Novel Mechanism of the Co-regulation of Nuclear Transport of SmgGDS and Rac1

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The armadillo protein SmgGDS promotes guanine nucleotide exchange by small GTPases containing a C-terminal polybasic region (PBR), such as Rac1 and RhoA. Because the PBR resembles a nuclear localization signal (NLS) sequence, we investigated the nuclear transport of SmgGDS with Rac1 or RhoA. We show that the Rac1 PBR has significant NLS activity when it is fused to green fluorescent protein (GFP) or in the context of full-length Rac1. In contrast, the RhoA PBR has very poor NLS activity when it is fused to GFP or in the context of full-length RhoA. The nuclear accumulation of both Rac1 and SmgGDS is enhanced by Rac1 activation and diminished by mutation of the Rac1 PBR. Conversely, SmgGDS nuclear accumulation is diminished by interactions with RhoA. An SmgGDS nuclear export signal sequence that we identified promotes SmgGDS nuclear export. These results suggest that SmgGDS-Rac1 complexes accumulate in the nucleus because the Rac1 PBR has NLS activity and because Rac1 supplies the appropriate GTP-dependent signal. In contrast, SmgGDS-RhoA complexes accumulate in the cytoplasm because the RhoA PBR does not have NLS activity. This model may be applicable to other armadillo proteins in addition to SmgGDS, because we demonstrate that activated Rac1 and RhoA also provide stimulatory and inhibitory signals, respectively, for the nuclear accumulation of p120 catenin. These results indicate that small GTPases with a PBR can regulate the nuclear transport of armadillo proteins.

Armadillo (ARM) family proteins that contain multiple copies of the ~42-amino acid (aa) ARM motif include SmgGDS, p120 catenin (p120ctn), β-catenin, plakoglobin, APC, karyopherin α (also known as importin α), and several other proteins (reviewed in Refs. 1–3). Nucleocytoplasmic shuttling by many ARM proteins allows them to regulate events in different cellular compartments, including gene transcription and cell adhesion (reviewed in Refs. 2–7). ARM proteins enter the nucleus by different mechanisms (reviewed in Refs. 2–7). Karyopherin α enters the nucleus when it associates with proteins containing a nuclear localization signal (NLS) sequence consisting of a series of adjacent lysines or arginines (reviewed in Ref. 3). The NLS sequence is believed to anchor within the long surface groove formed by the multiple ARM repeats of karyopherin α, promoting the nuclear import of both the NLS-containing protein and karyopherin α (3, 5). APC possesses two NLS sequences and may enter the nucleus by associating with karyopherin α or related proteins (4, 7). The mechanisms by which other ARM proteins enter the nucleus are less clear, because some of these proteins neither possess classic NLS sequences, nor have they been reported to associate with NLS-containing proteins.

Several ARM proteins interact with the Rho family of small GTPases (8–15) or with guanine nucleotide exchange factors (GEFs) for these GTPases (16, 17). SmgGDS promotes guanine nucleotide exchange by small GTPases containing a C-terminal polybasic region (PBR), which is a series of adjacent lysines or arginines (11–15, 18). We noticed a striking sequence similarity between the PBR of small GTPases that interact with SmgGDS and the NLS sequence of proteins that associate with the ARM protein karyopherin α. It is possible that the 11 ARM repeats of SmgGDS form a surface groove that binds the PBR of small GTPases, just as the 10 ARM repeats of karyopherin α form a groove that binds the NLS sequences of different proteins (3). Based on this possibility, we hypothesized that the PBR of small GTPases acts as an NLS, promoting the association of these GTPases with SmgGDS and the nuclear accumulation of the SmgGDS-GTPase complex.

We tested this hypothesis by examining the nuclear accumulation of transiently transfected SmgGDS in cells co-transfected with mutant or wild-type Rac1 or RhoA. We show that the nuclear accumulation of SmgGDS is enhanced by interactions with Rac1 but diminished by interactions with RhoA. The PBR of Rac1, but not RhoA, was found to have NLS activity. These findings support a model in which the ability of the Rac1 PBR to act as an NLS promotes the nuclear accumulation of SmgGDS-Rac1 complexes. Conversely, the inability of the RhoA PBR to act as an NLS promotes the cytoplasmic accumulation of SmgGDS-RhoA complexes. This model may be applicable to other ARM proteins in addition to SmgGDS, because we demonstrate that the nuclear accumulation of endogenous p120ctn is similarly enhanced by Rac1 but not by RhoA. These findings identify a new function of Rac1 and RhoA and define a novel mechanism for the nuclear accumulation of SmgGDS and potentially other ARM proteins.

**EXPERIMENTAL PROCEDURES**

*cDNA Constructs*—The majority of the cDNA constructs coding for hemagglutinin (HA)- or myc-tagged proteins that were generated for...
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this study have been deposited in the Guthrie cDNA Resource Center (available at www.cDNA.org), which provides these and other cDNAs to the research community as a non-profit service. Three mammalian expression vectors were used to subclone cDNAs coding for wild-type or mutant SmgGDS, Rac1, or RhoA in this study. The myc-pcDNA3.1 vector was made by inserting cDNA coding for two copies of the myc epitope (EQKLISEEDL), which is recognized by the 9E10 monoclonal antibody (Stratagene, Santa Cruz, CA), into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The HA-pcDNA3.1 vector, which contains cDNA coding for three copies of the HA epitope (YPY-DVPDYA) that is recognized by HA antibody (Covance, Berkeley, CA), was a generous gift of the Guthrie cDNA Resource Center. The pEGFP-C1 vector was purchased from Clontech (Palo Alto, CA). The pEFBOSp120-3AB and pEFBOSp120-3A plasmids, coding for the 3AB peptide (MNWVDAEYVQARRGKKKSG) that is recognized by HA antibody (Covance, Berkeley, CA), was a generous gift of the Guthrie cDNA Resource Center. The pSRα-smgGDS plasmid (19), which was generously provided by Dr. Yoshimi Takai, Osaka University Medical School, was used as the original source of the full-length SmgGDS coding sequence (SmgGDS isoform 2, NCBI Protein Data base accession number AAA21876). Site-directed mutagenesis of the cDNA constructs was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

