The Specific Binding of Small Molecule Isoprenoids to *rho*GDP Dissociation Inhibitor (*rho*GDI)†

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Received August 9, 1999; Revised Manuscript Received November 5, 1999

ABSTRACT: The activities of small G-proteins are in part regulated by their interactions with GDI proteins. This binding is thought to be dependent on the C-terminal isoprenoid modification (geranylgeranyl or farnesyl) of these proteins. G-proteins are generally isoprenylated/methylated at their C-terminal cysteine residues. A quantitative fluorescence assay is reported here to evaluate the specificity of binding of *rho*GDI. A rhodamine-labeled geranylgeranylated/methylated cysteine derivative is used to measure its binding to *rho*GDI. Saturable binding in the low micromolar range is found with various geranylgeranylated/farnesylated analogues. Interestingly, the carboxymethylated derivatives bound significantly better than their free acid counterparts, suggesting that the state of methylation of the analogues is important for binding. The binding is also selective with respect to isoprenoid. Analogues containing hydrophobic modifications other than geranylgeranyl or farnesyl do not bind with significant affinities. These data demonstrate a substantial degree of specificity in the binding of isoprenoids to a protein important in signal transduction.

Isoprenylation/methylation refers to a C-terminal hydrophobic posttranslational modification which involves a substantial number of proteins central to signal transduction. Isoprenylated proteins can be either mono- or bis-isoprenylated *(1−9)*. In the mono-isoprenylated series, geranylgeranylation (C20) is the predominant modification *(10)*, although a small subset of mono-isoprenylated proteins are farnesylated (C15) *(1, 4, 11)*. All known G-proteins[1] are isoprenylated, and, where studied, isoprenylation has proved to be essential for the function of these proteins *(12−16)*.

In the mono-isoprenylated series of G-proteins, the isoprenylated C-terminal cysteine residue is generally found to be carboxymethylated *(4, 5, 17−20)*. Isoprenylation/methylation of these proteins renders them more hydrophobic than their unmodified counterparts. The modifications thus enhance the abilities of the proteins to associate with membranes. Indeed, membrane targeting is thought to be a central function of isoprenylation/methylation and essential for G-protein function *(15, 19)*. Here, methylation increases hydrophobicity by neutralizing an otherwise negatively charged carboxyl group *(15, 19)*. An important issue to address is whether isoprenylation/methylation may also be involved in protein−ligand recognition, and, if so, how specific is the recognition. The existence of membrane-bound receptors, which recognize the lipid modification of isoprenylated/methylated G-proteins, has been suggested *(16, 21, 22)*. These putative interactions could, of course, be important in directing specific G-proteins to particular membrane sites.

There is at least one example where protein−isoprenoid recognition has been experimentally demonstrated to occur, namely, in the interaction of geranylgeranylated/methylated small G-proteins in the *rho* series with their cognate GDI proteins *(23, 24)*. The GDI proteins solubilize their G-protein partners and render them inaccessible to the membrane sites where the G-proteins bind and are functionally active *(24, 25)*. Thus, GDI proteins play critical roles in signal transduction *(26, 27)*. In the case of *rho* and *rho*GDI, it is clear that interactions between the proteins depend on geranylgeranylation *(22, 23)*. The dissociation constants (K_D) for the binding of *rho*GDI to geranylgeranylated small G-proteins have been reported using various methodologies *(9, 24, 28)*. The K_D values obtained from these measurements are found to be in the range 1.6−30 nM *(9, 24, 28)*. Moreover, NMR experiments have indicated a hydrophobic pocket in *rho*GDI which could interact with the geranylgeranyl moiety *(9)*. The specificity of the binding interactions is unclear from these measurements, inasmuch as the molecular interactions between the protein and hydrophobic moiety are left undefined *(9, 24, 28)*. In addition, the quantitative role of methylation in the binding event is left unexplored, as is the role of

† The work described here was supported by the U.S. Public Health Service NIH Grant EY-03624.

