Direct Interaction of the Rho GDP Dissociation Inhibitor with Ezrin/Radixin/Moesin Initiates the Activation of the Rho Small G Protein*

(Received for publication, June 16, 1997)

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The small G proteins of the Rho family, consisting of the Rho, Rac, and Cdc42 subfamilies, are implicated in reorganization of actin filaments (7), which has also been implicated in reorganization of actin filaments (8–10). ERM are intracellular proteins with at least two functionally different domains, the N-terminal plasma membrane-interacting and C-terminal actin filament-interacting domains (8–10). ERM are translocated to the plasma membrane probably through the interaction with the cytoplasmic domain of integral plasma membrane proteins, such as CD44, providing the actin filament association sites (11–13). When cells are treated with agonists or Ca2+, the GDP-bound form of RhoA, a member of the Rho subfamily, staying in the cytosol in complex with Rho GDI is activated to the GTP-bound form and translocated to the same areas as ERM are translocated (6). We have recently found that Rho GDI and CD44 are co-immunoprecipitated with moesin when the crude lysate of baby hamster kidney cells is treated with an anti-moesin monoclonal antibody and that the Rho subfamily members stimulate the interaction of ERM with the plasma membrane (7). These results have raised a possibility that ERM or CD44 has an activity to make the GDP-bound form of the Rho subfamily members complexed with Rho GDI sensitive to the action of Rho GEFs. We have examined this possibility using RhoA as a substrate for Rho GDI and Db1 as a Rho GEF.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—A series of truncated mouse radixin fusion proteins were expressed and purified from Escherichia coli. Nt-Fragment (amino acids 1–280), Ct-Fragment (amino acids 281–584), and full-length radixin (amino acids 1–584) were expressed as GST fusion proteins from pGEX vectors. The fusion proteins were purified using glutathione-Sepharose 4B columns, and the GST carrier was cleaved off by digestion with thrombin (14). Bovine Rho GDI was also expressed and purified as a GST fusion protein from E. coli, and the GST carrier was cleaved off by digestion with thrombin. The C-terminal fragment of Dbl containing the catalytic domain was expressed and purified as a GST fusion protein from E. coli (4). Human lipid-modified RhoA, Rac1, and Cdc42 were purified from the membrane fraction of Spodoptera frugiperda cells overexpressing each protein (4, 15).

Isolation of RhoA-Rho GDI Complex—[3H]GDP- or GDP-RhoA complexed with Rho GDI was obtained by first incubating GDP-RhoA with or without [3H]GDP, followed by incubation with Rho GDI for 30 min at 4 °C. The sample was then subjected to gel filtration using a Superdex 75 PC3.2/30 column (Pharmacia Biotech Inc.) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% CHAPS. [3H]GDP- or GDP-RhoA complexed with Rho GDI was detected by protein staining. The [3H]GDP- or GDP-bound form of Rac1 complexed with Rho GDI and the [3H]GDP- or GDP-bound form of Cdc42 complexed with Rho GDI were similarly prepared.

Construction of Expression Plasmids—Mammalian expression plasmids (pSRneo-Myc and pEFBOS-HA) were generated to express fusion proteins with the N-terminal Myc and HA epitopes, respectively, as

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described (6, 16). To generate pSROKE-Myc-Rho GDI and pEFBOS-HA-Nr-Fragment, their cDNA constructs were made by the polymerase chain reaction using specific oligonucleotide primers and inserted into pSROKE-Myc or pEFBOS-HA.

