Unique in Vivo Associations with SmgGDS and RhoGDI and Different Guanine Nucleotide Exchange Activities Exhibited by RhoA, Dominant Negative RhoA\textsuperscript{Asn-19}, and Activated RhoA\textsuperscript{Val-14}\textsuperscript{*}

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We compared the in vivo characteristics of hemagglutinin (HA)-tagged RhoA, dominant negative RhoA\textsuperscript{Asn-19}, and activated RhoA\textsuperscript{Val-14} stably expressed in Chinese hamster ovary (CHO) cells. Proteins co-precipitating with these HA-tagged GTPases were identified by peptide sequencing or by Western blotting. Dominant negative RhoA\textsuperscript{Asn-19} co-precipitates with the guanine nucleotide exchange factor (GEF) SmgGDS but does not detectably interact with other expressed GEFs, such as Ost or Dbl. SmgGDS co-precipitates minimally with wild-type RhoA and does not detectably associate with RhoA\textsuperscript{Val-14}. The guanine nucleotide dissociation inhibitor RhoGDI co-precipitates with RhoA, and to a lesser extent with RhoA\textsuperscript{Val-14}, but does not detectably co-precipitate with RhoA\textsuperscript{Asn-19}. Wild-type RhoA is predominantly in the \([\text{32P}]\text{GDP}\)-bound form, RhoA\textsuperscript{Val-14} is predominantly in the \([\text{32P}]\text{GTP}\)-bound form, and negligible levels of \([\text{32P}]\text{GDP}\) or \([\text{32P}]\text{GTP}\) are bound to RhoA\textsuperscript{Asn-19} in 32\textsuperscript{P}-labeled cells. Immunofluorescence analyses indicate that HA-RhoA\textsuperscript{Asn-19} is excluded from the nucleus and cell junctions. Microinjection of SmgGDS cDNA into CHO cells stably expressing HA-RhoA causes HA-RhoA to be excluded from the nucleus and cell junctions, similar to the distribution of RhoA\textsuperscript{Asn-19}. Our findings indicate that the expression of RhoA\textsuperscript{Asn-19} may specifically inhibit signaling pathways that rely upon the SmgGDS-dependent activation of RhoA.

Rho proteins are small GTPases that regulate actin/myosin interactions, controlling such diverse processes as cell division, migration, contraction, and adhesion (reviewed in Refs. 1 and 2). Rho is activated by associating with guanine nucleotide exchange factors (GEFs),\textsuperscript{1} which stimulate GTP binding to Rho in exchange for GDP. RhoA is activated by several GEFs in vitro, including SmgGDS (3–6), Dbl (3), Ost (7), p115-RhoGEF (8), Lfc, Lbc, Lsc (9), mNET1 (10), and GEF-H1 (11). However, the GEFs that interact with Rho proteins in vivo are not well characterized. Identifying the GEFs that associate with Rho proteins in vivo will help determine how Rho activity is regulated.

