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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 46, pp. 35255–35266, November 12, 2010
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Splice Variants of SmgGDS Control Small GTPase Prenylation and Membrane Localization*[S]

Received for publication, April 2, 2010, and in revised form, August 6, 2010 Published, JBC Papers in Press, August 13, 2010, DOI 10.1074/jbc.M110.129916

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Ras and Rho small GTPases possessing a C-terminal polybasic region (PBR) are vital signaling proteins whose misregulation can lead to cancer. Signaling by these proteins depends on their ability to bind guanine nucleotides and their prenylation with a geranylgeranyl or farnesyl isoprenoid moiety and subsequent trafficking to cellular membranes. There is little previous evidence that cellular signals can restrain nonprenylated GTPases from entering the prenylation pathway, leading to the general belief that PBR-possessing GTPases are prenylated as soon as they are synthesized. Here, we present evidence that challenges this belief. We demonstrate that insertion of the dominant negative mutation to inhibit GDP/GTP exchange diminishes prenylation of Rap1A and RhoA, enhances prenylation of Rac1, and does not detectably alter prenylation of K-Ras. Our results indicate that the entrance and passage of these small GTPases through the prenylation pathway is regulated by two splice variants of SmgGDS, a protein that has been reported to promote GDP/GTP exchange by PBR-possessing GTPases and to be up-regulated in several forms of cancer. We show that the previously characterized 558-residue SmgGDS splice variant (SmgGDS-558) selectively associates with prenylated small GTPases and facilitates trafficking of Rap1A to the plasma membrane, whereas the less well characterized 607-residue SmgGDS splice variant (SmgGDS-607) associates with nonprenylated GTPases and regulates the entry of Rap1A, RhoA, and Rac1 into the prenylation pathway. These results indicate that guanine nucleotide exchange and interactions with SmgGDS splice variants can regulate the entrance and passage of PBR-possessing small GTPases through the prenylation pathway.

The Ras and Rho families of small GTPases participate in almost all aspects of cell biology by regulating cell survival, proliferation, and migration (1–4). Some of these small GTPases possess a C-terminal polybasic region (PBR)[3] that regulates their trafficking as they undergo prenylation (5, 6) and is required as a second signal for membrane localization (7). It is not known how the PBR promotes the trafficking of small GTPases through the prenylation pathway. Interestingly, the PBR is required for small GTPases to interact with SmgGDS, a protein consisting almost entirely of predicted Armadillo (ARM) domains (8). Although SmgGDS was previously reported to preferentially interact with small GTPases that possess a PBR, including K-Ras, Rap1, RhoA, and Rac1 (9–11), the functional significance of these PBR-dependent interactions has not been clearly defined.

It was recently found that SmgGDS promotes the malignant phenotype in non-small cell lung carcinoma (NSCLC) (12) and prostate carcinoma (13). The participation of SmgGDS in cancer has been attributed to its ability to increase the activities of multiple PBR-possessing small GTPases, many of which promote the malignant phenotype (reviewed in Ref. 12). This conclusion is supported by reports that many cellular processes regulated by small GTPases, including NF-κB activity, actin/myosin interactions, contractile responses, and cell migration and proliferation, are inhibited by diminishing SmgGDS expression in cancer cells (12, 13) or other cell types (14).

The mechanism by which SmgGDS promotes the activities of PBR-containing small GTPases is unclear. SmgGDS was previously reported to be a weak guanine nucleotide exchange factor (GEF) because of its ability to promote the dissociation of GDP and uptake of GTP by its small GTPase partners (9–11). However, SmgGDS does not possess any of the known catalytic domains found in other GEFs, leading to the suggestion that SmgGDS regulates small GTPases by a novel, unknown mechanism that might not involve classical guanine nucleotide exchange. In this study, we provide evidence of a previously undescribed mechanism utilized by SmgGDS to regulate small GTPases, involving their entrance and trafficking through the prenylation pathway.

Prenylation is necessary for optimal activity of members of the Ras and Rho families of small GTPases, increasing their

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 CA136799 from NCI (to C. L. W.), R01 GM069700 from NIGMS (to C. L. W.), and R01 CA081436-12 from NCI (to A. P. F.). This work was also supported by Rock River Cancer Research Foundation (to C. L. W.), Advancing a Healthier Wisconsin (to R. L. and C. L. W.), the Wisconsin Breast Cancer Showhouse (to C. L. W.), the V-Foundation (to A. P. F.), and the American Lung Association/LUNGevity (to A. P. F.).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3, Tables S1 and S2, and additional references.

[1] Recipient of a training grant from the National Cancer Center.

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[3] The abbreviations used are: PBR, polybasic region; ARM, armadillo; DN, dominant negative; ER, endoplasmic reticulum; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; NSCLC, non-small cell lung carcinoma; PM, plasma membrane; PTase, prenyltransferase; Rce1, Ras-converting enzyme 1; tet, tetracycline; WT, wild type; *A*, aliphatic amino acid.

hydrophobic character and thereby promoting their participation in membrane-localized signaling pathways and other biological interactions (3, 5, 6). The newly synthesized GTPases first interact with cytosolic prenyltransferases (PTases) (15–19) that add a farnesyl isoprenoid or geranylgeranyl isoprenoid to the cysteine in the C-terminal *CAAX* (where *A* indicates aliphatic amino acid) motif of the GTPases (20–23). The prenylated GTPases then move to the endoplasmic reticulum (ER) to interact with the Ras-converting enzyme I (Rce1) and the isoprenylcysteine carboxylmethyltransferase for post-prenylation processing (3, 5, 6).

The presence of a PBR influences which pathway a Ras or Rho family member will take to reach the plasma membrane (PM) after post-prenylation processing is completed in the ER. Small GTPases such as H-Ras and N-Ras, which lack a PBR, move from the ER to the Golgi, where they are palmitoylated, and then move by vesicular transport to the PM (5, 6). In contrast, small GTPases that possess a PBR, such as K-Ras, Rap1, RhoA, and Rac1, move directly from the ER to the PM by an uncharacterized mechanism that most likely involves unidentified chaperone proteins (5, 6).

Several crucial events in this prenylation pathway remain a mystery. It is not known how a newly synthesized small GTPase initially finds its PTase in the cytosol. In the absence of any known regulatory mechanisms, it is often assumed that small GTPases are prenylated as soon as they are synthesized and advance through the prenylation pathway unimpeded in a non-regulated manner. Recently, several models have emerged to define how the Rab escort protein Rep might regulate the interactions of newly synthesized Rab small GTPases with their PTase, geranylgeranyltransferase-II (24–26). However, the mechanisms that might regulate the interactions of newly synthesized Ras and Rho family members with their PTases have not been characterized.

Also unknown is how PBR-possessing small GTPases, once modified by the addition of a hydrophobic prenyl group, move through the cytosol to the ER for final processing by Rce1 and isoprenylcysteine carboxylmethyltransferase nor is it known how the fully processed PBR-possessing small GTPases move from the ER to the PM. It has been suggested that unidentified chaperone proteins might escort PBR-containing small GTPases between the cytosol, ER, and PM during the prenylation pathway (5, 6), but these suspected chaperones have not been identified.

Here, we report that the prenylation and trafficking of PBR-containing small GTPases is regulated by GDP/GTP exchange and by interactions with two splice variants of SmgGDS, which we have named SmgGDS-558 and SmgGDS-607. Our results demonstrate that SmgGDS-607 specifically interacts with non-prenylated small GTPases and regulates their entry into the prenylation pathway, whereas SmgGDS-558 specifically associates with prenylated small GTPases and regulates trafficking to the PM. These findings suggest that, in contrast to the previous view of unregulated entry of small GTPases into the prenylation pathway, prenylation of PBR-containing small GTPases is regulated in multiple ways. Interactions of PBR-containing small GTPases with SmgGDS splice variants in conjunction with GDP/GTP exchange presents, for the first time, a specific cellular mechanism to regulate the prenylation and subsequent membrane localization of these GTPases.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection of siRNAs and cDNAs—All cell lines were obtained from the American Type Tissue Collection (Manassas, VA). The NSCLC cell lines NCI-H1703 and NCI-H23 were maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and antibiotics; the HEK-293T cell line was maintained in DMEM with 10% heat-inactivated fetal bovine serum and antibiotics. All siRNA duplexes were purchased from Dharmacon (Lafayette, CO). Sequences of the siRNAs are presented in supplemental Table S1. Nontargeting siRNAs (Dharmacon siControl 3) were designed not to target any human mRNA. Cells were transfected with 25 nM siRNA using DharmaFECT-3 transfection reagent (Dharmacon, Lafayette, CO) according to the manufacturer's instruction. All cDNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

cDNA Constructs—A construct encoding human SmgGDS-607 (cDNA clone FLJ30470) was obtained from the National Institute of Technology and Evaluation Biological Resource Center (Chiba, Japan) and was subcloned into the pcDNA3.1(+) vector with a single C-terminal hemagglutinin (HA) epitope tag (YPYDVPDYA). Two amino acids in the original construct that did not agree with the sequences in the NCBI Database were changed by site-directed mutagenesis to agree with the sequences in the NCBI Database (accession number NP_001093897). These changes were T197C to produce V66A and C1154T to produce F385L. SmgGDS-558-HA was generated by overlap PCR from SmgGDS-607-HA in the pcDNA3.1(+) vector. The Myc-tagged and GFP-tagged small GTPase constructs were generated as described previously (27). The S*AAX* mutants of the small GTPases described in supplemental Table S2 were generated by site-directed mutagenesis to change the cysteine in the C*AAX* motif to a serine. Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocols, using primers purchased from Operon. The FLAG-tagged Rap1 GEF constructs MR-GEF, C3G, GRP3, and PDZ-GEF were the kind gifts from Dr. Lawrence Quilliam, Indiana University School of Medicine and Walther Oncology Center (28).

