Chapter 7

Analysis of the Role of RhoGDI1 and Isoprenylation in the Degradation of RhoGTPases

Etienne Boulter and Rafael Garcia-Mata

Abstract

RhoGDI1 is one of the three major regulators of the Rho switch along with RhoGEFs and RhoGAPs. RhoGDI1 extracts prenylated Rho proteins from lipid membranes, sequesters them in the cytosol, and prevents nucleotide exchange or hydrolysis. In addition, RhoGDI1 protects prenylated Rho proteins from degradation. Here, we describe techniques to monitor Rho proteins degradation upon depletion of RhoGDI1 and their dependence upon prenylation for degradation.

Key words: RhoA, RhoGDI, Prenylation, YopT, Degradation

1. Introduction

Rho proteins act as molecular switches by cycling between an active (GTP bound) and an inactive (GDP bound) state. The activation of Rho proteins is mediated by specific guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. In their active state, GTPases interact with one of several downstream effectors to modulate their activity and localization. The signal is terminated by hydrolysis of GTP to GDP, a reaction that is stimulated by GTPase-activating proteins (GAPs) (1). An additional layer of regulation for the RhoGTPases is mediated by the Rho guanine nucleotide dissociation inhibitor (GDI) family of proteins. RhoGDIs were initially characterized based on their ability to inhibit the dissociation of the bound nucleotide (usually, GDP) from the RhoGTPases (2–4). It was subsequently shown that RhoGDI can also interact with the GTP-bound form of the RhoGTPases preventing both intrinsic and GAP-mediated GTP hydrolysis, as well as effector interaction (5–7). One of the main functions of RhoGDIs

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is to modulate the cycling of RhoGTPases between the cytosol and cellular membranes (8). RhoGDIs form a high-affinity interaction with RhoGTPases, promoting their extraction from membranes and sequestering them in an inactive state in the cytosol.

RhoGDIs' interaction with Rho proteins requires the GTPases to be prenylated. RhoGTPases contain a conserved CAAX motif at their carboxyl terminus, which is posttranslationally modified by isoprenylation at the cysteine residue (9). In the case of RhoA, Rac1, and Cdc42, a 20-carbon geranylgeranyl group is added. Upon release from RhoGDI, this isoprenyl group can be inserted into the lipid bilayer and anchors the Rho proteins to cellular membranes. Membrane association is essential for the function of RhoGTPases. When the Rho proteins are extracted from the membrane, the lipid moiety is transferred into a hydrophobic pocket in the RhoGDI molecule that prevents its exposure to the solvent (7). Structural studies showed that the interaction between RhoGTPases and RhoGDI requires the association of the N-terminal domain of RhoGDI with the switch region of the GTPase, which inhibits nucleotide release, and the insertion of the prenyl group of the GTPase into the hydrophobic C-terminal pocket of the GDI (10).

There are three RhoGDIs in the human genome: RhoGDI1 (GDIα), RhoGDI2 (Ly/D4GDI or GDIβ), and RhoGDI3 (GDIγ) (8). RhoGDI1 is ubiquitously expressed while GDI2 and 3 expression is restricted to certain tissues (hematopoietic for GDI2 and lung, brain, testis for GDI3) (11–14). RhoGDI1 has orthologues in *Saccharomyces cerevisiae* (RDI1), *Caenorhabditis elegans* (rhi-1), and *Drosophila melanogaster* (RhoGDI)(15).

