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Quantifying Sphingosine-1-Phosphate-Dependent Activation of the RhoGTPases

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Abstract

The GPCR-coupled sphingosine-1-phosphate (S1P) receptors regulate a number of important cell functions, including proliferation, migration, and adhesion. Since these processes require dynamic regulation of the actin cytoskeleton, the ability to monitor S1P-dependent activation of the Rho family GTPases is critical for our understanding of S1P signaling. Herein, we provide methods for the GST pull-down-based assay used to measure Rho, Rac, and Cdc42 activity in cultured cells treated with S1P.

Keywords

Sphingosine-1-phosphate; S1P; RhoGTPase; Rho; Rac; Cdc42

1. Introduction

The lipid agonist, sphingosine-1-phosphate (S1P), has been shown to play an important role in cardiovascular homeostasis, leukocyte function, and cancer progression by regulating a variety of important cell processes, including growth, differentiation, adhesion, and migration. Although it is clear that S1P signals through a family of G-protein-coupled receptors (S1P₁ through S1P₅), our understanding of S1P-dependent signaling is complicated by differential and dynamic expression of the S1P receptor subtypes and by differential coupling of the S1P receptor subtypes to various G-proteins (see refs. 1–3 for reviews). Thus, the overall effect of S1P on cell function is determined by the relative expression of S1P receptor subtypes and the distinct downstream signaling pathways to which those subtypes are coupled (3, 4). For example, endothelial cells express high levels of the G_{ai}-coupled receptor, S1P₁, and stimulation of these cells with S1P induces migration (5). In contrast, smooth muscle cells express high levels of the G_{α_{12/13}}-coupled receptor, S1P₂ (and low levels of S1P₁) and stimulation of these cells with S1P inhibits migration (6).

The effect of S1P on these actin-dependent processes is mediated, in large part, by the Rho family of small GTPases. Rho, Rac, and Cdc42 function as molecular switches and promote the formation of stress fibers, lamellipodia, and filopodia, respectively, by catalyzing specific types of actin polymerization (see refs. 7, 8 for reviews). The RhoGTPases are activated by specific guanine exchange factors (GEFs) that enhance GTP binding, and are inactivated by GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis. In the GTP-bound state, each RhoGTPase interacts with specific sets of effectors through conserved GTPase-binding domains. These effectors include proteins that directly catalyze actin nucleation as well as a

variety of kinases. The general signaling mechanisms by which GPCR-coupled receptors activate the RhoGTPases are beginning to be described (9), and S1P receptors 1–3 have been examined most thoroughly in this regard (10). S1P₁ activates Rac through a mechanism that requires G α _i-dependent activation of PI3-Kinase (10). In contrast, S1P₂ activates Rho through G α _{12/13} and RGS family of Rho-specific GEFs that interact with this G-protein (11). S1P₃ can activate Rac through G α _i, but also couples to G α _{q/11}, and activates Rho through the Trio family of RhoGEFs (12, 13).

The ability to measure S1P's effects on the Rho GTPases is a powerful tool for delineating the signaling mechanisms by which S1P regulates particular cell functions. Fairly standard methods for measuring RhoGTPase activity have been developed that take advantage of the binding of activated GTPases to their respective effectors (14, 15). Thus, GST fusion proteins containing specific RhoGTPase-binding domains (RBDs) can be used to pull "active" GTPases out of cell lysates. In this chapter, we provide detailed methods for measuring GTPase activity and hope that this information will prove useful to investigators studying S1P-dependent signaling.

2. Materials

2.1. GST Fusion Protein Expression Constructs

For measuring the activity of Rho, use a GST fusion construct containing the RBD (AA of 7–89) of the Rho effector, Rhotekin (see Note 1). For measuring the activity of Rac or Cdc42, use a GST fusion containing the first 290 amino acids of p21-activated kinase, PAK. These constructs are widely available and most are cloned into the IPTG-inducible pGEX series of bacterial protein expression vectors (GE Healthcare).

