Modulation of Rho and Cytoskeletal Protein Attachment to Membranes by a Prenylcysteine Analog*

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The GTPases Rho regulate the assembly of polymerized actin structures. Their C-terminal sequences end with the CAAX motif that undergo a lipidation of the cysteine residue. Analogous to the C-terminal ends of Rho proteins, N-acetyl-S-all-trans-farnesyl-L-cysteine and N-acetyl-S-all-trans-geranylgeranyl-L-cysteine, were used to analyze the role of prenylation in their membrane association. Silver-stained gels indicated that N-acetyl-S-all-trans-geranylgeranyl-L-cysteine treatment released only a few proteins of 20, 46, and 60 kDa. Western blot analysis showed that N-acetyl-S-all-trans-geranylgeranyl-L-cysteine released RhoB (10%), RhoA (28%), and Cdc42 (95%) from membranes, whereas N-acetyl-S-all-trans and trans-farnesyl-L-cysteine did not. Rab1, which possesses two geranylgeranyl groups, was also strongly extracted by N-acetyl-S-all-trans-geranylgeranyl-L-cysteine, whereas Ras, which is farnesylated, was not. Furthermore, N-acetyl-S-all-trans-geranylgeranyl-L-cysteine was very efficient (95%) in dissociating actin and tubulin from membranes but not integral membrane protein P-glycoprotein and sodium/potassium co-transporter NaP-2. The extraction of Rho and cytoskeletal proteins occurred below the critical micellar concentration of N-acetyl-S-all-trans-geranylgeranyl-L-cysteine. Membrane treatments with 0.7 M KI totally extracted actin, whereas 70% of Cdc42 was released. Actin was, however, insoluble in Triton X-100-treated membranes, whereas this detergent extracted (80%) Cdc42. These data show that Rho proteins and actin are not physically bound together and suggest that their extraction from membranes by N-acetyl-S-all-trans-geranylgeranyl-L-cysteine likely occurs via different mechanisms.

Various proteins are isoprenylated at their C termini. Farnesylated proteins include yeast mating factors, nuclear lamins, the retinal heterotrimERIC G protein transducin, the retinal rhodopsin kinase, and Ras proteins (1–3). Geranylgeranylation occurs in all of the remaining heterotrimeric G proteins and small G proteins (2). Isoprenylation is particularly interesting with respect to the small G proteins involved in signal transduction because different families undergo specific modifications. For instance, Ras proteins are farnesylated, Rab proteins terminate with CC or CXC, and both cysteine residues are geranylgeranylated, whereas Rho proteins are singly geranylgeranylated with the exception of RhoB and RhoE, which can be farnesylated (4).

Following prenylation of Rho proteins at cysteine residues in the C-terminal sequence motif CXXX, proteolytic removal of the last three amino acid residues occurs, and subsequently the free α-carboxyl group of the cysteine residue undergoes carboxyl methylation (5). Prenylation and methylation of the C-terminal cysteine residues of small G proteins increase their attachment to cell membranes (5). In addition, several Rho- and Ras-related proteins also contain a number of basic residues arranged in a combination of lysine and arginine residues adjacent to the C terminus (5, 6). The increased hydrophobicity resulting from the lipidation and methylation of C-terminal cysteine residues and the neutralization of the negative carboxyl charge in the methylated cysteine of small G proteins are critically important in the targeting and anchoring of these proteins to plasma membranes and so to the normal functioning of such proteins.

The Rho family of proteins in mammals comprises several members including RhoA, RhoB, and Cdc42 (7). Rho proteins transduce signals from plasma membrane receptors and control many cell functions requiring the assembly and organization of the actin cytoskeleton such as those involved in cell polarity (8), motility (9), adhesion (10), cellular transformation (11), and apoptosis (12). Although considerable progress has been made recently in identifying the regulators and effectors of Rho proteins (7, 13–15), the means by which prenylation affects interaction with membrane targets and/or regulatory proteins remains largely unknown. A possible mechanism for prenyl-mediated Rho protein targeting is association with cell membranes by interaction between the prenyl groups and membrane lipids (16). Alternatively, the translocation of activated Rho proteins from the cytosol to specific membranes in polarized epithelial cells (17) suggests that receptors could be involved in the targeting process. In addition, Gβγ dimers of heterotrimeric G proteins bind to GST1-Rho fusion proteins in vitro (18) suggesting that Gβγ dimers facilitate the association of Rho proteins to plasma membranes and possibly with other membrane proteins as well.

The shuttling of many isoprenylated Rho proteins between the cytoplasmic and membrane compartments appears to be as an essential aspect of their function. This cycling is mainly dependent on the regulatory protein GDP dissociation inhibitor (GDI) and its homologs (19). In addition to inhibiting the exchange of guanine nucleotides bound to Rho proteins by forming complexes with them in the cytosol, GDI stimulates the

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1The abbreviations used are: GST, glutathione S-transferase; GDI, GDP dissociation inhibitor; BBM, brush border membrane; AFC, N-acetyl-S-all-trans-farnesyl-L-cysteine; AGCC, N-acetyl-S-all-trans-geranylgeranyl-L-cysteine; P-gp, P-glycoprotein; PAGE, polyacrylamide gel electrophoresis; CMC, critical micellar concentration.
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Carmine. Usually, blots were incubated with a 1:1000 dilution of different antibodies in Tris-buffered saline containing 0.1% (v/v) Tween (Carnation). Usually, blots were incubated with a 1:1000 dilution of different antibodies in Tris-buffered saline containing 0.1% (v/v) Tween and 3% bovine serum albumin for 1 h at room temperature, followed by a 1-h incubation with a 1:1000 dilution of donkey anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase in Tris-buffered saline containing 0.1% (v/v) Tween and 3% bovine serum albumin for 1 h at room temperature, followed by a 1-h incubation with a 1:1000 dilution of donkey anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase in Tris-buffered saline containing 0.1% (v/v) Tween.

