cdc42, Rac, and RhoA in vivo, the relationship between ExoS RhoGAP and the eukaryotic regulators of Rho GTPases is not clear. The present study investigated the roles of Rho GTPase guanine nucleotide disassociation inhibitor (RhoGDI) in the reorganization of actin cytoskeleton mediated by ExoS RhoGAP. A green fluorescent protein-RhoGDI fusion protein was engineered and found to elicit actin reorganization through the inactivation of Rho GTPases. Green fluorescent protein-RhoGDI and ExoS RhoGAP cooperatively stimulated actin reorganization and translocation of Cdc42 from membrane to cytosol, and a RhoGDI mutant, RhoGDI(1777D), that is defective in extracting Rho GTPases off the membrane inhibited the actions of RhoGDI and ExoS RhoGAP on the translocation of Cdc42 from membrane to cytosol. A human RhoGDI small interfering RNA was transfected into HeLa cells to knock down 90% of the endogenous RhoGDI expression. HeLa cells with knockdown RhoGDI were resistant to the reorganization of the actin cytoskeleton elicited by type III-delivered ExoS RhoGAP. This indicates that ExoS RhoGAP and RhoGDI function in series to inactivate Rho GTPases, in which RhoGDI extracting GDP-bound Rho GTPases off the membrane and sequestering them in cytosol is the rate-limiting step in Rho GTPase inactivation. A eukaryotic GTPase-activating protein, p50RhoGAP, showed a similar cooperativity with RhoGDI on actin reorganization, suggesting that ExoS RhoGAP functions as a molecular mimic of eukaryotic RhoGAPs to inactivate Rho GTPases through RhoGDI.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes life-threatening infections in cystic fibrosis patients, the immune compromised, and burn patients (1). P. aeruginosa pathogenesis involves cell-associated and secreted virulence factors as well as intrinsic antibiotic resistance. Secreted virulence factors include the type III cytotoxins ExoS, ExoT, ExoY, and ExoU, which are delivered by the bacterium into mammalian cells (2). ExoS is a bi-functional cytotoxin that contains a Rho GTPase-activating protein (RhoGAP) domain within the N terminus and a 14-3-3-dependent ADP-ribosyltransferase domain within the C terminus. Transfection of the C-terminal ADP-ribosyltransferase domain into cultured cells resulted in cell death (3, 4), whereas transfection of the N-terminal RhoGAP domain stimulated reorganization of the actin cytoskeleton (5). The N-terminal RhoGAP domain of ExoS organizes the actin cytoskeleton through inactivating Cdc42, Rac1, and RhoA in vitro and in vivo. Arg^146 is a residue that is required for the ExoS RhoGAP activity (5, 6).

Rho, Rac, and Cdc42 play critical roles in the organization of actin cytoskeleton. Activation of Cdc42 stimulates the formation of filopodia (microspikes) and is essential for cell polarity (7, 8), whereas Rac activation stimulates lamellipodia (membrane ruffling) and contributes to cell motility. Activation of RhoA stimulates the formation of stress fibers (8, 9). Rho GTPases are synthesized in the cytosol and are isoprenylated by the cytosolic heterodimeric prenyltransferase and geranylgeranyltransferase I at their C-terminal CAAX motif (10, 11). The isoprenyl moiety tethers Rho GTPases to the cell membrane (12, 13). On the cell membrane Rho GTPases function as molecular switches that are tightly regulated by guanine nucleotide exchange factors (GEFs), which activate Rho GTPases by stimulating the exchange of GDP for GTP, and by GTPase-activating proteins (GAPs), which inactivate Rho GTPases by catalyzing the hydrolysis of GTP to GDP. Rho GTPase guanine nucleotide disassociation inhibitor (RhoGDI) is a multifunctional regulator of Rho GTPases. RhoGDI inhibits GEF-stimulated GDP disassociation from Rho GTPases (14) inhibits both intrinsic and RhoGAP-stimulated GTP hydrolysis in vitro (15, 16) and extracts Rho GTPases from the membrane and forms GDI-Rho GTPases complex in cytosol (17). RhoGDI has two distinct domains, an N-terminal flexible regulatory domain, and a C-terminal immunoglobulin-like domain (18). The N-terminal regulatory domain binds to the switch I and switch II regions of Cdc42, which presumably inhibits both GDP dissociation and GTP hydrolysis through interfering with the interaction between Rho GTPases and GTPases. The hydrophobic binding pocket within the C-terminal immunoglobulin-like domain of RhoGDI binds to the isoprenyl moiety of Rho GTPases and extracts Rho GTPases from membrane and forms a tight GDI-Rho complex in the cytosol. The mechanism that stimulates release of the Rho GTPases from RhoGDI is unclear but may involve conformational changes upon the binding of phosphatidylinositol 1,4,5-bisphosphate

