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Measuring Rab GTPase-Activating Protein (GAP) Activity in Live Cells and Extracts

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Abstract

Mammalian cells encode a diverse set of Rab GTPases and their corresponding regulators. In vitro biochemical screening has proven invaluable in assigning particular Rabs as substrates for their cognate GTPase-activating proteins. However, in vitro activity does not always reflect substrate specificity in cells. This method describes a functional test of GAP activity in cells or extracts that takes into account the presence of other factors or conditions that might change observed in vitro specificity.

Keywords

GTPase-activating protein; Rab GTPase; Effector protein

1 Introduction

Rab GTPases are small Ras-like GTPases that function as master regulators of membrane trafficking [1]. In cells, they act as molecular switches, changing conformations between an active GTP-bound state and an inactive GDP-bound state. In their active form, Rab proteins bind so-called effector molecules that function in all steps of membrane trafficking. Against this background of the nucleotide cycle, there is also a membrane association cycle with almost all Rab-GDP found in the cytosol complexed with a protein named GDI, and Rab-GTP located exclusively on distinct membrane surfaces in organized units called microdomains [2]. Effector binding and Rab localization are thus dependent on the ability of Rab proteins to exchange and hydrolyze guanine nucleotides.

As the intrinsic rates of nucleotide exchange and hydrolysis are quite slow, cells have evolved opposing enzymatic activities to regulate the identity of the bound nucleotide. Guanine nucleotide exchange factors (GEFs) remodel the nucleotide-binding site of a Rab, which accelerates the off-rate of bound GDP [3]. GTPase-activating proteins (GAPs) accelerate GTP hydrolysis by a “dual-finger” mechanism whereby a catalytic arginine and glutamine are supplied in *trans* by the GAP to properly orient a water molecule for nucleophilic attack on the gamma phosphate group of GTP [4]. Both sets of enzymatic activities seem to be encoded in conserved domains: many RabGEF proteins include a DENN domain that is sufficient for GEF activity [5], while all but one known RabGAP protein possess a Tre2/Bub2/Cdc16 (TBC) domain sufficient to stimulate GTP hydrolysis in vitro [4].

A great deal of recent work has been undertaken to assign the various Rab and GAP proteins into linked pathways called Rab cascades [6]. In this model, Rabs help to recruit GEFs and GAPs for the Rab proteins that function before and after themselves in a particular trafficking pathway like secretion [6] or endocytosis [7]. This model predicts that GEFs and GAPs are likely to be Rab effectors themselves, and the coordinated recruitment of these regulators to membranes ensures directionality to a trafficking pathway by ordering adjacent Rab-organized microdomains according to their stepwise functions.

The human genome encodes approximately 70 Rab proteins and approximately 40 TBC domain-containing proteins. Assigning Rab/GAP substrate pairs can be undertaken through high-throughput biochemical screening [4]. However, activity seen in vitro is not always recapitulated in living cells [8, 9]. In this chapter, we detail methods used to determine the specificity of RabGAP proteins in cells by using a purified, immobilized effector “Rab-binding domain” as an affinity column to report on the amount of active Rab GTPase present in cells under different conditions. Cells are transfected with wild-type RabGAP proteins or GAP-deficient point mutants and the amount of active, GTP-bound Rab present is determined by incubating lysates with the Rab effector column. GAP overexpression in cells should lead to a decrease in the amount of Rab protein retained by the column when compared to a negative control or in the presence of the GAP-deficient mutant. Alternatively, recombinant GAP protein can be added directly to semi-intact cells to overcome low expression of the GAP or a high abundance of effectors. These methods have been applied to other Rab/GAP systems [10] where effectors have been identified.

2 Materials

2.1 Mammalian Expression Plasmids

1. GFP-Rab33: human Rab33b coding sequence was cloned into pEGFP-C1 yielding an N-terminal GFP-tagged protein.
2. Myc-Rab32: human Rab32 coding sequence was cloned into a modified pCDNA3.1(+) vector containing a single N-terminal Myc epitope tag.
3. 3xMyc-RUTBC1: human RUTBC1 (isoform 2) was cloned into a modified pCDNA3.1(+) vector containing an N-terminal triple Myc epitope tag. 3xMyc-RUTBC1 R803A was generated by site-directed mutagenesis.
4. 3xMyc-RUTBC2: human RUTBC2 (isoform 4) was cloned into a modified pCDNA3.1(+) vector containing an N-terminal triple Myc epitope tag. 3xMyc-RUTBC2 R829A was generated by site-directed mutagenesis.