2.2. Solutions

Bacteria lysis buffer: 50 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM DTT, 1× protease inhibitor cocktail (Pierce) 1. All solutions should be stored at 4°C with protease inhibitors and DTT added just prior to use (see Note 2).

Bead wash buffer: Same as bacteria lysis buffer but without Triton X-100.

Cell lysis buffer: 50 mM Tris, pH 7.4, 500 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid (DOC), 1% Triton X-100, 10 mM MgCl₂, 1× protease inhibitor cocktail.

Pull-down wash buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, 0.5× protease inhibitor cocktail.

2.3. Reagents/Equipment for GST-Fusion Protein Preparation

LB media containing ampicillin (100 µg/ml), LB/agar/amp plates, a shaker capable of maintaining 37°C and 270 rpm, a refrigerated centrifuge that holds 50-mL conical tubes, and a sonicator. Other specific reagents required are the following.

BL21 competent bacteria (Promega): BL21 bacteria have relatively low protease activity making them better suited for protein expression.

Isopropyl β -D-1 thiogalactopyranoside (IPTG) (Sigma): Sterile stock solution at 100 mM.

Glutathione Sepharose 4B (GE Healthcare).

2.4. Reagents/Equipment for Cell Culture and S1P Treatment

Sterile hood, cell culture media, tissue culture plates, ice-cold PBS, cell scrapers, a refrigerated microcentrifuge, and a tube rotator. Other specific reagents required are the following.

S1P (Cayman Chemical): For a 10 mM solution, resuspend 1 mg of S1P in 264 μ L 0.3 M NaOH. Store 20- μ L aliquots at -80°C and avoid repeated freeze/thawing.

Precision Red Advanced Protein Assay (Cytoskeleton): This kit is a relatively fast method for determining protein concentration.

2.5. Reagents/Equipment for Gel Electrophoresis and Western Blotting

Electrophoresis cell, gel-casting system, acrylamide, running buffers, power source, and 0.22 μ m nitrocellulose. Other specific reagents required are the following.

Primary antibodies:

Rho—(Cell signaling) rabbit monoclonal (67B9) (see Note 3).

Rac—(Millipore) mouse monoclonal (23A8).

Cdc42—(BD Biosciences) mouse monoclonal (610929).

Secondary antibodies—HRP-conjugated (GE Healthcare).

ECL reagents (Thermo Scientific).

3. Methods

The RhoGTPases have intrinsic activity that can limit the efficacy of this assay (especially for Rho). Thus, minimize handling time whenever possible and maintain tubes and reagents at 4°C throughout the protocol.

3.1. GST-Fusion Protein Preparation

1. Transform the appropriate GST-RBD fusion plasmid into BL21-competent bacteria, spread onto LB/Agar /Amp plates, and grow overnight (O/N) at 37°C .
2. Transfer one colony to 10 mL of LB media containing 100 $\mu\text{g}/\text{mL}$ ampicillin. Shake O/N at 37°C .
3. For Rhotekin beads, dilute the overnight culture into 400 mL of fresh LB/ampicillin media. Shake at 37°C for approximately 3 h. Growth conditions may

vary, but an OD₆₀₀ from 0.2 to 0.3 is optimal (see Note 4 for details on preparation of PAK beads).