NCI-H1703 Cells Stably Expressing Tet-inducible shRNA—NCI-H1703 cells were transduced with lentiviral vectors encoding the luciferase protein (a kind gift from Dr. Michael Dwinell, Medical College of Wisconsin) and the tetracycline repressor protein (Invitrogen) and selected for stable expression of the vectors. Lentiviral vectors expressing inducible shRNA for SmgGDS-558 (shRNA BD), SmgGDS-607 (shRNA C2), or a nontargeting control shRNA were generated using the "Block-iT" inducible H1 lentiviral RNAi system (Invitrogen, K4925-00). Cells were transduced with the lentiviral vectors containing the inducible shRNAs and selected for stable expression of the vectors. Cells were maintained in RPMI 1640 triple selection media (200 μg/ml Zeocin, 12 μg/ml blasticidin, 400 ng/ml puromycin). Expression of shRNA was induced using 2 μg/ml tetracycline (Invitrogen). Oligonucleotide

sequences encoding the shRNA were purchased from Operon. The forward sequences were as follows: nontargeting shRNA, 5′-CACCATGGTTTACATGTTTTCTGATTGAGAAATCAGAAAACATGTAAACCA-3′; C2 shRNA, 5′-CACCATTCTCATTGCTATAGTTCCGAAGAACTATAGCAATGAGAA-3′; and BD shRNA, 5′-CACCATGAAGCGAATGGCTATCGTGAGAACGATAGCCATTCGCTTCA-3′. Reverse complements of the sequences were also purchased for formation of the shRNA double-stranded DNA complexes.

Enhanced Chemiluminescence (ECL)-Western Blotting—Equal numbers of transfected cells were boiled in Laemmli sample buffer and subjected to SDS-PAGE. The proteins were transferred to polyvinylidene difluoride and immunoblotted using antibody to SmgGDS (BD Transduction Laboratories 612511), GAPDH (Santa Cruz Biotechnology sc-32233), mouse HA antibody (Covance, MMS-101P), rabbit HA antibody (Covance, PRB-101P), mouse Myc antibody (Santa Cruz Biotechnology, sc-40), rabbit Myc antibody (Covance, PRB-150P), mouse antibody to FLAG (Sigma, F3165), rabbit antibody to FLAG (Sigma, F7425), mouse antibody to Actin (Santa Cruz Biotechnology, sc-47778), rabbit antibody to tet-repressor (Chemicon, AB3541), rabbit antibody to lamin B1 (Abcam, ab16048), or goat antibody to nonprenylated Rap1 (29) (Santa Cruz Biotechnology, sc-1482). Bound antibodies were visualized using horseradish peroxidase-linked anti-mouse IgG, anti-rabbit IgG (Amersham Biosciences), or anti-goat IgG (Santa Cruz Biotechnology, sc-2056) and ECL reagents (PerkinElmer Life Sciences), as described previously (30).

Analysis of Immunoprecipitates from Cultured Cells—HEK-293T cells were transiently transfected with cDNAs encoding HA-tagged SmgGDS splice variants and Myc-tagged small GTPases. After 24 h, equal numbers of cells were lysed in buffer containing 0.5% Nonidet P-40 with protease and phosphatase inhibitors, and the lysates were centrifuged (2500 × *g*, 5 min, 4 °C). A portion of the resulting supernatants was reserved for total cell lysates, and the remainder was immunoprecipitated using mouse HA antibody (Covance, MMS-101P) or mouse Myc antibody (Santa Cruz Biotechnology, sc-40). Immunoprecipitates and total cell lysates were subjected to ECL-Western blotting as described above.

Analysis of mRNA and Protein Expression in Human Tumors—Using an approved Institutional Review Board protocol (Medical College of Wisconsin Institutional Review Board PRO5554), lysates of archival lung tumors and their matched normal lung tissue were homogenized in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, and 10% glycerol with protease inhibitors (Complete Mini Tablets, Roche Applied Science) and then centrifuged (18,000 × *g* for 10 min at 4 °C). The resulting supernatants were then normalized to a protein concentration of 1 mg/ml protein, boiled in Laemmli sample buffer, and subjected to ECL-Western blotting using antibody to SmgGDS or actin. The expression of mRNAs for SmgGDS-558 and SmgGDS-607 in a panel of archival lung tumors and matched normal tissue was determined by quantitative real time PCR using methods described previously (31).

Clonogenic Assay—Anchorage-independent proliferation of the NSCLC cells NCI-H1703 and NCI-H23 was analyzed by their ability to form colonies in soft agar. 7×10^4 cells were transfected with siRNA or treated with transfection reagents only (mock transfection). After 24 h, cells were lifted, and 9.9×10^3 cells were suspended in 0.3% low-melt agarose (Bio-Rad) in complete RPMI. Triplicate wells were plated with 1 ml of the cell suspension over 1 ml of solidified 0.6% low-melt agarose in RPMI. After the 0.3% agarose layer set, each well was overlaid with 2 ml of complete RPMI. Digital images of the cells were collected 4 weeks (NCI-H1703) or 5 weeks (NCI-H23) later, and the numbers of visible colonies in the agar were counted using ImageJ.

Triton X-114 Fractionation—As described previously (32), cells were lysed in 1% Triton X-114 in TBS (50 mM Tris, 150 mM NaCl, pH 7.5), incubated 15 min on ice, and then centrifuged at 25,000 × *g* at 4 °C to remove insoluble debris. An aliquot of the cleared lysate was retained as total cell lysate. The remaining lysate was subjected to temperature-dependent separation of the aqueous and detergent phases (2 min of incubation at 37 °C followed by room temperature centrifugation at 1500 × *g*). After separation of the aqueous and detergent phase, the aqueous phase was transferred to a separate microcentrifuge tube, and 11% Triton X-114 was added to a final concentration of 1%. Any remaining aqueous sample was then scavenged from the detergent phase and discarded. This step resulted in a loss of both detergent and aqueous sample relative to total cell lysate, visible as a disproportionate isolation of lamin B1 in the detergent fractions and GAPDH in aqueous fractions compared with the lamin B1 and GAPDH in the total cell lysate. TBS was then added to the detergent phase to a final concentration 1% Triton X-114. The detergent fractions, aqueous fractions, and total cell lysate were combined 1:1 with 2× Laemmli sample buffer, and equal volumes of each fraction and the total cell lysate were separated by SDS-PAGE and examined by ECL-Western blotting. Proteins were detected with mouse HA antibody to detect SmgGDS isoforms and small GTPases, GAPDH antibody (an aqueous phase loading control), and rabbit antibody to lamin B1 (a detergent phase loading control).

OD values of the GTPases detected in the aqueous, detergent, and total cell lysate samples were measured on an AlphaImager HP and analyzed using AlphaView SA software (Alpha Innotech, San Leandro, CA). As discussed above, we observed in some experiments that there was disproportionate isolation of the detergent fraction (containing lamin B1) compared with the aqueous fraction (containing GAPDH). Therefore, all OD values for the detergent fraction were normalized by the factor (OD lamin in detergent phase/OD lamin in total cell lysates). Similarly, all OD values in the aqueous phase were normalized by the factor (OD GAPDH in aqueous phase/OD of GAPDH in the total cell lysate).

Intracellular Localization of Proteins—NCI-H1703 cells stably expressing tet-inducible shRNAs were cultured with or without tetracycline for 72 h, transfected with cDNA encoding GFP-tagged Rap1A, and 24 h later examined by fluorescence microscopy as described previously (30). The sample identities were masked, and two investigators independently ranked the membrane localization of GFP-Rap1A on a scale of 1–3, a score of 1 representing undetectable, 2 moderate, and 3 extensive membrane localization.