2.2 Bacterial Expression Plasmids

1. Rab-Binding Domain (RBD) expression plasmids:
 - (a) GST-Atg16L1: human Atg16L1 coding sequence (isoform 1, aa. 80–265) was ligated into pGEX 4T-1 resulting in a GST-tagged fusion protein.
 - (b) GST-Varp: human Varp coding sequence (aa. 451–730) was also ligated into pGEX 4T-1 resulting in a GST-tagged fusion protein.

2. RUTBC1-C: human RUTBC1 coding sequence (aa. 533–1,066) containing the TBC domain was ligated into pET28a resulting in a 6x-histidine-tagged protein (6xHis-RUTBC1-C). 6xHis-RUTBC1-C R803A was generated by site-directed mutagenesis.

2.3 Protein Purification

All protein expression plasmids were transformed into Rosetta2 (DE3) cells for purification.

1. Luria broth (LB): 10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl.
2. Ampicillin or carbenicillin (2,000×): 100 mg/mL in water; stored at –20 °C.
3. Kanamycin (1,000×): 50 mg/mL in water; stored at –20 °C.
4. Chloramphenicol (1,000×): 34 mg/mL in 100 % ethanol; stored at –20 °C.
5. 1 M isopropyl beta-D-thiogalactopyranoside (IPTG); stored at –20 °C.
6. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.03 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4.
7. PMSF: 100 mM in 100 % ethanol; stored at –20 °C.
8. Protease inhibitors (100×): 100 µg/mL each aprotinin, leupeptin, and pepstatin A; stored at –20 °C.
9. RBD Lysis Buffer: 25 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 1 mM DTT.
10. RBD Elution Buffer: 25 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 1 mM DTT, 20 mM reduced glutathione; stored at 4 °C.
11. GAP Lysis Buffer: 25 mM HEPES–NaOH, pH 7.4, 300 mM NaCl, 50 mM imidazole.
12. GAP Elution Buffer: 25 mM HEPES–NaOH, pH 7.4, 300 mM NaCl, 250 mM imidazole.
13. Glutathione-agarose (RBDs) and Ni-NTA agarose (GAPs).

2.4 GAP Assays in Cells and Extracts

1. HEK293T cells (ATCC CRL-3216).
2. Cell transfection reagents.
3. Cell Lysis Buffer: 50 mM HEPES–NaOH, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 1 % Triton X-100.
4. Binding Buffer: 50 mM HEPES–NaOH, pH. 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.2 % Triton X-100.
5. Swelling Buffer: 10 mM HEPES–NaOH, pH 7.4, 15 mM NaCl.
6. Scraping Buffer: 10 mM triethanolamine, 10 mM acetic acid, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM ATP, 15 mM creatine phosphate, 21 U/mL creatine

phosphokinase, 200 μ M PMSF, 2 μ g/mL pepstatin A, 1 $\times$ Roche Complete EDTA-free protease inhibitor cocktail.

7. 10 $\times$ Reaction Buffer: 500 mM HEPES–NaOH, pH 7.4, 1.5 M NaCl, 20 mM MgCl_2 .
8. Antibodies for immunoblotting: mouse anti-Myc (9E10) culture supernatant, rabbit anti-GFP.