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[1] Abbreviations: ADDCME, *N*-acetyldodecyl methyl ester; AFC, *N*-acetyl-S-*trans*,*trans*-farnesylcysteine; AFCME, *N*-acetyl-*S*-*trans*,*trans*-farnesylcysteine methyl ester; AFTPA, *trans*,*trans*-farnesylthiopicolinic acid; AFTPAME, methyl *trans*,*trans*-farnesylthiopicolinate; AGC, *N*-acetyl-*S*-*trans*,*trans*-geranylcysteine; AGCME, *N*-acetyl-*S*-*trans*,*trans*-geranylcysteine methyl ester; AGGC, *N*-acetyl-*S*-*trans*,*trans*-geranylgeranylcysteine; AGGCME, *N*-acetyl-*S*-*trans*,*trans*-geranylgeranylcysteine methyl ester; 2-FTB, 2-*trans*,*trans*-farnesylthiobenzoic acid; 3-FTB, 3-*trans*,*trans*-farnesylthiobenzoic acid; 4-FTB, 4-*trans*,*trans*-farnesylthiobenzoic acid; 4-FTBME, methyl 4-*trans*,*trans*-farnesylthiobenzoate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; G-protein, GTP binding protein; GDI, GDP dissociation inhibitor; GDP, guanosine diphosphate; ggcdc42, geranylgeranyl cdc42; GGPP, geranylgeranyl pyrophosphate; GST, glutathione *S*-transferase; GTP, guanosine triphosphate; GGTase, geranylgeranyl transferase; IPTG, isopropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; RGGCME, rhodamine-labeled *N*-acetyl-*S*-*trans*,*trans*-geranylgeranylcysteine methyl ester.

10.1021/bi991856n CCC: $19.00
Published on Web 12/20/1999

protein−protein interactions. Qualitative experiments on the binding of *rho*GDI and various lipids in detergent under highly nonphysiological conditions suggested a low order of specificity in the binding *(29)*. The issue of specificity of binding, and the possible role of methylation in the binding process, is clearly important to address. A quantitative approach to this problem is required to reveal the nature of the specificity inherent in the binding of an isoprenoid to *rho*GDI. In this article, a quantitative fluorescence approach is reported which addresses this issue of specificity with respect to binding of both the isoprenoid moiety and the methyl group. In these experiments, small molecule isoprenylated cysteine analogues are shown to specifically bind to *rho*GDI, and the specificity of these interactions is determined. There is significant specificity inherent in the binding process, and methylation is quite important for binding.

MATERIALS AND METHODS

Materials. 6-Carboxytetramethylrhodamine was from Molecular Probes. Oleic acid, sodium oleate, and methyl oleate were purchased from Aldrich. Geranylgeraniol and geranylgeranyl pyrophosphate (GGPP) were purchased from American Radiolabeled Chemicals. *N*-Acetyl-*trans*,*trans*-geranylgeranylcysteine (AGGC), *N*-acetyl-*trans*,*trans*-farnesylcysteine (AFC), *N*-acetylgeranylcysteine (AGC), and Zwittergent 3-14 were purchased from Calbiochem. Isopropyl β-D-thiogalactoside (IPTG), octyl Sepharose, thrombin protease, and GST purification kit were from Pharmacia. Dithiothreitol (DTT) and protease inhibitors (aprotonin, leupeptin) were obtained from Boehringer Mannheim. Phenylmethanesulfonyl fluoride (PMSF) was from Alexis. Benzamidine and cholic acid were obtained from Sigma. Triton X-100 was from NEN. All other reagents were of analytical grade.

*Synthesis. (A) Synthesis of Rhodamine-Labeled Geranylgeranylcysteine Methyl Ester [RGGCME (**1**)].* To a stirred solution of 6-carboxytetramethylrhodamine succinimidyl ester (5.0 mg, 0.00948 mmol) in DMF (1.0 mL) was added geranylgeranylcysteine methyl ester (4.2 mg, 0.0095 mmol) and diisopropylethylamine (1.5 mg, 0.015 mmol). The solution was allowed to stir at room temperature for 12 h. The reaction mixture was concentrated and flash-chromatographed through a silica gel column eluting with 10% methanol/methylene chloride. The product-containing fractions were collected and concentrated to yield 6.0 g (76%) of **1** as a purple colored solid: $R_f = 0.21$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.28 (d, $J = 8.0$ Hz, 1H), 7.05 (d, $J = 8.0$ Hz, 2H), 6.79 (d, $J = 8.1$ Hz, 4H), 6.73 (s, 2H), 5.17 (m, 1H), 5.06 (m, 3H), 4.81 (m, 1H), 3.73 (s, 3H), 3.27 (s, 12H), 3.11−3.05 (m, 2H), 2.87−2.72 (m, 2H), 2.04−1.92 (m, 12H), 1.62 (s, 9H), 1.54 (s, 6H). HRMS(FAB) calcd for $C_{49}H_{61}N_3O_6S$ 819.4281, found 819.4280.