Immunoafﬁnity puriﬁed on a HiTrap protein A column and then on a peptide-Sepharose column as described (23). The mouse monoclonal antibody C219 against puriﬁed RhoA (26C4) and four afﬁnity-puriﬁed rabbit polyclonal antibodies directed against synthetic peptides corresponding to amino acid residues 119–132 of RhoB (119), amino acid residues 182–197 of Rab1b, amino acid residues 178–198 of GDI, and amino acid residues 19–21 of the C-terminal ends of prenylated Rho proteins, to analyze the role of prenylation in their association to BBM in rat kidney.

EXPERIMENTAL PROCEDURES

Materials

The prenylcysteine analogs AFC, N-acetyl-S-all-trans-geranyl-L-cysteine (AGC), and AGGC were obtained from Biomol (Plymouth Meeting, PA). Leucine p-nitroanilide and p-nitrophenyl phosphate were from Sigma. Aﬃnity-puriﬁed mouse monoclonal antibodies raised against a synthetic peptide corresponding to amino acid residues 120–150 of human RhoA (26C4) and four afﬁnity-puriﬁed rabbit polyclonal antibodies directed against synthetic peptides corresponding to amino acid residues 119–132 of RhoB (119), amino acid residues 182–197 of Rab1b (C-19), amino acid residues 178–198 of GDI, and amino acid residues 166–182 of Cdc42 (F1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies directed against synthetic peptides corresponding to the 14-amino acid C-terminal portion of the rat sodium/phosphate symporter NaPi-2 were raised in rabbits and afﬁnity-puriﬁed on a HiTrap protein A column and then on a peptide-Sepharose column as described (23). The mouse monoclonal antibody C219 against the membrane P-glycoprotein (p-gp) was obtained from ID Labs (London, Ontario). Anti-p-actin was from Sigma, pan-ras (Ab-3) antibodies (clone 10) were from Oncogene Research Products (Cambridge, MA), and anti-α-tubulin (AB-1) was from Calbiochem. Donkey anti-rabbit and donkey anti-mouse horseradish peroxidase-conjugated antibodies and an enhanced chemiluminescence (ECL) detection system were from Amersham Pharmacia Biotech.

Methods

Preparation of Kidney Brush Border Membranes and Total Membranes—Male Harlan Sprague-Dawley rats (300–350 g of body weight) were obtained from Charles River (Saint-Constant, Quebec). Animals were sacriﬁced by lethal exposure to carbon dioxide followed by decapitation. The kidneys were quickly removed and chilled in an ice-cold 0.85% NaCl solution. Slices of outer cortex were cut, and BBM was isolated with an MgCl2 precipitation method (24). The pellet containing the BBM was resuspended in 50 mm mannitol, 2 mm Hepes/Tris, pH 7.5, to a concentration of about 10 mg of protein/ml and stored at −80 °C. To determine the purity of the membrane preparations, alkaline phosphatase activity was assayed in homogenates and BBM as described below. The speciﬁc activity was enriched 10–12-fold over that of cortex homogenate, depending on the preparation.

To isolate a crude membrane fraction, various tissues were removed into an ice-cold 0.85% NaCl solution, then minced in 250 mm sucrose, 5 mm Hepes/Tris, pH 7.5, and homogenized with a tight-ﬁtting glass Teflon motorized homogenizer. Unbroken cells and nuclei were removed by centrifugation at 3000 × g for 10 min. The supernatants were centrifuged at 100,000 × g for 60 min to sediment the membranes. The pellets were resuspended in 250 mm sucrose, 5 mm Hepes/Tris, pH 7.5. Protein concentration was measured with the method of Bradford (Fierce) using bovine serum albumin as the standard.

Measurement of Enzymatic Activities in BBM—Alkaline phosphatase and leucine aminopeptidase activities were assayed by spectrophotometrically measuring, at 410 nm, the formation of p-nitrophenol using p-nitrophenyl phosphate (25) and leucine-p-nitroanilide (26) as substrates.

Expression of Recombinant GST-GDI in Escherichia coli—Expression of the recombinant GST-GDI fusion protein was carried out as described elsewhere (27). GST-GDI was isolated by afﬁnity puriﬁcation on glutathione-Sepharose beads obtained from Amersham Pharmacia Biotech, and the purity of the fusion protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Protein concentration was measured as described above.