Dominant negative Rho mutants are used to define the signaling pathways that regulate Rho activity (12–15). Substitution of asparagine for threonine at amino acid 19 in RhoA is believed to induce a dominant negative function because of the increased association of RhoA\textsuperscript{Asn-19} with a GEF. Competitive binding of a specific GEF by RhoA\textsuperscript{Asn-19}\textsuperscript{19} may inhibit the GEF from interacting with endogenous RhoA. This event would disrupt any signal transduction pathway that normally utilizes the GEF. This model of how RhoA\textsuperscript{Asn-19} induces a dominant negative phenotype is based on previous studies using mutant Ras proteins with the analogous substitution of asparagine for serine at amino acid 17 (16, 17). These studies support the model that Ras\textsuperscript{Asn-17} has a dominant negative function because it competitively binds a GEF needed for the activation of endogenous Ras (16, 17). However, the GEFs that are preferentially bound by Ras\textsuperscript{Asn-17} or RhoA\textsuperscript{Asn-19} in vivo have not been characterized. The potential dependence of endogenous RhoA on the proteins competitively bound by RhoA\textsuperscript{Asn-19} provides a strong rationale for identifying the proteins associating with RhoA\textsuperscript{Asn-19} in vivo. For this reason, we investigated the in vivo characteristics of hemagglutinin (HA)-tagged wild-type and mutant RhoA stably expressed in Chinese hamster ovary (CHO) cells.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—The generation of the clonal CHO-m3 sublines stably expressing HA-tagged wild-type or mutant RhoA was described previously (10). HA-RhoA is expressed by the m3WTRho-1 and m3WTRho-11 cell lines. Activated HA-RhoA\textsuperscript{Val-14} is expressed by the m3CARho-1 and m3CARho-4 cell lines, and dominant negative HA-RhoA\textsuperscript{Asn-19} is expressed by the m3DN-Rho-2, m3DN-Rho-4, and m3DN-Rho-6 cell lines. The m3Zeo-2 cell line is a clonal CHO-m3 subline that does not express HA-tagged Rho proteins (12). All CHO-m3 sublines used in this study also express transfected human \(M\) muscarinic acetylcholine receptors (mACHR) (12). The cells were cultured in complete medium (12) to promote exponential proliferation. Exponentially proliferating Jurkat and HeLa cells were provided by Drs. John Noti and Robert Aronstam (Guthrie Research Institute), and freshly collected rat brain tissue was a gift from Dr. Seong-Woo Jeong (Guthrie Research Institute). The pCEV29-Ost-\(\alpha\) (P) plasmid coding for full-length Ost-\(\alpha\) (19) was provided by Dr. Toru Miki (NCI, National Institutes of Health). The pCVTH3-protob/\(d\)l plasmid coding for HA-tagged full-length Dbl (20) was a gift from Dr. Channing Der (University of North Carolina).

Levels of \([\text{32P}]\text{GTP}/[\text{32P}]\text{GDP}\) Bound to Wild-type or Mutant RhoA in Vivo—Cells were cultured for 90 min in phosphate-free Dulbecco’s modified Eagle’s medium containing Hesper (10 mM) and \([\text{32P}]\text{orthophosphate}\) (50 \(\mu\)Ci/ml) (ICN Biochemicals, Inc.) and scraped from the culture dishes after the addition of ice-cold extraction buffer (50 mM Heps, 500 mM NaCl, 15 mM MgCl\(_2\), 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 \(\mu\)M leupeptin, 0.3 \(\mu\)M aprotinin, pH 7.4). The cell lysates were centrifuged (13,000 \(\times\) g, 10 min, 4 \(^\circ\)C), and the resulting
superнатants were incubated (90 min, 4 °C) with HA antibody (5 μg/ml) (Babcо, Berkeley, CA) and Protein A-agarose (Life Technologies, Inc.). The 32P-labeled nucleotides were eluted from the immunoprecipitated HA-RhoA proteins and separated by thin layer chromatography as described previously (21).

Binding of [35S]GTP·S by Wild-type or Mutant RhoA in Cell Membranes—Cells were subjected to a −70 °C freeze/thaw cycle and dounced in ice-cold homogenization buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 430 μM leupeptin, pH 7.4), followed by centrifugation (300 × g, 10 min, 4 °C). The resulting supernatant was centrifuged (150,000 × g, 20 min, 4 °C), and the membrane pellet was incubated (10 min, 30 °C) in reaction buffer (50 mM Hepes, 100 mM NaCl, 6 mM MgCl2, 2 mM EDTA, 10 μM GDP, 150 mM GTP·S, pH 7.4) containing 150 mM [35S]GTP·S (1030 Ci/mmol) (Amersham Pharmacia Biotech). The membrane proteins were solubilized (30 min, 4 °C) in 50 mM Hepes, 20 mM MgCl2, 10 mM GDP, 100 μM GTP, 0.5% Nonidet P-40, 1 mM PMSF, 200 μg/ml leupeptin, pH 7.4. After centrifugation (13,000 × g, 10 min, 4 °C), the resulting supernatant were immunoprecipitated with HA antibody. The amounts of [35S]GTP·S bound to the immunoprecipitated HA-RhoA proteins were determined by liquid scintillation counting using an LS-60001C β-counter (Beckman Instruments, Fullerton, CA).