As depicted below in Fig. 1a, we generated cDNAs coding for wild-type SmgGDS (Sg) or mutant SmgGDS containing alanine substitutions in the N-terminal NES sequence (SgNA2 or SgNA4), the C-terminal NES sequence (SgCA2 or SgCA4), or both in the N-terminal and C-terminal NES sequences (SgCA2A4). These cDNAs were inserted into the HA-pcDNA3.1 vector to generate constructs coding for proteins with an N terminus 3× HA tag. Alternatively, the cDNAs were inserted into the myc-pcDNA3.1 vector to generate constructs coding for proteins with an N terminus 2× myc tag. As depicted below in Fig. 2a, the pEGFP-C1 vector was used to generate constructs coding for green fluorescent protein (GFP) fused to the N terminus of the following aa sequences: PPPVKRRKKKK to generate the GFP-PBR(Rac1) construct, PPPVKRRKRRKCLLL to generate the GFP-PBR(Rac1)CAAX construct, and QARRGKKKSG to generate the GFP-PBR(RhoA) construct, and QARRGKKKSGGLVL to generate the GFP-PBR(RhoA)CAAX construct. To facilitate expression, a codon for methionine was inserted at the C terminus of the GFP coding sequence in the pEGFP-C1 vector, immediately preceding the cDNA insert.

We obtained from the Guthrie cDNA Resource Center the HA-pcDNA3.1 vector containing cDNA insertions coding for wild-type Rac1 (Rac1), constitutively active Rac1 containing a valine substitution at aa 12 (CA-Rac1), or dominant negative Rac1 containing an asparagine substitution at aa 17 (DN-Rac1). We replaced the six basic aa comprising the N-terminal PBR in these cDNAs with glutamine to yield the Rac1(PBRQ), CA-Rac1(PBRQ), and DN-Rac1(PBRQ) cDNAs, as depicted below in Fig. 3a. We also obtained the HA-pcDNA3.1 vector containing cDNA insertions coding for wild-type RhoA (RhoA), constitutively active RhoA containing a valine substitution at aa 14 (CA-RhoA), and dominant negative RhoA containing an asparagine substitution at aa 19 (DN-RhoA) from the Guthrie cDNA Resource Center. The five basic aa comprising the RhoA PBR in these constructs were replaced with glutamine to yield the RhoA(PBRQ), CA-RhoA(PBRQ), and DN-RhoA(PBRQ) cDNAs, as depicted below in Fig. 3d. The cDNA insertions were placed into the pEGFP-C1 vector to generate constructs coding for proteins with an N terminus 3× HA tag. Transfection of Cells with cDNA Constructs—The CHO-m3 cell line, which was used for all of the experiments in this study, is a Chinese hamster ovary (CHO) cell line stably transfected with the Mscruscinacrylactone resistant receptor. This extensively characterized cell line (15, 20–22) was used because we are investigating the effects of muscarinic acetylcholine receptor (M3) agonist activation on the observed current responses, which will be the subject of a future report. In the absence of agonist for the muscarinic acetylcholine receptor, which is the case in this study, the CHO-m3 cells do not exhibit any detectable differences from parental CHO cells (20, 21). The cells were transfected by suspension in Ham’s F-12 medium (6 × 10⁶ cells/200 μl of medium) containing 8 μg of the indicated cDNA, and electroporation by a single electric pulse (200 V, 50 ms) using a BTX Electro Square Porator (Genetronics, Inc., San Diego, CA). The electroporated cells were transferred to tissue culture plates and incubated for 16–24 h (37 °C, 5% CO₂) in complete CHO medium consisting of Ham’s F-12 medium, heat-inactivated fetal bovine serum (5%), glutamine (0.3 μg/ml), peni-

cillin (20 units/ml), and streptomycin sulfate (20 μg/ml). The cells were then used in the indicated assays or plated onto glass coverslips in complete CHO medium (2 × 10⁶ cells/ml medium) and incubated for an additional 16–24 h (37 °C, 5% CO₂) before being examined for the intracellular localization of the indicated proteins.

Intracellular Localization of Proteins—Cells expressing GFP-tagged proteins were examined by fluorescence microscopy while the cells were permeabilized with TX-100-permeabilized cells or with endogenous p120ctn, the cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) (15 min, 4 °C) and incubated with 50 mM ammonium chloride in PBS to quench formaldehyde fluorescence (10 min, 25 °C). The fixed cells were permeabilized with acetone (10 s, 4 °C) or with 0.2% Triton X-100 (TX-100) in PBS (10 min, 25 °C), as described previously (15, 22). After incubating with PBS containing 1% bovine serum albumin (30 min), respectively, the cells were incubated with mouse antibody to HA (Covance) or to p120ctn (BD Transduction Laboratories, San Diego, CA) (1 h, 25 °C), followed by incubation with TRITC-anti-mouse IgG (1 h, 25 °C). The cells were mounted in PBS containing 90% glycerol and 0.1% p-phenylenediamine and examined using a Nikon Optiphot fluorescence microscope, as previously described (22). Digital images of the cells were collected using a Kodak DC 290 zoom digital camera and Adobe Photoshop software.

In each assay, investigators ranked the nuclear localization of proteins in at least 20 different cells transfected with the same cDNA, the identity of which was unknown to the investigators. The relative amount of a protein that was detectable in the nucleus of each cell was ranked using the following scale: 0 = undetectable, 1 = very low nuclear level and high cytoplasmic level; 2 = moderate nuclear level and high cytoplasmic level; 3 = similar nuclear and cytoplasmic levels; 4 = high nuclear level and moderate cytoplasmic level; 5 = high nuclear level and undetectable or very low cytoplasmic level.