3 Methods

3.1 Expression and Purification of GST-RBDs

1. A 50 mL overnight culture of each GST-RBD (LB supplemented with 50 μ g/mL carbenicillin and 34 μ g/mL chloramphenicol) is used to inoculate a 1 L culture of LB/antibiotics.
2. The culture is grown at 37 °C until it reaches an OD_{600} of ~ 0.6 . The cultures are then transferred to 22 °C and induced by bringing them to a final concentration of 0.1 mM IPTG. Cultures are incubated at 22 °C for an additional 4 h and collected by centrifugation (4,000 $\times g$, 10 min, 4 °C). The pellet is resuspended in PBS and then repelleted. The pellets can be snap frozen in liquid nitrogen and stored at -20 °C for later processing, if desired. All buffers used below should be chilled to 4 °C.
3. The washed pellet is then resuspended in 25 mL RBD Lysis Buffer supplemented with 1 mM PMSF and 1 $\times$ protease inhibitors. The cells are broken by passing twice through an EmulsiFlex-C5 apparatus at 20,000 psi (Avestin) (*see Note 1*).
4. The homogenate is diluted to 50 mL with RBD Lysis Buffer and transferred to chilled centrifuge tubes. The diluted homogenate is spun at 30,000 $\times g$ for 30 min at 4 °C.
5. Transfer the clarified supernatant to fresh 50 mL conical tubes and incubate them with glutathione-agarose beads (equilibrated in RBD Lysis Buffer) for 2 h at 4 °C with rotation (*see Note 2*).
6. Pour supernatant/bead solution into an empty column and wash the beads with 50 column volumes of RBD Lysis Buffer (*see Note 3*).
7. GST-RBDs are eluted by incubating the beads with 2 column volumes of RBD Elution Buffer for 10 min at 4 °C. This step is repeated another four times. Eluates are analyzed by SDS-PAGE and fractions containing GST-RBDs are then pooled and dialyzed overnight against RBD Lysis Buffer. The pool is brought to 10 % (v/v) glycerol and yield is determined by Bradford assay (*see Note 4*). The pool is then aliquoted, snap frozen in liquid nitrogen, and stored at -80 °C.

3.2 Expression and Purification of 6x-His-RUTBC1-C and 6xHis-RUTBC1-C R803A

1. A 100 mL overnight culture of either the wild-type or mutant construct (LB supplemented with 50 μ g/mL kanamycin and 34 μ g/mL chloramphenicol) is used to inoculate 3 $\times$ 2 L cultures of LB/antibiotics.

2. The culture is grown at 37 °C until it reaches an OD₆₀₀ of ~0.5. Transfer the cultures to 22 °C and induce expression by bringing them to a final concentration of 0.4 mM IPTG. Cultures are incubated at 22 °C for an additional 4 h and collected by centrifugation at 4 °C (4,000 × g, 10 min, Beckman SX4750A rotor). The pellets are resuspended in PBS and then repelleted as earlier. At this point the pellets can be snap frozen in liquid nitrogen and stored at –20 °C. All buffers used below should be chilled to 4 °C.
3. The washed pellet is resuspended in 100 mL GAP Lysis Buffer supplemented with 1 mM PMSF. The cells are broken by passing twice through an EmulsiFlex-C5 apparatus at 20,000 psi.
4. The homogenate is diluted to 200 mL with GAP Lysis Buffer and transferred to chilled centrifuge tubes and the homogenate is spun at 30,000 × g for 45 min at 4 °C.
5. Transfer the clarified supernatants to fresh 50 mL conical tube and incubate them with 3.0 mL Ni-NTA agarose (equilibrated in GAP Lysis Buffer) for 1 h at 4 °C with end-over-end rotation.
6. Pour supernatant/bead solution into a column and wash the beads with 2 × 25 column volumes (2 × 75mL) of cold GAP Lysis Buffer.
7. Elute the column with 5 × 1 column volumes (3.0 mL) cold GAP Elution Buffer. Eluates are analyzed by SDS-PAGE and fractions containing 6xHis-RUTBC1-C are then pooled and dialyzed overnight against GAP Storage Buffer to remove imidazole. The pool is brought to 10 % (v/v) glycerol and yield is determined by Bradford assay. The pool is then aliquoted, snap frozen in liquid nitrogen, and stored at –80 °C.

3.3 GAP Assay in Cells

1. Seed 100 mm dishes of HEK293T (*see Note 5*) cells at approximately 40 % confluency.
2. On the next day, cotransfect the cells with GFP-Rab33b or Myc-Rab32 and either 3xMyc-RUTBC1 or 3xMyc-RUTBC1 R803A (*see Note 6*).
3. At approximately 22 h posttransfection, immobilize GST-RBDs by incubating them with glutathione-Sepharose for at least 2 h at 4 °C with end-over-end rotation. At the end of the incubation, pellet the glutathione-Sepharose (1,000 rpm, 1 min, 4 °C, microcentrifuge) and remove the supernatant. Add Binding Buffer to obtain a 50 % slurry and keep the immobilized RBDs on ice.
4. After 1 h of the above RBD incubation, transfer dishes to a chilled steel plate on ice. Wash the cells twice with 5 mL PBS by aspiration and drain the dishes of excess PBS by holding the dish nearly vertical for 10 s.
5. Add 0.5 mL of Cell Lysis Buffer supplemented with protease inhibitor cocktail and incubate on ice for 10 min. Scrape the cells using a rubber policeman and transfer lysates to fresh 1.5 mL tubes.