Immunoprecipitation and Enhanced Chemiluminescence (ECL) Western Blotting—Rat brain tissue or cells which were either unlabeled or labeled with [35S]methionine for 16 h were lysed in ice-cold lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, pH 7.4) containing protease inhibitors (200 μg/ml PMSF, 10 μM leupeptin) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 0.2 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 10 mM EDTA). The lysates were centrifuged (13,000 × g, 10 min, 4 °C), and the resulting supernatants were subjected to ECL Western blotting (12) using antibodies to HA (Babco), Dbl, Ost (L-20), Vav (C-14) (Santa Cruz Biotechnology, Santa Cruz, CA), or β-catenin (Transduction Laboratories, Lexington, KY). Alternatively, the supernatants were immunoprecipitated with HA antibody (4 μg/ml) as described previously (22). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography or ECL Western blotting using antibodies to HA (Babco), Dbl, Ost (L-20), and Vav (C-14) (Santa Cruz Biotechnology). The microinjected cells were stained with 2′,7′-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) and examined for co-precipitating proteins by SDS-polyacrylamide gel electrophoresis followed by autoradiography or ECL Western blotting using antibodies to HA, Dbl, or Ost (L-20). The 150-kDa protein that co-precipitates with HA-RhoAAsn-19 was collected in a piece of excised gel and frozen at −20 °C until it was enzymatically digested and sequenced at the Protein Core Facility, Mayo Clinic (Rochester, MN), using previously described techniques (23).

Microinjection and Immunofluorescence—The cells were cultured for 2 days on glass coverslips etched with labeled grids (Eppendorf, Hamburg, Germany) before being microinjected as described previously (24) using an Eppendorf Model 5171 micromanipulator and Model 3246 transrector. The cells were microinjected with plasmids coding for full-length SmgGDS, Ost-α, or HA-Dbl at a concentration of 100 ng of DNA/μl of injection buffer (24). The success of injection was determined by co-injecting some cells with the pEGFP-N1 plasmid containing the green fluorescent protein (GFP) coding sequence (CLONTECH Laboratories, Palo Alto, CA), at a concentration of 5 ng of DNA/μl of injection buffer. Control cells were microinjected either with injection buffer or the pEGFP-N1 plasmid alone. The cells were cultured in complete medium (12) for 18–24 h after injection, and then immunofluorescently labeled with HA or RhoA antibody (25). The microinjected cells were identified by their location on the grids on the coverslips or by their expression of GFP prior to fixation for immunofluorescent labeling.

RESULTS

The in vivo guanine nucleotide exchange activities of the HA-tagged wild-type and mutant RhoA proteins were determined by measuring the amounts of [32P]GTP and [32P]GDP bound to these proteins in 32P-labeled cells (Fig. 1A). Wild-type RhoA is in the predominantly [32P]GDP-bound form, whereas activated RhoAVal-14 is in the predominantly [32P]GTP-bound form in the 32P-labeled cells (Fig. 1A). In contrast, only minimal levels of [32P]GDP and [32P]GTP are bound to RhoAAsn-19 in the 32P-labeled cells (Fig. 1A, lane 3). Both wild-type RhoA and activated RhoAVal-14 were found to bind significant levels of [35S]GTP·S after a 10-min incubation of cell membranes with [35S]GTP·S (Fig. 1B). In contrast, minimal levels of [35S]GTP·S are bound to dominant negative RhoAAsn-19 (Fig. 1B).

HA-tagged RhoA proteins were immunoprecipitated from 35S-labeled cells and examined for co-precipitating proteins (Fig. 2A). A 28-kDa protein co-precipitates with wild-type RhoA (lane 2, Fig. 2A). Western blotting identified this protein as RhoGDI (Fig. 2B). RhoGDI does not associate with dominant negative RhoAAsn-19 (lane 3, Fig. 2A), and it associates only weakly with activated RhoAVal-14 (lane 4, Fig. 2A). The 150-kDa protein that associates only with wild-type RhoA (lane 2, Fig. 2A) has not yet been identified.