We found that TX-100 solubilizes some cytosolic proteins, causing the ratio of nuclear protein to cytosolic protein in TX-100-permeabilized cells to sometimes be greater than the value of these ratios in non-permeabilized and acetone-permeabilized cells. This effect of TX-100 becomes apparent when the subcellular distributions of GFP and GFP-tagged proteins in TX-100-permeabilized cells (see Figs. 6–8) are compared with those in living cells (see Figs. 2b, 3b, and 3c). This effect also becomes apparent when the subcellular distributions of HA-Sg and HA-SgNA4 in TX-100-permeabilized cells (see Figs. 6 and 7) are compared with those in acetone-permeabilized cells (see Fig. 1, b and c). Despite this limitation, TX-100 permeabilization was used because it was found to be significantly better then acetone permeabilization in preserving the subcellular distributions of the expressed wild-type or mutant Rac1 and RhoA proteins.

Intracellular Localization of HA-Labeled Proteins—Twenty-four hours after electroporation, the transfected cells were suspended in labeling medium consisting of methionine- and cysteine-free Dulbecco’s modified Eagle’s medium, [³⁵S]methionine (10 μCi/ml), and 2% heat-inactivated fetal calf serum. The cells were cultured for an additional 16 h (37 °C, 5% CO₂) in the presence or absence of compactin (Sigma, St. Louis, MO) and then suspended in ice-cold lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, pH 7.4) containing phosphatase inhibitors (15). The cells were incubated in the lysis buffer for 20 min on ice with periodic vigorous vortexing of the cell suspension, followed by centrifugation (13,000 × g, 10 min, 4 °C). Nuclear proteins were immunoprecipitated by this method as indicated by our ability to detect nuclear proteins such as RCC1 in Western blots of the lysate supernatants (data not shown). The supernatants from the centrifuged lysates were immunoprecipitated using the 9E10 myc antibody (Santa Cruz Biotechnology), the HA antibody (Covance), or the p120ctn antibody (BD Transduction Laboratories), as previously described (15). The immunoprecipitates were subjected to SDS-PAGE followed by autoradiography as previously described (15).

Binding of [⁵⁷S]GTP·S by HA-tagged Wild-type and Mutant Rac1 Proteins—These assays were performed exactly as described by Strassheim et al. (15). Twenty-four hours after electroporation, the cells were permeabilized by a freeze/thaw cycle, incubated for 10 min (30 °C) with buffer containing 150 mM [⁵⁷S]GTP·S (1 μCi/ml), solubilized with detergent and centrifuged (13,000 × g, 10 min, 4 °C). The resulting supernatants were immunoprecipitated with HA antibody, and the amounts of [⁵⁷S]GTP·S bound to the immunoprecipitated HA-tagged proteins were determined by liquid scintillation counting.

Statistical Analyses—The means of the measured values of each treatment group were compared by using Student’s t test. Means were
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FIG. 1. SmgGDS has a functional NES at the N terminus. a, the cDNAs that were used to test the function of the putative NES sequences in SmgGDS are depicted. The relevant leucines and isoleucines in the putative NES sequences near the N terminus (aa 4–13) and C terminus (aa 465–473) of wild-type SmgGDS (Sg) are shown in the top diagram. The alanines that replace these relevant leucines and isoleucines in the different SmgGDS mutants (SgNA2, SgNA4, SgCA2, SgCA4, and SgNA4CA4) are shown in the lower diagrams. b, CHO-m3 cells transiently expressing the indicated HA-tagged wild-type or mutant SmgGDS proteins were incubated in the absence (top panels) or presence (bottom panels) of 10 μM leptomycin B for 90 min. The fixed and acetone-permeabilized cells were immunofluorescently stained with HA antibody. The intracellular distribution of HA-SgCA4 (left panels) was found to be indistinguishable from that of HA-SgCA2 (not shown). The intracellular distribution of HA-SgNA4 (center panels) was found to be indistinguishable from those of HA-SgNA2 and HA-SgNA4CA4 (not shown). Results shown are representative of four independent experiments. The bar represents 10 μm.

RESULTS

SmgGDS Has a Nuclear Export Signal Sequence—Because SmgGDS interacts with nuclear proteins (23, 24) and cytoplasmic proteins (11–15, 18), we looked for sequences in SmgGDS that might promote nucleocytoplasmic shuttling. Although classic NLS sequences are not apparent in SmgGDS, putative NES sequences, consisting of LXX(L/I)XX(L/I)X(L/I) in which the leucines are sometimes replaced by isoleucines (reviewed in Ref. 6), are present near the N terminus at aa 4–13 (LS-DTLKKLKL) and near the C terminus at aa 465–473 (LA-LIAALEL), as indicated by the underlined aa. We generated cDNA constructs coding for HA-tagged wild-type SmgGDS (HA-Sg) or HA-tagged mutant SmgGDS proteins containing alanine substitutions in the putative N-terminal or C-terminal NES sequences (Fig. 1a). Transient expression of these cDNA constructs in CHO-m3 cells indicates that the nuclear accumulation of SmgGDS is significantly increased by disrupting the N-terminal NES sequence (aa 4–13) but not by disrupting the C-terminal NES sequence (Fig. 1a). These results indicate that SmgGDS shuttles between the nucleus and cytoplasm. The nuclear export of SmgGDS is dependent on the N-terminal NES sequence and functional exportin 1.