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‡The abbreviations used are: RhoGAP, Rho GTPase-activating protein; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; RhoGDI, Rho GTPase guanine nucleotide disassociation inhibitor; GFP, green fluorescent protein; HA, hemagglutinin; CHO, Chinese hamster ovary; siRNA, small interfering RNA.
and Ezrin/Radixin/Moesin to the Rho GTPase-RhoGDI complex (19–21). GDI displacement factors are also involved in Rho and Rab release from RhoGDI (22, 23). Although GEFs appear to initiate Rho GTPase activation, current studies showed that Rho GTPases complexed with RhoGDI were resistant to activation by GEFs (14, 24) and that release of Rho GTPases from RhoGDI was required for Rho GTPase membrane translocation and activation by GEFs (25), suggesting that RhoGDI plays an important role in Rho GTPase regulation.

Rho GTPases are preferred targets for bacterial toxins (26). Through modulation of Rho GTPases activity, bacterial pathogens may either facilitate invasion into nonprofessional cells or promote macrogotysis by professional phagocytes. Transiently modulate Rho GTPase activity through covalent modification or noncovalent mechanisms that regulate the nucleotide binding state (reviewed in Ref. 27). Toxins that covalently modify Rho GTPases include: C3 exoenzyme, which inactivates RhoA signaling through ADP-ribosylation of RhoA-C at Arg41; Clostridium difficile toxins A and B, which inactivate RhoA, Rac, and Cdc42 through glycosylation of Thr2735, cytotoxic necrotizing factor, which activates RhoA, Rac, and Cdc42 through deamidation of Rho GTPases at Glu1463; and YopT, which inactivates RhoA as a cytosolic protease that cleaves the isoprenoid moiety of RhoA. Toxins that regulate the nucleotide binding states of Rho GTPases include ExoS, ExoT, SptP, and YopE, which are GAPs for various Rho GTPases, and SopE, which is a GEF for Rac and Cdc42.

Although the enzymatic activities of the bacterial GAPs or GEFs toward their specific Rho GTPases are well characterized, little is known about how these bacterial GAPs and GEFs interact with the host regulators of Rho GTPases. Therefore, the present study investigated the roles of RhoGDI (RhoGDI-1) in reorganization of the actin cytoskeleton stimulated by ExoS RhoGAP.

MATERIALS AND METHODS

Plasmid Construction—Glutathione S-transferase-RhoGDI expressing vector, pGEX-RhoGDI, was a gift from Dr. Klaus Aktories. The cDNA encoding RhoGDI-1 in pGEX-RhoGDI was subcloned into the BamHI sites of pEGFP-C1 to produce a GFP-RhoGDI-1 fusion protein. The mutations I177D and L55S,L56S on pGFP-RhoGDI were obtained through standard site-directed mutagenesis procedures using a QuickChange mutagenesis kit (Stratagene). The PCR products were confirmed by DNA sequencing. pEXV-p50RhoGAP was a gift from Dr. Alan Hall. The DNA encoding p50RhoGAP was amplified by PCR using primers containing HindIII/EcoRI sites and C-terminal HA tag (positive primer: 5'-agt ctc gag aag ctt gcc atg gat ccg ctc tca-3'; negative primer: 5'-gtc gac gaa ttc tca caa gct ggc gta gtc ggg cac gtc gta ggg gta gag ccc-3'). The PCR product was confirmed by sequencing and cloned into the HindIII/EcoRI sites of pEGFPN1 vector to produce p50RhoGAP-HA. Mammalian expression vectors encoding ExoS1–234, ExoS1–234/R146K, ExoS/E381ID, and GFP-Cdc42 have been described previously (6).