A 60-kDa protein preferentially associates with dominant negative RhoAAsn-19 (lane 3, Fig. 2A). Enzymatic digestion and peptide sequencing of this 60-kDa protein yielded a 17-amino acid peptide identical to residues 320–336 of SmgGDS (Fig. 2C). The high performance liquid chromatography fraction containing this peptide also contained a small amount of a secondary peptide. The sequence of this secondary peptide corresponds to residues 379–393 of SmgGDS (Fig. 2C). These results indicate that SmgGDS is the 60-kDa protein that preferentially associates with dominant negative RhoAAsn-19. This conclusion is supported by previous reports that SmgGDS is a 61-kDa (558 amino acids) GEF that physically associates with RhoA (3–6). SmgGDS and a 57-kDa protein preferentially co-precipitate with RhoAAsn-19 in all three sublines expressing dominant negative RhoAAsn-19 (lanes 3–5, Fig. 2D).

Small amounts of these proteins also co-precipitate with HA-tagged wild-type RhoA expressed in m3WT/Rho-1 and m3WT/Rho-11 cells (lanes 1 and 2, Fig. 2D).

We found that both Dbl and Ost are expressed by m3DNRho-4 cells (Fig. 2E) and by the other CHO-m3 sublines (data not shown). Interestingly, dominant negative RhoAAsn-19 does not detectably co-precipitate with these GEFs nor with other proteins of similar relative molecular mass (55–115 kDa) (Fig. 2, A and D). The co-precipitation of Dbl or Ost with RhoAAsn-19 was also not observed when Western blots of immunoprecipitated RhoAAsn-19 were probed with Dbl or Ost antibodies (n = 3, data not shown).

Wild-type RhoA is diffusely distributed throughout the nucleus and cytoplasm, with increased localization at cell junctions and in the perinuclear area of some cells (Fig. 3A). Activated RhoAVal-14 is similarly distributed throughout the
cell, although some cells appear to have greater levels of RhoAVal-14 in the nucleus compared with the cytoplasm (Fig. 3B). This appearance may result from increased nuclear localization of RhoAVal-14 or because increased cell spreading causes a more diffuse and apparently less intense staining of cytoplasmic RhoAVal-14. Dominant negative RhoAAsn-19 is confined to the perinuclear area (Fig. 3C). Microinjection of SmgGDS cDNA into m3WT-Rho-1 cells resulted in the exclusion of HA-RhoA from the nucleus and from cell-cell junctions (Fig. 3E). In contrast, wild-type RhoA remains in the nucleus and at junctions after the cells are microinjected with Ost-α cDNA (Fig. 3F). Microinjection of HA-Dbl cDNA into CHO-m3 cells did not alter the junctional and nuclear staining of endogenous RhoA detected by RhoA antibody (data not shown). Microinjection of buffer or GFP cDNA also did not alter the distribution of HA-RhoA or endogenous RhoA in the cells (data not shown).

**DISCUSSION**

This study demonstrates that dominant negative RhoAAsn-19 has increased association with SmgGDS and reduced interaction with RhoGDI. RhoAAsn-19 also does not detectably bind GTP in the CHO-m3 sublines. This result is consistent with *in vitro* studies demonstrating that H-RasAsn-17 has a 40-fold lower affinity for GTP compared with that of wild-type H-Ras (16). The binding of GTP by small GTPases may promote their disassociation from GEFs such as SmgGDS (6, 26). Thus, RhoAAsn-19 may remain associated with SmgGDS because RhoAAsn-19 cannot bind GTP. These possibilities are consistent with a previously developed model suggesting that RasAsn-17 exerts a dominant negative phenotype by nonproductively associating with a GEF (16, 17).

