

[31] Analysis of Activated GAPs and GEFs in Cell Lysates

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Abstract

An assay was developed that allows the precipitation of the active pools of Rho-GEFs, Rho-GAPs, or effectors from cell or tissue lysates. This assay can be used to identify GEFs, GAPs, and effectors involved in specific cellular pathways to determine their GTPase specificity and to monitor the temporal activation of GEFs and GAPs in response to upstream signals.

Introduction

Rho GTPases control many aspects of cellular behavior, including the organization of the cytoskeleton, cell migration, cell adhesion, cell cycle, and gene expression (Burrige and Wennerberg, 2004; Hall, 1998; Van Aelst and D'Souza-Schorey, 1997). Like all GTPases, Rho proteins act as molecular switches by cycling between an active (GTP bound) and an inactive (GDP bound) state. Exchange of GTP for GDP allows the GTPase to interact with downstream effectors to modulate their activity and localization. Cycling between the GDP-bound and GTP-bound state is regulated primarily by two distinct families of proteins: guanine-nucleotide exchange factors (GEFs) activate Rho proteins by catalyzing the exchange of GDP for GTP, the GTPase activating proteins or GAPs negatively regulate GTPase function by stimulating GTP hydrolysis.

Since the development of the Rho pull-down assay in 1999 (Ren *et al.*, 1999), a lot of progress has been made in determining the identity of the Rho-GTPases that are activated in response to certain stimuli or signals. However, much less has been done in terms of identifying the molecules involved in the activation and inactivation of each particular Rho-GTPase. The issue becomes more complicated considering that the human genome contains more than 60 RhoGEFs and approximately 80 RhoGAPs (Moon and Zheng, 2003; Peck *et al.*, 2002; Rossman *et al.*, 2005; Schmidt and Hall, 2002).

We have developed an affinity precipitation assay that allows us to specifically pull down RhoGEFs, RhoGAPs, or effectors that are being

activated in the cell at any given time or condition. The assay takes advantage of constitutively active and dominant negative Rho-family mutants that bind either to Rho-GAPs and effectors or to RhoGEFs, respectively. Constitutively active Rho mutants were originally designed based on their analogous Ras mutants (G12V and Q61L) (Garrett *et al.*, 1989; Ridley and Hall, 1992; Ridley *et al.*, 1992). These mutants have lost both their intrinsic capacity and their GAP-mediated ability to hydrolyze GTP, and they bind with high affinity to GAPs and effectors (Barbacid, 1987; Trahey and McCormick, 1987). Traditional dominant negative mutants like S17N-Ras and the analogous Rho mutants have been shown to bind GDP with similar affinity to the corresponding wild-type form. However, their affinity for GTP is extremely low, so virtually all of the protein is found in the GDP-bound form (Feig, 1999; Ridley *et al.*, 1992). Another dominant negative Ras mutant (RasG15A) has been previously shown to bind very poorly to both GDP and GTP, existing virtually in a nucleotide free state (Chen *et al.*, 1994). A nucleotide-free GTPase is one of the intermediates of the nucleotide exchange reaction and is able to form a high affinity binary complex with the GEF (Cherfils and Chardin, 1999). This intermediate is rapidly dissociated by GTP and does not accumulate in cells. We took advantage of the properties of these mutants and used them to pull down Rho effectors and GAPs from tissue and cell lysates (Arthur *et al.*, 2002; Noren *et al.*, 2003). We generated mutant versions of various representative GTPases of the Rho subfamily that harbor mutations equivalent to the Q61L and G15A mutations in Ras. We then used GST fusion proteins of these mutants to specifically pull down Rho family GEFs, GAPs, or effectors from cell or tissue lysates. This assay can be used to determine Rho protein specificity on GEFs, GAPs, or effectors (Arthur *et al.*, 2002; Ellerbroek *et al.*, 2004; Noren *et al.*, 2003; Wennerberg *et al.*, 2003). It can also be used as a simple way to find interactors such as GEFs, GAPs, and effectors to GTPases where little is known about upstream and downstream regulation. In addition, many GEFs and GAPs seem to be activated by making the binding site to their target GTPase available. This can be achieved either by unmasking an intramolecular inhibitory domain (Vav1, Dbl, Asef), by association with or dissociation from other proteins (p115-RhoGEF, Sos, Dock180), or by release of the protein from a sequestering cellular compartment (GEF-H1/Lfc, Net1, Ect2) (Schmidt and Hall, 2002; Rossman *et al.*, 2005). Given this type of regulation, it is likely that the activated and nucleotide-free mutants of Rho proteins will have highest affinity toward “activated” GAPs and GEFs, respectively, and that our assay can, indeed, be used to detect activation of GEFs and GAPs by specific stimuli.