Cell Culture and Transfection—CHO cells were cultured in Ham's F-12 medium (Invitrogen) supplemented with 10% newborn calf serum, 1.4% sodium bicarbonate, and 0.5% penicillin-streptomycin at 37 °C in 5% CO2. Subconfluent lawns of CHO cells were transfected with the indicated plasmids, using LipofectAMINE Plus (Invitrogen) as suggested by the manufacturer. Total transfected DNA was normalized with pCVM-luciferase.

Subcellular Fractionation—Twenty-four hours post-transfection, CHO cells were harvested and suspended in cold homogenization buffer 1 (250 mM sucrose, 3 mM imidazole, pH 7.4). The cells were centrifuged at 250 × g for 5 min and suspended in cold homogenization buffer 2 (250 mM sucrose, 3 mM imidazole, pH 7.4, 1% protease inhibitors mixture III (Roche Applied Science), and 0.5 mM EDTA). The cells were broken by passage (14–20 times) through a 25-gauge ½-inch needle in homogenization buffer 2 followed by centrifugation at 250 × g for 5 min at 4 °C. The post-nuclear supernatant was centrifuged at 100,000 × g for 30 min at 4 °C. The insoluble material (membrane fraction) was dissolved in homogenization buffer 2 containing 1% Triton X-100 and mixed with an equal volume of SDS sample buffer. The fractions were boiled for at least 5 min and subjected to SDS-PAGE and Western blot analysis.

Fluorescent Microscopy—At 24 h post-transfection, CHO cells were washed with cold phosphate-buffered saline twice, fixed with 4% paraformaldehyde for 15 min at room temperature, and examined by fluorescence microscopy. Actin cytoskeleton was detected by fixing cells with 4% paraformaldehyde, followed by 0.1% Triton X-100 for 3–5 min and staining with Alexa-546 phallolidin (Molecular Probes) in phosphate-buffered saline with 1% bovine serum albumin for 20 min. The cells were washed and visualized by fluorescent microscopy.

SDS-PAGE and Western Blotting Analysis—The proteins were subjected to SDS-PAGE (13.5% separating gel) followed by ECL Western blot analysis (PicoWest, Pierce), using the indicated primary antibody and corresponding secondary antibody. The images were developed by FluorChem8900, and the densities of immunoreactive signals were quantified by densitometry.

Knockdown RhoGDI Expression Using RhoGDI siRNA—RhoGDI siRNA, control siRNA with fluorescein conjugate, transfection reagent, and transfection medium were purchased from Santa Cruz Biotechnology, Inc. siRNA transfection was performed following the manufacturer's protocol. Briefly, HeLa cells at 40–50% confluence were transfected with a mixture of siRNA and transfection reagent in complete medium without antibiotics. The expression of RhoGDI was monitored for 24–72 h post-transfection, during which cells were resplit and retransfected with siRNA when the transfected cells reach confluency.

Immunofluorescent Staining of the Actin Cytoskeleton, RhoGDI, and p50RhoGAP—Cells—To investigate the roles of RhoGDI in reorganization of the actin cytoskeleton by ExoS RhoGAP, pEGFP-RhoGDI was engineered to express RhoGDI as a GFP fusion protein. This is the first reported GFP fusion protein of RhoGDI-1 that allowed live visualization of RhoGDI expression in mammalian cells. When transiently transfected into CHO cells at 20 ng/ml DNA concentration, GFP-RhoGDI was expressed and present in the cytosol of cultured CHO cells (Fig. 1A). The expression and cytosolic localization of GFP-RhoGDI was further confirmed in a fractionation analysis using anti-GFP antibody (Fig. 1B). Anti-GFP antibody detected a 55-kDa cytosolic protein, which is similar to the predicted size of GFP-RhoGDI, indicating native RhoGDI, GFP-RhoGDI was expressed as a stable cytosolic protein. Unlike GFP, which was diffuse in both cytosol and nucleus, GFP-RhoGDI did not localize in the nucleus (Fig. 1A). Although transient transfection with 20 ng/ml pEGFP-RhoGDI (Fig. 1A) did not elicit cell rounding, transfection with higher concentrations of pEGFP-RhoGDI stimulated a dose-dependent cell rounding (Fig. 1C).