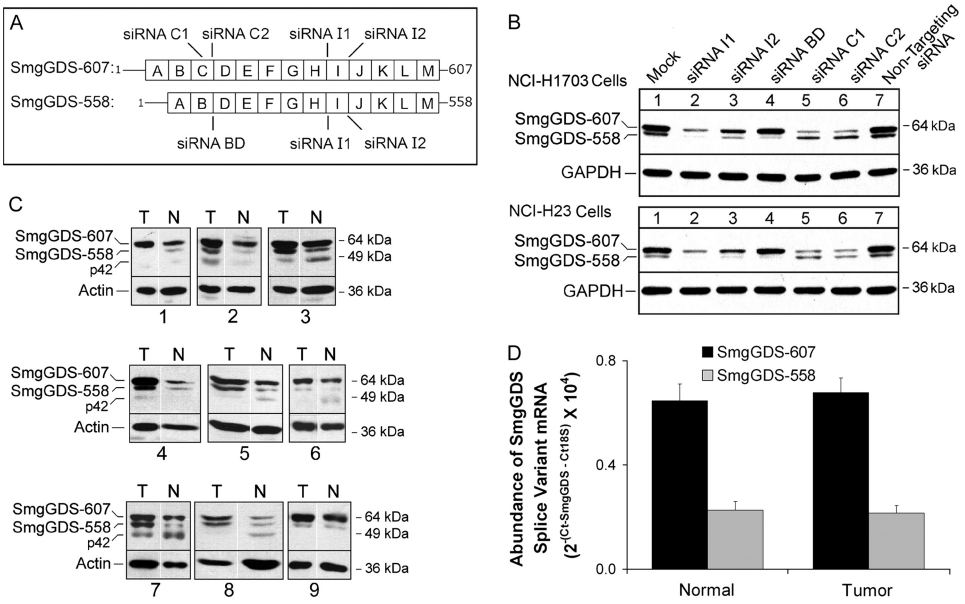

FIGURE 1. **Two splice variants of SmgGDS with distinct functions are expressed in NSCLC.** *A,* SmgGDS-607 and SmgGDS-558 contain multiple predicted ARM domains (represented by the *letters A–M*); SmgGDS-558 is missing ARM domain C. siRNAs *C1* and *C2* target only SmgGDS-607; siRNA BD targets only SmgGDS-558, and siRNAs I1 and I2 target both SmgGDS splice variants. *B,* NCI-H1703 or NCI-H23 cells were transfected with the indicated siRNAs or reagent only (*Mock*), lysed after 72 h, and subjected to ECL-Western blotting using antibodies to SmgGDS and GAPDH. Results are representative of five independent experiments. *C,* archival lung tumors (*T*) and matched normal lung tissue (*N*) from nine patients (*1–9*) were subjected to ECL-Western blotting using antibodies to SmgGDS and actin. *D,* quantitative real time PCR using primers that specifically amplify SmgGDS-558 or SmgGDS-607 mRNA was conducted using lung tumor tissue and matched normal lung tissue isolated from 50 patients. Values were normalized to expression of 18 S RNA as described previously (31).

Statistics—All results are the means ± S.E. Symbols above a column indicate a statistical comparison between the bracketed sample by analysis of variance with Dunnett's post hoc multiple comparison's test, by Student's *t* test, or by χ^2 analysis, as indicated in the figure legends. *p* values less than 0.05 were considered significant.

RESULTS

SmgGDS Is Expressed as Two Splice Variants in NSCLC Cell Lines and Tumors and Normal Lung Tissue—SmgGDS (also known as smgGDS and Rap1GDS1) was originally isolated from bovine brain (33) and human brain (34) as a cDNA encoding a 558-residue protein (NCBI accession NP_001093899, isoform 5). In addition to this previously described protein, which we refer to as SmgGDS-558, the NCBI Database also lists a 607-residue SmgGDS splice variant (NCBI accession NP_001093897, isoform 3), which we designate as SmgGDS-607. Both SmgGDS-607 and SmgGDS-558 consist mainly of predicted ARM repeats, with SmgGDS-607 differing from SmgGDS-558 by the addition of only one ARM repeat (Fig. 1*A*; supplemental Fig. S1).

Western blotting of NSCLC cell lysates using a monoclonal SmgGDS antibody detects two proteins with apparent molecular masses of 55 and 60 kDa (Fig. 1*B*). We suspected that the 55-kDa protein corresponds to SmgGDS-558 and the 60-kDa protein corresponds to SmgGDS-607. To confirm the identities of these immunoreactive proteins, we designed siRNA duplexes to target either both SmgGDS splice variants simultaneously (siRNAs I1 and I2), only SmgGDS-607 (siRNAs C1 and C2), or only SmgGDS-558 (siRNA BD) (Fig. 1*A*; supplemental Table S1). Transfection of two NSCLC cell lines, NCI-H1703 and NCI-H23 (Fig. 1*B*), with siRNAs I1 and I2 decreases expression of both proteins detected by the SmgGDS antibody. siRNA BD decreases only the 55-kDa protein, and siRNAs C1 and C2 decrease only the 60-kDa protein. These results confirm the identities of the 55- and 60-kDa immunoreactive proteins and demonstrate that both SmgGDS-558 and SmgGDS-607 are expressed in NSCLC cell lines (Fig. 1*B*).

In our previous immunohistochemical analysis of lung tumors and tissues, we found that SmgGDS protein is significantly elevated in NSCLC tumors compared with normal lung tissue (12). To determine whether both SmgGDS splice variants are expressed in lung tumors and tissues, we conducted Western blotting of lung tumors and matched normal lung tissue from nine patients (Fig. 1*C*). Two proteins are detected by the SmgGDS antibody in most of these immunoblotted tissue samples, and the migration of the immunoreactive proteins corresponds to the migration of SmgGDS-607 and SmgGDS-558 (Fig. 1*C*). The SmgGDS antibody also detects an unidentified 42-kDa protein (p42) in some of the tumor or lung tissue samples (Fig. 1*C*, *samples 2–8*), which may be an SmgGDS degradation product or potentially another uncharacterized SmgGDS splice variant.

We examined the expression of mRNA transcripts for SmgGDS-558 and SmgGDS-607 using quantitative RT-PCR in a panel of 50 matched samples of lung tumors and normal lung tissues (Fig. 1*D*). In both the normal tissue samples and in the tumor samples, quantitative RT-PCR analysis detected more abundant SmgGDS-607 mRNA than SmgGDS-558 mRNA (Fig. 1*D*). This detection of more SmgGDS-607 mRNA than SmgGDS-558 mRNA is consistent with the immunoblotting results, which indicated more detectable SmgGDS-607 protein than SmgGDS-558 protein in the majority of the tumors and normal tissue samples examined (Fig. 1*C*) as well as in the cultured cells (Fig. 1*B*). Somewhat surprisingly, we observed that neither SmgGDS-558 mRNA expression nor SmgGDS-607 mRNA expression is elevated in the tumors compared with the matched normal lung tissues (Fig. 1*D*). The finding that SmgGDS mRNA is not significantly elevated in the tumors was somewhat unexpected, based on our earlier report that SmgGDS protein is elevated in lung tumors (12). The lack of SmgGDS mRNA overexpression in the tumors suggests that SmgGDS protein may be elevated in lung tumors due to post-translational events, such as stabilization of SmgGDS protein.

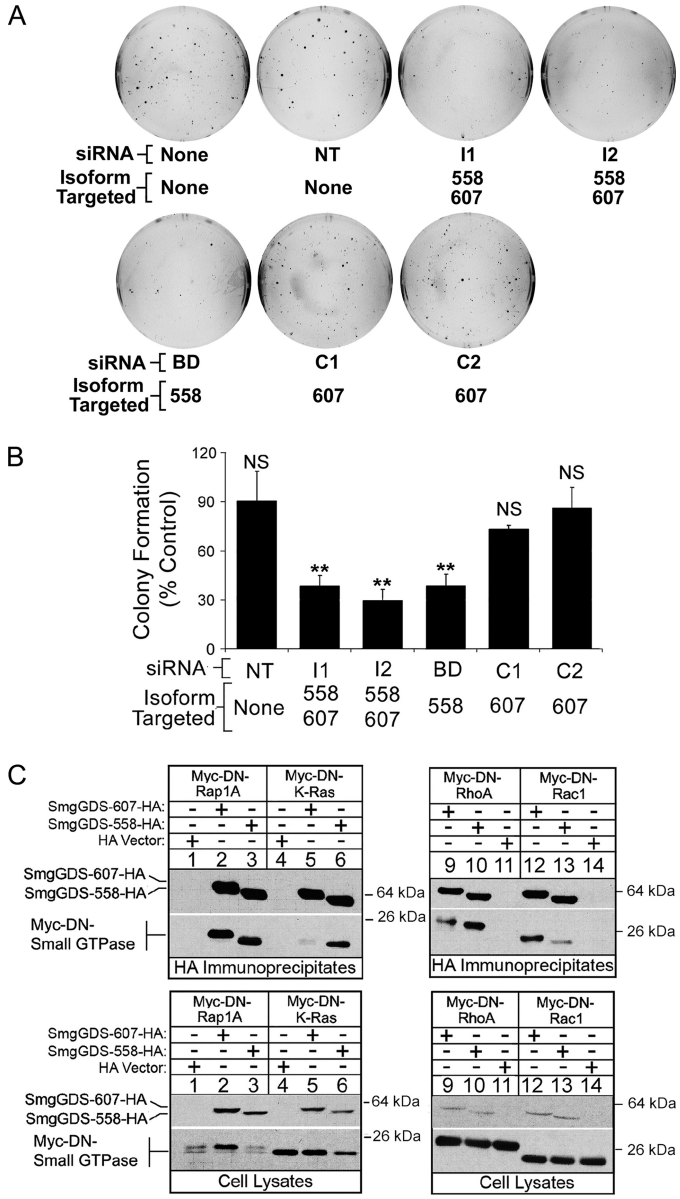

FIGURE 2. **SmgGDS splice variants are functionally distinct proteins and interact differently with small GTPases.** *A* and *B,* NCI-H1703 cells were transfected in the absence (control) or presence of the indicated siRNAs and 24 h later re-plated in soft agar. Colonies were imaged (*A*) and quantified (*B*) after 1 month. Results are the means ± S.E. from three independent experiments. (*NT*, nontargeting siRNA; **, $p < 0.001$; *NS*, not significant by analysis of variance with Dunnett's post hoc multiple comparisons test). *C,* HEK-293T cells were transfected with a cDNA encoding the HA vector, SmgGDS-607-HA, or SmgGDS-558-HA plus a cDNA encoding the indicated Myc-tagged DN small GTPases and then immunoprecipitated 24 h later with HA antibody. Immunoprecipitates and cell lysates were subjected to ECL-Western blotting using HA antibody and Myc antibody. Results are representative of at least three independent experiments.

