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A Quantitative Fluorometric Approach for Measuring the Interaction of RhoGDI with Membranes and Rho GTPases

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Abstract

Tight regulation of Rho GTPase-signaling functions requires the proper localization of proteins to the membrane and cytosolic compartments, which can themselves undergo reconfiguration in response to signaling events. The importance of lipid-mediated membrane signal transduction continues to emerge as a critical event in many Rho GTPase-signaling pathways. Here we describe methods for the reconstitution of lipid-modified Rho GTPases with defined lipid vesicles and how this system can be used as a real-time assay for monitoring protein–membrane interactions.

Keywords

Cdc42; Rho; Fluorescence resonance energy transfer; Synthetic lipid vesicles; Guanine nucleotide dissociation inhibitor

1. Introduction

Membrane localization of signaling proteins and their interaction with constituent lipid headgroups underlies the recruitment of reactive partners to the membrane surface. It has been well documented that proteins often harbor binding domains such as those that bind to specific lipid headgroups (e.g., pleckstrin homology (PH) domains exhibit general specificity for phosphoinositide lipids) (1–6). Green fluorescent protein probes fused to lipid-specific domains have been used successfully to demonstrate the asymmetric distribution of different lipids in living cells and to follow their accumulation and redistribution during cellular stimulation (7–9). In order to study membrane–protein interactions more quantitatively, we have been developing reconstituted lipid/protein systems where one can measure in real time the association–dissociation kinetics of Rho GTPases (10–12) interacting with membranes and with one of their key regulatory proteins, RhoGDI (for *Rho*-*G*uanine nucleotide *D*issociation *I*nhibitor). RhoGDI forms a cytosolic complex with the isoprenylated forms of the Rho GTPases, having the overall effect of stabilizing a soluble form of the Rho GTPase and affecting its translocation from the membrane to the solution compartments (13, 14).

The X-ray crystal structure of the complex formed between Cdc42 and RhoGDI provides a structural basis for the stabilization of the cytosolic form of Cdc42, as it shows the C-terminal lipid modification of Cdc42 (geranylgeranyl) nestled in a hydrophobic pocket in the immunoglobulin-like domain of RhoGDI (15). The membrane-cytosolic partitioning of Rho GTPases is dictated by the relative affinity of the lipid surface or RhoGDI, respectively, for the GTPase. How do different lipid headgroups, nucleotide-bound states, and protein–protein interactions other than those involving RhoGDI influence the reactivity and distribution of Rho GTPases and their signaling properties? In order to address these

questions, we have used a reconstitution approach, which uses fluorescence resonance energy transfer (FRET) as a spectroscopic method for monitoring the degree of Rho GTPase association with membranes. Our approach uses the fluorescent guanine nucleotide analog 2'-(or-3')-*O*-(*N*-methylantraniloyl)guanosine 5'-diphosphate (MANT-GDP) or 2'-(or-3')-*O*-(*N*-methylantraniloyl)- β : γ -imidoguanosine 5'-triphosphate (MANT-GMPPNP) as the fluorescence donor and hexadecanoylaminofluorescein incorporated into synthetic vesicles of defined composition as the fluorescence resonance energy acceptor (16, 17). Monitoring changes in the fluorescence of the MANT probes bound to Cdc42 provides an extremely sensitive assay for studying the kinetics of Cdc42–membrane interactions that has allowed for the delineation of discrete mechanistic steps in the membrane-to-cytosolic transition of this GTPase and how RhoGDI plays an important role in regulating these events.