(B) Synthesis of Prenylcysteine Analogues. *N*-Acetyl-*S*-geranylgeranylcysteine methyl ester (AGGCME) and *N*-acetyl-*S*-farnesylcysteine methyl ester (AFCME) were synthesized following the reported methods *(30−32)*. The syntheses of 2-farnesylthiobenzoic acid (2-FTB), 3-farnesylthiobenzoic acid (3-FTB), 4-farnesylthiobenzoic acid (4-FTB), and farnesylthiopicolinic acid(AFTPA) *(33)* have been described previously.

(C) Methylfarnesyl Thiopicolinate (AFTPAME) and Methylfarnesyl 4-Thiobenzoate (4-FTBME). These compounds were prepared by methylation of the corresponding acid, FTPIC and 4-FIB, respectively, with diazomethane in ether.

(D) Methylfarnesyl Thiopicolinate (AFTPAME). The product was purified by flash chromatography, eluting with 10% ethyl acetate/hexanes: $R_f = 0.35$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, $J = 4.5$ Hz, 1H), 8.19 (dd, $J = 7.5$, 2.0 Hz, 1H), 7.04 (m, 1H), 5.39 (m, 1H), 5.09 (m, 2H), 3.92 (s, 3H), 3.84 (d, $J = 7.5$ Hz, 2H), 2.09−1.94 (m, 8H), 1.75 (s, 3H), 1.68 (s, 3H), 1.59 (s, 6H).

(E) Methylfarnesyl Thiobenzoate (4-FTBME). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (d, $J = 8.5$ Hz, 2H), 7.29 (d, $J = 8.5$ Hz, 2H), 5.31 (m, 1H), 5.08 (m, 2H), 3.90 (s, 3H), 3.62 (d, $J = 7.0$ Hz, 2H), 2.07−1.96 (m, 8H), 1.68 (s, 6H), 1.60 (s, 3H), 1.59 (s, 3H).

(F) N-Acetyldodecyl Methyl Ester (ADDCME). *N*-Acetyldodecyl methyl ester was prepared by alkylation of the cysteine methyl ester with 1-bromodecane followed by acetylation with acetic anhydride in methylene chloride. The product was purified by flash chromatography, eluting with 50% ethyl acetate/hexanes: $R_f = 0.24$; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.26 (m, 1H), 4.82 (m, 1H), 3.77 (s, 3H), 2.99 (t, $J = 5.0$ Hz, 2H), 2.50 (t, $J = 7.5$ Hz, 2H), 2.05 (s, 3H), 1.26 (s, 18H), 0.88 (t, $J = 6.5$ Hz, 3H).

Expression and Purification of rhoGDI and CDC42. pGEX2T expression vectors containing *rho*GDI and cdc42 coding regions were gifts from A. Hall and C. Carpenter, respectively. These proteins were purified as glutathione *S*-transferase (GST) fusion product from *E. coli* following the reported methods *(34, 35)*. In brief, a 50 mL overnight culture of *E. coli* containing plasmids for *rho*GDI or cdc42 was diluted to a 500 mL Luria broth medium, and incubated at 37 °C at 220 rpm. Protein expression was induced by addition of 100 μM IPTG after the optical density of the medium reached ~0.6 at 600 nm. The cells were pelleted by centrifugation at 5000 rpm, and stored at −80 °C. The pellets were thawed into 5 mL of cold lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$) containing 5 mM DTT, 10 μg/mL aprotonin, 10 μg/mL leupeptin, 3 mM benzamidine, and 100 μM PMSF. The suspension was sonicated in a Branson Sonifier-250 with 4 × 30 s bursts and 30 s pauses. The resulting supernatant was centrifuged at 10 000 rpm, and the supernatant was incubated with glutathione Sepharose beads for 30 min. These beads were washed extensively with lysis buffer, and resuspended in cleavage buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM DTT). The GST domain of the fusion protein was cleaved by incubating protein with thrombin for 12−14 h. Protein concentrations were determined using the Bradford method *(36)* with the BioRad protein assay kit.