SmgGDS Splice Variants Are Functionally Distinct Proteins and Interact Differently with Small GTPases—Soft agar colony formation by NCI-H1703 cells is significantly reduced by diminishing the expression of either SmgGDS-558 alone (Fig. 2, *A* and *B*, *siRNA BD*) or both SmgGDS isoforms simultaneously (Fig. 2, *A* and *B*, *siRNAs I1* and *I2*). In contrast, diminishing only SmgGDS-607 expression has little effect (Fig. 2, *A* and *B*, *siRNAs C1* and *C2*). These results indicate that SmgGDS-558 and SmgGDS-607 are functionally different proteins.

To begin to determine how signaling through the SmgGDS splice variants might diverge, we compared the abilities of SmgGDS-607 and SmgGDS-558 to co-precipitate different small GTPases. We used dominant negative (DN) small GTPases in these assays because of the possibility that the SmgGDS splice variants might act as GEFs for the small GTPases. DN GTPases do not readily bind GTP and thus do not readily dissociate from GEFs, resulting in a stable DN-GTPase-GEF complex that can be immunoprecipitated (35). In contrast, wild type (WT) GTPases form a transient, unstable complex with GEFs because GDP/GTP exchange rapidly dissociates the WT GTPase from the GEF. The use of DN GTPases in these assays is supported by our previous finding that SmgGDS co-precipitates more with DN-RhoA than with WT-RhoA expressed in cells (36).

When the proteins were co-expressed in HEK293T cells, both SmgGDS-558 and SmgGDS-607 co-precipitated the DN forms of Rap1A, K-Ras, RhoA, and Rac1 (Fig. 2*C*). Very interestingly, SmgGDS-607 co-precipitates a slower migrating form of all of the small GTPases tested (Fig. 2*C*, *HA immunoprecipitates, lanes 2, 5, 9,* and *12*), whereas SmgGDS-558 co-precipitates a faster migrating form of all of the small GTPases (Fig. 2*C*, *HA immunoprecipitates, lanes 3, 6, 10,* and *13*). This finding suggests that the SmgGDS splice variants form complexes with different post-translationally modified forms of the small GTPases. Prenylation is the major post-translational modification that is shared by all four of these small GTPases. Furthermore, prenylated small GTPases typically migrate faster than nonprenylated small GTPases in SDS-PAGE (37). Based on these characteristics, we hypothesized that SmgGDS-558 associates with prenylated small GTPases, whereas SmgGDS-607 associates with nonprenylated small GTPases.

SmgGDS-607 Co-precipitates Nonprenylated Small GTPases, whereas SmgGDS-558 Co-precipitates Prenylated Small GTPases—To test the hypothesis of prenylation-specific interactions of SmgGDS isoforms with small GTPases, we compared the abilities of the SmgGDS splice variants to co-precipitate GTPases that have a normal C*AAX* motif *versus* mutant GTPases that cannot be prenylated due to serine-for-cysteine substitution in the C*AAX* motif where the prenyl group is attached. When expressed in cells, GTPases with a normal C*AAX* motif will be present in both the nonprenylated and prenylated forms, and each of these different forms could selectively co-precipitate with a specific SmgGDS splice variant. In contrast, GTPases with the mutant S*AAX* motif (termed K-Ras-S*AAX*, Rap1A-S*AAX*, Rac1-S*AAX*, and RhoA-S*AAX*) will only be present in the nonprenylated form, and will co-precipitate only with the SmgGDS splice variant(s) that can form complexes with nonprenylated GTPases.

We first compared the interactions of SmgGDS splice variants with DN GTPases that have a normal or mutant C*AAX* motif (Fig. 3, *A*, *C, E,* and *G*, *HA immunoprecipitates*). We found that SmgGDS-558 co-precipitates DN GTPases that have a normal C*AAX* motif but not DN GTPases that lack prenylation due to the S*AAX* mutation (Fig. 3, *A*, *C, E,* and *G, HA immunoprecipitates, lanes 3* and *4*). These results indicate that SmgGDS-558 is restricted to interacting with prenylated small GTPases. In contrast, SmgGDS-607 co-precipitates nonprenylated DN GTPases that have the S*AAX* mutation (Fig. 3, *A*, *C, E,*

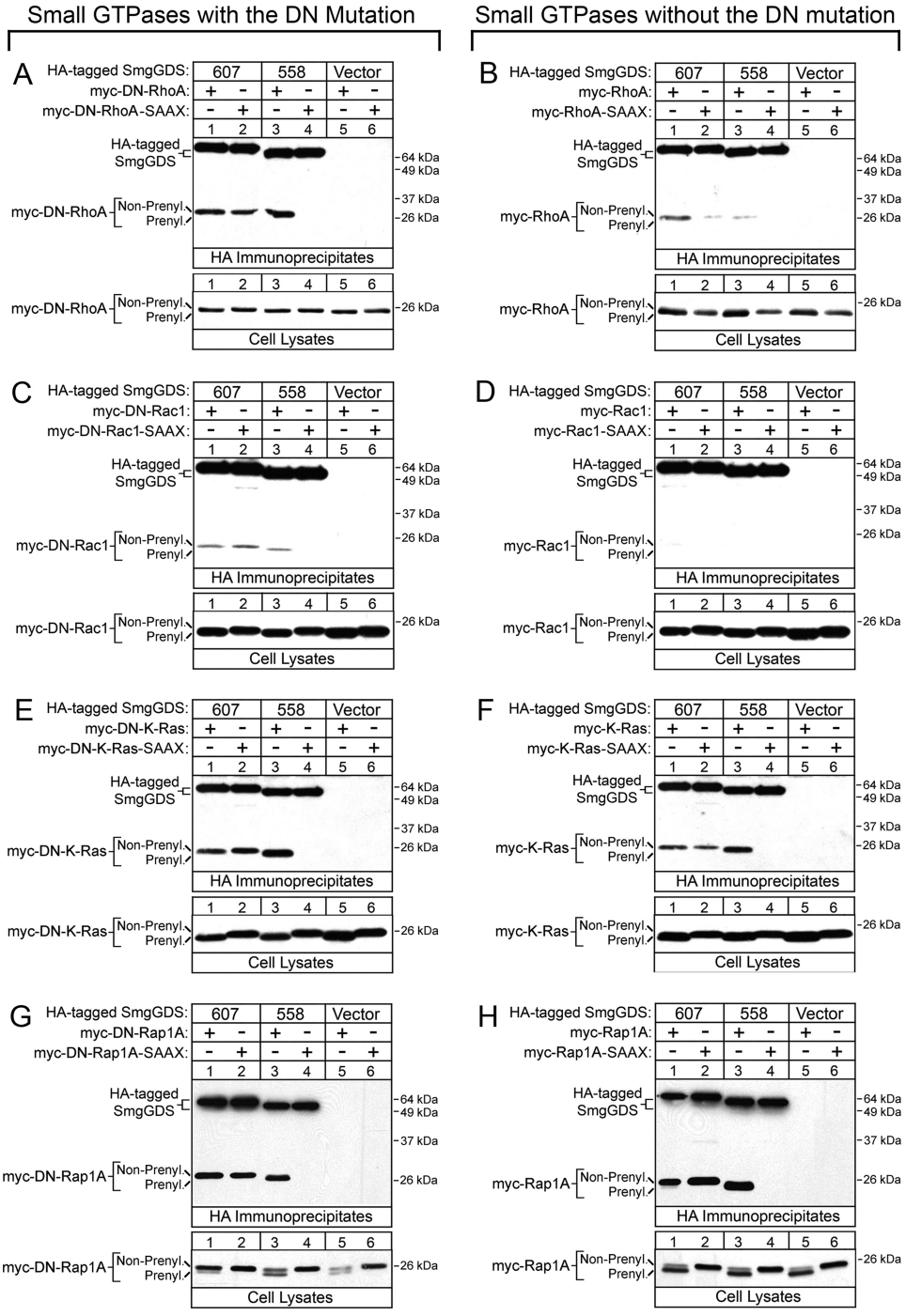

FIGURE 3. **SmgGDS-558 associates with prenylated small GTPases, whereas SmgGDS-607 associates with nonprenylated small GTPases.** HEK-293T cells were transfected with a cDNA encoding SmgGDS-607-HA (607), SmgGDS-558-HA (558), or the HA vector (*Vector*) plus a cDNA encoding the indicated Myc-tagged small GTPase possessing either a normal C*AAX* motif (*odd lanes*) or the S*AAX* mutation (*even lanes*), and containing the DN mutation (*A, C, E,* and *G*) or lacking the DN mutation (*B, D, F,* and *H*). After 24 h, the cells were lysed, and an aliquot of each lysate was subjected to ECL-Western blotting using Myc antibody (*Cell Lysates*). The remaining volume of each cell lysate was immunoprecipitated with HA antibody, followed by ECL-Western blotting using HA antibody and Myc antibody (*HA Immunoprecipitates*). Results are representative of three independent experiments.