**Transient Expression and Immunoprecipitation of Expressed Proteins**—Transient expression of Myc-Rho GDI with or without HA-Nr-Fragment was carried out using pSROKE-Myc-Rho GDI and pEFBOS-HA or pEFBOS-HA-Nr-Fragment in COS-7 cells. The cells were plated at a density of 5 × 10^6 cells/60-mm dish and were incubated for 18 h. The cells were then cotransfected with 2 µg of pSROKE-Myc-Rho GDI and 2 µg of pEFBOS-HA or pEFBOS-HA-Nr-Fragment using the DEAE-dextran method. Immunoprecipitation was performed at 48 h after the transfection. The cells were washed with PBS twice, lysed in lysis buffer (containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM p-aminophenylmethanesulfonyl fluoride), and sonicated. The cell lysate was centrifuged at 100,000 × g for 1 h to prepare the cytosolic fraction. Myc-Rho GDI was precipitated with 5 µg of anti-Myc monoclonal antibody bound to 20 µl of protein A-Sepharose, followed by centrifugation and extensive washing with lysis buffer in the presence of 1% Nonidet P-40.Comparable amounts of the pellets were subjected to SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrotransferred to a nitrocellulose membrane sheet. The sheet was processed using the ECL detection kit (Pharmacia Biotech Inc.) to detect Myc-Rho GDI, RhoA, and HA-Nr-Fragment with the anti-Rho GDI polyclonal, anti-RhoA polyclonal, and anti-HA monoclonal antibodies as primary antibodies, respectively.

**Microinjection and Immunofluorescence Microscopy**—Swiss 3T3 cells were plated at a density of 1 × 10^5 cells/35-mm grid dish in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and were incubated for 3 days. Then, the medium was changed to a serum-free medium, and the cells were further incubated for 24 h. After the incubation, microinjection was performed as a GST fusion protein and purified from E. coli. C3 was kindly supplied by S. Narumiya (Kyoto University, Kyoto, Japan). All proteins used were concentrated to 5 mg/ml with a Centricon-10 concentrator (Amicon, Inc.). Each sample to be tested was co-microinjected with 2.5 mg/ml rat IgG into cells as described (17). GST-Nr-Fragment was microinjected at 4 mg/ml, and its intracellular concentration was ~10 µM. C3 was microinjected at 40 µg/ml, and its intracellular concentration was ~0.23 µM. The cells were fixed at 30 min after the microinjection with 3.7% paraformaldehyde in PBS for 10 min. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. After being soaked in 10% fetal bovine serum/PBS for 1 h, the cells were treated for 1 h with fluorescein isothiocyanate-conjugated goat anti-rat IgG (Chemicon International, Inc., Temecula, CA) and rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, OR) in 10% fetal bovine serum/PBS for detection of the microinjected cells and actin filaments, respectively. After being washed with PBS three times, the cells were examined using an LSM 410 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