Recently, our understanding of RhoGDI's function has progressed as we showed that it protects prenylated Rho proteins from misfolding and degradation (16). Prenylation is a major feature of Rho proteins which generates a biological dilemma: isoprenylation of Rho proteins is absolutely required for their proper subcellular localization and signaling (17), but simultaneously, this lipid moiety disturbs Rho protein folding in solution and triggers their degradation (16). Prenylation of RhoGTPases can be inhibited using general inhibitors of the cholesterol/isoprenoid biosynthetic pathway or specific inhibitors for the geranyl-geranyl-transferase enzyme (GGTaseI). 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase inhibitors, such as statins, inhibit the conversion of HMG-CoA to mevalonate in the biosynthesis pathway for both isoprenoids and cholesterol (18). Alternatively, the isoprenoid group can be cleaved using YopT, a cystein protease expressed by pathogenic species of *Yersinia*. YopT cleaves N-terminal to the prenylated cysteine in RhoA, Rac, and Cdc42. This cleavage results in the irreversible removal of the lipid modification from the GTPases and their subsequent membrane detachment (19). YopT cleaves GTP- and GDP-bound forms of RhoA equally, suggesting that the cleavage does not depend upon the conformation status of the GTPases (19).

Here, we describe techniques to monitor the degradation of Rho proteins upon depletion of RhoGDI1 and to characterize the role of prenylation in the RhoGDI-mediated degradation of Rho-GTPases.

2. Materials

Prepare all solutions using deionized water unless specified otherwise. Similarly, prepare and store all reagents at room temperature unless indicated otherwise.

2.1. SiRNA and RNA/DNA Ttransfection

1. We routinely order siRNAs as desalted dsRNA oligos from Sigma-Genosys. No further purification seems to be required for efficient silencing. SiRNAs are resuspended in ultrapure sterile water at a stock concentration of 20 μM.
2. We design siRNAs using the neural network technology-based designer BIOPREDsi at http://www.biopredsi.org (20) which is now part of Qiagen.
3. The sequence of the siRNA against human RhoGDI1 is 5′-UCAAUCUUGACGCCUUUCCTT-3′ and the mismatch containing control siRNA is 5′-UCACUCGUGCCGCAUUUCCTT-3′ (16).
4. 2× HBSP buffer: 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 50 mM Hepes, pH 7.05, 12 mM glucose. Filter on a 0.45-μm pore-size filter. Store at room temperature.
5. 2.5 M $CaCl_2$. Filter on a 0.45-μm pore-size filter.

2.2. Cell Culture

Growth medium is Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% fetal bovine serum and antibiotics: penicillin 100 U/mL and streptomycin 100 μg/mL (Invitrogen).

2.3. Lysis and Western Blotting

1. 10× PBS: Dissolve 80 g of NaCl, 2 g of KCl, 14.4 g of NaH_2PO_4 $2H_2O$, and 2 g of KH_2PO_4 in 900 mL of water. Complete to 1 L with water. Filter on 0.8-μm pore-size membrane. Store at room temperature.
2. Laemmli lysis buffer: 100 mM Tris–HCl, pH 6.8, 2.5% SDS, 10% glycerol. Beta-mercaptoethanol (5% final concentration) and bromophenol blue (0.0025% final concentration) are added after cell lysis.
3. Sonicator: Branson Sonifier S150D.
4. 2× resolving gel buffer: 750 mM Tris–HCl, pH 8.8, 0.2% SDS. Dissolve 90.8 g of Tris in 800 mL of water. Adjust pH to 8.8. Complete to 1 L with water. Add 2 g of SDS. Filter on a 0.8-μm pore-size membrane. Store at room temperature.