and *G, HA immunoprecipitates, lane 2*), indicating that unlike SmgGDS-558, SmgGDS-607 can interact with nonprenylated small GTPases. SmgGDS-607 also co-precipitates DN GTPases that have a normal C*AAX* motif (Fig. 3, *A, C, E,* and *G, HA immunoprecipitates, lane 1*). Intriguingly, these DN GTPases with normal C*AAX* motifs that are co-precipitated by SmgGDS-607 co-migrate in SDS-PAGE with the S*AAX* mutants that are co-precipitated by SmgGDS-607 (Fig. 3, *A*, *C, E,* and *G, HA immunoprecipitates, lanes 1* and *2*). This finding suggests that when SmgGDS-607 is co-expressed with GTPases containing a normal C*AAX*, SmgGDS-607 selectively co-precipitates the nonprenylated GTPase from among the different prenylated and nonprenylated forms. The co-migration of C*AAX*- and S*AAX*-containing small GTPases in SDS-PAGE after their immunoprecipitation with SmgGDS-607 is most detectable among GTPases whose migration is most affected by the S*AAX* mutation, such as the DN-Rap1A proteins (Fig. 3*G*, *HA immunoprecipitates, lanes 1* and *2*); this effect is least detectable among GTPases whose migration is least affected by the S*AAX* mutation, such as the DN-RhoA proteins (Fig. 3*A*, *HA immunoprecipitates, lanes 1* and *2*).

We next examined whether these interactions are restricted to DN small GTPases or also occur among WT small GTPases that can undergo guanine nucleotide exchange. As discussed above, if SmgGDS splice variants act as GEFs, they would be expected to form more stable complexes with DN GTPases than with WT GTPases (35). Consistent with this expectation, both SmgGDS splice variants detectably associate more with DN-RhoA than with WT-RhoA (Fig. 3, *A* and *B*, *HA immunoprecipitates*), and more with DN-Rac1 than with WT-Rac1 (Fig. 3, *C* and *D*, *HA immunoprecipitates*). However, we unexpectedly observed that both SmgGDS splice variants associate well with the DN and WT forms of K-Ras (Fig. 3, *E* and *F*, *HA immunoprecipitates*) and with the DN and WT forms of Rap1A (Fig. 3, *G* and *H*, *HA immunoprecipitates*). These results indicate that SmgGDS acts more like a classical GEF when it is interacting with RhoA and Rac1 than when it is interacting with K-Ras and Rap1A. When co-precipitation of the WT GTPases was detectable, the WT GTPases exhibited interactions similar to those exhibited by

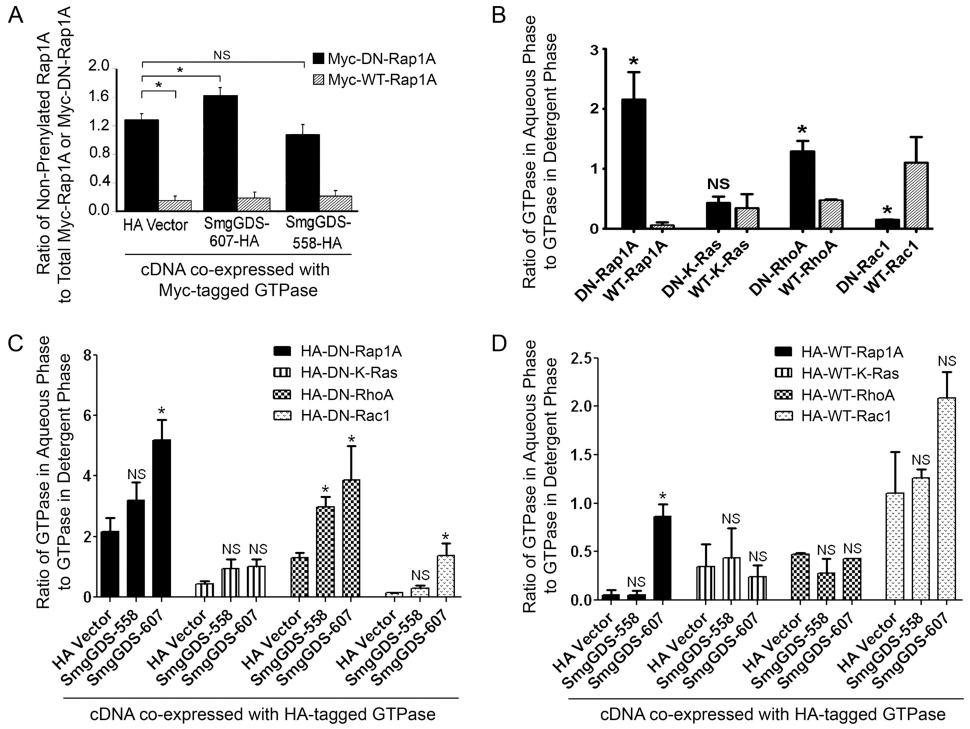

FIGURE 4. **Prenylation of small GTPases is regulated by GDP/GTP exchange and SmgGDS-607 expression.** *A,* HEK-293T cells were transfected with myc-Rap1A or myc-DN-Rap1A in the presence of SmgGDS-558-HA, SmgGDS-607-HA, or the HA vector. After 24 h, the cell lysates were subjected to ECL-Western blotting using an antibody that recognizes the nonprenylated form of Rap1 and an antibody that recognizes the Myc epitope (to detect total myc-Rap1A or myc-DN-Rap1A). The ratio of the proteins detected by the nonprenylated Rap1 antibody and the Myc antibody was quantified by densitometry. Results are the mean ± S.E. for three independent experiments. (*NS*, not significant; *, $p < 0.05$ by Student's *t* test). A representative Western blot is presented in supplemental Fig. S2*A*. *B–D,* HEK-293T cells were transfected with either SmgGDS-607-HA, SmgGDS-558-HA, or the HA vector in the presence of one of the indicated HA-tagged DN GTPases or WT GTPases. After 24 h, the cell lysates were subjected to Triton X-114 fractionation, and equal volumes of the aqueous phase, detergent phase, or total cell lysate were separated by SDS-PAGE and subjected to ECL-Western blotting using HA antibody. The average ratio (± S.E.) of the HA-tagged GTPases detected in the aqueous phase (nonprenylated GTPases) compared with the detergent phase (prenylated GTPases) was calculated from the OD values as described under "Experimental Procedures" (*, $p < 0.05$; *NS*, not significant). Representative Western blots are presented in supplemental Fig. S2, *B–I*.

the DN GTPases (Fig. 3, *right panels* and *left panels,* respectively), indicating that the prenylation-specific interactions of the GTPases with the SmgGDS splice variants are not an artifact of the DN mutation.

Prenylation Is Regulated by Guanine Nucleotide Exchange—An examination of the total cell lysates reveals that the S*AAX* mutation significantly slows the migration of the Rap1A proteins in SDS-PAGE (Fig. 3, *G* and *H*, *cell lysates, lanes 2, 4,* and *6*), indicating that changes in prenylation dramatically alter Rap1A migration in SDS-PAGE. When Rap1A containing a normal C*AAX* motif is expressed in cells, it migrates as two distinctly resolving immunoreactive bands in the Western blot of the cell lysates (Fig. 3, *G* and *H*, *cell lysates, lanes 1, 3,* and *5*). The upper immunoreactive band is predicted to be nonprenylated Rap1A due to its co-migration with the nonprenylated Rap1A S*AAX* mutant, whereas the lower immunoreactive band is predicted to be prenylated Rap1A due to the faster migration of prenylated than nonprenylated GTPases in SDS-PAGE (37). Based on these predictions, WT Rap1A is expressed mainly as the prenylated form in the cells, as indicated by the predominance of the lower immunoreactive band of WT Rap1A in the immunoblot of the cell lysates (Fig. 3*H*, *cell lysates, lane 5*). In contrast, DN-Rap1A is expressed equally as prenylated and nonprenylated forms, as indicated by similar amounts of the upper and lower immunoreactive bands of DN-Rap1A in the Western blot of the cell lysates (Fig. 3*G*, *cell lysates, lane 5*). These differences in the amounts of the two resolving upper and lower immunoreactive bands of WT-Rap1A *versus* DN-Rap1A support the hypothesis that the DN mutation diminishes prenylation of Rap1A.