2. Materials

2.1. Buffers, Reagents, and Equipment

1. *Spodoptera frugiperda* (Sf21) cells for adherent cultures (Invitrogen). Our contract supplier, Kinnakeet Biotechnology (Midlothian, VA), performs large-scale infections on suspension cultures of Sf9 cells with the viruses we provided.
2. Bacterial strain *E. coli* BL21(DE3) (Novagen).
3. Parental bacterial expression vectors pET28a (Invitrogen) and pGEX-KG (ATCC).
4. Hypotonic buffer: 20 mM sodium borate, pH 10.2, 5 mM MgCl₂, 200 μ M phenylmethylsulfonyl fluoride (PMSF), and 1 μ g/mL each of aprotinin and leupeptin.
5. Tris buffered saline (TBSM): 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 5 mM MgCl₂. For solubilizing membranes, this buffer is supplemented with 1% Triton X-100. For eluting GST-RhoGDI this buffer is supplemented with 10 mM glutathione.
6. High salt wash buffer: 50 mM Tris–HCl, pH 7.5, 700 mM NaCl, 5 mM MgCl₂, 0.1% CHAPS, and 20 mM imidazole, for immobilized metal ion affinity chromatography (IMAC).
7. Elution buffer for IMAC: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1% CHAPS, and 500 mM imidazole TEDA buffer: 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM sodium azide.
8. Chelating Sepharose beads charged with Ni²⁺ (Qiagen).
9. Glutathione Sepharose beads (GE Healthcare).
10. 40-mL glass homogenizer (Wheaton).
11. Sonicator: Sonic Dismembrator Fisher Scientific 550.
12. Fritted glass columns, 10 $\times$ 1 cm bore (BioRad).
13. Amicon Ultra 10 K concentrators (Millipore).
14. Colloidal Blue (Boston Scientific).
15. Polyacrylamide gel electrophoresis is carried out using a Novex Mini-Cell (Invitrogen) with precast 4–20% Tris–glycine gradient gels (1.5 mm $\times$ 10 well).

16. Lipids are obtained as lyophilized powders (Avanti Polar Lipids) or as reconstituted samples in chloroform (Sigma Aldrich). Between uses, the aliquots are stored under argon at -20°C . Cholesterol is from Nu-Chek Prep (see Fig. 1).
17. Hexadecanoylaminofluorescein (HAF), MANT-GDP, and Mant-GMPPNP (Invitrogen, formerly Molecular Probes). Store the 5 mM stock in a readily accessible dark location at room temperature.
18. Lipid vesicles for fluorescence spectroscopy are prepared using an Avanti mini-extruder (Avanti Polar Lipids) that comes with easy to follow instructions. Use 1- μm diameter pore polycarbonate membranes (Avanti, Cat. No. 610010).
19. 1 M MgCl_2 solution.
20. Fluorescence spectrometer Varian Cary Eclipse (Varian, Inc.) operated in the counting mode. To be used with a 1-mL quartz cuvette (Hellma Analytics) and a magnetic stirrer.

2.2. Proteins

1. Rho GTPases must be expressed in eukaryotic cells in order to be post-translationally modified (more specifically, isoprenylated), as bacteria do not possess the geranylgeranyl transferase enzyme necessary for C-terminal lipid modification. Viruses that enable expressing modified Rho GTPases in cultured Sf21 cells are produced following the Bac-2-Bac Baculoviral expression system (Invitrogen) according to the manufacturer's instructions. Sf21 cells can be grown in 175 cm² screw cap flasks (Corning, Inc) and infected with the baculovirus encoding the Rho GTPase of interest at a multiplicity of infection (MOI) of $\sim 3:1$ (see Note 1). Once test infections demonstrate acceptable levels of protein expression, as viewed on Western blots with the polyhistidine tags, Sf21 cell numbers can be scaled up according to the needs of the experiment. Intact cells can be stored as pellets indefinitely at -80°C and worked up as needed. Large scale expression of Rho GTPases in insect cells can be performed in spinner culture where a typical yield for Cdc42 is approximately 5 mg protein/L of culture medium (see Note 2). We routinely contract out large scale expression of protein to our outside supplier, Kinnakeet Biotechnology (Midlothian, VA).
2. Bacterial expression of GST or polyhistidine fusion proteins of RhoGDI or Cdc42, respectively, is carried out by growing *E. coli* that harbor expression plasmids for each protein. Cells are grown at 37°C until an $\text{OD}_{600} \sim 0.6$ is achieved, at which time protein expression is initiated by adding 1 mM isopropyl 1-thio- β -D-galactopyranoside. Cells are grown for an additional 3 h prior to pelleting by centrifugation ($6,000 \times g$ for 10 min). Cell pellets are resuspended in TBSM and sonicated immediately for protein purification or frozen as pellets at -80°C for later use.