6. Clarify the lysates by spinning at $16,000 \times g$ in a microcentrifuge for 15 min at 4 °C. Clarified supernatants were then diluted at least five-fold with Binding Buffer to decrease detergent concentration and ensure all supernatants had equal protein concentrations.
7. Aliquot the diluted supernatants to fresh tubes and add 20 μ L of 50 % RBD bead slurry (10 μ L bed volume) and incubate for 2 h at 4 °C with end-over-end rotation.
8. Pellet the beads as above and remove the supernatant completely (*see Note 7*). Wash the beads (in batch) four times with 1.0 mL cold Binding Buffer and elute the bound material in 30 μ L 2 $\times$ SDS-PAGE sample buffer.
9. Analyze the amount of GFP- or 3xMyc-tagged Rab bound in the presence or absence of wild-type RUTBC1 and RUTBC1 R803A by immunoblot (Fig. 1) (*see Note 8*).

3.4 Biochemical GAP Assay in Cell Extracts

1. HEK293T cells are transfected and GST-RBDs are immobilized as above.
2. 24 h post-transfection, the cells are transferred to a chilled steel plate on ice and are washed once with PBS and once with Swelling Buffer and then incubated in Swelling Buffer for 5 min (*see Note 9*). Swelling can be confirmed by observing the cells using an inverted light microscope.
3. Aspirate the Swelling Buffer from cells and drain dishes vertically for 10 s.
4. Add 500 μ L of 1 $\times$ SEAT buffer supplemented with ATP-regenerating system and protease inhibitors to each 100 mm dish. Scrape the cells with a trimmed rubber policeman, pipette the cell suspension three times with a 1 mL micropipette, and transfer suspension to a chilled 1.5 mL microcentrifuge tube.
5. Pass the cells through a 25G needle ten times using a 1 mL syringe. Confirm cell breakage by microscopy. Aliquot the cell suspension into three equal volumes of 150 μ L.
6. Assemble the GAP reactions as follows: 150 μ L lysate, 20 μ L 10 $\times$ Reaction Buffer, the volume required for the desired final concentration of purified GAP or equivalent volume of 1 $\times$ Reaction Buffer and ddH₂O up to 200 μ L. Incubate the reactions at 37 °C for 5 min.
7. Stop the reactions by transferring them to ice and fully solubilize cells by adding 10 μ L 20 % Triton X-100. Pipette to mix and incubate on ice for 10 min. Spin the lysates for 15 min at $16,000 \times g$ in a microcentrifuge at 4 °C.
8. Dilute the clarified supernatants five-fold with Binding Buffer and store on ice until GST-RBD immobilization is complete.
9. Clarify the lysates by spinning at $16,000 \times g$ in a microcentrifuge for 15 min at 4 °C. Clarified supernatants are then diluted at least five-fold with Binding Buffer to decrease detergent concentration and ensure all supernatants had equal protein concentrations.

10. Aliquot the diluted supernatants to fresh tubes and add 20 μ L of 50 % RBD bead slurry (10 μ L bed volume, ~10 μ g GST-RBD) and incubate for 2 h at 4 °C with end-over-end rotation.
11. Pellet the beads as above and remove the supernatant completely. Wash the beads (in batch) four times with 1.0 mL cold Binding Buffer and elute the bound material in 30 μ L 2 $\times$ SDS-PAGE sample buffer.
12. Analyze the amount of GFP- or 3xMyc-tagged Rab bound in the presence or absence of wild-type RUTBC1 and RUTBC1 R803A by immunoblot (Fig. 2).