Analysis of the Ability of Prenylcysteine Analogs and GST-GDI to Extract Rho Proteins from Kidney BBM—Membranes (20 μg protein) in a ﬁnal volume of 30 μl were incubated in 20 mm Hepes/Tris, pH 7.0, in the presence of prenylcysteine analogs dissolved in a ﬁnal concentration of 1% dimethyl sulfoxide (Me2SO) or, alternatively, with 0.36 μM GST or GST-GDI at 25 or 37 °C for various periods of time. Soluble proteins were separated from membranes by centrifugation at 40,000 × g for 30 min at 4 °C. Supernatants and membrane pellets were solubilized in Laemmli sample buffer (28), and proteins were analyzed by SDS-PAGE and Western blotting.

Irradiation Procedure—Irradiation was carried out at −78 °C in a Gammacell Model 220 60Co irradiator at a dose rate of ~10 kilorads/h as described previously (29). The target size of Rho proteins in BBM was calculated from the rate of disappearance of immunodetected RhoA and Cdc42 as a function of irradiation dose using the following empirical equation (50),

\[
D_{37} = \frac{1.29 \times 10^7}{D_{37}} (\text{Eq. 1})
\]

where \( D_{37} \) was the irradiation dose (kilotars) at which the measured immunodetected band has been decreased to 37% of its initial value.

Gel Electrophoresis and Western Blot Analysis—SDS-PAGE was performed according to Laemmli (28) with a Mini-Protein II apparatus (Bio-Rad). Proteins were heated at 95 °C in Laemmli sample buffer for 3 min before loading onto 12% polyacrylamide gels. The proteins were electroblotted onto 0.45-μm pore diameter polyvinylidene diﬂuoride membranes (Immobilon-P, Millipore) in transfer buffer (96 mm glycine, 10 mm Tris, and 10% methanol) at 80 mA/gel for 1–1.5 h. The blots were blocked overnight at 4 °C in Tris-buffed saline (137 mm NaCl, 20 mm Tris, pH 7.5) containing 0.1% (v/v) Tween 20 and 5% nonfat dried milk (Carnation). Usually, blots were incubated with a 1:1000 dilution of different antibodies in Tris-buffed saline containing 0.1% (v/v) Tween and 3% bovine serum albumin for 1 h at room temperature, followed by a 1-h incubation with a 1:1000 dilution of donkey anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase in Tris-buffed saline containing 0.1% (v/v) Tween. Immune reactive bands were detected with the ECL Western blotting kit as described in the manufacturer’s instructions (Amersham Pharmacia Biotech). Blots were exposed to Fuji ﬁlm, and the autoradiograms were scanned with a Personal Densitometer (Molecular Dynamics).

Determination of AGGC Critical Micellar Concentration—The critical micellar concentration (CMC) of AGGC was measured by a colorimetric method, which solubilized a blue hydrophobic dye from stained beads as described in the manufacturer’s instructions (ProChem, Rockford, IL).

RESULTS

The Prenylcysteine Analog AGGC Solubilizes Speciﬁc Members of Rho Proteins—Initial experiments were performed to investigate the effects of different prenylcysteine analogs mimicking the C-terminal ends of prenylated Rho proteins on the release of Cdc42 from cell membranes. Incubation of kidney BBM with increasing concentrations of AGGC progressively promoted the extraction of Cdc42, whereas AGC and AFC were ineffective at concentrations up to 250 μM (Fig. 1). Thus the prenylcysteine analogs AGC and AFC, which possess aliphatic chains shorter than that of AGGC, were unable to dissociate Cdc42 from BBM. The speciﬁcity of AGGC on the release of Cdc42 is of interest because this protein, as the majority of small GTP-binding proteins, is geranylgeranylated (2). Subsequently, AGGC was used to characterize the effect of prenylcysteine analogs on the association of Rho proteins to membranes.

We next measured the ability of AGGC to solubilize various Rho proteins from kidney BBM. A concentration of 200 μM
AGGC completely extracted Cdc42 (Fig. 2). Membrane dissociation of RhoA by AGGC was much lower and reached a plateau of 28% extraction at 200 μM AGGC (Fig. 2). In the case of RhoB, less than 10% of this protein was extracted by 250 μM AGGC (Fig. 2). Thus, the solubilization activity was very different for the three Rho proteins at AGGC concentrations greater than 100 μM. Because only a fraction of the RhoA and very little RhoB were released by AGGC treatment, these results show that only a subset of the Rho proteins is sensitive to the extraction activity of AGGC.

Specificity of Solubilization of BBM-bound Proteins by AGGC—We subsequently determined the specificity of the solubilizing effect of AGGC on BBM-bound proteins. The extracted proteins by AGGC were separated by SDS-PAGE, visualized by silver staining, and then analyzed by densitometry (Fig. 3). In untreated (control) and Me2SO-treated BBM, the patterns of released proteins were similar. Interestingly, upon incubation of BBM with AGGC, only a few proteins were recovered into supernatants. In order of abundance, the three major polypeptides were found to migrate at about 46, 20, and 60 kDa. Thus, AGGC was able to solubilize only particular BBM proteins.

The effects of AGGC on the activity and dissociation of specific enzyme markers were also measured in BBM. Alkaline phosphatase activity in pelleted membranes and in solubilized material was slightly reduced when BBMs were treated with AGGC compared with those observed in control or MeS0-treated membranes, whereas the activity of leucine aminopeptidase was similar under all conditions (Table I). Both enzyme activities were similar whether or not AGGC was present in the assay mixtures (Table I). Because the proportions of both alkaline phosphatase (13–15%) and leucine aminopeptidase (10–12%) activities, which were found in supernatants, were similar for both untreated and AGGC-treated BBM, these two enzyme markers were not solubilized by AGGC. Thus, AGGC appeared to have a minor perturbing affect on the binding of proteins to membranes because only a small number of membrane proteins was released (Fig. 3). Furthermore, two BBM markers remained attached to membranes during AGGC treatment, whereas their specific activities were weakly or not affected by the prenylcysteine analog (Table I).