3. Methods

3.1. Protein Purification

3.1.1. Purification of His-Tagged Proteins—Carry out all procedures at 4°C unless otherwise noted.

¹The MOI refers to the ratio of the number of virus particles to the number of Sf21 cells adherent to the flask or growing in spinner culture. A MOI of 3 is a good starting point for optimizing protein expression using baculovirus/Sf21 protein expression systems.

²The 5 mg per liter of spinner culture is on the high end of what we expect typical yields to be for Rho GTPases in insect cell expression. Despite the similarity of the proteins, varying yields of the different Rho GTPases were consistently observed.

The protocol described below is for IMAC of His₆-tagged proteins from insect cells. For IMAC of bacterially expressed His₆-tagged proteins proceed as in Subheading 3.1.2, steps 1–3 and continue with the supernatant from step 5 below.

1. The insect cell pellet harvested from a liter of culture is resuspended in 40 mL of hypotonic buffer and Dounce homogenized, using a 40-mL glass homogenizer (see Note 3).
2. Particulates are pelleted from this homogenate at $150,000 \times g$ for 20 min. The supernatant from this spin contains the non-prenylated form of the Rho GTPase (e.g. Cdc42), which can be recovered by affinity chromatography at this stage if desired or discarded. The pellet contains the geranylgeranylated form of Cdc42 and this is resuspended and repelleted twice in order to thoroughly clear the membranes of unmodified, aqueous soluble protein.
3. Resuspend the pellet a third time with a final 50 mL of TBSM supplemented with 1% Triton X-100 and rotate at 4°C for 30 min to solubilize the membranes completely.
4. Spin down the remaining particulates for 20 min at $9,000 \times g$ at 4°C and save the supernatant. This supernatant contains the isoprenylated, His₆-tagged Cdc42 and is used for further purification using standard Ni²⁺ charged Sepharose beads.
5. Carry out IMAC by incubating the supernatant from step 1 with 2 mL of Ni²⁺ bead slurry with gentle rocking at 4°C for 30 min.
6. Allow the beads to settle on a 10 × 1 cm bore fritted glass column and allow the liquid to drain from the column (save this for later protein analysis).
7. Wash the column with 400 mL of ice-cold high salt buffer.
8. Elute the His₆-Cdc42 with 10 mL of elution buffer. Divide eluate into two samples and concentrate one sample down to 1 mL (see Note 4) using Amicon Ultra 10 K concentrators. If you are unable to concentrate the protein to this low of a volume, due to precipitation, take note of the specific concentration at which this occurred and concentrate the remaining fraction down to a safe volume.
9. Analyze fractions collected at different stages of the purification by SDS-PAGE analysis using Colloidal Blue staining to visualize protein (see Note 5).
10. Divide the eluates into 100 µL aliquots, snap freeze the tubes and store at –20°C (see Note 6).

³A thorough dissolution of the insect pellet requires both its immersion in the hypotonic buffer (described above) and repeated disruption with a microspatula. To hasten this process, it is useful to break the pellet into multiple pieces and expose as much surface area to the solvent as possible. The homogenization process that follows is most efficient when the pellet has completely dissolved.

⁴In purifying lipid-modified proteins, be wary of hydrophobic-induced aggregation. The protein concentration step, at the end of the purification, is most vulnerable to this. Once white fiber-like particulates are observed, the sample is no longer usable for functional assays. Therefore, approach this step with caution and concentrate only a fraction of the eluate in an Amicon 10 K MWKO membrane at 5 min intervals of $5,000 \times g$, monitoring its status. If it precipitates from the solution, take note of the point at which this occurred and be careful when concentrating the remaining fractions.

⁵Despite encompassing the majority of protein in this purification, the GTPase is not the only protein present in the eluate. Thus, the Bradford assay overestimates its concentration. A SDS-PAGE-assisted densitometric analysis, comparing the Coomassie blue-stained band from a particular volume of your concentrated sample with a known amount of BSA is an alternative way to determine the concentration of protein.