Fluorescence Anisotropy Measurements. *(A) Direct Binding Assays.* The rhodamine-labeled geranylgeranylcysteine derivative (RGGCME) was titrated with increasing concentrations of *rho*GDI. The concentration of the rhodamine-labeled derivative was determined spectrophotometrically by using a molar extinction coefficient of 8.8×10^4 M^{-1} cm^{-1} for RGGCME at 547 nm. The measurements were performed on a Perkin-Elmer LS-50B luminescence spectrometer equipped with a thermostat. Rhodamine-labeled derivatives were excited at 550 nm, and the emission was monitored at 580 nm with an integration time 5 s. Four measurements were made for each point, and their average values were

used for calculation. Measurements were performed in 20 mM Tris buffer, pH 7.5, using a cuvette of 0.5 × 0.5 cm dimension. The cuvette was equipped with an appropriate adapter. RGGCME was prepared as a concentrated stock solution in DMSO. Small amounts (typically in the range 1−2 μL) of this concentrated stock solution were taken in the cuvette, and appropriate dilutions were made by addition of Tris buffer. A desired amount (in the microliter range) of the concentrated stock of *rho*GDI in Tris buffer was added immediately to the solution in the cuvette, and mixed thoroughly. The resulting increase in anisotropy due to addition of protein was measured after 2−3 min of incubation. Fresh samples were prepared for measurements at each concentration of *rho*GDI.

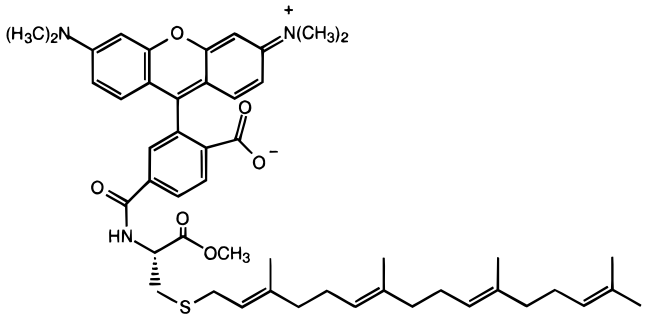
(B) Competitive Binding Assays. The dissociation constants (K_D) between geranylgeranyl/farnesyl cysteine analogues and *rho*GDI were determined by titration of RGGCME-bound *rho*GDI with analogue molecules. Initially, it was observed that the interaction of RGGCME and the analogue increases the background anisotropy in the absence of protein. The following approach was adopted to determine the change in anisotropy when the analogue and RGGCME compete for binding to *rho*GDI. First, the change in anisotropy was measured when the analogue molecule was added to a mixture of *rho*GDI and RGGCME. Second, the background increase in anisotropy due to the interaction RGGCME and the analogue molecule was measured under the same condition but in the absence of *rho*GDI. The resultant difference between these two anisotropies yields the anisotropy change due to competition between RGGCME and the analogue molecule for *rho*GDI. The experimental data obtained by this method show dose-dependent decrease in anisotropies with increasing concentration of the analogue molecule. Following this approach, we performed all competition experiments using RGGCME as fluorescent probe. Experiments were performed at 20 mM Tris buffer, pH 7.5. In a typical set of experiments, the concentrated DMSO stock of RGGCME was diluted in the cuvette with buffer. This buffer contained a desired concentration of *rho*GDI when experiments were performed in the presence of the protein. Concentrated stocks of analogue molecules were then added to this mixture. The resulting change in anisotropy was measured after thorough mixing of the solutions. The change in anisotropy at each concentration of analogue molecule was measured by making fresh solutions.

Stock solutions of geranylgeranyl/farnesyl derivatives were prepared in DMSO and stored in the dark at −20 °C. The titrations were performed by adding the desired amount of stock solutions of the analogues directly in the cuvette. The final concentrations of DMSO in the cuvette were less than 2.5%. These amounts of DMSO did not have any effect in the titration experiments.

Determination of Dissociation Constants. The experimental data from the direct binding assay allow determination of the dissociation constants between RGGCME and *rho*GDI (K_d). The following equation was used to analyze these data *(37)*:

$$i - i_0 = \Delta i \left[\left([\mathrm{GDI}]_0 + [\mathrm{RGGCME}]_0 + K_d \right) - \left(\left([\mathrm{GDI}]_0 + [\mathrm{RGGCME}]_o + K_d \right)^2 - 4[\mathrm{GDI}]_0[\mathrm{RGGCME}]_0 \right)^{1/2} \right] / 2 \quad (1)$$

Scheme 1: Structure of Rhodamine-Labeled *N*-Acetyl-*S*-*trans*,*trans*-geranylgeranylcysteine Methyl Ester (RGGCME)



where i and i_0 are the fluorescence anisotropy of [RGGCME] in the presence and in the absence of *rho*GDI, respectively. Δi is the difference between fluorescence anisotropy of 1 nM [RGGCME] in the presence of an infinite concentration of *rho*GDI, and fluorescence anisotropy in the absence of *rho*GDI. $[\mathrm{GDI}]_0$ and $[\mathrm{RGGCME}]_0$ are their initial concentrations.