To gain some insight into the identity of the most abundant proteins released by AGGC (Fig. 3) and to further investigate the specificity of AGGC for BBM protein dissociation, we examined the release of particular membrane proteins by West-
Membrane proteins were incubated for 60 min at 37 °C in 20 mM Hepes/Tris, pH 7.0, alone (control) or with either 1% Me$_2$SO or 250 μM AGGC. Following the incubation period, BBMs were centrifuged at 40,000 × g for 30 min at 4 °C to separate soluble proteins from the pelleted membranes. Protein concentrations were estimated with the Bradford assay as described under “Experimental Procedures.” To measure the activities of BBM markers, pelleted membranes were resuspended in 30 μl of 20 mM Hepes/Tris, pH 7.0. Aliquots from solubilized and resuspended membranes were used to assay leucine aminopeptidase and alkaline phosphatase activities as described under “Experimental Procedures.”

| Conditions | Alkaline phosphatase | Leucine aminopeptidase |
|------------|---------------------|------------------------|
|            | nmol/min             | nmol/min |
| Control*   |                     |           |
| Supernatants | 10.9 ± 0.7          | 0.90 ± 0.30          |
| Pellets    | 62.9 ± 4.1           | 7.24 ± 0.44          |
| Me$_2$SO*  |                     |           |
| Supernatants | 9.8 ± 0.6           | 0.95 ± 0.15          |
| Pellets    | 62.1 ± 2.3           | 7.39 ± 0.10          |
| AGGC*      |                     |           |
| Supernatants | 7.7 ± 0.7           | 0.99 ± 0.15          |
| Pellets    | 55.1 ± 1.0           | 6.97 ± 0.06          |
| AGGC*      |                     |           |
| Supernatants | 7.5 ± 2.2           | 0.90 ± 0.02          |
| Pellets    | 52.1 ± 5.9           | 7.87 ± 0.23          |

* The enzyme activities were measured in the absence of 250 μM AGGC in assay mixtures.

The enzyme activities were measured in the presence of 250 μM AGGC in assay mixtures.

Effect of AGGC on the release of prenylated, cytoskeletal, or integral membrane proteins from kidney BBM. Membranes (20 μg of protein) were incubated in 20 mM Hepes/Tris, pH 7.0 (control), and either 1% Me$_2$SO (DMSO) or 250 μM AGGC for 60 min at 37 °C. After incubation, BBMs were centrifuged at 40,000 × g for 30 min at 4 °C. Soluble (S) and membrane proteins (P) were dissolved in Laemmli sample buffer and separated by SDS-PAGE. Ras, Rab1, actin, tubulin, P-gp, and NaPi-2 were detected by Western blot analysis as described under “Experimental Procedures.” These results are representative of two experiments done in duplicate.

![Fig. 4](http://www.jbc.org/) Effect of AGGC on the release of prenylated, cytoskeletal, or integral membrane proteins from kidney BBM. Membranes (20 μg of protein) were incubated in 20 mM Hepes/Tris, pH 7.0 (control), and either 1% Me$_2$SO (DMSO) or 250 μM AGGC for 60 min at 37 °C. After incubation, BBMs were centrifuged at 40,000 × g for 30 min at 4 °C. Soluble (S) and membrane proteins (P) were dissolved in Laemmli sample buffer and separated by SDS-PAGE. Ras, Rab1, actin, tubulin, P-gp, and NaPi-2 were detected by Western blot analysis as described under “Experimental Procedures.” These results are representative of two experiments done in duplicate.

![Fig. 5](http://www.jbc.org/) Effect of AGGC on the release of prenylated, cytoskeletal, or integral membrane proteins from kidney BBM. Membranes (20 μg of protein) were incubated in 20 mM Hepes/Tris, pH 7.0 (control), and either 1% Me$_2$SO (DMSO) or 250 μM AGGC for 60 min at 37 °C. After incubation, BBMs were centrifuged at 40,000 × g for 30 min at 4 °C. Soluble (S) and membrane proteins (P) were dissolved in Laemmli sample buffer and separated by SDS-PAGE. Ras, Rab1, actin, tubulin, P-gp, and NaPi-2 were detected by Western blot analysis as described under “Experimental Procedures.” These results are representative of two experiments done in duplicate.

Tissue Distribution of the Capacity of AGGC to Solubilize Rho and Cytoskeletal Proteins from Membranes—Kidney epithelial cells possess highly polarized apical and basolateral membranes that show typical lipid and protein compositions (32). Thus, to determine whether the ability of AGGC to release RhoA and Cdc42 was a general phenomenon, we analyzed its effect on crude membranes from different rat tissues. AGGC in the presence of urea and benzyl alcohol was highly effective and similar to that of actin, we examined whether AGGC could modulate the interaction of cytoskeletal proteins to membranes. Interestingly, the addition of AGGC to BBM totally solubilized both actin and tubulin (Fig. 4).