⁶Mix the concentrate extensively prior to aliquoting, to avoid a heterogeneous configuration of sample concentrations. Producing many small volume aliquots helps to prevent the need to reuse a sample, as repeated freeze–thaw cycles reduce the quality of these modified proteins. 500-µL eppendorf tubes, with indented lids, are labeled by sample and date and placed on ice. After the concentrated protein is aliquoted and snap frozen, it can be stored indefinitely at –80°C.

3.1.2. Purification of GST RhoGDI

1. Resuspend the bacterial pellet (see Subheading 2.2, item 2) in 25 mL of ice-cold TEDA buffer.
2. Sonicate with a Sonic Dismembrator set at level 5 for 5 min with 15 s bursts followed by 15 s off. Carry out sonication on ice.
3. Clarify the resuspended, sonicated pellet by centrifugation at $20,000 \times g$ for 30 min at 4°C.
4. Transfer the supernatant to a fresh tube with 3 mL of glutathione bead slurry pre-equilibrated with TEDA buffer.
5. Rock at 4°C for 30 min to allow binding of the GST-RhoGDI and then load on to a 10×1 cm bore fritted glass column and allow the liquid to drain from the column.
6. Wash with 20 column volumes of TEDA supplemented with 500 mM NaCl.
7. Elute the GST-RhoGDI with TBSM supplemented with 10 mM glutathione and analyze the fractions for purity by SDS-PAGE. Choose the most concentrated and purest fractions and concentrate these with an Amicon Ultra 10 K concentrator.

3.2. Preparation of Lipid Vesicles for Fluorescence Spectroscopy

The standard lipid composition as mole fractions for “control” vesicles is 0.35 phosphatidylethanolamine (PE), 0.25 phosphatidylserine (PS), 0.05 phosphatidylinositol, and 0.35 cholesterol (see Note 7). See Fig. 1 for tabulated information concerning lipid constituents used in the preparation of vesicles.

1. Just prior to the experiment, lightly vortex the lipid stock and transfer 100 μ L of the lipid/chloroform mixture of desired composition to a clean glass vial. Take care to immediately argon seal the lipid stock and return to -20°C . Direct a stream of argon gas to the 100 μ L of lipid mixture until the chloroform is evaporated leaving a translucent lipid film (see Note 8). Add 1 mL of TBSM to the dried lipid film and incubate for 1 h at room temperature in order to rehydrate the lipids.
2. After incubation and gentle flicking of the tube, the film should detach and be ready for extrusion (see Note 9). Pass the hydrated lipid solution through the 1 μ m pore polycarbonate membrane mounted on the mini-extruder 15 times (see Note 10), as an odd number of passes ensures that the liposomes are collected on the opposite side of the loading side of the filter.
3. Finally, transfer the liposomes to a clean 1.5-mL microcentrifuge tube. The final concentration of lipids is 1 mg/mL.

⁷This composition is aimed to mimic the inner leaflet of the plasma membrane, which is relevant for our purposes as the majority of Rho Family GTPases make important signaling contributions at this location.

⁸Take care not to perturb the lipids during the solvent-evaporation process. We typically prepare at least two identical liposome samples per experiment, as liposomes will occasionally aggregate inappropriately, making them less ideal for extrusion.

⁹This consists mostly of large multilamellar lipid bilayers. It should have a semi-translucent appearance, with no visible white particulates.

¹⁰Always perform an odd number of passes, as it selects against any larger particulates that cannot pass through the membrane. Those particulates could be a source of scattering in the subsequent fluorescence experiments.