The data from the competitive binding assays can be used to determine the dissociation constant between the geranylgeranyl/farnesyl cysteine analogue and *rho*GDI (K_D). The following equation was used for analysis of these results *(37)*:

$$[\mathrm{analog}]_0 = \left[\left(K_D(i_\alpha - i)/K_d(i - i_o) + 1 \right] \times \left([\mathrm{GDI}]_0 - K_d(i - i_0)/(i_\alpha - i) - [\mathrm{RGGCME}]_0(i - i_0)/(i_\alpha - i_0) \right) \right) \quad (2)$$

where $[\mathrm{analog}]_0$ is the initial concentrations of geranylgeranyl/farnesyl cysteine analogues. K_D was determined by the nonlinear curve fitting of the experimental data using eq 2.

RESULTS

Direct Binding of Rhodamine-Labeled Geranylgeranylcysteine (RGGCME) Methyl Ester to rhoGDI As Monitored by Fluorescence Anisotropy. A fluorescence anisotropy methodology was developed in order to quantify binding of *rho*GDI. In the current studies, the rhodamine-labeled geranylgeranylated molecule RGGCME (Scheme 1) was chosen as the fluorescence probe to monitor *rho*GDI−isoprenoid interactions. This analogue incorporates important features of a ligand expected to strongly interact with *rho*GDI, since the L-cysteine derivative is both geranylgeranylated and methylated. *rho*GDI is expected to bind to geranylgeranylated/methylated G-proteins, since most small G-proteins are so modified *(10)*. As shown in Figure 1, saturable binding is observed in the interactions of *rho*GDI with RGGCME. The equations used in all fluorescence calculations are provided under Materials and Methods. The data are fit to a 1:1 binding isotherm and yield a $K_d = 1.7 \pm 0.2$ μM. These data demonstrate a specific mode of binding between the *rho*GDI protein and the small molecule fluorescent probe. As a control to this binding study, experiments were also performed using free rhodamine as a probe. No measurable binding of free rhodamine to *rho*GDI was observed in these experiments.

Small Molecule Interactions with rhoGDI As Monitored by Competition with RGGCME. Using RGGCME as a probe, it is a simple matter to evaluate the binding between *rho*GDI and isoprenylated ligands that bind to it. Competition

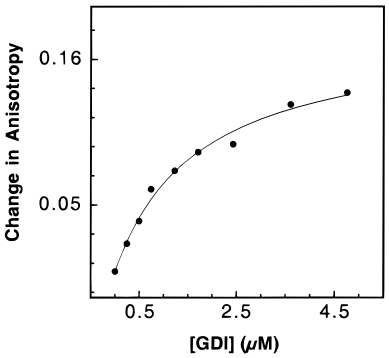
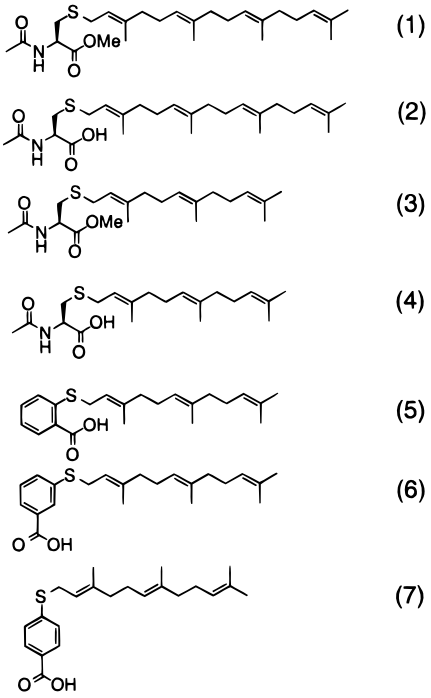


FIGURE 1: Plot of change in fluorescence anisotropy of RGGCME solutions (14 nM) as a function of *rho*GDI concentrations. The solid line is the computer-generated fit to experimental data using eq 1. The dissociation constant was determined to be 1.7 ± 0.2 μM.