Because Rho proteins play crucial roles in the organization of actin microfilaments (7, 13–15) and the major peripheral proteins, we examined whether AGGC could modulate the interaction of Rho proteins with membranes. We tested the ability of AGGC to solubilize small G proteins that are isoprenylated differently than are Rho proteins at their C-terminal ends. Cdc42 and RhoA are singly geranylgeranylated, and Rab1, which is doubly geranylgeranylated, was also easily solubilized by AGGC (Fig. 4).

Similarly, benzyl alcohol (40 mM), known to increase membrane fluidity, did not solubilize these proteins (Fig. 6).

Denaturing reagents such as urea (6 M) and guanidine-HCl (6 M), which unfold proteins, did not extract Cdc42 and RhoA (Fig. 6). Similarly, benzyl alcohol (40 mM), known to increase membrane fluidity, did not solubilize these proteins (Fig. 6).

Thus, various compounds known to extract membrane proteins did not solubilize Cdc42 and RhoA from kidney BBM, whereas AGGC partially released Cdc42 and RhoA (Fig. 6).

Because AGGC released actin and tubulin from kidney BBM, we examined whether AGGC released Rho proteins by AGGC in the presence of denaturing reagents. Because a high salt concentration (1 M KCl) was unable to extract them (Fig. 6), we analyzed the effects of these different chaotropic reagents on the membrane release of Rho proteins by AGGC. Urea and benzyl alcohol did not increase the proportions of Cdc42 and RhoA extracted by AGGC, whereas KCl (1 M) totally abolished the Rho protein extraction mediated by AGGC (Fig. 6).

Because AGGC released actin and tubulin from kidney BBM (Fig. 4), we also studied the effect of the chaotropic reagents on the extraction of these proteins. In the absence of AGGC, the releasing patterns of actin and tubulin were identical to those of Cdc42 and RhoA. For example, urea and benzyl alcohol released very little actin and tubulin, whereas KCl did not extract these proteins from BBM (Fig. 6). When AGGC was added to BBM, the release of actin and tubulin in the presence of urea and benzyl alcohol was highly effective and similar to...
that obtained by AGGC alone, but AGGC-mediated release was again abolished by KCl (Fig. 6). Together, these results show that the extraction of actin and tubulin parallels that of Cdc42 and RhoA for all conditions tested with chaotropic reagents in the absence as well as in the presence of AGGC.

In the absence of AGGC, treatment of BBM with guanidine-HCl strongly decreased the amounts of RhoA and Cdc42 recovered in both the soluble and membrane fractions (Fig. 6). Furthermore, the total amounts of RhoA and Cdc42 detected in soluble and membrane fractions from BBM treated with AGGC plus urea or guanidine-HCl were lower than those observed in control BBM (Fig. 6). A degradation product was also recognized by antibodies directed against Cdc42 in both fractions from BBM treated with urea, which became predominant when AGGC was included (Fig. 6). Some proteolysis of RhoA and Cdc42 also occurred in BBM treated with AGGC because the immunodetected amounts in soluble and pelleted fractions were lower than those found in BBM incubated with Me2SO alone (Fig. 6). These results show that unfolding of proteins by strong denaturants, such as urea or guanidine-HCl, promotes the proteolysis of RhoA and particularly that of Cdc42 during the 60-min incubation at 37 °C. Furthermore, the addition of AGGC together with urea favors the degradation of Rho proteins by BBM proteases.

**AGGC Extracts Rho Proteins from Kidney BBM below Its Critical Micellar Concentration.** AGGC appears to dissociate geranylgeranylated Rho proteins from membranes by some means other than simply unfolding them, because the addition of denaturing reagents did not increase their extraction from BBM (Fig. 6). We next determined whether AGGC, which possesses a negatively charged carboxyl group linked to a long hydrophobic chain, could act by a detergent-like mechanism to extract RhoA and Cdc42. Determination of the AGGC CMC in the buffer used to extract Rho proteins from BBM showed that micelles were formed at a concentration of 340 μM (Fig. 7), whereas the maximum extraction of Cdc42 and RhoA was reached at 200 μM AGGC (Fig. 2).

Cholesterol-sequestering agents such as digitonin and filipin have been shown to release cortical cytoskeletal proteins from membranes in mammalian cultured cells (33). Treatment of kidney BBM with digitonin (0.01%) and filipin (0.001%) at concentrations below their CMC were unable to extract Cdc42, RhoA, and actin (data not shown). These results strongly suggest that AGGC does not extract Rho proteins by solubilizing the lipid bilayer or by sequestering the cholesterol. This conclusion was supported by the observation that integral membrane proteins such as P-gp and NaPi-2 were not extracted by AGGC (Fig. 4).
BBM (20 μg of protein) were incubated in 20 mM Hepes/Tris, pH 7.0 (control), and either 1% Me₂SO (DMSO) or 250 μM AGGC, geranyl linalool, acetyl cysteine, and geranylgeranyl pyrophosphate for 60 min at 37 °C. Following incubation, BBM were centrifuged at 40,000 × g for 30 min at 4 °C to sediment the membranes. Laemmli sample buffer was added to the soluble proteins, and they were separated by SDS-PAGE. Released Cdc42 and RhoA were detected by Western blot analysis as described under “Experimental Procedures.” These results are representative of two experiments done in duplicate.