4 Notes

1. Alternatively, the cells can be broken by use of a French pressure cell or the lysozyme/sonication method. We have found the EmulsiFlex-C5 to yield comparable breakage to the French press with good temperature control and without an inherent volume limitation.
2. We routinely use glutathione-Sepharose 4 FF (GE Healthcare). Due to differences in protein expression, GST-Atg16L1 required 3.0 mL bed volume per liter of culture while GST-Varp required 1.0 mL bed volume.
3. The beads can also be washed in batch using centrifugation (1,000 $\times$ g, 5 min, 4 °C).
4. If desired, the protein pool can be concentrated using Amicon spin concentrators with the appropriate molecular weight cut-off. As the GST-RBDs are later immobilized on a resin, it is likely not necessary for those constructs.
5. Any cell line that is easily transfectable could be used—we had good experience with HEK293T cells but one could also use HeLa, Vero, or COS-7.
6. Our lab routinely uses polyethyleneimine (PEI) to transfect HEK293T cells as it yields high transfection efficiency at low cost. It should be prepared in 1 mg/mL stock concentration (pH 7.4) and stored at –20 °C. Thawed aliquots can be stored at 4 °C for up to 2 weeks. For a 100 mm dish, 6 μ g total DNA is mixed in 970 μ L of opti-MEM and then 30 μ L PEI stock solution is added to the DNA/medium mixture and incubated at room temperature for 15 min. The transfection mix was added drop-wise to the cells. We have also routinely tested other reagents including cationic lipid-based reagents and found them suitable.
7. The use of a Hamilton syringe (point style 3, 22S needle) is very helpful in removing virtually all supernatant above the bead bed in a microcentrifuge tube.
8. As a reference, so-called dominant negative mutants of Rab GTPases (GDP-preferring mutants, G1 motif S/T to N) can be used as a negative control to give an idea of background binding in the experiment. Additionally, so-called constitutively active mutants (G3 motif Q to L/A) could be used as a positive control. However, be aware that these mutants do not function identically for all Rabs [11] and that TBC domain proteins have been shown to be able to inactivate constitutively active Rabs in vitro [4, 8].

9. Various cell types will respond differently to the hypo-osmotic swelling. COS-7 cells, for example, stay attached to the tissue culture dish for longer time periods (10–15 min) while HEK293T cells start to become unattached from the dish with incubations longer than 5 min.

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Fig. 1.

RUTBC1 can act as a Rab32 GAP in cells. (a) Lysates of HEK293T cells transfected with Myc-Rab32 alone or Myc-Rab32 with either 3xMyc-RUTBC1 wildtype or R803A for 24 h were incubated with GST-Varp Rab binding domain. Shown are 2 % input (below) and 100 % of the affinity column eluate (above). (b) Lysates of HEK293T cells transfected with GFP-Rab33B alone or GFP-Rab33B with either 3xMyc-RUTBC1 wild type or R803A for 24 h were incubated with GST-Atg16L1 Rab-binding domain. Shown are 2 % input (below) and 100 % of the affinity column eluate (above). Rab32 and RUTBC1 were detected with anti-Myc antibody; Rab33B was detected with anti-GFP antibody. GST-Rab-binding domains were detected by Ponceau S staining. This research was originally published in *Journal of Biological Chemistry*. Nottingham, R.M. et al. RUTBC1 protein, a Rab9A effector that activates GTP hydrolysis by Rab32 and Rab33B proteins. *J. Biol. Chem.* 2010. 286, 33213–33222. © the American Society for Biochemistry and Molecular Biology

Fig. 2.

RUTBC1 GAP activity in crude extracts. **(a)** HEK293T cell extracts from cells transfected with Myc-Rab32 were incubated with purified His-RUTBC1-C and then incubated with GST-Varp Rab-binding domain. Shown are 4 % input (below) and 100 % affinity column eluate (above). **(b)** HEK293T extracts from cells transfected with GFP-Rab33B were incubated with purified His-RUTBC1-C and then incubated with GST-Atg16L1 Rab binding domain. Shown are 4 % input (below) and 100 % affinity column eluate (above). Rab32 was detected with anti-Myc antibody; Rab33B was detected anti-GFP antibody. GST-tagged Rab-binding domains and His-RUTBC1-C were detected by Ponceau S staining. This research was originally published in Journal of Biological Chemistry. Nottingham, R.M et al. RUTBC1 protein, a Rab9A effector that activates GTP hydrolysis by Rab32 and Rab33B proteins. *J. Biol. Chem.* 2010. 286, 33213–33222. © the American Society for Biochemistry and Molecular Biology