To test the hypothesis that Rap1A prenylation is diminished by the DN mutation, changes in Rap1A prenylation were investigated using an antibody that detects only nonprenylated Rap1 (29). Cells were transfected with myc-Rap1A or myc-DN-Rap1A and co-transfected with either the HA vector, SmgGDS-607-HA, or SmgGDS-558-HA. After 24 h, the cell lysates were immunoblotted with the nonprenylated Rap1 antibody and with the Myc antibody, which detects both prenylated and nonprenylated forms of the Myc-tagged GTPases. The ratio of the proteins detected by these two antibodies was quantified by densitometry to define the relative amount of nonprenylated Myc-tagged GTPase expressed in the cells (Fig. 4*A* and supplemental Fig. S2*A*). We found that the ratio of nonprenylated myc-GTPase to total myc-GTPase expressed in cells that were co-transfected with the HA vector was 1.3 ± 0.2 for myc-DN-Rap1A, and it was 0.2 ± 0.1 for myc-WT-Rap1A ($p = 0.02$) (Fig. 4*A*). This result indicates that the cells express a greater amount of nonprenylated DN-Rap1A than nonprenylated WT-Rap1A (Fig. 4*A*), supporting the conclusion that the DN mutation reduces Rap1A prenylation.

Having found that the DN mutation diminishes the prenylation of Rap1A, we next tested whether the DN mutation affects the prenylation of K-Ras, Rac1, and RhoA. We could not examine the prenylation status of these GTPases by examining their migration rates in SDS-PAGE, because unlike Rap1A, the prenylated and nonprenylated forms of K-Ras, Rac1, and RhoA do not readily resolve into two distinct immunoreactive bands under our SDS-PAGE conditions (Fig. 3, *A–F, cell lysates, lanes 1, 3,* and *5*). Additionally, antibodies to specifically detect the nonprenylated forms of these GTPases are not currently available. Therefore, to characterize the prenylation status of these small GTPases, cells expressing the HA-tagged GTPases were lysed in Triton X-114 to solubilize the prenylated proteins into the detergent phase and the nonprenylated proteins into the

aqueous phase. The fractions were subjected to Western blotting using the HA antibody, followed by densitometry to define the ratio of nonprenylated HA-GTPase in the aqueous phase *versus* prenylated HA-GTPase in the detergent phase (Fig. 4*B*). Representative immunoblots can be found in supplemental Fig. S2, *B–I*. We observed that DN-Rap1A accumulates more than WT-Rap1A in the aqueous phase (Fig. 4*B*), consistent with our previous results indicating reduced prenylation of DN-Rap1A (Fig. 4*A*). Similarly, DN-RhoA accumulates more than WT-RhoA in the aqueous phase (Fig. 4*B*), indicating reduced prenylation of DN-RhoA. In contrast, WT-Rac1 accumulates more than DN-Rac1 in the aqueous phase (Fig. 4*B*), indicating reduced prenylation of WT-Rac1 compared with DN-Rac1. Equal amounts of DN-K-Ras and WT-K-Ras accumulate in the aqueous phase (Fig. 4*B*), indicating that the DN and WT forms of K-Ras do not significantly differ in their prenylation status. Taken together, these results indicate that the DN mutation significantly diminishes the prenylation of Rap1A and RhoA, enhances the prenylation of Rac1, and does not detectably alter the prenylation of K-Ras.

Prenylation Is Regulated by SmgGDS-607—Because SmgGDS-607 forms stable complexes with the nonprenylated form of all small GTPases examined (Fig. 3), we tested whether SmgGDS-607 regulates the prenylation of small GTPases. The results of the Triton X-114 fractionation assay indicated that co-expression of SmgGDS-607 causes DN-Rap1A, DN-RhoA, and DN-Rac1 to accumulate in the aqueous phase (Fig. 4*C*), indicating reduced prenylation of these DN GTPases when SmgGDS-607 is overexpressed.

The results of several other assays also indicate that SmgGDS-607 regulates DN-Rap1A prenylation. Western blotting using the nonprenylated Rap1 antibody indicated that co-expression of SmgGDS-607 significantly diminishes the prenylation of DN-Rap1A (Fig. 4*A*). Evidence that SmgGDS-607 regulates DN-Rap1A prenylation is also provided by the immunoblots of the total cell lysates shown in Figs. 2*C* and 3*G*, which indicated that the upper immunoreactive band of nonprenylated DN-Rap1A is more prominent in the total cell lysates prepared from cells co-transfected with SmgGDS-607 compared with cells co-transfected with the HA vector (Fig. 2*C*, *cell lysates, lanes 1* and *2*, and Fig. 3*G*, *cell lysates, lanes 1* and *5*). Taken together, these findings indicate that SmgGDS-607 expression diminishes the prenylation of DN-Rap1A.

Having found that SmgGDS-607 co-expression diminishes the prenylation of DN-Rap1A, DN-RhoA, and DN-Rac1, we next examined the effects of SmgGDS-607 on the WT forms of these GTPases. In the Triton X-114 fractionation assay, SmgGDS-607 co-expression significantly diminishes the prenylation of the WT form of Rap1A, but not K-Ras, RhoA, or Rac1 (Fig. 4*D*). The reduced prenylation of WT-Rap1A in cells co-expressing SmgGDS-607 (Fig. 4*D*) was somewhat surprising, because Western blotting using the nonprenylated Rap1A antibody did not indicate altered WT-Rap1A prenylation in the presence of co-expressed SmgGDS-607 (Fig. 4*A*). Additionally, we did not detect any change in the migration rate of WT-Rap1A in Western blots of cells co-transfected with SmgGDS-607 *versus* the HA vector (Fig. 3*H*, *cell lysates, lanes 1* and *5*). The Triton X-114 fractionation assay might detect a SmgGDS-607-dependent change in the prenylation of WT-Rap1A because this method is a more sensitive indicator of prenylation than the other methods employed to detect changes in prenylation.

Because SmgGDS has been reported to be a weak GEF for small GTPases (9–11), we examined the possibility that other GEFs might also regulate small GTPase prenylation. We focused on GEFs for Rap1, including MR-GEF, GRP3, PDZ-GEF, and C3G, because many of these GEFs specifically interact with Rap1. None of the overexpressed Rap1 GEFs detectably altered prenylation of DN-Rap1A (supplemental Fig. S3*A*), even though they are capable of forming stable complexes with nonprenylated DN-Rap1A (supplemental Fig. S3*B*). Thus, SmgGDS-607 is unique among all of the tested proteins in its ability to alter prenylation of the tested small GTPases.

In contrast to the responses induced by SmgGDS-607, the co-expression of SmgGDS-558 has little effect on the prenylation of small GTPases. This conclusion is supported by our observations that SmgGDS-558 co-expression does not alter the migration of DN-Rap1A in immunoblotted cell lysates (Figs. 2*C* and 3*G*) nor does SmgGDS-558 co-expression increase the amount of Rap1A detected by the nonprenylated Rap1 antibody (Fig. 4*A*). Furthermore, in the Triton X-114 fractionation assays, SmgGDS-558 co-expression does not detectably alter the prenylation of the WT GTPases (Fig. 4*D*) or most of the DN GTPases (Fig. 4*C*). However, SmgGDS-558 co-expression caused an unexpected shift of DN-RhoA into the aqueous phase in the Triton X-114 fractions (Fig. 4*C*). It is unclear why SmgGDS-558 overexpression would reduce prenylation of DN-RhoA, based on our finding that SmgGDS-558 does not detectably interact with nonprenylated GTPases.

SmgGDS-558 Uniquely Interacts with Prenylated GTPases and Regulates Rap1A Membrane Localization—In striking contrast to SmgGDS-607, which interacts with nonprenylated small GTPases, SmgGDS-558 exhibits exceptional specificity for prenylated small GTPases. Among all of the potential GEFs that we tested, we found that SmgGDS-558 is the only protein that does not detectably co-precipitate with the nonprenylated *SAAX* mutant of DN-Rap1A (supplemental Fig. S3*B*, *lane 4*). SmgGDS-558 is likewise confined to interacting with prenylated K-Ras, Rac1, and RhoA and does not detectably interact with their corresponding nonprenylated *SAAX* mutants (Fig. 3). This observation is consistent with the original description by Takai and co-workers (38) that SmgGDS-558 interacts only with prenylated small GTPases.