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hypothesize that SmgGDS enters the nucleus when it associates with small GTPases containing a PBR that functions as an NLS. The PBR is present in the 10 amino acids immediately preceding the terminal CAAX region in Rac1 (PPPVKKRRKRK) and RhoA (QARRGKKKSG), as indicated by the underlined basic aa. cDNA constructs coding for these 10 aa fused to GFP were generated (Fig. 2a) and expressed in CHO-m3 cells (Fig. 2b). GFP accumulates in both the nucleus and cytoplasm of the transfected cells (Fig. 2b), probably reflecting the unfacilitated diffusion of GFP (~27 kDa) through nuclear pores. The nuclear localization of the GFP chimeric protein is increased significantly by the PBR of Rac1 but only modestly by the PBR of RhoA (Fig. 2b). cDNA constructs coding for GFP fused to the last 14 amino acids of each GTPase were also generated, to yield GFP chimeras containing the PBR plus the CAAX sequence of each GTPase (Fig. 2b). Expression of these cDNA

**Fig. 3.** The PBR enhances the nuclear accumulation of Rac1, but not RhoA. a, the cDNAs that were used to test the function of the PBR in Rac1 are depicted. The top diagram shows the lysines and arginines in the PBR of Rac1 (aa 183–188), and the glycine and threonine (aa 12 and 17) that regulate Rac1 activity. The lower diagrams show the valine substitution at aa 12 in the CA-Rac1 mutants, the asparagine substitution at aa 17 in the DN-Rac1 mutants, and the glutamine substitutions at aa 183–188 in the Rac1(PBRQ) mutants. b, the distributions of the transiently transfected GFP-tagged wild-type or mutant Rac1 proteins in living CHO-m3 cells were determined by fluorescence microscopy. The results shown are representative of four independent experiments. The bar represents 10 μm. c, the nuclear localization of the GFP-tagged proteins in fixed and TX-100-permeabilized CHO-m3 cells was scored by investigators who did not know the identities of the GFP-tagged proteins expressed by the cells. Results shown are the means ± 1 S.E. from 60 cells scored in three independent experiments. Brackets above the columns indicate a statistical comparison between the two bracketed samples. Symbols within a column indicate a statistical comparison between the sample and the control sample of cells transfected with GFP-Rac1 (first column). (**, *p < 0.001; NS, not significant.) d, the cDNAs that were used to test the function of the PBR in RhoA are depicted. The top diagram shows the lysines and arginines in the PBR of RhoA (aa 182–187), and the glycine and threonine (aa 14 and 19), which regulate RhoA activity. The lower diagrams show the valine substitution at aa 14 in the CA-RhoA mutants, the asparagine substitution at aa 19 in the DN-RhoA mutants, and the glutamine substitutions at aa 182–187 in the RhoA(PBRQ) mutants. e, the intracellular distributions of the transiently expressed GFP-tagged wild-type or mutant RhoA proteins in living CHO-m3 cells were determined by fluorescence microscopy. The results shown are representative of four independent experiments. The bar represents 10 μm. f, the nuclear localization of the indicated GFP-tagged proteins in fixed and TX-100-permeabilized CHO-m3 cells was scored by investigators who did not know the identities of the GFP-tagged proteins expressed by the cells. The results shown are the means ± 1 S.E. from 60 cells scored in three independent experiments. Brackets above the columns indicate a statistical comparison between the two bracketed samples. Symbols within a column indicate a statistical comparison between the sample and the control sample of cells transfected with GFP-RhoA (first column). (**, *p < 0.001; *, p < 0.005; NS, not significant.)
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Fig. 4. Mutation of the PBR alters Rac1 protein interactions and guanine nucleotide exchange but not prenylation. a, to assess Rac1 protein interactions, the indicated proteins were immunoprecipitated with HA antibody from lysates of equal numbers of 35S-labeled CHO-m3 cells transiently expressing the HA-tagged proteins (lanes 1–6) or transiently transfected with the HA-pcDNA3.1 vector (control, lane 7). A representative autoradiograph from five independent experiments is shown. Proteins that were identified by Western blotting or peptide sequencing previously (22) are indicated at the left. b, to assess Rac1 prenylation, the indicated HA-tagged proteins were immunoprecipitated from transiently transfected CHO-m3 cells that were incubated for 16 h with [35S]methionine in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 5 μM compactin. A representative autoradiograph from three independent experiments is shown. c, the guanine nucleotide exchange activities of the wild-type and mutant Rac1 proteins were assessed by measuring [35S]GTP·S binding by the HA-tagged proteins transiently expressed in CHO-m3 cells. The control value in each experiment was the amount of [35S]GTP·S bound to HA-Rac1 (first column). Results shown are the means ± 1 S.E. from three independent experiments conducted with triplicate samples in each experiment. Brackets above the columns indicate a statistical comparison between the two bracketed samples. Symbols within a column indicate a statistical comparison between the sample and the control sample of cells transfected with HA-Rac1 (first column). (**, p < 0.005; *, p < 0.05; NS, not significant.) d, the effects of co-transfected SmgGDS on Rac1 guanine nucleotide exchange was assessed by measuring [35S]GTP·S binding by the HA-tagged proteins in CHO-m3 cells that were co-expressing the indicated myc-tagged proteins. In each experiment, the control value was the amount of [35S]GTP·S bound to HA-Rac1 in cells co-transfected with the myc-pcDNA3.1 vector (first column). Results shown are the means ± 1 S.E. from three independent experiments conducted with triplicate samples in each experiment. Brackets above the columns indicate a statistical comparison between the two bracketed samples. (*, p < 0.05; NS, not significant.)

Constructs in CHO-m3 cells indicates that the isolated PBR of Rac1, but not RhoA, acts as a potent NLS even in the presence of the CAAX sequence (Fig. 2A).