5. 2× stacking gel buffer: 250 mM Tris–HCl, pH 6.8, 0.2% SDS. Dissolve 30.3 g of Tris in 800 mL of water. Adjust pH to 6.8. Complete with water to 1 L. Add 2 g of SDS. Filter on a 0.8-µm pore-size membrane. Store at room temperature.
6. Stacking gel: 125 mM Tris–HCl, pH 6.8, 0.1% SDS, 4% acrylamide–bisacrylamide (29:1) (Biorad). Mix 50 mL of 2× stacking gel buffer, 20 mL of 30% acrylamide–bisacrylamide (29:1) solution, and 30 mL of water. Store at 4°C.
7. Ammonium persulfate (APS): 10% solution (w/v) in water.
8. *N*,*N*,*N*,*N*′-tetramethyl-ethyldiamine (TEMED). Store at 4°C.
9. 10× SDS-PAGE running buffer: 250 mM Tris–HCL, pH 8.5, 2.2 M glycine, 1% SDS. Dissolve 30.5 g of Tris and 164 g of glycine in 800 mL of water. Check that pH is between 8.3 and 8.8. Dissolve 10 g of SDS. Complete to 1 L with water. Store at room temperature.
10. 10× transfer buffer: 250 mM Tris, pH 8.5, 1.92 M glycine. Dissolve 30 g of Tris and 144 g of glycine in 800 mL of water. Complete to 1 L with water. Store at room temperature.
11. Polyvinylidene fluoride (PVDF) membrane.
12. Ponceau red solution: 0.5% Ponceau red, 1% acetic acid in water.
13. Blocking buffer: PBS supplemented with 5% milk powder.
14. Antibodies: Anti-RhoA monoclonal antibody 26C4 (Santa Cruz Biotechnology). Anti-RhoGDI1 polyclonal antibody A20 (Santa Cruz Biotechnology). Mouse monoclonal anti-HA antibody (clone 16B12) (Covance). Secondary horseradish peroxidase (HRP)-coupled anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch).
15. Immobilon Western chemiluminescent HRP substrate (Millipore).
16. Protein gel electrophoresis and transfer equipment: Mini-PROTEAN tetracell (Biorad).

2.4. Prenylation Inhibition

1. Lovastatin (Axxora): Resuspend in ethanol at a concentration of 2.5 mM.
2. HA-tagged YopT: pPTuner IRES2 HA-YopT was engineered in our laboratory. (16) (avaliable from the authors upon request).

3. Methods

3.1. Transfection and Lovastatin Treatment

1. Plate HeLa cells at a density of 50% for RNA/DNA transfection or 80% for pharmacological inhibitor treatment in a 100-mm culture dish with 5 mL of growth medium. Perform transfection the next morning.

2. Mix 12.5 µL of siRNA (20 µM stock) with 225 µL of sterile ultrapure water. Add 25 µL of 2.5 M $CaCl_2$ (see Note 1). Vortex and add 250 µL of 2× HBSP buffer starting from the bottom of the tube (see Note 2). Wait for 30–60 s and dispense the mix on the cells. This should give a final concentration of siRNA of around 50 nM.
3. Approximately 8 h after transfection, aspirate the medium, wash once with PBS, and add 5 mL of growth medium (see Note 3).
4. Perform transfection again on the next day according to the same protocol and then wait for 24–48 h before assessing RhoGDI1 silencing.
5. Alternatively, it is possible to cotransfect YopT cDNA by adding 2 µg of HA-YopT plasmid to the transfection mix either during the first or second transfection. Single transfection of YopT can also be achieved by mixing 2 µg of HA-YopT cDNA with 225 µL of sterile ultrapure water and 25 µL of 2.5 M $CaCl_2$. Like previously (step 2), add 250 µL of 2× HBSP buffer and add to the cells for 8 h.
6. Alternatively, cells can be treated with lovastatin, which inhibits HMG-CoA reductase resulting in impaired prenylation. Add 10 µL of 2.5 mM lovastatin to 10 mL of growth medium (final concentration of lovastatin is 2.5 µM) for 24–48 h maximum (see Note 4).

3.2. Cell Lysis

1. Discard growth medium and wash cells with ice-cold PBS (see Note 5).
2. For cell lysis, add 1 mL of Laemmli buffer to a 100-mm dish (see Note 6). Incubate Laemmli buffer on cells for 5 min at room temperature (see Note 7) and then harvest cell lysate.
3. Sonicate cell lysate for 5–10 s (see Note 8). Add β-mercaptoethanol to a final concentration of 5% and bromophenol blue to a final concentration of 0.0025%.