Rho and Cytoskeletal Proteins—We also compared various molecules mimicking different structural elements of AGGC on the extraction of Rho and cytoskeletal proteins. None of the tested compounds, N-acetyl-l-cysteine, geranylgeranyl pyrophosphate, and geranyl linalool, released Cdc42, actin, and tubulin from BBM (Fig. 8). However, geranyl linalool and AGGC showed a similar efficiency to release RhoA (Fig. 8). Together, these results indicate that AGGC acts selectively to release actin, tubulin, and Cdc42, whereas it preferentially extracts RhoA from cell membranes.

Detergent and KI Treatment Effects on the Extraction of Actin and Cdc42 from BBM—A major question raised by our results was whether actin was solubilized because AGGC released Rho proteins to which it is bound or alternatively whether AGGC by itself decreases of immunodetected Cdc2 and RhoA. Their calculated target sizes were 141 ± 10 kDa and 129 ± 29 kDa, respectively (Fig. 10). Because the molecular weights of Cdc42 and RhoA as predicted from their cDNA sequences are about 21 kDa, the measured sizes clearly support the hypothesis that these proteins could form homo- or heteromeric complexes rather than by a simple co-elution.

BBM-bound RhoA and Cdc42 Are Multimeric Complexes—To determine whether membrane receptors could be involved in the targeting and binding of Rho proteins to BBM, we used the radiation inactivation technique. This method is very useful for determining the size of membrane-bound proteins, because it does not require purification of the studied protein and is unaffected by associated lipids (34). Exposure of BBM vesicles to ionizing radiation resulted in a dose-dependent decrease of immunodetected Cdc42 and RhoA. Their calculated target sizes were 141 ± 10 kDa and 129 ± 29 kDa, respectively (Fig. 10). Because the molecular weights of Cdc42 and RhoA as predicted from their cDNA sequences are about 21 kDa, the measured sizes clearly support the hypothesis that these proteins could form homo- or heteromeric complexes when bound to renal BBM.

Comparison of AGGC and GDI Abilities to Extract Rho Proteins from BBM—GDI is a regulatory cytosolic protein that interacts with Rho proteins and inhibits the dissociation of GDP. Recently, it was shown that GDI also releases Rho proteins from membranes (20). Given that the ability of AGGC to extract RhoA from BBM rapidly reached a plateau (Fig. 2), suggesting two subpopulations of membrane-bound forms, we assessed the capacity of both GDI and AGGC to extract Rho proteins from BBM.
proteins by sequentially treating BBM with GDI then with AGGC. When the fusion protein GST-GDI was added to BBM followed by centrifugation, some Cdc42 and RhoA were recovered in supernatants whereas neither GST nor the buffer alone (control) could extract these proteins (Fig. 11, S1). Then membranes were resuspended and treated under selected conditions to extract the remaining BBM-bound Rho proteins. Interestingly, the addition of GST-GDI or, even more so, AGGC partly extracted Cdc42 and RhoA from BBM, which had been pretreated with buffer alone (control) or with GST (Fig. 11, S2). With BBM that had been pretreated with GST-GDI, however, no Cdc42 nor RhoA were released by a second treatment with GST-GDI, although AGGC extracted additional Rho proteins (Fig. 11, S2). These results show that AGGC is a more powerful reagent than GDI for the extraction of Rho proteins from BBM. The difference between AGGC and GDI abilities in extracting RhoA and Cdc42 could be because of the nucleotide bound to the Rho proteins because it is usually assumed that GDI preferentially extracts GDP-bound forms, whereas AGGC could act independently of the nucleotide state.

Effect of Ionic Strength on the Extraction of Rho Proteins from BBM by AGGC or GST-GDI—We also investigated the effects of physiological osmolality and ionic strength conditions on the extraction of BBM-bound Rho proteins by AGGC and GST-GDI. Mannitol (300 mM) slightly reduced the extraction of Cdc42 and RhoA by both GST-GDI and AGGC (Fig. 12). However, a physiological concentration of KCl (150 mM) strongly inhibited the extraction of Cdc42 and RhoA by GST-GDI and totally abolished the release mediated by AGGC (Fig. 12). Thus, the ability of GST-GDI and AGGC to extract Rho proteins is similarly affected by KCl or by mannitol. It is interestingly that incubation of BBM in isosmotic concentrations of mannitol or KCl shows that only ionic strength blocks the dissociation of RhoA and Cdc42 that is mediated by either AGGC or GST-GDI.

Together, these results suggest that the two releasing activities could share common mechanisms in the process of membrane extraction of Rho proteins.

**DISCUSSION**

Rho proteins regulate key signal transduction events in normal and transformed cells during growth and proliferation (7, 14, 35). These small GTPases exert this signaling activity while they are associated with plasma membranes. This process of membrane binding is primarily mediated by post-translational isoprenylation of their C terminus (1–3, 5, 6). In the current study, we used prenylcysteine analogs mimicking the C-terminal ends of Rho proteins to examine the role of prenylation in their membrane association.