Transient transfection of CHO cells with pEGFP-RhoGDI at concentrations from 50 to 400 ng/ml yielded 60–380 ng of GFP-RhoGDI/106 cells, respectively, which was 0.5–4-fold greater than the reported endogenous RhoGDI (28). Overexpression of GFP-RhoGDI was not cytotoxic to CHO cells, because the expression of GFP, which served as a cell viability marker (4), was not affected by GFP-RhoGDI expression (Fig. 1C, inset). Together, these data indicated that GFP-RhoGDI was functional as native RhoGDI, as observed by microinjection of RhoGDI–γ or transfection of DNA encoding nonfusion forms of RhoGDI (RhoGDI-1) in mammalian cells (29–31).

To further test whether the rounding phenotype elicited by GFP-RhoGDI is due to disruption of actin cytoskeleton through
inactivation of Rho GTPases, GFP-RhoGDI was co-transfected into CHO cells with GFP fusion Rho GTPases. The actin cytoskeleton structure was visualized by phalloidin staining after transfection. As previously described (6), expression of GFP-Cdc42, GFP-Rac1, or GFP-RhoA stimulated unique structures of actin cytoskeleton, whereas expression of GFP-RhoGDI disrupted the unique phenotypes elicited by the respective Rho GTPases (data not shown). The reorganization of actin cytoskeleton elicited by GFP-RhoGDI was similar to that elicited by ExoS (1–234), the RhoGAP domain of ExoS, indicating that ExoS RhoGAP and RhoGDI may function in the same signal pathway to modulate Rho GTPase activity.

**ExoS RhoGAP and GFP-RhoGDI Cooperatively Stimulated Cell Rounding in CHO Cells**—To investigate the role of RhoGDI in ExoS RhoGAP-stimulated reorganization of the actin cytoskeleton, the combined effect of GFP-RhoGDI and ExoS RhoGAP on actin cytoskeleton was tested. Both ExoS RhoGAP and GFP-RhoGDI stimulated a dose-dependent cell rounding (Fig. 2A). At high concentrations of DNA transfection (>50 ng/ml), ExoS RhoGAP elicited ~80% cell rounding, whereas GFP-RhoGDI elicited ~50% cell rounding. At lower concentrations of DNA transfection (5–20 ng/ml), neither GFP-RhoGDI nor ExoS RhoGAP effectively elicited cell rounding, but upon co-transfection of GFP-RhoGDI and ExoS RhoGAP, an additive effect on cell rounding was observed (Fig. 2A). Controls showed that ExoS(1–234/R146K, a mutated form of the ExoS RhoGAP domain that does not possess RhoGAP activity, failed to stim-
Cdc42 was calculated. The data from three independent experiments indicated that the ExoS RhoGAP activity was required to stimulate membrane-cytosol translocation of GFP-Cdc42 (Fig. 3). In addition, co-transfection of pExoS(1–234)R146K and pEGFP-RhoGDI at 10 and 20 ng/ml did not stimulate the translocation of GFP-Cdc42 from membranes to cytosol (data not shown).

**RhoGDI(I177D) Is a Dominant-negative Mutant That Inhibits RhoGDI and ExoS RhoGAP-stimulated Membrane-Cytosol Translocation of GFP-Cdc42**—Interestingly, studies performed by other groups showed that RhoGDI inhibited both intrinsic and RhoGAP-stimulated GTP hydrolysis of Rho GTPases in vitro (16, 32). These in vitro data were further supported by structural studies showing that the N-terminal regulatory arm of RhoGDI binds to switch I and switch II regions of Rho GTPases and interferes with RhoGAP binding to the same regions (18). In the present study, ExoS RhoGAP and RhoGDI cooperatively inactivated Rho GTPases in vitro, which indicates that RhoGDI does not inhibit binding of ExoS RhoGAP to Rho GTPases in vivo; instead, RhoGDI and ExoS RhoGAP function in series to interact and inactivate Rho GTPases. That is, ExoS RhoGAP first converted GTP-Rho to GDP-Rho on the cell membrane and RhoGDI subsequently extracts GDP-Rho from the cell membrane to the cytosol, inhibiting the reactivation of Rho GTPases by GEFs on membrane.