3.3. SDS-PAGE and Western Blotting

1. Cast a 15% mini-gel by mixing 5 mL of 2× resolving gel buffer with 5 mL of acrylamide–bisacrylamide solution. Add 100 µL of 10% APS solution and 10 µL of TEMED. Mix by inverting the tube. Dispense the gel immediately between the glass plates (see Note 9), leaving 1–1.5 cm of empty space on top of the gel and carefully layer it with 100–200 µL of water (see Note 10). The gel usually polymerizes within 5–10 min.
2. When the resolving gel is polymerized, remove the top layer of water. Add 30 µL of 10% APS and 3 µL of TEMED to 3 mL of stacking gel solution. Dispense on top of the resolving gel and insert the comb.
3. Remove the comb and rinse the wells with distilled water to remove any excess of nonpolymerized acrylamide. Assemble

the gel electrophoresis unit and add SDS-PAGE running buffer on top of the gel and in the wells.

4. Heat the samples at 95°C for 5 min. Load the samples (15–25 μlL/lane) according to your loading scheme and run the gel at 140 V for approximately 1 h 40 min (until the blue dye exits the gel).
5. Soak the PVDF membrane in ethanol 95% to rehydrate it. Equilibrate the PVDF membrane and the pieces of Whatman paper in 1× transfer buffer with 20% ethanol. Disassemble the gel electrophoresis unit and the glass plates to recover the gel. Assemble the transfer sandwich as follows: Starting from the cathode-facing side of the sandwich, place two pieces of Whatman paper, one piece of PVDF membrane, the gel, and two pieces of Whatman paper (see Note 11). Transfer the gel in 1× transfer buffer with 20% ethanol 95% at 100 mA for 2 h. Stain the membrane to check transfer efficiency with Ponceau red solution (optional).
6. Block the membrane in blocking buffer for 10 min and incubate overnight at 4°C with the appropriate antibodies. We use the 26C4 antibody at a dilution of 1/1,000, anti-GDI1 antibody at 1/10,000, and anti-HA antibody at 1/5,000 in blocking buffer.
7. Wash the membrane three times in PBS for approximately 10 min each. Incubate with the secondary antibody at the dilution (in blocking buffer) recommended by the manufacturer for 1 h (see Note 12).
8. Wash the membrane three times in PBS for approximately 10 min each, and develop using the Immobilon Western chemiluminescent HRP substrate following the manufacturer's instructions (see Note 13). Expose the membrane to an X-ray film.

A typical result from such an experiment is shown in Fig. 1. By western blot, the RhoA band is observed around 21 kDa while RhoGDI1 is slightly higher at around 28–30 kDa. HA-YopT can be seen much higher on the membrane. RhoGDI1 silencing triggers degradation of RhoA (Fig. 1a). This can be rescued either by expression of YopT which cleaves prenylated RhoA C-terminal tail (Fig. 1b) or by treating cells with lovastatin (Fig. 1c).

4. Notes

1. The $CaCl_2$ solution has a higher density than the water/DNA mix; it sinks in the water/DNA solution and requires thorough mixing.
2. The HBSP solution is less dense than the DNA/$CaCl_2$ mix; therefore, it is better to add it starting at the bottom of the