In contrast with AGC and AFC, which possess shorter hy-
drophobic chains than AGGC (Fig. 1), and with geranylgeranyl pyrophosphate, which shares the same hydrophobic chain as AGGC (Fig. 8), we demonstrate that AGGC easily extracts Cdc42 from kidney BBM and from membranes of other tissues (Figs. 1 and 5). Furthermore, AGGC is also able to partly release RhoA and Rab1, two geranylgeranylated small GTPases, whereas it does not significantly extract RabB or Ras from BBM (Figs. 2 and 4). While RhoA and Cdc42 cycle between cytosolic and membrane compartments, Ras and RabB are localized exclusively to membranes (4). In addition, RabB was reported to undergo both farnesylation and geranylgeranylation, and Ras is constitutively farnesylated (4, 36). However, attempts to extract Ras from kidney BBM with the farnesylceine analog AFC were fruitless (data not shown). Together, these results with various small GTPases and different prenylceine analogs demonstrate that the ability of AGGC to extract membrane-bound small GTPases seems specific to geranylgeranylated forms including RhoA and, predominantly, Cdc42.

Our results show that Cdc42 is more easily extracted than RhoA by AGGC (Figs. 2, 5, 6, 8, and 12). Several small GTPases contain basic residues adjacent to the C-terminal end, and these polybasic domains participate in membrane binding (5, 6). These polybasic domains could favor the binding of Rho proteins to membranes through interactions with phospholipids. The polybasic domain of Cdc42 contains two basic amino residues, whereas that of RabB possesses five basic amino residues (5). Thus, the number of basic residues suggests that RhoA should bind to membranes stronger than Cdc42 and would explain the higher efficiency of AGGC to extract Cdc42 than RhoA. Similarly, although Rab1 is doubly geranylgeranylated, it is easily extracted by AGGC because this small GTPase contains only a lysine residue at its C-terminal end.

Interestingly, when investigating by SDS-PAGE the capacity of AGGC to extract proteins from membranes, we observed that three major kidney BBM proteins of 20, 46, and 60 kDa were released (Fig. 3). From the molecular weight values and from the results obtained by immunoblotting showing the efficiency of AGGC to extract Rho proteins, actin and tubulin, from membranes plus the known role of Rho proteins to regulate the organization of actin cytoskeleton, we conclude that the protein peaks seen in Fig. 3 correspond to tubulin (50 kDa), actin (43 kDa), and various small GTPases (21 kDa). This conclusion is also supported by the observation that immunodetected tubulin and actin comigrated with the same AGGC-extracted proteins at 60 and 46 kDa as visualized by Coomassie Blue staining of polyvinylidene difluoride membranes (data not shown).

A crucial point about the effect of AGGC was to determine whether the prenylceine analogs act by a specific or a detergent-like mechanism. Our results suggest that AGGC releases the membrane-bound Rho proteins by a specific mechanism. This conclusion is supported by several observations. RhoA and Cdc42 are released from kidney BBM at AGGC concentrations (200–250 μM) below its CMC, which is estimated at 340 μM under the conditions used (Fig. 7). SDS-PAGE analysis demonstrates that AGGC solubilizes only a small set of proteins. Alkaline phosphatase and leucine aminopeptidase, two enzyme markers attached to BBM by means of hydrophobic interactions between their anchoring domains and lipid components of these apical membranes, are not solubilized by AGGC (Table I). Furthermore, two integral membrane proteins, the sodium/phosphate cotransporter NaP-2 and the P-gp, which causes resistance to multiple chemotherapeutic drugs, remain membrane-bound in BBM incubated with AGGC (Fig. 4). Denaturing or chaotropic reagents such as urea, guanidine-HCl, and benzyl alcohol are unable to extract Rho proteins from kidney BBM (Fig. 6). The addition of these denaturing reagents together with AGGC does not increase the extraction of Rho proteins because of the prenylceine analog (Fig. 6). The small GTPases, Ras, which is farnesylated, and RhoB, which could be geranylgeranylated or farnesylated, are not extracted by AGGC (Figs. 2 and 4). Taken together, these results agree with the hypothesis that the extraction of membrane-bound Rho proteins by AGGC occurs by a specific mechanism. However, because of the hydrophobic chain of AGGC, the contribution of a nonspecific detergent-like mechanism could not be excluded. For example, extraction of Cdc42 occurs over a 2-fold span of AGGC concentration (Fig. 2). Thus, the release of Rho proteins by AGGC could be a complex process. It would involve a competition by AGGC for a specific prenyl group binding site on the membrane, a possible solubilization by a detergent-like mechanism, and a modulation by other factors such as the presence of polybasic domains and different post-translational modifications of small G proteins.

A major observation in our study is that extraction of RhoA and Cdc42 from cell membranes by AGGC is accompanied by the release of actin and tubulin as well (Figs. 4, 5, 6, and 8). This raises the question whether actin is solubilized because AGGC releases Rho proteins or conversely whether AGGC by solubilizing actin induces the release of Rho proteins. We observed that membrane-bound actin is totally extracted by a treatment with 0.7 M KI and that this was accompanied by the release of 70% Cdc42 (Fig. 9A). On the other hand, actin was insoluble in Triton-treated membranes, whereas Cdc42 was extracted at 80% by the detergent (Fig. 9A). These results show that actin extraction does not necessarily induce that of Rho proteins and that the strong release of a Rho protein is not accompanied by that of actin. These latter results agree with the fact that GDI extracts BBM-bound Rho proteins without releasing actin and tubulin (data not shown). Because the release of Rho proteins by GDI is not involved in actin and tubulin extraction, this is also in agreement with the observations that Rho proteins need effectors to modulate the cytoskeletal organization (7, 14). Together, these results suggest that AGGC extracts Rho and cytoskeletal proteins likely via different mechanisms rather than by a simple co-elution.