**RESULTS AND DISCUSSION**

We first examined the direct physical interaction of Rho GDI with ERM or CD44 using the highly purified recombinant proteins. Rho GDI did not directly interact with full-length radixin or the cytoplasmic fragment of CD44 containing the ERM-interacting domain (amino acids 391–462) (data not shown), suggesting that Rho GDI indirectly interacts with these proteins through an unidentified protein or directly interacts with the specific region of ERM that is masked by these proteins through an unidentified protein or directly interacts with the specific region of ERM that is masked by these proteins. We have previously shown that the GEF-independent GDP/GTP exchange reaction of RhoA at low Mg²⁺ concentrations (micromolar range) is much faster than that at high Mg²⁺ concentrations (millimolar range) (21), that Rho GDI inhibits the reactions both at low and high Mg²⁺ concentrations, but that the inhibitory effect of Rho GDI is apparently more obvious at low Mg²⁺ concentrations than at high Mg²⁺ concentrations (14). We first examined the effect of the interaction of Rho GDI with Nr-Fragment on its activity to inhibit the GDP/GTP exchange reaction of RhoA at low Mg²⁺ concentrations. This reaction was estimated by measuring the dissociation of [3H]GDP from [3H]GDP-RhoA complexed with Rho GDI and the binding of [35S]GTPγS to GDP-RhoA complexed with Rho GDI. Nr-Fragment reduced this Rho GDI activity in a dose-dependent manner (Fig. 2). Under comparable conditions, neither Cr-Fragment nor full-length radixin affected the Rho GDI activity. The same inhibitory effect of Nr-Fragment was also observed when Rac1 or Cdc42 was used as a substrate for Rho GDI. The amino acid sequence of the N-terminal fragment is highly conserved within ERM (−85% identical for any pair) (9, 10). Consistently, the N-terminal fragments of ezrin (amino acids 1–280) and moesin (amino acids 1–280) showed the same inhibitory effects on the Rho GDI activity for RhoA, Rac1, and Cdc42 (data not shown). These results indicate that the N-terminal region of ERM has a potency to directly interact with Rho GDI and to reduce its activity to inhibit the GDP/GTP exchange reactions of all the Rho GDI substrate small G proteins.
In the [3H]GDP dissociation assay, the [3H]GDP-RhoA-Rho GDI complex (50 nM) was incubated for 20 min at 30 °C with various concentrations of Nr-Fragment, Cr-Fragment, or full-length radixin in a reaction mixture (50 µl) containing 30 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.1 mM GTP, 10 mM EDTA, 1 mM diethiothreitol, 0.12% CHAPS, and 0.2 mg/ml bovine serum albumin. After the incubation, the dissociation of [3H]GDP from [3H]GDP-RhoA-complexed with Rho GDI was assayed by measuring the radioactivity of [3H]GDP bound to RhoA by the filtration method using a nitrocellulose filter (26). In the [35S]GTP·S binding assay, the GDP-RhoA-Rho GDI complex (50 nM) was incubated as described above except that 1 µM [35S]GTP·S was used instead of 0.1 mM GTP, and the binding of [35S]GTP·S to GDP-RhoA-complexed with Rho GDI was assayed by measuring the radioactivity of [35S]GTP·S bound to RhoA. The same experiments were also performed using Rac1 and Cdc42 instead of RhoA. A, [3H]GDP dissociation; B, [35S]GTP·S binding; ●, Cr-Fragment; ○, Nr-Fragment. The [3H]GDP-RhoA-Rho GDI or GDP-RhoA-Rho GDI complex (50 nM each) was incubated for 20 min at 30 °C in the presence of the indicated concentrations of 90 nM GST-Dbl and 1.5 µM Nr-Fragment as described in the legend to Fig. 2 except that 5 mM MgCl₂ and 1 mM EDTA were used instead of 5 mM MgCl₂ and 10 mM EDTA. ○, [3H]GDP-RhoA free of Rho GDI; ●, [3H]GDP-RhoA free of Rho GDI plus Dbl; ▲, [3H]GDP-RhoA complexed with Rho GDI plus Dbl; □, [3H]GDP-RhoA complexed with Rho GDI plus Dbl plus Nr-Fragment. B, dose-dependent effect of Nr-Fragment. The [3H]GDP-RhoA-Rho GDI or GDP-RhoA-Rho GDI complex (50 nM each) was incubated for 20 min at 30 °C with 90 nM GST-Dbl in the presence of various concentrations of Nr-Fragment. Panel a, [3H]GDP dissociation; panel b, [35S]GTP·S binding; ●, with Dbl; ○, without Dbl. The values are means ± S.E. of three independent experiments.

We have shown that Rho GEFs, such as Dbl and Rom1/2, stimulate the GDP/GTP exchange reaction of GDP-RhoA free of Rho GDI, but not that of GDP-RhoA complexed with Rho GDI, at high Mg²⁺ concentrations (4, 5). We therefore next examined the effect of Nr-Fragment on the Rho GDI activity to inhibit the Dbl-stimulated GDP/GTP exchange reaction of RhoA at high Mg²⁺ concentrations. Dbl stimulated the dissociation of GDP from GDP-RhoA, but the dissociation of GDP from GDP-RhoA complexed with Rho GDI was markedly reduced, and this reaction was restored by Nr-Fragment (Fig. 3A). This inhibitory effect of Nr-Fragment on the Rho GDI activity was dose-dependent and also observed in the Dbl-dependent binding of GTP·S to GDP-RhoA complexed with Rho GDI (Fig. 3B). Nr-Fragment also reduced the Rho GDI activity to inhibit the Dbl-independent GDP/GTP exchange reaction of RhoA, but the level of this reduction was apparently small due to the slow rate of the GDP/GTP exchange reaction of RhoA at high Mg²⁺ concentrations. These results indicate that the interaction of Rho GDI with the N-terminal region of ERM reduces its activity in both the Rho GEF-independent and -dependent GDP/GTP exchange reactions of RhoA.