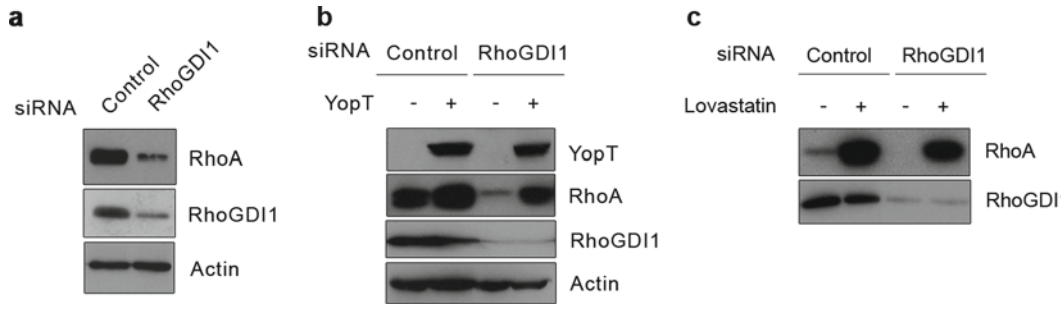

Fig. 1. (**a**) RhoGDI1 depletion triggers degradation of Rho proteins. Control or RhoGDI1 siRNA-transfected HeLa cells were analyzed by western blotting. Upon depletion of RhoGDI1, many Rho proteins including RhoA are degraded. (**b**) Prenylation removal rescues Rho protein degradation in the absence of RhoGDI1. HeLa cells were cotransfected with control or RhoGDI1 siRNA and a cDNA encoding the bacterial protease YopT. Cell lysates were resolved by SDS-PAGE and analyzed by western blotting (reproduced with permission from Nature Publishing Group). (**c**) Inhibition of RhoA prenylation prevents its degradation in the absence of RhoGDI1. HeLa cells were transfected with control or RhoGDI1 siRNA and treated with lovastatin 2.5 μM for 24 h. Cell lysates were resolved by SDS-PAGE and analyzed by western blotting. Reproduced with permission from Nature Publishing Group (16).

tube with a circular motion going up in the tube. Additional mixing can be achieved by generating bubbles from the bottom of the tube.

3. At this point, the medium may look like the cells are contaminated with bacteria. Indeed, the calcium phosphate method is based on the generation of a precipitate of calcium phosphate which traps DNA/RNA molecules. This precipitate looks like contamination although it is not. Usually, the remaining precipitate after washing is finally engulfed by cells during the following 10–12-h period.
4. It can be noticed that during treatment with lovastatin cells tend to round up after 24 h. Cells need to be carefully monitored since they finally detach after rounding up. A similar effect is observed upon expression of YopT cDNA.
5. Washing the cells with ice-cold PBS washes out proteins from the growth medium, such as bovine serum albumin, and cools down the cells to inhibit any signaling during lysis.
6. The volume of lysis buffer is to be modified according to the size of the culture vessel. We usually use 1 mL for 100-mm dish and 300 μL for each well of a 6-well plate.
7. We perform cell lysis with this high SDS concentration buffer at room temperature to avoid SDS precipitation. It is worth noting that cell lysis with such a high concentration of SDS results in an almost immediate cell lysis and denaturation of proteins blocking all enzymatic activities.
8. Lysis with SDS disrupts the nuclear envelope and releases genomic DNA which results in an extremely viscous lysate almost impossible to pipet. A simple way to reduce viscosity of

the lysate is to sonicate it for a few seconds at lowest settings in order to shear the genomic DNA.

9. Any gel thickness can be used, but the present protocol refers to 1.5-mm-thick gels.
10. During polymerization, polyacrylamide gels have a tendency to retract at the interface between the gel and surrounding air, leaving an irregular interface. In order to avoid this, it is common to create an artificial liquid interface. This can be done using water-saturated butanol (which tends to be avoided since it is toxic), a viscous solution of SDS, or just plain water. Water is the simplest way to get a straight interface but requires extra caution when dispensing over the gel.
11. Cut pieces of Whatman paper and PVDF membrane slightly larger than the gel itself. When placing the gel on the PVDF membrane, make sure to soak the gel with 1× transfer buffer to avoid tearing it as it may stick to the gloves when drying. Use a test tube or pipet as a rolling pin and roll over the membrane carefully in both directions to remove any air bubbles trapped between the gel and the membrane. Air bubbles block the current and impair protein transfer at the site of the bubble.
12. The recommended dilution for secondary antibodies to start off with is 1/10,000. Alternatively, if the background is too high, primary and secondary antibodies can be washed using TBS or PBS with 0.5% Tween 20. For these antibodies, a 1-h incubation at RT is usually enough to get a good signal.
13. The Immobilon HRP substrate can be used to amplify very weak signals; therefore, since blotting for RhoA or RhoGDI1 leads to very strong signals, we usually dilute the HRP substrate to 1:4 in water.

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