To determine whether the PBR acts as an NLS in the context of full-length Rac1 or RhoA, we generated cDNA constructs coding for GFP-tagged wild-type, constitutively active, or dominant negative Rac1 or RhoA proteins containing either a normal or mutant PBR (Fig. 3, a and d). Transient transfection of these constructs in CHO-m3 cells indicates that Rac1 nuclear accumulation is enhanced by activation of Rac1, as indicated by the enhanced nuclear accumulation of GFP-CA-Rac1 (Fig. 3, b and c), and diminished by disruption of the PBR, as indicated by the reduced nuclear accumulation of the GFP-tagged Rac1(PBRQ) proteins (Fig. 3, b and c). Thus, Rac1 nuclear accumulation is promoted by both its conversion to the GTP-bound state and by an intact PBR, consistent with the PBR acting as an NLS. The unique intracellular distributions of the wild-type and mutant Rac1 proteins are not changed by replacing the GFP tag with a much smaller 3 kDa HA tag (~3 kDa) (data not shown). This finding indicates that the PBR enhances Rac1 nuclear accumulation even when Rac1 is presumably small enough to diffuse through nuclear pores. As expected, the GFP-tagged RhoA proteins exhibit much less nuclear accumulation than do the GFP-tagged Rac1 proteins (Fig. 3, e and f), consistent with the inability of the RhoA PBR to act as an NLS. Disruption of the RhoA PBR does not diminish, but instead can actually enhance, the nuclear accumulation of GFP-tagged RhoA proteins (Fig. 3f), providing further evidence that the RhoA PBR does not act as an NLS.

It is possible that Rac1(PBRQ) proteins do not accumulate in the nucleus because they are sequestered in the cytoplasm due to abnormal interactions with cytoplasmic proteins. To examine this possibility, HA-tagged wild-type and mutant Rac1 proteins were immunoprecipitated from 35S-labeled cells and examined for co-precipitating proteins (Fig. 4a). RhoGDI preferentially co-precipitates with HA-Rac1, whereas IQGAP1-calmodulin complexes preferentially co-precipitate with HA-CA-Rac1 (Fig. 4a, lanes 1 and 3), consistent with our previous findings (22). These proteins do not co-precipitate with HA-Rac1(PBRQ) or HA-CA-Rac1(PBRQ) (Fig. 4a, lanes 2 and 4).
indicating that disruption of the PBR diminishes the interaction of Rac1 with cytoplasmic proteins. The reduced interaction of Rac1(PBRQ) proteins with cytosolic proteins may account for our observations that mutation of the PBR diminishes the ability of CA-Rac1 to localize at membrane ruffles (Fig. 3d) and releases DN-Rac1 from a restricted juxtanuclear localization (Fig. 3b). The reduced interaction of Rac1(PBRQ) proteins with cytoplasmic proteins also lessens the possibility that Rac1(PBRQ) proteins remain in the cytoplasm because they are sequestered by cytoplasmic proteins. Instead, these findings are consistent with the model that Rac1(PBRQ) proteins remain in the cytoplasm because the NLS has been lost by disrupting the PBR.

The prenylation state of the Rac1(PBRQ) proteins was determined by examining their migration rates in SDS-PAGE gels. Non-prenylated small GTPases migrate slower than prenylated small GTPases in SDS-PAGE gels (11, 26). We found that Rac1 and Rac1(PBRQ) proteins exhibit similar rates of migration on SDS-PAGE gels (Fig. 4b). Rac1 and Rac1(PBRQ) proteins isolated from cells treated with the prenylation inhibitor compactin (27) migrate slower than proteins isolated from untreated cells (Fig. 4b). These results indicate that the Rac1(PBRQ) proteins are prenylated. Thus, the unique intracellular distributions of the Rac1(PBRQ) proteins are probably not due to changes in prenylation of these proteins.

Mutation of the PBR Alters Rac1 Protein Interactions and Guanine Nucleotide Exchange—To further define the functions of the Rac1 PBR, we compared the abilities of the HA-tagged Rac1 and Rac1(PBRQ) proteins to bind [35S]GTPyS (Fig. 4, c and d). Constitutively active HA-CA-Rac1 binds less [35S]GTPyS than does HA-Rac1 (Fig. 4e), consistent with constitutively active CA-Rac1 having a slower rate of GTP hydrolysis, which would result in a slower rate of [35S]GTPyS binding (22). Dominant negative HA-DN-Rac1 also binds less [35S]GTPyS than does HA-Rac1 (Fig. 4e), consistent with the mutation of aa 17 in DN-Rac1 diminishing the ability of DN-Rac1 to bind GTP (22). Interestingly, HA-Rac1(PBRQ) binds more [35S]GTPyS than does HA-Rac1 (Fig. 4, c and d), indicating that disruption of the PBR does not diminish [35S]GTPyS binding but can actually enhance it. Consistent with reports that SmgGDS promotes guanine nucleotide exchange by Rac1 (12–14), we found that expression of either myc-Sg or myc-SgNA4 enhances [35S]GTPyS binding by HA-Rac1 and HA-Rac1(PBRQ) in transiently transfected CHO-m3 cells (Fig. 4d).

The results of the [35S]GTPyS binding assays indicate that Rac1 and Rac1(PBRQ) proteins are able to interact with either SmgGDS or mutant SmgGDS containing a disrupted N-terminal NES. To investigate the stability of these protein interactions, we co-purified the HA-tagged Rac1 proteins with either myc-Sg or myc-SgNA4 transiently expressed in 35S-labeled CHO-m3 cells (Fig. 5a). We found that myc-Sg weakly co-purifies HA-Rac1 and HA-CA-Rac1 but strongly co-purifies HA-DN-Rac1 (Fig. 5a, lanes 2, 4, and 6). These results agree with the generally accepted model that GTpases form relatively unstable complexes with SmgGDS, because the GTpases only transiently associate with SmgGDS during guanine nucleotide exchange (15, 22). Conversely, dominant negative GTpases form relatively stable complexes with SmgGDS, because dominant negative GTpases do not readily bind GTP and thus do not readily dissociate from SmgGDS during guanine nucleotide exchange (15, 22).