Since the C-terminal fragment of ezrin has been shown to interact with the N-terminal fragment longer than amino acids 1–296 (20), and the C-terminal fragment of radixin has been shown to interact with the N-terminal fragment containing amino acids 1–318 (19), Nr-Fragment used here was not expected to interact with Cr-Fragment. We therefore prepared a longer N-terminal fragment (Nr1-Fragment, amino acids 1–318). Nr1-Fragment also reduced the Rho GDI activity in a dose-dependent manner, with an efficacy similar to that of Nr-Fragment (data not shown). We then examined the effect of Cr-Fragment on the Nr-Fragment and Nr1-Fragment activities to reduce the Rho GDI activity. Cr-Fragment reduced the Nr1-Fragment activity, but not the Nr-Fragment activity (Fig. 4).
Myc-Rho GDI, suggesting that the RhoA complexed with Rho GDI was replaced by HA-Nr-Fragment to form the Rho GDI-HA-Nr-Fragment complex in intact cells.

We further examined whether overexpression of Nr-Fragment mimics the functions of the Rho family members. For this purpose, we used Swiss 3T3 cells because the GTP-bound forms of RhoA, Rac1, and Cdc42 have been shown to induce the formation of stress fibers, lamellipodia, and filopodia, respectively, in this cell line (22). The GTP-bound forms of Rac1 and Cdc42 are also known to induce membrane ruffling (22). Serum-starved Swiss 3T3 cells had very few stress fibers, but microinjection of GST-Nr-Fragment into these cells induced the formation of prominent stress fibers (Fig. 5B). This response was inhibited by co-microinjection with C3, which is known to ADP-ribosylate the Rho subfamily members and to inhibit their functions (1, 2). However, microinjection of GST-Nr-Fragment did not induce the formation of lamellipodia and filopodia or membrane ruffling. Microinjection of GST did not show any effect (data not shown). These results have provided another line of evidence that ERM indeed initiate the activation of the Rho subfamily members through Rho GDI in intact cells. Moreover, these results, together with the in vitro results described above, show that the N-terminal region of ERM has a potency to activate all the Rho, Rac, and Cdc42 subfamily members, have raised a possibility that there is a mechanism by which ERM induce the selective activation of each Rho GDI substrate small G protein in intact cells. This mechanism is currently unknown, but one possible factor involved in this mechanism is a Rho GEF specific for each Rho family member, such as Lhc, Tiam-1, and FGDI (23–25). Elucidation of this mechanism is one of the important issues to be addressed next.

ERM have been shown to be translocated from the cytosol to the plasma membrane at least partly through the interaction with CD44 in response to agonists (6, 13). This translocation has been shown to be accompanied by reorganization of actin filaments (8–10). Our previous result, that CD44 and Rho GDI are co-immunoprecipitated with moesin (7), indicates that these three proteins are able to form a ternary complex. Moreover, in a cell-free system, we have demonstrated that ERM directly interact with the cytoplasmic fragment of CD44 in the presence of phosphatidylinositol 4,5-bisphosphate at a physiological salt concentration (150 mM KCl), but that ERM directly interact with it in the absence of phosphatidylinositol 4,5-bisphosphate at a low salt concentration (40 mM KCl) (7). We therefore finally examined whether full-length radixin complexed with CD44 at 40 mM KCl reduces the Rho GDI activity. The cytoplasmic fragment of CD44 did not make full-length radixin reduce the Rho GDI activity in intact cells

FIG. 5. Nr-Fragment-induced activation of RhoA in intact cells. A, Nr-Fragment-induced dissociation of RhoA from Rho GDI and subsequent formation of the Rho GDI-Nr-Fragment complex in intact COS-7 cells. Myc-Rho GDI was expressed with or without HA-Nr-Fragment in COS-7 cells and immunoprecipitated with an anti-Myc antibody. Immunoprecipitated Myc-Rho GDI, RhoA, and HA-Nr-Fragment were detected with anti-Rho GDI polyclonal, anti-RhoA polyclonal, and anti-HA monoclonal antibodies, respectively. The results shown are representative of three independent experiments. B, Nr-Fragment-induced formation of stress fibers in intact Swiss 3T3 cells. GST-Nr-Fragment or GST-Nr-Fragment plus C3 were microinjected with rat IgG into Swiss 3T3 cells. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG or rhodamine-labeled phalloidin and analyzed by confocal microscopy. The results shown are representative of three independent experiments. Bar = 25 μm.
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doi: 10.1074/jbc.272.37.23371

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