4. Induce fusion protein expression by adding IPTG to a final concentration of 0.3 mM. Shake at room temperature for approximately 18 h.
5. Aliquot the 400 mL of culture into 50-mL conical tubes and pellet bacteria at 2,700×g for 15 min. Decant supernatant completely. At this point, bacteria pellets can be stored at −80°C for future use.
6. Completely resuspend one pellet in 2 mL of bacteria lysis buffer using trituration and vortexing as necessary (see Note 5)
7. To ensure complete bacteria lysis, sonicate the sample on ice. Due to variability in sonication equipment, it is likely that this step will need to be optimized empirically using fusion protein yield and stability as end points (see Note 6).
8. Split the sonicated lysate evenly into two microfuge tubes and clear by centrifugation at 32,000×g for 10 min at 4°C. Transfer supernatants to clean tubes.
9. Prewash the Glutathione Sepharose beads once with bacteria lysis buffer. Resuspend beads in bacteria lysis buffer to give 500 µL of a 50% bead slurry. Make sure to use a wide-bore pipette tip for bead transfers.
10. Add 200 µL of bead slurry to each tube and rotate at 4°C for 2 h. Be sure to mix beads completely before pipetting.
11. Wash 3× in 1 mL of bead wash buffer combining beads into a single tube after the first wash (see Note 7).
12. After the last wash, add 250 µL of bead wash buffer and store at 4°C. Beads are best used when fresh but can be stored for up to a week (2 weeks for PAK beads) without a dramatic reduction in utility (see Note 8).
13. Analyze fusion protein yield and stability using gel electrophoresis. In short, add 2, 4, 8, and 12 µL of beads to sample buffer to elute fusion protein. After boiling, load samples on a 10% SDS-PAGE gel along with 0.5, 1, 2, and 4 µg of BSA standards. After staining the gel with coomassie blue for 10 min, destain the gel for 5 min in a large volume of boiling water (see Fig. 1).
14. The GST-Rhotekin RBD fusion protein runs at 34 kD, while the GST-PAK fusion protein runs at 60 kD. Determine the approximate fusion protein concentration by comparing to the BSA standards. A good preparation will yield a fusion protein concentration of approximately 2–5 µg/µL (see Note 9). Less robust preparations or preparations that show substantial breakdown can be used, but are typically less effective.

3.2. Cell Culture, Treatment, and Lysis

1. Grow cells in 100-mm culture dishes to approximately 75–80% confluence (see Note 10).

2. Serum-starve cells: It is important to note that tolerance to serum-free conditions varies widely by cell type and that the precise conditions that allow for detection of GTPase activation without significant changes in cell viability need to be determined empirically (see Note 11).
3. Treat cells with a fresh 10 mM stock of S1P to obtain final concentrations of 1–10 μ M.
4. At specific times following treatment (see Note 12), place plates on ice and rinse 2 $\times$ with ice-cold PBS. Remove as much PBS as possible after the final wash.
5. Scrape cells in cell lysis buffer using a volume that will produce a final protein concentration of approximately 2 μ g/ μ L (about 250 μ L for a 100-mm dish; about 500 μ L for a 150-mm dish).
6. After dispersing cells by trituration, collect lysates in chilled microfuge tubes and immediately centrifuge at 32,000 $\times g$ for 5 min at 4°C. Transfer cleared supernatant to a new microfuge tube.
7. (Optional) Determine protein concentration using the Precision Red Advanced Protein Assay per protocol. As stated previously, it is important to minimize handling time. Thus, unless significant differences in cell number/protein concentration are possible (e.g., when comparing S1P signaling in Wt and knockdown cell lines), skipping protein determinations often leads to more consistent results.

3.3. Pull-Downs

1. Before incubating with fusion protein, save 5–10% of cleared lysate (by volume or protein concentration) to use as a control for total GTPase protein levels. Add an equal volume of 2 $\times$ sample buffer, boil for 3 min, and save on ice for Western Blotting.
2. Prewash the appropriate amount of GST fusion protein beads (30–50 μ g of protein per sample) in cell lysis buffer and resuspend beads in cell lysis buffer as a 50% slurry.
3. Combine 30–50 μ g of fusion protein with equal amounts of cell lysate and rotate at 4°C for 30 min. We use at least 500 μ g of lysate protein in our pull-downs, and usually 1 mg when measuring RhoA activity.
4. During the 30-min incubation, prepare and chill the pull-down wash buffer.
5. Pellet beads by tap-down centrifugation and wash 3 $\times$ with 1 ml of pull-down wash buffer. Be careful not to disturb pellets (see Note 7).
6. On the final wash, remove as much supernatant as possible. Resuspend pellets in 25 μ L of 2 $\times$ sample buffer, boil for 3 min to elute protein, and save on ice for Western Blotting.