To test this hypothesis, two RhoGDI mutations were engineered, RhoGDI(I177D) and RhoGDI(L55S,L56S). Ile<sup>177</sup> lies in the center of hydrophobic binding pocket of RhoGDI and is essential for RhoGDI to bind to the hydrophobic isoprenyl moiety of Rho GTPases (18). The residues Leu<sup>55</sup> and Leu<sup>56</sup> lie in the N-terminal flexible regulatory arm of RhoGDI that binds to the switch I and switch II regions of Rho GTPases. The double mutation L55S,L56S on RhoGDI decreases the affinity of RhoGDI for the nonisoprenylated form of Rac1 (33, 34). When transiently transfected into CHO cells, pGFP-RhoGDI(I177D) and pGFP-RhoGDI(L55S,L56S) were expressed as stable GFP fusion proteins (Fig. 4A). GFP-RhoGDI(I177D) showed a different subcellular distribution relative to GFP-RhoGDI(wt) and GFP-RhoGDI(L55S,L56S). GFP-RhoGDI(wt) and GFP-RhoGDI(L55S,L56S) are primarily localized in cytosol, whereas GFP-RhoGDI(I177D) is localized in both membrane and cytosol fractions (Fig. 4A). Compared with GFP-RhoGDI(wt), GFP-RhoGDI(I177D) did not translocate GFP-Cdc42 from membrane to cytosol (Fig. 4B). This is consistent that binding of RhoGDI to the isoprenyl moiety of Rho GTPases is required for extracting Rho GTPases from the cell membrane. Interestingly, GFP-RhoGDI(I177D) inhibited the membrane-cytosol translocation of GFP-Cdc42 by GFP-RhoGDI(wt), suggesting that RhoGDI(I177D) is a dominant-negative mutant of RhoGDI(wt). The N-terminal flexible regulatory arm of RhoGDI(I177D) binds to Cdc42 at switch I and switch II regions, but the defective hydrophobic binding pocket fails to extract the isoprenyl moiety of Cdc42 from the cell membrane; thus, RhoGDI(I177D) forms complex with Cdc42 on the cell membrane, which further blocks the interaction between Cdc42 and wild type RhoGDI. As a control, GFP-RhoGDI(L55S,L56S) did not translocate GFP-Cdc42 from membrane to cytosol and did not inhibit the action of GFP-RhoGDI(wt) on translocation of GFP-Cdc42 from membrane to cytosol (Fig. 4B). These data are consistent with a two-step model for RhoGDI-mediated Rho GTPase membrane release, in which the N-terminal regulatory arm of RhoGDI binds to the switch I and switch II regions of Rho GTPases on membrane (fast step), and subsequently the C-terminal hydrophobic binding pocket of RhoGDI extracts the membrane-buried isoprenyl moiety of Rho GTPases off membrane (slow step) (18, 35).

This dominant-negative mutant of RhoGDI was used to test the role of RhoGDI in the actin reorganization by ExoS RhoGAP. GFP-RhoGDI(I177D) inhibited the membrane-cytosol translocation of GFP-Cdc42 stimulated by ExoS RhoGAP.
Actin Reorganization by ExoS and p50RhoGAP through RhoGDI

Fig. 4. GFP-RhoGDI(I177D) inhibited the membrane-cytosol translocation of GFP-Cdc42 by GFP-RhoGDI(WT) and ExoS RhoGAP. A, upper panels, CHO cells were transfected with pGFP, pGFP-RhoGDI(I177D), or pGFP-RhoGDI(L55S,L56S) at a concentration of 20 ng/ml. At 24 h post-transfection, the cells were harvested, lysed, and fractionated into post-nuclear supernatant (P), membrane (M), and cytosol (C) fractions. The equivalent fractions were subjected to SDS-PAGE followed by Western blot using anti-GFP monoclonal antibody as primary antibody. B and C, CHO cells were transfected with pGFP, pGFP-Cdc42 (Cdc42), pGFP-RhoGDI (GDI-wt), pGFP-RhoGDI(I177D) (GDI-DN), pGFP-RhoGDI(L55S,L56S) (GDI(LL/SS)), and pExoS(1-234)HA (ExoS-GAP) at the indicated concentrations (ng/ml). The total transfected DNA was normalized with pCMV-luciferase.

A.

B.