The HA-tagged Rac1(PBRQ) proteins do not detectably co-purify with myc-Sg (Fig. 5a, lanes 3, 5, and 7), indicating that mutation of the Rac1 PBR diminishes the stability of the Rac1-SmgGDS complex. Diminished stability of the Rac1(PBRQ)-SmgGDS complex is consistent with an enhanced

![Fig. 5. The ability of SmgGDS to form strong complexes with RhoA and weak complexes with Rac1 is affected by mutating the PBR of the GTpases or the N terminus NES of SmgGDS.](image-url)
Previous studies indicate that SmgGDS interacts more effectively with RhoA than with Rac1 (12–15, 22). Consistent with these studies, we found that both myc-Sg and myc-SgNA4 co-precipitate HA-tagged RhoA proteins (Fig. 5b) much more effectively than they co-precipitate HA-tagged Rac1 proteins (Fig. 5a), indicating that SmgGDS associates more strongly with RhoA than with Rac1. These findings are consistent with our previous reports that endogenous SmgGDS co-precipitates more readily with HA-DN-RhoA than with HA-DN-Rac1 in stably transfected CHO-m3 cells (15, 22). Mutation of the RhoA PBR or the SmgGDS N terminus NES diminishes the stability of RhoA/SmgGDS complexes (Fig. 5b), similar to the effects of these mutations on Rac1/SmgGDS complexes (Fig. 5a).

Rac1 and RhoA Regulate SmgGDS Localization—Our hypothesis predicts that SmgGDS will accumulate in the nucleus when it interacts with Rac1, but not with RhoA, because the PBR of Rac1, but not RhoA, has NLS activity. To test this prediction, we examined the nuclear localization of HA-Sg and HA-SgNA4 in cells co-expressing GFP-tagged Rac1 or RhoA proteins with a normal or mutant PBR (Figs. 6 and 7). The nuclear accumulation of HA-Sg is significantly enhanced by GFP-CA-Rac1 (Fig. 6a, panel 4 and b), suggesting that Rac1-GTP supplies a signal that promotes SmgGDS nuclear accumulation. Mutation of the PBR diminishes the ability of CA-Rac1 to stimulate SmgGDS nuclear accumulation (Fig. 6a, panel 5 and b), consistent with the Rac1 PBR acting as an NLS for Rac1/SmgGDS complexes. Expression of dominant negative GFP-DN-Rac1 (PBRQ), which supplies neither a GTP-dependent signal nor a PBR-dependent signal, diminishes HA-Sg nuclear accumulation (Fig. 6a, panel 7 and b). The inhibitory effects of GFP-DN-Rac1 and GFP-DN-Rac1 (PBRQ) on SmgGDS nuclear accumulation are most noticeable in the presence of diminished SmgGDS nuclear export, as exhibited by HA-SgNA4 (Fig. 6a, panels 13 and 14) and c).

Interestingly, the nuclear accumulation of GFP-Rac1 is greater in cells transfected with HA-SgNA4 (Fig. 6a, panel 9)
Rac1 and RhoA Regulate p120ctn Localization—The abilities of Rac1 and RhoA to regulate the nuclear localization of other ARM proteins were tested by examining the distribution of endogenous p120ctn in CHO-m3 cells transiently transfected with the wild-type or mutant GTPases (Fig. 8). The nuclear accumulation of p120ctn is increased significantly by GFP-CA-Rac1 (Fig. 8, a (panel 2) and b) but only modestly by GFP-CA-Rac1(PBRQ) (Fig. 8, a (panel 3) and b). This finding indicates that both GTP-dependent and PBR-dependent signals from Rac1 enhance the nuclear accumulation of p120ctn. In contrast, the nuclear accumulation of p120ctn is diminished by GFP-CA-RhoA (Fig. 8, a (panel 4) and b). This inhibitory effect of GFP-CA-RhoA on p120ctn nuclear accumulation is most noticeable when the cells are treated with leptomycin B (Fig. 8, a (panel 9) and c), which was previously reported to inhibit p120ctn nuclear export (6).

To investigate the physical association of p120ctn with Rac1, we transiently co-expressed either the 3A or 3AB isoform of p120ctn (Ref. 6) with HA-tagged Rac1, CA-Rac1, or DN-Rac1 in CHO-m3 cells. Although we could immunoprecipitate the endogenous p120ctn and the expressed 3A and 3AB isoforms of p120ctn with the wild-type or mutant GTPases (Fig. 8). The nuclear accumulation of p120ctn is increased significantly by GFP-CA-Rac1 (Fig. 8, a (panel 2) and b) but only modestly by GFP-CA-Rac1(PBRQ) (Fig. 8, a (panel 3) and b). This finding indicates that both GTP-dependent and PBR-dependent signals from Rac1 enhance the nuclear accumulation of p120ctn. In contrast, the nuclear accumulation of p120ctn is diminished by GFP-CA-RhoA (Fig. 8, a (panel 4) and b). This inhibitory effect of GFP-CA-RhoA on p120ctn nuclear accumulation is most noticeable when the cells are treated with leptomycin B (Fig. 8, a (panel 9) and c), which was previously reported to inhibit p120ctn nuclear export (6).
p120ctn from the 35S-labeled cells using the p120ctn antibody, we could not detect any co-precipitation of the HA-tagged Rac1, CA-Rac1, or DN-Rac1 proteins with the immunoprecipitated p120ctn proteins (data not shown). Similarly, although we could immunoprecipitate the HA-tagged Rac1, CA-Rac1, or DN-Rac1 proteins using the HA antibody, we could not detect any co-precipitation of endogenous p120ctn or the expressed 3A or 3AB isoforms of p120ctn with the immunoprecipitated HA-tagged proteins (data not shown).