Activation and inactivation of GEFs and GAPs can be studied either by analyzing the precipitation of known candidate components by

immunoblot or by determining the identity of the bound proteins by mass spectrometry. In addition, GEF and GAP pull-downs can be an extremely useful tool to follow the pattern of GAP or GEF activation over time or in response to different upstream signals.

Materials

DNA Constructs

Human cDNA for RhoA, Rac1, and Cdc42 were subcloned into pGEX 4T-1 (Amersham) between the *EcoRI* and *XhoI* sites. Empty-nucleotide mutants, G17A (RhoA), G15A (Rac1 and Cdc42), and constitutively active mutants, Q63L (RhoA), and Q61L (Rac1 and Cdc42) were generated by site-directed mutagenesis using the Quick Change Site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions.

Antibodies

Antibodies against p190RhoGAP, ROCK, Sos1, and GFP were from BD Biosciences; Lsc, LARG, and RhoGDI were from Santa Cruz Biotechnology; and anti-myc was from Invitrogen.

Solutions

Lysis Buffer: 20 mM HEPES, pH 7.5; 150 mM NaCl; 5 mM MgCl₂; 1% Triton X-100; 1 mM DTT with protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). DTT and protease inhibitors should be added fresh before use. In addition to these, other inhibitors can be added. If pervanadate is added, DTT should be omitted, because DTT will reduce it and inactivate it.

HBS: 20 mM HEPES, pH 7.5; 150 mM NaCl.

Methods

Expression and Purification of GST-Rho Constructs

1. Transform and grow up *Escherichia coli* with the appropriate pGEX construct. For RhoA, Rac1, and Cdc42 proteins, DH5α or any regular strain will work. For some less-soluble Rho proteins (RhoB, RhoC, RhoG), it is good to use a codon optimized strain such as codon plus BL21 (Stratagene).

2. Grow up a 50-ml culture overnight to full density in LB with 50 μg/ml ampicillin (LB-Amp)(O.D. > 1.0).

3. Dilute the culture into 450 ml of LB-Amp and let grow for 30 min at 37°.
4. Induce the protein by adding IPTG to a final concentration of 100 μ M and shake the culture at room temperature over night (~16 h).
5. Spin down bacteria and resuspend in 10 ml of lysis buffer. All purification steps are carried out at 4°.
6. Sonicate the resuspended bacteria for 1 min.
7. Spin at 15,000–20,000g for 15 min.
8. Transfer supernatant to a new tube and add 100–500 μ l of glutathione-sepharose (Amersham) pre-equilibrated in lysis buffer (amount depends on the solubility of the protein). Rotate tube at 4° for 45–60 min (RhoA ~200 μ l of 50% Glutathione sepharose slurry, Rac1, and Cdc42: 500 μ l).
9. Spin down sepharose and wash it with 2 \times 10 ml lysis buffer and 2 \times 10 ml with HBS with 5 mM MgCl₂ and 1 mM DTT.
10. Aspirate sufficient wash buffer to get initial sepharose volume (i.e., get approximately 50% slurry). Add 0.5 volumes of glycerol.
11. Estimate protein concentration (see following) directly on beads using Coomassie Plus protein reagent (Pierce).
12. (Optional) If possible, dilute the beads to a final concentration of 1 mg/ml with glutathione-sepharose in 2/3 HBS w/5 mM MgCl₂, 1 mM DTT, and 1/3 glycerol. (It is advantageous to have the different fusion proteins at the same concentration so you add equal amounts of sepharose to each sample in the pull-down.)
13. Store the beads at –20°. Typically, the activated mutants are stable for at least a month, whereas the nucleotide-free mutants are stable 1–2 weeks. For longer storage, the beads can be aliquoted, snap frozen and stored at –70°.

Estimation of GST-Rho Protein Concentration on Sepharose Beads

The amount of purified protein can be estimated without the need of eluting the proteins from the beads. We use the Coomassie Plus reagent (Pierce) following the manufacturer's directions and use a small aliquot of the purified GST-beads (2.5 μ l is usually enough) to measure protein concentration.

Pull-Down of GEFs or GAPs

1. Treat the cells in the desired way. How many cells you will need for each sample will have to be optimized for each different situation, depending on cell type, level of expression of protein of interest, its affinity

for the Rho protein tested, and also if it is going to be used for immunodetection or mass-spectrometry analysis. The quality and detection level of the antibody used to detect the protein is also relevant. A good starting point is a 100-mm dish for Western blot experiments and $1-2 \times 150$ -mm dishes for silver stain/mass-spectrometry analysis.