C.

alone and also inhibited the cooperative effect of GFP-RhoGDI and ExoS RhoGAP on translocation of GFP-Cdc42 from membrane to cytosol (Fig. 4C). This indicates that GFP-RhoGDI(I177D) serves as a dominant-negative mutant for both endogenous RhoGDI and GFP-RhoGDI to inhibit the membrane-cytosol translocation of Rho GTPases. Together, the cooperativity between ExoS RhoGAP and RhoGDI on the actin reorganization suggests that ExoS RhoGAP and RhoGDI function in series to interact and inactivate Rho GTPases, and extraction of GDP bound Rho GTPases on the cell membrane by RhoGDI was the rate-limiting step of Rho GTPase inactivation.

RhoGDI Is Required for the ExoS RhoGAP-mediated Reorganization of the Actin Cytoskeleton—Although RhoGDI(I177D) inhibited the ability of RhoGDI and RhoGAP to translocate Rho GTPases from membrane to cytosol, it did not inhibit cell rounding stimulated by RhoGAP and GFP-RhoGDI. Similar to GFP-RhoGDI, expression of GFP-RhoGDI(I177D) in CHO cells stimulated cell rounding in a dose-dependent manner, whereas GFP-RhoGDI(L55S,L56S) did not stimulate cell rounding (data not shown). This indicates that the binding of the N-terminal regulatory arm of RhoGDI to GDP-bound Rho GTPases on the membrane inhibits GEF-stimulated activation of Rho GTPases.

To further investigate the roles of RhoGDI in the ExoS RhoGAP-mediated actin cytoskeleton reorganization, siRNA technology was applied to knock down the expression of endogenous RhoGDI in HeLa cells. Using a control siRNA, which has a fluorescent conjugate, the siRNA transfection efficiency reached ~90%. Transfection of RhoGDI-specific siRNA into HeLa cells, at 68–72 h post-transfection, reduced expression of RhoGDI to 10–20% of endogenous levels (Fig. 5A). The knockdown in expression of RhoGDI was confirmed by immunofluorescent staining with α-GDI antibody (Fig. 5B). Compared with wild type HeLa cells, the cells with knockdown RhoGDI appeared flatter and produced extended actin cytoskeleton structures (Fig. 5B). Although type III-delivered ExoS RhoGAP, ExoS(1-234)-HA, efficiently elicited cell rounding and actin reorganization in wild type HeLa cells, the HeLa cells with knockdown expression of RhoGDI were resistant to the cell rounding elicited by ExoS RhoGAP (Fig. 5C), and cells with knockdown expression of RhoGDI and infected with ExoS RhoGAP retained extended actin cytoskeleton structures, such as stress fibers, lamellipodia, and filopodia, indicating the presence of active Rho, Rac, and Cdc42 (Fig. 5D). Quantitative analysis showed that type III-delivered ExoS RhoGAP elicited ~90% actin reorganization in wild type HeLa cells, whereas it elicited only ~30% actin reorganization in RhoGDI knockdown cells (Fig. 5E). This indicated that RhoGDI is a major regulator of Rho GTPases, and RhoGDI is required for ExoS RhoGAP to elicit reorganization of the actin cytoskeleton and showed that depletion of RhoGDI is critical for cellular resistance to ExoS RhoGAP effect.

Expression and Characterization of p50RhoGAP in CHO Cells—To gain insight into the functional similarity between the bacterial RhoGAPs and their mammalian homologues, the interaction between a mammalian prototype RhoGAP, p50RhoGAP, and GFP-RhoGDI were tested. The cDNA encoding p50RhoGAP was expressed with a C-terminal HA epitope tag for detection and normalization. p50RhoGAP-HA was expressed as a ~48-kDa cytosolic protein (Fig. 6B) and stimulated a dose-dependent rounding phenotype in CHO cells (Fig. 6, A and C). Transient transfection of CHO cells with...
p50RhoGAP-HA at concentrations from 20 to 320 ng/ml yielded 60–370 ng of p50RhoGAP/10⁶ cells, respectively. Like GFP-RhoGDI, transient expression of p50RhoGAP-HA was not cytotoxic to host cells (Fig. 6C, inset).