AGGC efficiency to extract Rho proteins is higher than that of GDI, and in that case, actin and tubulin are totally released from the membranes. A possible explanation is that Rho proteins extracted by AGGC but resistant to the action of GDI are responsible for the release of actin and tubulin. The radiation inactivation data indicate that Rho proteins bound to BBM form complexes of about 130–140 kDa (Fig. 10). The membrane-bound Rho proteins are mainly localized to the attachment sites anchoring the actin microfilaments to membranes, and several proteins are known to attach the actin filaments to membranes (37). The strong release of Rho proteins from these complexes by AGGC could possibly disrupt them and consequently liberates actin and tubulin indirectly. An alternative explanation is that the capacity of AGGC compared with GDI to extract membrane-bound cytoskeletal proteins operates directly by acting on proteins anchoring the actin and the tubulin to membranes, but the identity of those that are able to bind AGGC remains to be established.

The estimated target sizes of Cdc42 and RhoA in BBM vesicles exposed to ionizing radiations are 141 ± 10 kDa and 129 ± 29 kDa, respectively (Fig. 10). Because the target size of a membrane-bound protein is unaffected by associated lipids (34), these results suggest that Rho proteins may bind to membrane receptors in kidney BBM. Several recently published studies support this assumption. For instance, we showed that activated Rho proteins translocate to the apical membranes of
polarized epithelial cells in kidney (17). Treatment of neutrophil membranes with either heat or trypsin significantly reduced the translocation of Rho proteins (36), suggesting the existence of a membrane receptor. Furthermore, Gβy dimers of heterotrimeric G proteins associate to various GST-Rho proteins in vitro (18) suggesting that these subunits participate in the binding of Rho proteins to membranes. Association of RH1, the Saccharomyces cerevisiae homolog of the mammalian RhoA, with cortical actin patches is saturable and requires prenylation (38). In addition, we reported that RhoA and Cdc42 are associated with caveolae-enriched membrane domains in endothelial cells and that RhoA interacts with caveolin-1 in vitro (39). Although the hypothesis of a membrane receptor for the small Rho proteins is supported by several observations, its existence remains to be clearly demonstrated. However, by assuming the involvement of a membrane receptor, in this case the molecular mechanism used by AGGC to extract small GT-Pases would be in competition with the geranylgeranylated ends of Cdc42 and RhoA for the binding sites on these receptors and under saturating conditions the prenylcytochrome analog would extract Rho proteins from membranes.

Current models propose that Rho proteins are in an inactive form when complexed with GDI in the cytosol to sequester the isoprenoid group and thus prevent nonspecific association of Rho proteins with inappropriate membranes or with other proteins (40). Upon cell stimulation, these complexes translate to the plasma membrane or to the submembranous cytoskeleton where Rho proteins transduce extracellular signals via membrane receptors. Following activation of signaling pathways, the inactive Rho proteins are recognized by GDI, and complexes are released from cell membranes (20). Consequently, we compared the efficiency of both the regulatory protein GDI and the prenylcytochrome analog AGGC for the extraction of Rho proteins. When kidney BBM are sequentially treated with GST-GDI and then AGGC, the prenylcytochrome analog extracts additional Cdc42 and RhoA (Fig. 11). These results suggest that AGGC could possess a more general re-leasing activity than GDI. Thus, different subtypes of Rho proteins, because of the type of bound guanine nucleotide or various post-translational modifications, are extracted by each agent. Alternatively, the smaller size of AGGC (436 Da) allows its better accessibility to Rho proteins than GST-GDI (46,000 Da). The small size of AGGC would also help it to compete with the binding sites for the two geranyleranyl groups on Rab1.

The abilities of GST-GDI and AGGC to extract Rho proteins from kidney BBM behave similarly under conditions of physiological osmolality and ionic strength. Whereas mannitol slightly inhibits the extraction of Cdc42 and RhoA that is dependent on GST-GDI or AGGC, a physiological concentration of KCl strongly blocks the release of these proteins (Fig. 12). These results suggest that electrostatic interactions or modulation of protein conformation by ionic strength limit the accessibility of Cdc42 and RhoA to GST-GDI and AGGC. Alternatively, a physiological ionic strength could strengthen the binding of these proteins to membranes. Nevertheless, the parallel effects of ionic strength and osmolality on the abilities of both AGGC and GDI to reduce the release of Cdc42 and RhoA from kidney BBM strongly suggest that GDI and AGGC share convergent mechanisms in the processes required to extract these proteins from membranes.

In conclusion, the present study clearly demonstrates that the prenylcytochrome analog AGGC preferentially extracts membrane-bound geranylgeranylated small GTPases including RhoA and Cdc42 as well as submembranous tubulin and actin. The extraction efficiency of Rho proteins by AGGC is even higher than that obtained by the regulatory protein GDI. These observations indicate that AGGC could be a very useful tool for investigating the mechanisms regulating the interactions of Rho proteins with membranes.

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