These findings provide an explanation for the dominant negative function of RhoAAsn-19. The association of SmgGDS with RhoAAsn-19 may prohibit SmgGDS from interacting with endogenous RhoA. This event may disrupt signaling pathways that involve the SmgGDS-dependent activation of RhoA. RhoAAsn-19 expression diminishes myosin reorganization induced by stimulating M3 mAChR in the CHO-m3 sublines (12). It is possible that M3 mAChR stimulation cannot activate RhoA when SmgGDS-dependent activation of RhoA. RhoAAsn-19 expression diminishes myosin reorganization induced by stimulating M3 mAChR in the CHO-m3 sublines (12). It is possible that M3 mAChR stimulation cannot activate RhoA when SmgGDS-dependent activation of RhoA. RhoAAsn-19 expression diminishes myosin reorganization induced by stimulating M3 mAChR in the CHO-m3 sublines (12). It is possible that M3 mAChR stimulation cannot activate RhoA when SmgGDS-dependent activation of RhoA.
these perinuclear organelles. We found that microinjection of SmgGDS cDNA causes wild-type HA-RhoA to be excluded from the nucleus and from cell junctions. The absence of HA-RhoA from the junctions of cells overexpressing SmgGDS is consistent with previous reports that SmgGDS causes small GTPases to dissociate from plasma membranes (5, 30). Previous studies indicate that signals transduced by surface receptors cause RhoA to enter the nucleus (31). Our results suggest that interactions with SmgGDS may prohibit RhoA from entering the nucleus.

It is somewhat surprising that dominant negative RhoAVal-14 interacts with SmgGDS but does not detectably associate with other GEFs such as Dbl or Ost. It is possible that Dbl and Ost are expressed at significantly lower levels than SmgGDS, causing the co-precipitation of RhoAVal-14 with Dbl or Ost to be significantly less detectable than its co-precipitation with SmgGDS. We are currently addressing this possibility by comparing the expression of SmgGDS with other GEFs in the CHO-m3 cell lines.

SmgGDS can activate several other small GTPases in addition to RhoA in vivo, including Rac1 (3, 5, 6), Rac2 (6), Cdc42 (3, 6), K-Ras (3, 4), and Rap1B (3, 4). The binding of SmgGDS by RhoAVal-14 could conceivably diminish the interaction of SmgGDS with these different GTPases. This event would disrupt signaling pathways that involve the SmgGDS-dependent activation of these other GTPases. However, several lines of evidence suggest that the expression of RhoAVal-14 does not inhibit the activity of GTPases different from RhoA. First, these different GTPases are believed to be selectively activated by GEFs other than SmgGDS in vivo (6, 2). Thus, the activity of these GTPases would only be minimally affected by their inability to interact with SmgGDS that is competitively bound to RhoAVal-14. Secondly, SmgGDS is significantly more active toward RhoA than toward other GTPases (3–5). The potential loss of SmgGDS interactions would therefore affect RhoA more profoundly than other GTPases. Finally, we did not detect the co-precipitation of SmgGDS with wild-type Rac1, activated Rac1Val-12, or dominant negative Rac1Asn-17, stably expressed in other CHO-m3 sublines. This finding indicates that Rac1 does not interact with SmgGDS in these cells, even though Rac1 can be activated by SmgGDS in vivo (3, 5, 6).

In contrast to dominant negative RhoAAsn-19, activated RhoAVal-14 has reduced association with both SmgGDS and RhoGDI. This result is consistent with reports that SmgGDS and RhoGDI have reduced affinity for the GTP-bound form of small GTPases (6, 26). We did not detect any proteins that co-precipitate more effectively with RhoAVal-14 compared with wild-type RhoA. The interaction of RhoAVal-14 with potential effectors may be too labile to be detected by our immunoprecipitation techniques. Alternatively, the effectors that interact with RhoAVal-14 may not be solubilized by our immunoprecipitation techniques.

These findings help define how the expression of RhoAVal-14 or RhoAAsn-19 alters certain signaling pathways. The predom-

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2 C. L. Williams, unpublished data.