3.4. GTPase Detection by Western Blotting (Fig. 2)

1. Separate pull-down and loading control samples on a 15% SDS-PAGE gel. The GTPases run at approximately 20 kD, so be careful not to overrun the gel.
2. Transfer protein to 0.22 μ m nitrocellulose.
3. Subject membrane to standard Western Blotting procedures using the appropriate Abs and reagents.

4. Notes

1. RBDs from other Rho effectors have been used effectively in this assay.
2. Buffers can be made from the appropriate stocks. A 1 M DTT stock solution can be stored in aliquots at -20°C . After thawing, make sure that DTT is completely dissolved before addition. Triton X-100 is extremely viscous. Cutting approximately 0.5 in. off a P-1000 pipette tip can help when pipetting.
3. Antibodies specific for RhoB (Santa Cruz; sc-180) or RhoC (Santa Cruz; sc-26480) are available and could be used to specifically measure the activation of these Rho family members.
4. For preparing PAK beads, dilute the 10 ml culture (1:20) in 200 ml of fresh LB/ampicillin media and shake at 37°C for 2 h (reaching an $\text{OD}_{600} \sim 0.6$). Add IPTG to a final concentration of 0.3 mM. Shake at 37°C for an additional 3 h. Continue with step 5 of Subheading 3.1.
5. Depending upon yield, one 50-ml bacteria pellet should provide enough fusion protein for approximately 15–20 lysate samples. However, the protocol can be expanded as needed as long as reagent ratios are maintained.
6. Using a Sonics Vibracell Sonicator with a 6-mm tip set at an amplitude of 40, we sonicate in 1-s pulses for 1 min. This procedure is then repeated three more times with 1-min breaks between sonications. Be sure that the sonicator tip is completely submerged at all times to avoid frothing of the sample.
7. When aspirating supernatants, we use a gel-loading tip connected to a vacuum flask. This allows finer control of aspiration and less disturbance of bead pellets.
8. We recommend that fusion protein beads be stored in a location that is not frequently accessed to avoid temperature fluctuations.
9. If GST fusion protein concentrations are low, one or more of the following should be tried: start with newly transformed colonies, make fresh IPTG, increase IPTG induction time, use a slightly higher bacteria density, use a slightly higher temperature during induction, and increase sonication. If significant fusion protein breakdown is apparent, try shorter induction times, lower induction temperatures, decreased sonication, or better temperature control during fusion protein preparation.

10. Baseline and S1P-stimulated GTPase activity can vary dramatically between cell types, so adjustments in plate size may need to be optimized to obtain consistent GTPase activity measurements. Also, because RhoA activity is usually more difficult to detect, we recommend starting with 150-mm culture dishes. Finally, variations in confluence have significant effects on RhoGTPase activity and should be avoided.
11. As a general rule, starve cells for as long as possible (up to 24 h) in the lowest possible concentration of serum. If serum is required, bioactive lipids can be removed by activated charcoal treatment.
12. GTPase activity is dynamic. It is good to begin with 5- and 15-min time points and to expand this time course if necessary.

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Fig. 1.

Analysis of a GST Rhotekin RBD fusion protein preparation. The indicated amounts of BSA (*left*) and GST RBD fusion protein beads (*right*) were separated on a 10% SDS-PAGE gel. Following staining with coomassie blue, fusion protein concentration was estimated to be approximately 2 μg/μL. Note the modest level of fusion protein breakdown products below the 36-kD band.

Fig. 2. S1P dependent activation of Rho in smooth muscle cells. Primary rat aortic smooth muscle cells were grown to 75% confluence, serum starved for 24 h, and treated with 10 μ M S1P (or vehicle) for 10 min. Cell lysates (1 mg) were incubated with 30 μ g of GST RBD fusion protein. Precipitated protein and 10% loading controls were run on a 15% SDS-PAGE gel, transferred to nitrocellulose, and subjected to Western Blotting using an antibody to Rho.