3.3. FRET Measurements for Monitoring the Interaction of Rho GTPases with Lipid Membranes and RhoGDI

1. Add 100 μL of liposomes (see Subheading 3.2) to a 0.5-mL eppendorf tube and add 0.4 μL of 5 mM HAF with a P2 pipetman. Immediately vortex the mixture at a maximum speed for 5 s (see Note 11).
2. Briefly vortex the HAF/liposome or liposome control mixtures at maximum speed and transfer 20 μL to a new 0.5-mL eppendorf tube. If liposomes are not to be included this early, then add 20 μL of TBSM instead. Add at least a twofold greater molar concentration of Mant-nucleotides over the GTPase. For our samples, 1 μL of 50 μM Cdc42 is added along with 2.5 μL of 50 μM Mant-nucleotide (see Note 12). Add the appropriate amount of 500 mM EDTA to achieve a final concentration of 8.5 mM. Pipet mixture and set a timer for 5 min (see Note 13).
3. During or before nucleotide exchange, clean the fluorescent cuvette to be used, by rinsing thoroughly with distilled water, cleaning the stir bar as well. This is best done with a new pair of powder-free gloves, as particulates that wash off into the cuvette can lead to undesirable light scattering (see Note 14).
4. Fluorescence monitoring of the association between Cdc42:Mant-GDP and HAF-labeled vesicles using a fluorimeter in counting mode. Use a magnetic stirrer with a 1-mL quartz cuvette with 1,000 μL of TBSM at 25°C; the photomultiplier tube is set to 750 V. The excitation wavelength is set at 365 nm (5 nm slit width) and emission is monitored continuously at 440 nm (20 nm slit width) (Fig. 2) (see Note 15).
5. With 10 s left on the timer, add 1 M MgCl_2 to the GTPase–Mant-nucleotide–liposome mixture to achieve a final concentration of 15 mM. Immediately start the fluorescence run and add 80% of the total sample volume to the cuvette at a precise time point (see Note 16).
6. Many configurations of the FRET assay can provide information regarding the kinetics of protein exchange between membrane vesicles. To observe the rate of Mant-Cdc42 incorporation into HAF-labeled vesicles, do not include any liposome during the *in vitro* nucleotide exchange reaction. Stir 1 μM Mant-Cdc42 in 1,000 μL of TBSM and monitor the Mant emission at 440 nm. At a later time point, carefully add the desired volume of HAF-labeled vesicles. The decrease in 440 emission reflects the degree of Mant-GDP:Cdc42 quenching and therefore the association and insertion of Cdc42 into the liposome.

¹¹The liposome mixture should become yellow upon vortexing with HAF. This liposome preparation will be optimal for four trials. It is best not to let the HAF-liposome mixture sit for too long a period of time, as deviations from initial observations become evident over time. Preparing a fresh mixture of HAF and liposome every four trials allows for the most accurate and reproducible measurements.

¹²Prenylated GTPases are best examined directly upon thawing, as they begin to behave differently when allowed to sit on ice for extended periods. Prior to an experiment, we always thaw one of our 100- μL aliquots of insect cell-expressed Cdc42 on ice and divide it further into eighteen 5- μL aliquots (in 500- μL indented eppendorfs). These are snap-frozen and stored in the -80°C freezer. Once Cdc42 is needed, it can be directly added to the membranes without delay.

¹³This step is GTPase specific and depends on both the protein and lipid composition. Certain Rho GTPases, in our experience, have been found to slowly associate with membranes, requiring a longer incubation period in order to bind to liposomes.

¹⁴Cleaning the cuvette, after each experiment, involves rinsing with distilled water and drying the sides with Kimwipes. Avoid allowing tissue fibers from entering the cuvette, as they can cause erratic signals from light scattering.

¹⁵Prior to use, perform a background analysis on the cuvette, filled with 1 mL TBSM. Set it as the blank and make sure the noise level is minimal. This ensures the experimental data will be as clean as possible. Once the noise level is acceptable, leave the cuvette to await the addition of your samples.

¹⁶Depending on the particular assay, speed is often crucial at this point. Thus, it is useful to have all the micropipets, to be used, preset to their intended volumes.

3.3.1. Monitoring the Association of RhoGDI with Rho GTPases—To monitor the association of RhoGDI with MantGDP:Cdc42 bound to membranes and the subsequent translocation to the surrounding bulk medium, include 20 μ L of the HAF/liposome mixture during the in vitro nucleotide exchange reaction of Cdc42 with Mant-nucleotide. As before, add this mixture to 1,000 μ L of TBSM with stirring. After equilibration and signal stabilization (see Note 17), add a stoichiometric amount of RhoGDI. The subsequent increase in fluorescence provides a real-time read-out for the release of Cdc42 from the lipid surface as a complex of RhoGDI and Cdc42:Mant-GDP. A sample fluorescence emission trace following the above protocol is shown in Fig. 3.