**RhoGDI and p50RhoGAP Cooperatively Stimulated Actin Reorganization in CHO Cells**—Because the relationship between mammalian RhoGAPs and RhoGDI-1 on actin reorganization has not been addressed, p50RhoGAP was co-expressed with GFP-RhoGDI in CHO cells. As observed for ExoS RhoGAP with GFP-RhoGDI, co-expression of p50RhoGAP-HA and GFP-RhoGDI cooperatively stimulated reorganization of actin cytoskeleton in CHO cells (Fig. 7A) and translocated GFP-Cdc42 from membrane to cytosol (Fig. 7B).

**DISCUSSION**

As a multifunctional regulator of Rho GTPases, RhoGDI plays an important role in actin reorganization by bacterial toxins that covalently modify Rho GTPases. Monoglucosylation of Rho, Rac, and Cdc42 by *C. difficile* toxins A and B blocks the binding of Rho GTPases to RhoGDI and subsequently results in accumulation of modified Rho GTPases at membrane. This inhibits Rho GTPases cycling between membrane and cytosol (36). Although ADP-ribosylation of RhoA, B, and C by C3 exoenzyme does not alter nucleotide binding, effector binding, and intrinsic and GAP-stimulated GTP hydrolysis, it increases the stability of the Rho-RhoGDI complex, which prevents release of Rho GTPases from RhoGDI and entraps Rho GTPases in an inactive state (37). Cleavage of the isoprenyl moiety of RhoA by YopT inhibits RhoA binding to RhoGDI and subsequently blocked the RhoA cycling between the membrane and cytosol (38, 39).

The present study is the first report addressing the roles of RhoGDI in reorganization of the actin cytoskeleton stimulated by a bacterial RhoGAP that noncovalently modulates the nucleotide state of Rho GTPases, which linked the action of this bacterial RhoGAP to the host regulators of Rho GTPases. Although previous biochemical and structural studies indicated that RhoGDI inhibited both intrinsic and RhoGAP-stimulated GTP hydrolysis (16, 32), the observed cooperative effect between ExoS RhoGAP and GFP-RhoGDI in reorganization of actin cytoskeleton suggests that, in vivo, ExoS RhoGAP and
RhoGDI function in series to inactivate Rho GTPases. That is, ExoS RhoGAP converts GTP-Rho to GDP-Rho, and RhoGDI subsequently extracts GDP-Rho from the cell membrane and forms a tight complex with GDP-Rho in cytosol. This effectively inhibits the reactivation of Rho GTPases by GEFs on the cell membrane. This model is supported by the previous studies showing that RhoGDI prefers to bind to GDP-Rho than GTP-Rho (40), and GDP-Rho complexed with RhoGDI is resistant to the actions of GEFs (25).

The mechanism of cooperativity between ExoS RhoGAP and RhoGDI is studied by using a dominant-negative mutant of RhoGDI, RhoGDI(I177D), which is capable of binding to switch I and switch II regions of Rho GTPases with the N-terminal flexible regulatory arm but is defective in extracting the isoprenyl moiety of GDP-Rho off the membrane through the hydrophobic binding pocket. RhoGDI(I177D) inhibited the membrane-cytosol translocation of Rho GTPases by GFP-RhoGDI and ExoS RhoGAP, suggesting that RhoGDI plays essential roles in ExoS RhoGAP-stimulated Rho GTPase inactivation, and extraction of the isoprenyl moiety of GDP-Rho off the membrane by the hydrophobic binding pocket of RhoGDI is the rate-limiting step. The present study is consistent with the previously proposed two-step model for the interaction of RhoGDI with the membrane-bound Cdc42 based on the kinetics and structural studies, in which the N-terminal flexible regulatory arm of RhoGDI binds to the switch I and switch II regions of Cdc42 (fast step), and the C-terminal hydrophobic binding pocket of RhoGDI extracts the isoprenyl moiety of Cdc42 off the membrane (slow step) (18, 35). Although RhoGDI(I177D) inhibited the ability of RhoGDI and RhoGAP to translocate Rho GTPases from membrane to cytosol, it did not inhibit cell rounding stimulated by RhoGAP and GFP-

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**Fig. 6. Expression and characterization of p50RhoGAP-HA.**