**DISCUSSION**

Our findings support a model in which SmgGDS nuclear accumulation is regulated by opposing Rac1- and RhoA-dependent pathways (Fig. 9). According to our model, the association of SmgGDS with Rac1-GDP converts it to Rac1-GTP, which generates a GTP-dependent signal that promotes the nuclear accumulation of the Rac1-SmgGDS complex (Fig. 9A). This GTP-dependent signal may involve the activation of Rac1 effectors. Our model depicts Rac1-GTP entering the nucleus with SmgGDS (Fig. 9A). This proposed entry of Rac1-GTP into the nucleus is consistent with the enhanced nuclear accumulation of GFP-CA-Rac1 in CHO-m3 cells (Fig. 3, b and c) and the nuclear accumulation of Rac1 in other cell types (28–30).

Our model proposes that Rac1-SmgGDS complexes dissociate in the nucleus (Fig. 9A). This proposal is consistent with our
inability to co-precipitate any of the HA-tagged Rac1 or Rac1(PBRQ) proteins with myc-SgNA4 (Fig. 5a, lanes 5–10), which is retained in the nucleus (Fig. 1, b and c). According to our model, the retention of SgNA4 in the nucleus contributes to its reduced association with Rac1 and Rac1(PBRQ) proteins.

We propose that Rac1(PBRQ) is less effective than Rac1 in promoting SmgGDS nuclear accumulation because Rac1(PBRQ) has a more transient interaction with SmgGDS and because Rac1(PBRQ) does not provide an NLS for SmgGDS (Fig. 9b). These attributes of Rac1(PBRQ) may also contribute to the diminished nuclear accumulation of Rac1(PBRQ) compared with Rac1 (Fig. 3, b and c).

According to our model, DN-Rac1 associates with SmgGDS but cannot convert to the GTP-bound form, resulting in the prolonged association of DN-Rac1 with SmgGDS (Fig. 9c). This proposal is consistent with our ability to co-precipitate stable complexes of DN-Rac1-SmgGDS (Fig. 5a). The association of DN-Rac1 with SmgGDS may reduce the nuclear accumulation of SmgGDS because DN-Rac1 cannot provide a GTP-dependent signal and because DN-Rac1 competitively inhibits the association of endogenous Rac1 with SmgGDS (Fig. 9c). The stable association of DN-Rac1 with SmgGDS in the cytoplasm may also contribute to the reduced nuclear accumulation of DN-Rac1 (Fig. 3, b and c).

Our model also provides an explanation for the inhibitory effects of RhoA on SmgGDS nuclear accumulation (Fig. 9d). Our results indicate that SmgGDS associates more strongly with RhoA than with Rac1 (Fig. 5). We propose that RhoA diminishes the nuclear accumulation of SmgGDS because the PBR of RhoA does not provide an NLS and because RhoA competitively inhibits the association of endogenous Rac1 with SmgGDS (Fig. 9d). The conversion of RhoA-GDP to RhoA-GTP may release SmgGDS, allowing SmgGDS to interact with Rac1 (Fig. 9d). Our finding that SmgGDS associates more strongly with RhoA than with RhoA(PBRQ) (Fig. 5b) may account for our observations that, in some cases, the nuclear accumulation of SmgGDS is inhibited more by RhoA proteins than by RhoA(PBRQ) proteins (Fig. 7).

Our findings indicate that Rac1 supplies both an NLS- and a GTP-dependent signal to promote the nuclear accumulation of Rac1-SmgGDS complexes. The importance of these signals in relation to each other has not yet been established. Interestingly, there is greater nuclear accumulation of SmgGDS in the presence of CA-Rac1(PBRQ) than in the presence of Rac1(PBRQ) or DN-Rac1(PBRQ) (Fig. 6). This finding indicates that the enhanced GTP-dependent signal from CA-Rac1(PBRQ) can promote the nuclear accumulation of SmgGDS even when CA-Rac1(PBRQ) cannot supply an NLS. One possible explanation for this finding is that CA-Rac1(PBRQ) activates an effector that subsequently interacts with complexes of SmgGDS associated with endogenous Rac1. According to this proposal, endogenous Rac1 supplies the NLS for the SmgGDS-endogenous Rac1 complex, whereas CA-Rac1(PBRQ) activates an effector to enhance the nuclear accumulation of the SmgGDS-endogenous Rac1 complex. In this way, CA-Rac1(PBRQ) may enhance the nuclear accumulation of SmgGDS-endogenous Rac1 complexes, even though CA-Rac1(PBRQ) lacks an NLS and may not accompany these complexes into the nucleus.

An important aspect of our model is that the Rac1 PBR functions as an NLS for complexes of Rac1-SmgGDS, promoting the nuclear entry of Rac1-SmgGDS complexes. This proposed mode of SmgGDS nuclear entry is very similar to the enhanced nuclear entry of karyopherin α when it associates with NLS-containing proteins such as open reading frame 57 (5). The 11 ARM repeats of SmgGDS may create a platform that has structural and functional similarities to the NLS-binding platform created by the 10 ARM repeats of karyopherin α. Karyopherin α is believed to bind two NLS-containing proteins simultaneously at NLS binding sites indicated by the aa sequence WXXYN (reviewed in Ref. 3). Karyopherin α has three WXXYN sequences at the major NLS binding site, and two WXXN
sequences at the minor NLS binding site (reviewed in Ref. 3). We observed that SmgGDS has one WXXN sequence (WIPSN), which is located at aa 275–279 in SmgGDS isoform 2 and at aa 324–328 in SmgGDS isoform 1 (NCBI Protein Data base accession number P52306). The presence of the WXXN sequence in SmgGDS is consistent with the Rac1 PBR acting as an NLS that promotes the formation and nuclear accumulation of SmgGDS-Rac1 complexes. The fact that SmgGDS has less WXXN sequences than karyopherin α is consistent with SmgGDS interacting with a unique subset of NLS-containing proteins (only small GTPases with PBRs), compared with karyopherin α, which interacts with a large variety of NLS-containing proteins.