We hypothesized that SmgGDS-558 regulates the trafficking of prenylated small GTPases to the PM. To test this hypothesis, we examined the membrane localization of transiently transfected GFP-Rap1A in NCI-H1703 cells that stably express tet-inducible shRNA targeting either SmgGDS-558 (shRNA BD) or SmgGDS-607 (shRNA C2) or shRNA that is nontargeting (Fig. 5). Incubation with tet for 72 h induces shRNA expression in these cells, resulting in specific knockdown of the targeted SmgGDS splice variant (Fig. 5*A*). Membrane localization of GFP-Rap1A is not detectably altered by the tet-induced expression of either the nontargeting shRNA (Fig. 5, *B*, *panels a* and *b*, and *C*) or the C2 shRNA that knocks down SmgGDS-607 (Fig. 5, *B*, *panels e* and *f*, and *C*). In contrast, membrane localization

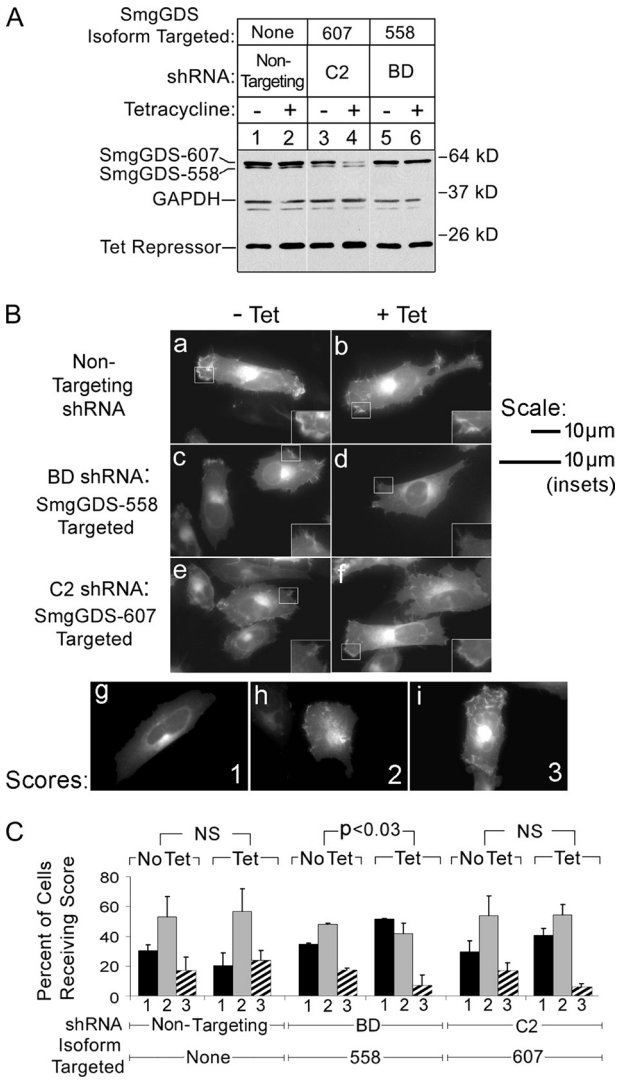

FIGURE 5. **SmgGDS-558 contributes to Rap1A membrane localization.** *A* and *B,* NCI-H1703 cells stably transfected with shRNAs in a tet-inducible system were incubated with or without tet (*Tet*) for 72 h. The cells were then lysed and subjected to ECL-Western blotting using the indicated antibodies (*A*), or alternatively, they were transfected with GFP-Rap1A and then imaged by fluorescence microscopy 24 h later (*B*). Membrane localization of GFP-Rap1A was scored using criteria shown in *panels g–i*. The distribution of cells receiving scores of 1–3 in the absence or presence of tet are shown (*C*). Results are the mean ± S.E. of two independent experiments. χ^2 analysis was used to determine whether the distribution of scores in each cell population significantly differed in the absence or presence of tet (*NS,* not significant).

of GFP-Rap1A is diminished by the tet-induced expression of the BD shRNA that knocks down SmgGDS-558 (Fig. 5, *B*, *panels c* and *d*, and *C*). These results indicate that SmgGDS-558 plays a greater role than SmgGDS-607 in promoting the membrane localization of Rap1A, consistent with our model that SmgGDS-558 promotes the trafficking of prenylated small GTPases to cell membranes, whereas SmgGDS-607 interacts with small GTPases before they are prenylated.

DISCUSSION

Our results indicate that prenylation and subsequent membrane trafficking of small GTPases is a dynamically regulated process defined by changes in the nucleotide-bound state of the GTPase and by interactions with SmgGDS splice variants. The assumption that newly synthesized small GTPases immediately enter the prenylation pathway, without any regulatory controls, is no longer tenable. This newly defined ability to regulate prenylation might provide many advantages to cells, including the ability to store nonprenylated small GTPases that could be rapidly released into the prenylation pathway when they are needed, rather than depending on energy-dependent transcription and translation.

The most striking and consistent pattern we observed is that SmgGDS-607 interacts with the nonprenylated forms of all of the small GTPases tested, whereas SmgGDS-558 is restricted to recognizing the prenylated forms of these small GTPases. The finding that SmgGDS-607 co-expression slows the prenylation of the DN forms of Rap1A, RhoA, and Rac1 indicates that SmgGDS-607 regulates the entrance of these GTPases into the prenylation pathway. It might seem perplexing that SmgGDS-607 regulates the prenylation of DN GTPases more than WT GTPases, especially because DN GTPases are not endogenously expressed in cells. However, the biological significance of this observation is explained by reports that the DN mutation prolongs the time that small GTPases remain in the nucleotide-free state or the GDP-bound state (36, 39), which are states attained by endogenous small GTPases in cells. Thus, the preferential regulation of DN GTPases by SmgGDS-607 indicates that SmgGDS-607 preferentially regulates small GTPases in the nucleotide-free or GDP-bound forms. Consistent with this interpretation, it was previously found that WT-Rac1 remains in the nucleotide-free form when it is bound to SmgGDS (40). Based on these observations, it is intriguing to speculate that SmgGDS-607 intercepts newly synthesized, nucleotide-free, or GDP-bound GTPases to regulate their entrance into the prenylation pathway.

The demonstration that insertion of the DN mutation alone alters prenylation indicates that changes in the nucleotide-bound state control the entrance of small GTPases into the prenylation pathway. We found that the DN mutation slows the prenylation of Rap1A and RhoA but enhances the prenylation of Rac1. Because it is unlikely that the actual mechanism of prenylation differs between Rap1A, RhoA, and Rac1, all of which are geranylgeranylated (20–22), these dissimilarities likely represent differences in how these GTPases enter the prenylation pathway in response to unique signals and differing interactions with SmgGDS-607 or other proteins. Interestingly, we found that neither the DN mutation nor interactions with SmgGDS-607 detectably alter the prenylation of K-Ras, which is farnesylated (23). It is possible that K-Ras is less responsive than the other GTPases because farnesylated GTPases are regulated differently than geranylgeranylated GTPases.

Our observation that co-expression of SmgGDS-607 diminishes, rather than enhances, the prenylation of RhoA, Rac1, and Rap1A suggests that SmgGDS-607 functions as a gatekeeper that prohibits these GTPases from entering the prenylation pathway until the correct signals are received. Due to this regulatory function, it might be expected that silencing SmgGDS-607 expression would allow the unimpeded entrance of small GTPases into the prenylation pathway. Consistent with the possibility that small GTPases can continue to be prenylated when SmgGDS-607 expression is diminished, we found that