3.3.2. Monitoring the Rate of Exchange of Rho GTPases Between Lipids—To monitor the rate of exchange of Cdc42 between lipid residues, prepare two populations of liposomes: one population that contains the HAF and a population of control liposomes that lack HAF. Pre-incubate the liposomes, devoid of HAF, with Cdc42:Mant-GDP as above and add to the cuvette with stirring. Upon addition of HAF-incorporated liposomes, the decrease in 440-nm fluorescence emission reflects the loss of Cdc42:Mant-GDP from one vesicle population to the other (see Fig. 4).

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¹⁷This is a common variation among different protein and liposome preparations. For reasons that are not completely clear, Mant fluorescence can sometimes decline, failing to equilibrate to a steady value within a reasonable time frame. Occasionally, this can be remedied by preparing fresh liposomes. Otherwise, interpretable measurements can still be made, provided the signal reduction is slow relative to the measurement of interest.

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Liposome Stock Preparation (10 mg/mL)

Components:

Avanti (<http://avantilipids.com/>)

- 840032C L- α -phosphatidylserine (PS) 25mg
- 840026C L- α -phosphatidylethanolamine (PE) 25mg
- 840042C L- α -phosphatidylinositol (PI) 10mg

Nu Chek Prep (<http://www.nu-chekprep.com/>)

- Ch-800 Cholesterol 1g (dissolved in ultrapure (>99.99%) chloroform)

Lipids (concentration in chloroform)	Molecular Weight (g/mol)	Final Molar Percentage	Mass needed for 1 mole of lipid mix ^a	Fraction of the total in the previous column
Phosphatidylethanolamine 10 mg/mL	768	40	307.2 g	0.47
Phosphatidylserine 10 mg/mL	812	20	162.4 g	0.25
Phosphatidylinositol 10 mg/mL	909	5	135.5 g	0.07
Cholesterol 10 mg/mL	387	35	45.5 g	0.21

^a Molecular Weight (g/mole) x molar percentage (1/100) x 1 mole

Determine Limiting Reagent *

Lipid	Amount available	Mass Fraction	Limiting Reagent Column 2 ÷ Column 3	Multiply fractional masses by the conversion factor (53)	Volume needed for a 10 mg/mL stock
PE	25 mg	0.47	53 *	25 mg	2.5 mL
PS	25 mg	0.25	100	13.25 mg	1.325 mL
PI	10 mg	0.07	143	3.71 mg	371 μ L
Chol.	∞	0.21	∞	11.13 mg	1.113 mL

Fig. 1.

A table of supplier information for components of the vesicle system described in this study.

Fig. 2.

FRET measurements for monitoring the interaction of Rho GTPases with lipid membranes.

(a) The basis of the experiment is shown in this illustration where the co-localization of donor and acceptor probes on the surface of liposomes results in the quenching of donor fluorescence. (b) Elements of the synthetic lipid vesicle reconstitution and FRET pairing used to monitor the association–dissociation kinetics of Cdc42 and HAF-labeled vesicles in the presence and absence of RhoGDI. The chemical structures of the fluorophores used here and their excitation and emission spectra are shown.

Fig. 3. Translocation experiment. **(a)** This schematic outlines the sequence of events in a typical translocation experiment. **(b)** Fluorescence emission trace showing the HAF-labeled vesicle quenching of Mant-GDP:Cdc42 upon HAF vesicle addition followed by the reversal of Mant emission with the addition of RhoGDI.

Fig. 4. Vesicle-vesicle exchange experiment. **(a)** Schematic for the vesicle-vesicle exchange observed in the absence of RhoGDI. **(b)** The kinetics of vesicle-vesicle exchange exhibits a well-behaved dose dependence on the amount of HAF vesicles added.