Although there are appealing similarities between the nuclear entry of SmgGDS associated with Rac1 and the nuclear entry of karyopherin α associated with an NLS-containing protein, there are also some interesting differences. Karyopherin α must interact with importin β at the nuclear pore to enter the nucleus. This interaction depends on the importin β-binding domain of karyopherin α (reviewed in Ref. 3). We have not detected this domain in SmgGDS, suggesting that SmgGDS does not directly interact with importin β when entering the nucleus.

It was recently suggested that the interaction of importin β with the small GTPase Ran provides a model for the interaction of SmgGDS with small GTPases (31). This suggestion, which is based on the binding of Ran-GTP to the 10 ARM repeats of importin β (reviewed in Ref. 31), has parallels to our model. However, the absence of a PBR in Ran diminishes the similarities between the Ran/importin β interaction and the Rac1/SmgGDS interaction. Despite this limitation, Ran/importin β interactions may provide additional insights into the structural features of small GTPases and SmgGDS, which allow these proteins to interact.

The Rac1 PBR shares striking structural and functional similarities with a classic monopartite NLS sequence. However, the Rac1 PBR also has other functions, including regulating the ability of Rac1 to physically associate with RhoGDI, IQGAP1-calmodulin complexes (Fig. 5a) and the Rac1 effector PKA (32). It is possible that the diminished interactions of Rac1(PBRQ) with these proteins contribute to the diminished ability of Rac1(PBRQ) to accumulate in the nucleus, and the diminished ability of Rac1(PBRQ) to promote the nuclear accumulation of SmgGDS. Nevertheless, the ability of the Rac1 PBR to act as an NLS for SmgGDS and the inability of the RhoA PBR to act as an NLS provide a plausible explanation for our findings.

Our findings indicate that, like SmgGDS, the nuclear accumulation of p120ctn is enhanced by GTP-dependent and PBR-dependent signals supplied by Rac1 but not by RhoA. Thus, our model of the Rac1- and RhoA-dependent regulation of SmgGDS nuclear accumulation (Fig. 9) generally applies to the Rac1- and RhoA-dependent regulation of 120ctn nuclear accumulation. If Rac1 regulates p120ctn nuclear localization in the same manner that it regulates SmgGDS nuclear localization, Rac1 should physically interact with p120ctn. However, we have been unable to precipitate complexes of Rac1 associated with either endogenous p120ctn or with the transiently expressed 3A or 3AB isoforms of p120ctn. Complexes of Rac1-p120ctn may form in the cells, but they may be too unstable to isolate by immunoprecipitation. Complexes of Rac1-p120ctn may be as unstable as complexes of Rac1-SmgGDS, which are also difficult to immunoprecipitate (Fig. 5a). Even though Rac1-SmgGDS and Rac1(PBRQ)-SmgGDS complexes are difficult to immunoprecipitate (Fig. 5a), both Rac1 and Rac1(PBRQ) interact with SmgGDS in the cells, as indicated by the enhanced binding of $[^{35}S]GTP\gamma S$ by Rac1 and Rac1(PBRQ) in the presence of SmgGDS (Fig. 4d). Interestingly, it was previously reported that p120ctn interacts with RhoA in vitro (9), consistent with our model that p120ctn interacts with RhoA, as well as with Rac1, in vivo. However, we cannot currently assess whether these potential interactions involve the direct association of p120ctn with RhoA and Rac1 or involve the participation of additional intermediary proteins.

We found that DN-Rac1 inhibits the nuclear accumulation of SmgGDS (Fig. 6) but does not inhibit the nuclear accumulation of p120ctn (Fig. 8). This difference may occur because of the different abilities of SmgGDS and p120ctn to serve as a GEF for Rac1. According to our model, the formation of stable DN-Rac1-SmgGDS complexes during attempted guanine nucleotide exchange by DN-Rac1 inhibits the ability of SmgGDS to interact with endogenous Rac1 and enter the nucleus (Fig. 9C). In contrast, DN-Rac1 may not form a stable complex with p120ctn, because p120ctn reportedly does not act as a GEF for Rac1 (9). The inability of DN-Rac1 to stably bind p120ctn would make DN-Rac1 a much less effective inhibitor of p120ctn nuclear accumulation, compared with the ability of DN-Rac1 to inhibit SmgGDS nuclear accumulation.

Our model depicts the conversion of Rac1-GDP to Rac1-GTP occurring in the cytoplasm, before complexes of Rac1-SmgGDS or Rac1-p120ctn arrive at the nuclear pore (Fig. 9A). However, the nuclear localization of several GEFs for Rac1 (33–35) and for other Rho family members (36, 37) suggests the interesting possibility that Rac1-GDP converts to Rac1-GTP when the Rac1-p120ctn complex arrives at the nuclear pore, where the Rac1-p120ctn complex might have access to nuclear GEFs. This possibility is consistent with p120ctn associating with Vav (16), which is a Rac1 GEF that is present in the nucleus (33, 34).

Nucleocytoplasmic shuttling allows ARM proteins to participate in many cellular activities, including the regulation of small GTPases, gene transcription, cell adhesion, and neoplastic transformation (reviewed in Refs. 2, 4, 6, and 10). Our demonstration that Rac1 and RhoA regulate the nucleocytoplasmic shuttling of SmgGDS and p120ctn raises the intriguing possibility that these GTPases affect the nuclear localization of other ARM proteins. Other small GTPases with a PBR, which have been reported to interact with SmgGDS, include Cdc42 (13, 14), K-Ras4B (11, 14), Rap1A (18), Rap1B (11, 14), and Rap1A (18). The PBRs of these GTPases may have functions similar to those of Rac1 and RhoA. This possibility is supported by a previous report that the ability of Rap1 to interact with SmgGDS requires a Rap1 C-terminal region containing the PBR (38). The roles of these small GTPases in regulating the nuclear accumulation of SmgGDS and other armadillo proteins need to be determined.

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