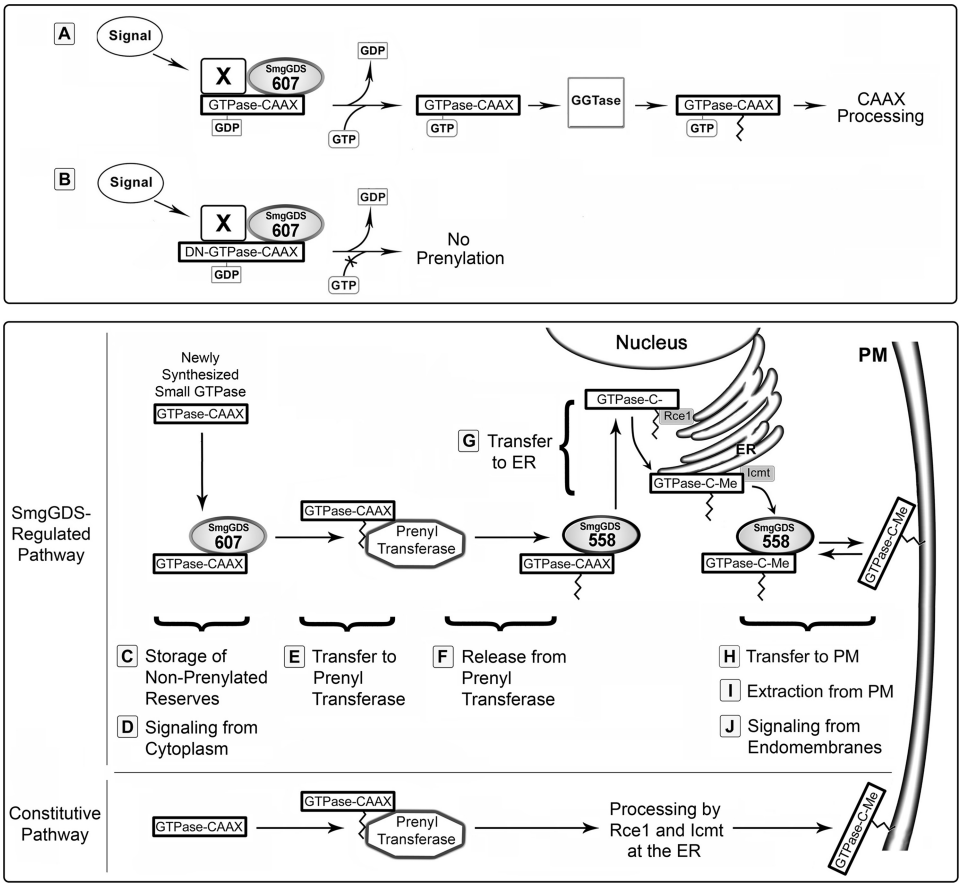

FIGURE 6. **Models of the regulation of prenylation and trafficking of PBR-containing small GTPases by SmgGDS splice variants.** *A,* SmgGDS-607 may act directly or in cooperation with another protein (*X*) to stimulate GDP/GTP exchange and promote entry of nonprenylated GTPases into the prenylation pathway. A nonprenylated GTPase may remain bound to SmgGDS-607 until a signal allows it to undergo GDP/GTP exchange and be released to the PTase. Alternatively, a signal may stimulate binding of the nonprenylated GTPase to SmgGDS-607, where it is retained in the nonprenylated form until GDP/GTP exchange occurs. *B,* inability of a DN GTPase to bind GTP may inhibit its release from SmgGDS-607, thereby diminishing the interaction of the GTPase with the PTase. Overexpression of SmgGDS-607 will generate more complexes of SmgGDS-607 bound to nonprenylated DN GTPases, causing further retention of DN GTPases in the nonprenylated form. *C–J,* ability of SmgGDS-607 and SmgGDS-558 to selectively bind nonprenylated and prenylated small GTPases, respectively, suggests multiple roles for SmgGDS splice variants in the regulation of small GTPase prenylation and localization. Because prenylation is not reversible, SmgGDS-607 must intercept newly synthesized small GTPases before prenylation occurs. SmgGDS-607 may then store nonprenylated small GTPases in the cytoplasm as a reserve for rapid prenylation (*C*) or stimulate nonconventional signaling from cytosolic GTPases (*D*). SmgGDS-607 may also transfer nonprenylated small GTPases to the PTase (*E*). SmgGDS-558, which binds only prenylated small GTPases, may facilitate release of newly prenylated GTPases from the PTase (*F*) or transport GTPases to the ER (*G*). SmgGDS-558 may also transport fully processed GTPases from the ER to the PM (*H*) or extract these GTPases from the PM (*I*), potentially for transport to endomembranes where the small GTPases participate in different signaling pathways (*J*).

silencing SmgGDS-607 does not significantly alter events that depend on prenylated GTPases, including NSCLC soft agar colony formation (Fig. 2, *A* and *B*) and the membrane localization of GFP-tagged Rap1A in NSCLC cells (Fig. 5).

Multiple signals might release small GTPases from SmgGDS-607 and allow their entrance into the prenylation pathway. One of the most likely signals to release a GTPase from SmgGDS-607 is GDP/GTP exchange, because SmgGDS has been reported to act as a weak GEF for all of the small GTPases tested in this study (9–11). However, it is possible that SmgGDS splice variants do not have intrinsic GEF activity but instead act as scaffolds that bind GEFs for small GTPases. A scaffold function for SmgGDS is supported by the discovery that SmgGDS forms complexes with Rac1 and β-Pix, which is a GEF for Rac1 (41). The presence of ARM repeats in SmgGDS also supports its role as a scaffold, because ARM repeats often occur in proteins with scaffold functions (42). In their role as scaffold proteins, SmgGDS splice variants may form complexes with small GTPases and different regulatory proteins at specific steps in the prenylation pathway. Release into the prenylation pathway, or subsequent transport to or from the ER, may depend on GDP/GTP exchange induced by a GEF in the SmgGDS-GTPase complex. Alternatively, signaling events such as phosphorylation of proteins in the SmgGDS-GTPase complex may trigger release of GTPases to allow their processing or transport.

To incorporate our findings into a model of signal-dependent prenylation (Fig. 6), we propose that SmgGDS-607 retains nonprenylated GTPases in a state that slows their entry into the prenylation pathway. When the appropriate signal is delivered, SmgGDS-607 facilitates GDP/GTP exchange by the GTPase either directly or in complex with another protein, promoting the entry of the GTPase into the prenylation pathway (Fig. 6*A*). Because WT GTPases can undergo GDP/GTP exchange, they can be released from SmgGDS-607 and enter the prenylation pathway (Fig. 6*A*). In contrast, because DN GTPases do not bind GTP, DN GTPases are relatively nonresponsive to the signals that release the GTPase from the SmgGDS-607 complex (Fig. 6*B*). Overexpression of SmgGDS-607 would increase the formation of these nonresponsive DN-GTPase-SmgGDS-607 complexes. This possibility explains why SmgGDS-607 overexpression reduces the prenylation of DN-Rap1A, DN-RhoA, and DN-Rac1 (Fig. 4*C*). Overexpressed SmgGDS-607 would also slow the prenylation of WT GTPases, if the required signal is not delivered to release the WT GTPase from the overexpressed SmgGDS-607. This possibility explains why SmgGDS-607 overexpression reduces the prenylation of WT-Rap1A, as indicated by the results of the Triton X-114 fractionation assay (Fig. 4*D*).

In this model, SmgGDS-607 might sequester newly synthesized small GTPases in the cytoplasm as reserves for rapid prenylation (Fig. 6*C*), consistent with reports of regulated activation of PTases (43–46) and rapid prenylation of small GTPases

in response to different signals (47). SmgGDS-607 might also act as a scaffold or GEF to stimulate nonconventional cytoplasmic signaling by nonprenylated small GTPases (Fig. 6*D*). Our results also support the possibility that SmgGDS-607 transfers nonprenylated small GTPases to the PTase or facilitates a guanine nucleotide exchange event necessary for prenylation (Fig. 6*E*), likely in response to a signal.

The binding of prenylated PBR-containing small GTPases by SmgGDS-558 suggests several possible mechanisms by which SmgGDS-558 might promote signaling and membrane localization of small GTPases. SmgGDS-558 might promote the release of newly prenylated small GTPases from PTases (Fig. 6*F*), consistent with a recently proposed model that unidentified cellular proteins assist in the release of farnesylated proteins from farnesyltransferase (48). SmgGDS-558 might also escort newly prenylated small GTPases to the ER for C*AAX* processing (Fig. 6*G*). The possibility that SmgGDS-558 transfers PBR-containing GTPases from the ER to the PM (Fig. 6*H*) is consistent with SmgGDS-558 preferentially interacting with PBR-containing small GTPases, because PBR-containing GTPases follow a unique ER to PM route that differs from the Golgi to PM route followed by small GTPases lacking a PBR (6). Finally, SmgGDS-558 might also escort prenylated GTPases from the PM to endomembranes (Fig. 6*I*), thereby promoting signaling from endomembranes (Fig. 6*J*). These multiple mechanisms provide novel ways to regulate small GTPase prenylation and membrane trafficking that merit further investigation.

Our discovery that SmgGDS splice variants participate in the prenylation of small GTPases has potential therapeutic implications for NSCLC and prostate cancer, which are cancers that overexpress SmgGDS (12, 13). There is much interest in developing chemotherapeutic drugs that block the passage of small GTPases through the prenylation pathway or disrupt the functioning of the isoprenoid moiety. Drug targets include PTases (49, 50), Rce1, and isoprenylcysteine carboxylmethyltransferase (3), enzymes that synthesize the isoprenoid moiety (51) and the attached isoprenoid moiety itself (52). Unfortunately, some of the developed drugs can disrupt other metabolic pathways and block the prenylation of proteins unrelated to small GTPases, which may result in unwanted side effects. In contrast, SmgGDS splice variants are potentially more specific therapeutic targets, because they interact only with PBR-containing small GTPases (11, 38). Further studies are needed to validate SmgGDS splice variants as potential drug targets in cancer.

Our study supports the view that, in addition to the widely accepted model that prenylation of small GTPases occurs constitutively, cells possess the capability of regulating the prenylation of small GTPases. Expression of SmgGDS-607 and SmgGDS-558 provides cells with a mechanism to potentially coordinate the entry, transit, and exit of small GTPases moving through the prenylation pathway to their final sites of activity at membranes. The participation of SmgGDS splice variants in these events raises the possibility that SmgGDS splice variants, as well as other uncharacterized regulatory proteins, could be exploited to therapeutically control the prenylation and localization of small GTPases.

Acknowledgments—We thank Dr. Lawrence Quilliam for cDNAs encoding MR-GEF, C3G, GRP3, and PDZ-GEF and Dr. Michael Dwinell for the lentiviral vector encoding luciferase.

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