Scheme 2[a]



[a] Structures of (1) *N*-acetylgeranylgeranylcysteine methyl ester (AGGCME), (2) *N*-acetylgeranylgeranylcysteine acid (AGGC), (3) *N*-acetylfarnesylcysteine methyl ester (AFCME), (4) *N*-acetylfarnesylcysteine acid (AFC), (5) 2-farnesylbenzoic acid (2-FTB), (6) 3-farnesylbenzoic acid (3-FTB), and (7) 4-farnesylbenzoic acid (4-FTB)

experiments with AGGCME and its congeneric acid, AGGC, both shown in Scheme 2, show a substantial difference in binding affinities (Figure 2). The methyl ester exhibits a $K_D = 1.6 \pm 0.1$ μM while the free acid binds with a $K_D > 20$ μM (Table 1). The K_D of AGGC cannot be accurately determined due to its comparatively weak affinity and limited aqueous solubility in buffer, which is a general property of isoprenylated compounds. The same pattern of binding is also observed with the farnesyl analogues AFCME and AFC (Scheme 2), as shown in Figure 3. While AFCME binds with a $K_D = 4.8 \pm 0.2$ μM, AFC binds with a $K_D > 27$ μM (Table 1). These data suggest that the methyl group is important for binding, at least in the small molecule series, and that the hydrophobicity of the isoprenyl side chain may also be important. The latter view is also consistent with the observation that neither AGCME nor AGC displaces RGGCME from *rho*GDI, and thus do not appear to specifically

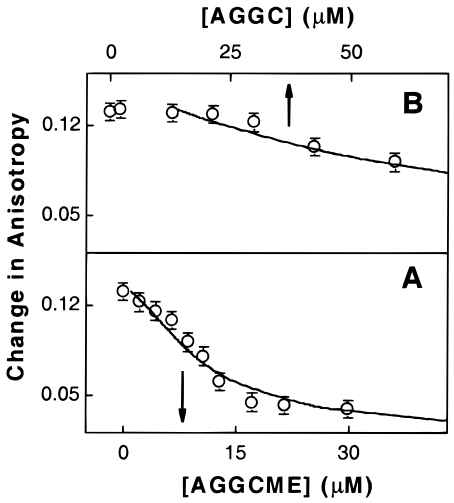


FIGURE 2: (A) Plot of change in fluorescence anisotropy of RGGCME solutions (14 nM) containing 5 μM *rho*GDI as a function of AGGCME (A) and AGGC (B) concentrations. The solid line is the computer-generated fit to experimental data using eq 2.

Table 1: Dissociation Constants (K_D) for the Binding of Small Molecules to *rho*GDI As Measured by Competitive Fluorescence Anisotropy Experiments at 20 °C

molecules	K_D (μM)
Ac-GG-Cys-OMe (AGGCME)	1.6 ± 0.1
Ac-Farn-Cys-OMe (AFCME)	4.8 ± 0.2
Ac-GG-Cys-Acid (AGGC)	>20
Ac-Farn-Cys-Acid (AFC)	>27
4-Farn-Thio-Benz-Acid (4-FTB)[a]	nb[b]
4-Farn-Thio-Benz-OMe (4-FTBME)	6.2 ± 0.5
Farn-Thio-PIC-Acid (AFTPA)	7.6 ± 0.4
Farn-Thio-PIC-OMe (AFTPAME)	3.3 ± 0.2
methyl oleate[a]	nb
Ac-G-Cys-OMe (AGCME)[a]	nb
Ac-dodecyl-Cys-OMe (ADDCME)	>18

[a] The following molecules were also tested that showed no measurable binding: 2-Farn-Thio-Benz-Acid (2-FTB), 3-Farn-Thio-Benz-Acid (3-FTB), oleic acid, sodium oleate, and Ac-G-Cys-Acid (AGC). [b] nb: no measurable binding.

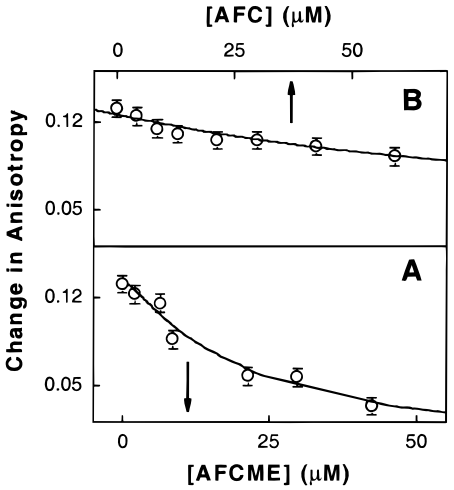


FIGURE 3: (A) Plot of change in fluorescence anisotropy of RGGCME solutions (14 nM) containing 5 μM *rho*GDI as a function of AFCME (A) and AFC (B) concentrations. The solid line is the computer-generated fit to experimental data using eq 2.