A, CHO cells were transfected with pEGFP (50 ng/ml) alone or co-transfected with pEGFP (50 ng/ml) and p50RhoGAP-HA (320 ng/ml). Total transfected DNA was normalized with pCMV-luciferase. At 24 h post-transfection, the cells were examined by fluorescent microscopy. B, pEGFP was co-transfected with either p50RhoGAP or GFP-RhoGDI into CHO cells. At 24 h post-transfection, the cells were lysed and fractionated into post-nuclear supernatant (P), membrane (M), and cytosol (C), which was followed by ECL Western blotting using α-GFP or α-HA antibody as the primary antibodies. C, CHO cells were co-transfected with pEGFP (50 ng/ml) along with the indicated amount of p50RhoGAP-HA. Total transfected DNA was normalized with pCMV-luciferase. At 24 h post-transfection, the percentage of cell rounding was calculated by the fluorescent microscopy. The cells were lysed, and the whole cell lysates were subjected to SDS-PAGE followed by Western blot with anti-HA antibody or anti-GFP antibody (inset). The expression level of p50RhoGAP-HA was determined similarly as in Fig. 1C, by using recombinant ExoS-HA as standard in FluorChem®8900. The data from three independent experiments are shown.
RhoGDI. Similar to GFP-RhoGDI, expression of GFP-RhoGDI(I177D) in CHO cells stimulated cell rounding in a dose-dependent manner, whereas GFP-RhoGDI(L55S,L56S) did not stimulate cell rounding (data not shown). This indicates that the binding of the N-terminal regulatory arm of RhoGDI to GDP-bound Rho GTPases on the membrane also inhibits GEF-stimulated activation of Rho GTPases.

Small interfering RNA provides a powerful and convenient tool to investigate the roles of RhoGDI in reorganization of the actin cytoskeleton mediated by ExoS RhoGAP. HeLa cells with knockdown expression of RhoGDI were resistant to the actin reorganization elicited by type III-delivered ExoS RhoGAP.

This indicates that RhoGDI is required for ExoS RhoGAP-mediated actin reorganization. Therefore, RhoGDI appears to be a major regulator of Rho GTPases activity and actin cytoskeleton. RhoGDI extracting Rho GTPases off the membrane and sequestering them in cytosol may be the rate-limiting step in Rho GTPases regulation.

The cooperativity between p50RhoGAP and GFP-RhoGDI in reorganization of the actin cytoskeleton suggests the presence of a similar mechanism of Rho GTPase inactivation by eukaryotic RhoGDI and RhoGDI. Apparently, ExoS RhoGAP functions as a molecular mimic of eukaryotic RhoGAPs to modulate host actin cytoskeleton, and this modulation is through coordinating with eukaryotic RhoGDI. Although ExoS RhoGAP mimics eukaryotic RhoGAP, direct comparison between ExoS RhoGAP and p50RhoGAP indicated that ExoS RhoGAP is ~3-fold more potent than p50RhoGAP in eliciting actin reorganization (data not shown). ExoS RhoGAP also appears more resistant to the actions of dominant active Rho GTPases (data not shown). This could be due to the different subcellular localization between ExoS RhoGAP and p50RhoGAP. ExoS localized to membranes through an N-terminal membrane localization domain (MLD) (41), whereas p50RhoGAP is primarily localized in cytosol. Therefore, ExoS RhoGAP can constitutively inactivate Rho GTPases in membrane, whereas p50RhoGAP may only act on Rho GTPases on membrane through temporal membrane localization (42). The differences between ExoS and p50RhoGAP in reorganization of actin cytoskeleton and in resistance to the actions of dominant active Rho GTPases could also be due to their different specificity toward Rho GTPases. Both in vitro and in vivo studies showed that ExoS RhoGAP targeted RhoA, Rac, and Cdc42 equally well (5, 6), whereas p50RhoGAP prefers to target Cdc42 relative to Rho and Rac (43–45). The crystal structures of p50RhoGAP (46, 47) and ExoS RhoGAP (48, 49) showed that although the two proteins shared the common catalytic arginine finger, there is little similarity between the two structures, indicating a convergent evolution between the eukaryotic RhoGAPs to ExoS RhoGAP.

Like ExoS, YopE of *Yersinia* and SptP of *Salmonella* are type III cytotoxins that possess RhoGAP activity. YopE is a GAP for Cdc42, Rac1, and RhoA (50, 51), whereas SptP is a GAP for Cdc42 and Rac1 (52). It is likely that YopE and SptP also modulate the actin cytoskeleton through RhoGDI.

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