2. Immediately after treatment, wash the cells twice with ice-cold HBS.
3. Lyse the cells in $400 \mu\text{l}$ lysis buffer/100-mm dish.
4. Scrape off the cells and spin down debris at $16,000g$ for 1 min.
5. Measure protein concentration using BioRad DC Protein Assay. It is important to equalize both the total amount of protein and also the volume used for each condition.

6. Pre-clear the lysate by rotating it with $50 \mu\text{l}$ of GST bound to glutathione-sepharose (1 mg/ml) for 10 min at 4° . (This step is optional and can be left out if you are looking for conformational states of binding proteins that might be short-lived.)

7. Spin down the GST beads and transfer the cleared lysate to a new tube. Save $10-20 \mu\text{l}$ of lysate for a loading (mix this with an equal volume of $2 \times$ SDS-PAGE sample buffer).

8. Add $10 \mu\text{g}$ of fusion protein on beads (GST or GST-Rho protein) to each lysate. Rotate for $45-60$ min at 4° .

9. Wash the beads three times with lysis buffer, aspirate off all buffer, and dissolve protein complexes by adding $20-40 \mu\text{l}$ of $1 \times$ SDS-PAGE sample buffer and boil. For mass spectrometry analysis, it is recommended to wash more extensively (5-6 times).

10. Run the lysate and the pull-down samples on appropriate SDS-PAGE gels, transfer, and blot for the protein of interest.

11. For the identification of novel proteins, stain the gels with Coomassie blue or silver stain and submit the samples for mass spectrometry analysis.

Results

GAP and GEF Pull-Downs

Wild-type RhoA, nucleotide-free RhoA (G17A-RhoA), and constitutively active Q63L-RhoA were expressed as GST fusion proteins, and affinity precipitations were performed on lysates of CHO cells to assess the ability of these GST fusion proteins to bind to known regulators of Rho proteins. We immunoblotted the precipitated proteins with antibodies against a RhoA-GAP (p190RhoGAP), a RhoA effector ROCK (ROK or Rho kinase), a RhoA-GEF (Lsc/p115 RhoGEF), and RhoGDI. Our results revealed that constitutively active Q63L-RhoA selectively bound both the

RhoA-specific GAP p190RhoGAP and the RhoA effector ROCK but not the RhoGEF Lsc or RhoGDI. In contrast, wtRhoA bound and RhoGDI and G17-Rho bound to Lsc but neither was unable to precipitate p190-RhoGAP and the effector ROCK (Fig. 1A).

As mentioned in the "Introduction," the GEF pull-down assay can also be used to characterize the specificity of a Rho-GEF. We tested the ability of three RhoGEFs of known specificity to bind the empty nucleotide mutants of RhoA, Rac1, and Cdc42. We transiently expressed XPLN (RhoA), an activated mutant of Tiam1 (Rac1), and the DH-PH tandem domains of Intersectin (Cdc42) in NIH 3T3 cells. We found that XPLN coprecipitated only with G17A-RhoA but not with G15A-Rac1 or G15A-Cdc42, Tiam1 coprecipitated only with G15A-Rac1 but not with G17A-RhoA or G15A-Cdc42, and Intersectin coprecipitated only with G15A-Cdc42 but not with G17A-RhoA or G15A-Rac1 (Fig. 1B).