bind to *rho*GDI. The geranyl analogue contains a C10 moiety, while geranylgeranyl contains a C20 moiety. However, it is not simply hydrophobicity that is important for

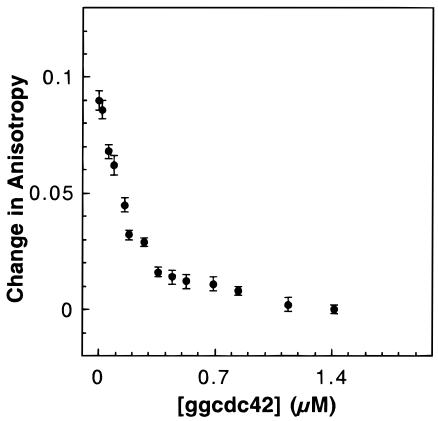


FIGURE 4: Change in the fluorescence anisotropy of RGGCME solutions (14 nM) containing 2.0 μM *rho*GDI as a function of geranylgeranylated CDC42 concentrations.

binding since the dodecyl analogue ADDCME only weakly binds to *rho*GDI and has a K_D value >18 μM (Table 1). In addition, neither methyl oleate nor oleic acid proved to have any measurable affinity for *rho*GDI (Table 1). These binding experiments demonstrate a fair degree of specific binding.

Small molecule isoprenylated cysteine analogues can have substantial effects on signal transduction processes, which could be due to interactions between the isoprenylated analogue and a cognate receptor *(31)*. It was of interest to determine if some of these analogues could specifically bind to *rho*GDI. The acids 2-FTB, 3-FTB, and 4-FTB (Scheme 2) were unable to bind to *rho*GDI. However, the methyl ester of 4-FTB bound with a $K_D = 6.2$ μM (Table 1), a result consistent with the general preference for esters over acids in the binding reaction. However, in at least one instance, AFTPA (Table 1), a free acid can also competitively bind ($K_D = 7.6$ μM). Even in this case, the methyl ester was bound with higher affinity (Table 1).

The studies described above are focused on small molecule isoprenylated analogues able to bind to *rho*GDI. To link the observed binding behavior to CDC42 binding, further competition experiments were performed with geranylgeranylated CDC42. The importance of the geranylgeranylation in binding of CDC42 to *rho*GDI was determined using the above competition method. CDC42 was prepared in its unmodified and its geranylgeranylated forms. The geranylgeranylated form was prepared by incubating CDC42, geranylgeranyl pyrophosphate, and GGTase 1 (*38*). Geranylgeranylated CDC42 was readily separated from unmodified CDC42 by chromatography on an octyl Sepharose column. The *rho*GDI could be readily bound to a glutathione column and shown to interact with geranylgeranylated, but not unmodified, CDC42 (unpublished experiments). Interestingly, only geranylgeranylated CDC42, but not unmodified CDC42, competed with RGGCME for binding to *rho*GDI. As shown in Figure 4, a saturable competition curve was obtained for geranylgeranylated CDC42 and the fluorescent probe RGGCME. These observations further validate the use of RGGCME as a probe to specifically monitor the binding of molecules to the *rho*GDI isoprenoid binding site. However, it was not possible to obtain a good fit to these data using eq 2, making a precise calculation of K_D impractical. This is doubtless in part because it is difficult to determine the precise dissociation constant of a competing binder when its binding affinity is significantly greater than that of the probe molecule. Under the present experimental conditions with $K_d = 1.7$ μM for the probe molecule (RGGCME), it can be calculated that the competition curve remains nearly insensitive to any K_D value between 1 and 100 nM (see eq 2). The dissociation constant for geranylgeranylated CDC42 binding to *rho*GDI has already been reported to be in the low nanomolar range (1.6−30 nM) *(9, 24, 28)*, and, hence, no further attempts were made to determine the K_D value here.