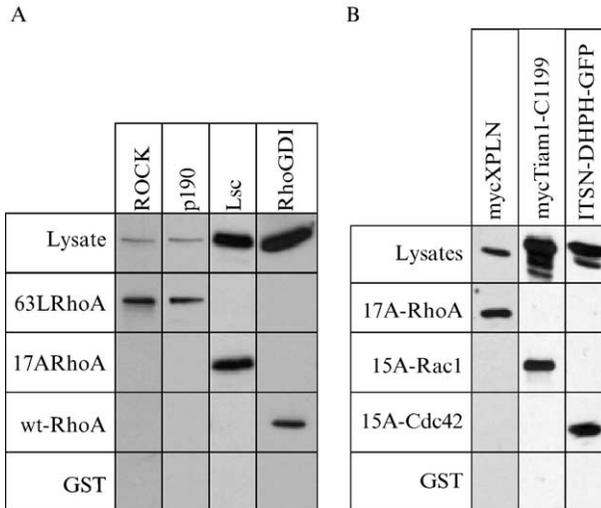


FIG. 1. (A) Selective precipitation of Rho-GEFs, GAPs, and effectors from cell lysates. CHO lysates were subjected to pull-down analysis with GST alone, GST-wt-RhoA, GST-G17A-RhoA, and GST-Q63L-RhoA as described in "Methods." The precipitated material was run on an SDS-PAGE gel and immunoblotted for ROCK, p190RhoGAP, Lsc, and RhoGDI. (B) Analysis of Rho-GEF specificity. NIH 3T3 cells were transiently transfected with mycXPLN, mycTiam1-C1199, or ITSN-DHPPH-GFP. After 24 h, cells were lysed, and the lysates were subjected to pull-down analysis with GST alone, GST-G17A-RhoA, and GST-G15A-Rac1 or GST-G15A-Cdc42 as described in "Methods." The precipitated material was run on an SDS-PAGE gel and immunoblotted for the transfected proteins with either anti-myc or anti-GFP antibodies.

These results are consistent with previous studies that demonstrated that XPLN, Intersectin, and Tiam1 are specific for RhoA, Cdc42, and Rac1, respectively (Arthur *et al.*, 2002; Hussain *et al.*, 2001; Michiels *et al.*, 1995).

To determine whether the pull-downs were specific enough and whether they could be used to identify true interactors of Rho GTPases, NIH 3T3 lysates were subjected to pull-downs with GST, GST-wtRhoA, GST-G15A-RhoA, and GST-Q63L-RhoA (10 μ g fusion protein/pull-down), and bands specifically precipitated with the different variants of RhoA were identified by mass spectrometry (Fig. 2). Of eight proteins identified, three well-known effectors (ROCK2, PKN1, and PKN2) and three well-known RhoA GEFs (Lfc, Lsc, and SmgGDS) were found. Two of the eight identified proteins did not obviously fall in to any Rho-regulatory class of proteins: tubulin in the G17A-RhoA pull-down and myosin heavy chain in the Q63L-RhoA pull-down. It is possible that tubulin coprecipitated with the tubulin-binding RhoGEF, Lfc/GEF-H1 and that myosin heavy chain coprecipitated with one of the Rho effectors. It should be noted that GEFs precipitated not only with the G17A mutant but also to a lesser degree with the Q63L mutant. Because GEFs are able to exchange both GDP and GTP *in vitro* (Cherfils and Chardin, 1999), it is possible that at the concentration used in the assay, some Rho-GEFs can bind and coprecipitate not only with the GDP-bound Rho but also with GTP-bound Rho. *In vivo*, this does not occur, because the concentration of GTP and the interaction of the GTP-bound form with the effectors drive the reaction in the GDP > GTP direction. Overall, these results indicate that the method is suitable for the identification of novel GEFs, GAPs, and effectors of less well-studied GTPases, as well as identification of changes in activity of GEFs or GAPs for well-studied GTPases.

One exciting application for this kind of pull-down assay is to follow the activation of a particular GEF or GAP in response to an upstream signal. We hypothesized that RhoGEFs and RhoGAPs will only be able to bind their target GTPases when they are in an active state and that this pull-down method, therefore, can distinguish between active and inactive GEFs. To test this, we treated CHO cells with PDGF that activates Rac1 and used GST-15A-Rac to pull-down binding partners (i.e., GEFs that had been activated by the PDGF treatment) from the lysate. One GEF that has been implicated in the activation of Rac downstream of PDGF is Sos1 (Nimmual *et al.*, 1998). We blotted for Sos1 in the pull-downs and observed an increase in the amount of Sos1 that was precipitated with G15A-Rac in response to PDGF (Fig. 3). In contrast, Tiam1, another Rac-specific GEF that was present in the lysates, did not bind to G15A-Rac in the presence or absence of PDGF. We have previously shown (Arthur *et al.*, 2002 and Fig. 1B) that a constitutively active mutant of Tiam1 can associate with