DISCUSSION

While receptor-mediated recognition of isoprenylated cysteine moieties has often been invoked as being important in G-protein-mediated signal transduction *(16, 21, 22, 29)*, scant evidence exists to support such hypotheses *(21, 22)*. However, the fact that isoprenylated cysteine analogues, such as AFC, show powerful effects on several different signal transduction pathways suggests that critical binding events may be mediated via isoprenoid recognition *(39−41)*. One possible target for isoprenylated cysteine analogues are the *rho*GDI proteins. GDI proteins are important in cell signaling because they bind to small G-proteins, solubilizing them and rendering them inactive *(24, 25)*. Small molecules capable of interfering with GDI−small G-protein binding would be expected to have important effects on signal transduction in cells, because these putative antagonists would have the effect of activating G-proteins. Importantly, in the case of GDI proteins, it has been demonstrated that G-protein isoprenylation is essential for strong binding *(10, 22, 42, 43)*, suggesting an isoprenoid binding site in GDI. In fact, NMR experiments on a 16 kDa carboxyl-terminal *rho*GDI fragment provide evidence for a hydrophobic binding site to which the isoprenoid might bind *(9)*. Interestingly, binding appears to be independent of whether the bound nucleotide is GTP or GDP *(35)*. In the current studies, interest focused on the specificity of the isoprenoid−GDI recognition event.

Several issues are addressed in these studies. Since all known mono-isoprenylated proteins are also carboxymethylated at the terminal cysteine residue, it is of interest to determine the role of carboxymethylation on binding. Since carboxymethylation is the only reversible step in the isoprenylation of posttranslational modification pathway, it is possible that reversible methylation could be important in modifying G-protein function. It is also of interest to determine the relationship between the structure of the hydrophobic (isoprenoid) moiety and binding. Finally, the question of whether *rho*GDI−CDC42 binding can be influenced by small molecule isoprenylated cysteine analogues is also addressed.

To approach the issues raised above, a quantitative fluorescence polarization-based assay was developed to monitor interactions between *rho*GDI and ligands. In these experiments, the rhodamine-labeled RGGCME is used to directly monitor binding to *rho*GDI. That this binding is biochemically relevant can be inferred from the observation that saturable competitive binding is observed with geranylgeranylated CDC42, but not with its nongeranylgeranylated counterpart. The fluorescence methodology used here, while useful for determining the binding of small molecules with K_D values in the micromolar range, proved not to be

useful for measuring the binding constant of geranylgeranylated CDC42 to *rho*GDI. The dissociation constant for geranylgeranylated CDC42 and *rho*GDI is in the low nanomolar range (1.6−30 nM) has been reported earlier by various other methods *(9, 24, 28)*. In any case, the greater affinity measured with geranylgeranylated CDC42 *(9, 24, 28)* compared to the small molecule analogues suggests that significant protein−protein interactions mediate the association between geranylgeranylated CDC42 and *rho*GDI.

Competitive binding experiments were then carried out with small molecule antagonists. Binding studies on a series of small molecule isoprenoids show that methylation appears to be important in the binding event. In most instances, the free isoprenylated acids bind rather weakly to *rho*GDI. Whether the enhanced binding of the methyl esters is due to a general preference for hydrophobic moieties or is related to a specific binding of the methyl ester is currently unknown. However, the finding that methyl esters are preferentially bound could be of physiological significance inasmuch as carboxymethylation is the only reversible step in the isoprenylation pathway.

While a thorough study of the role of the isoprenyl group in the binding event was not made, the data nevertheless suggest a general trend, with binding affinity increasing with chain length, and thus hydrophobicity, of the adducted isoprenoid. Since *rho*GDI is presumably selected to recognize the geranylgeranyl moiety, it is not surprising that binding affinity for the geranylgeranylated analogues was greater than for the farnesylated and geranylated analogues. However, the difference in binding between the farnesyl and geranylgeranylated methyl esters is not great (K_D values 4.8 and 1.6 μM, respectively). Interestingly, the *n*-dodecyl analogue (ADDCME) bound only very weakly to *rho*GDI, even though the *n*-dodecyl group is approximately as hydrophobic as the farnesyl group *(44)*, suggesting that isoprenoids may preferentially bind to *rho*GDI. It is also clear that unrelated hydrophobes, such as oleic acid and methyl oleate, do not measurably bind to *rho*GDI, further demonstrating specificity in the binding process.

As described above, free isoprenyl acids, such as AFC and FTB, only bind very weakly, if at all, to *rho*GDI. Their methyl esters, of course, do show binding. AFC *(39−41)*, FTB *(33, 45)*, and similar molecules *(46)* can show significant effects on signal transduction processes and even on growth control *(47)*. Whether these effects are specific or not is unknown. However, these analogues do not effectively bind to *rho*GDI, eliminating the latter as a target. Finally, the quantitative fluorescence assay methods described here should make it possible to uncover novel molecules which can uncouple GDI binding from its cognate small G-protein, and thus modulate paterns of signal transduction.

ACKNOWLEDGMENT

The GGTase used in this study was a generous gift of Dr. P. J. Casey.

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BI991856N