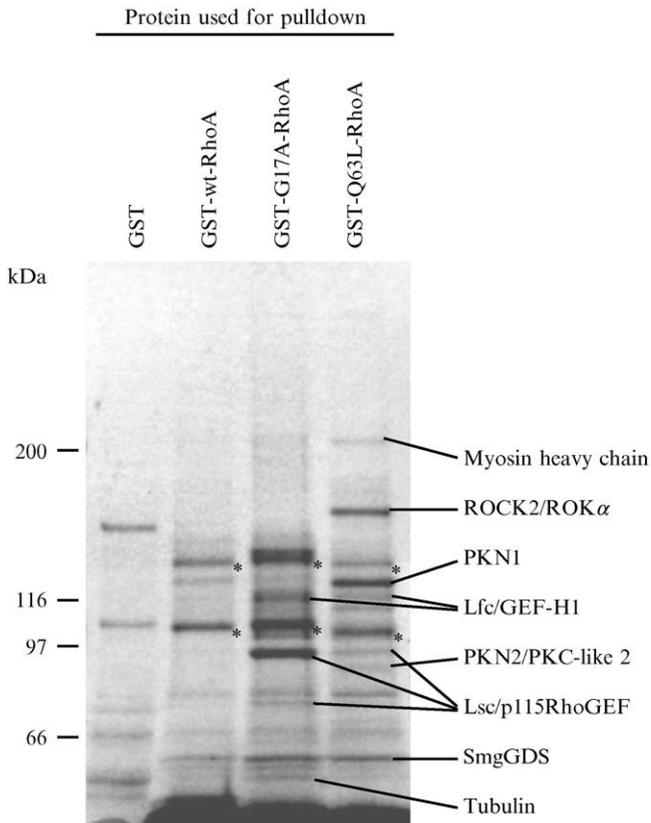


FIG. 2. Precipitation of RhoA-interacting proteins from NIH 3T3 cell lysates. NIH 3T3 lysates were subjected to pull-down analysis with GST alone, GST-wt-RhoA, GST-G17A-RhoA, and GST-Q63L-RhoA as described in “Methods.” The precipitated material was run on an SDS-PAGE gel and stained with Coomassie blue, and bands that were specifically precipitated with the RhoA variants were identified by mass spectrometry. Eight proteins were identified and are marked on the left side of the gel. The GST-RhoA fusion protein can be seen at the bottom of the gel image, as well as in higher migrating bands marked with asterisks (*). These are likely dimers and trimers of the fusion protein.

G15A-Rac, suggesting that in this case the Tiam1 present in the PDGF-treated cells is inactive and thus unable to bind the GTPase. Supporting these results, we have previously shown that only the active cellular pool of p190-RhoGAP is able to bind to Q63L-RhoA (Noren *et al.*, 2003).

In another experiment, we wanted to examine the RhoA-regulatory signals in nontransformed immortalized human embryonic kidney cells and their Ras-transformed counterparts (Hahn *et al.*, 1999). Several studies

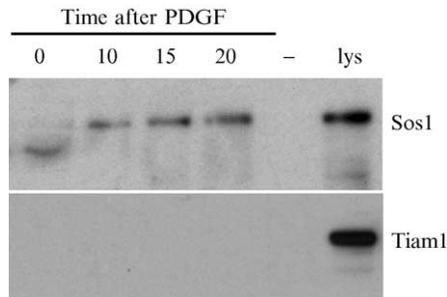


FIG. 3. Sos1 activation in PDGF-treated cells. After PDGF stimulation, CHO cells were lysed, and the lysates incubated with GST-G15A-Rac1 to pull down Rac1-specific GEFs. Samples were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

have suggested a role for RhoA regulation in Ras transformation of cells (Chen *et al.*, 2003; Khosravi-Far *et al.*, 1995; Zhong *et al.*, 1997). Interestingly, we did not detect any significant difference in overall RhoA-GTP levels between the two cell types (data not shown). However, in G17A-RhoA pull-downs, we noticed increased pull-down of a band of about 200 kDa from the Ras-transformed cells (Fig. 4A). This protein was identified as LARG, a RhoA-specific GEF. When lysates were blotted for the presence of LARG, we could detect an upregulation of LARG protein levels in the Ras-transformed cells (Fig. 4B). We also noticed a gel shift of the main isoform of LARG, suggesting that either the Ras-transformed cells mainly express a different splice variant of LARG than the nontransformed cells or that the LARG protein in the Ras-transformed cells is posttranslationally modified. Because p190RhoGAP has been suggested to play a role in Ras-mediated regulation of RhoA (Chen *et al.*, 2003), we did pull-downs with Q63L-RhoA to assess its activity in these cells (Noren *et al.*, 2003). We detected increased pull-down of p190RhoGAP without the protein being overexpressed (Fig. 4C), indicating that p190RhoGAP is activated in the Ras-transformed cells. Together, these results suggest that, at least in the immortalized HEK system, Ras transformation leads to both RhoA-activating and inactivating signals without drastically changing the overall levels of RhoA-GTP. This change in regulation could result in increased cycling between the GTP and GDP-bound states of RhoA in the Ras-transformed cells because of increased GEF and GAP activities. Alternatively, this could result in differential subcellular regulation of RhoA in the Ras-transformed cells where RhoA-GTP levels are high in cellular compartments where LARG is located and low in compartments

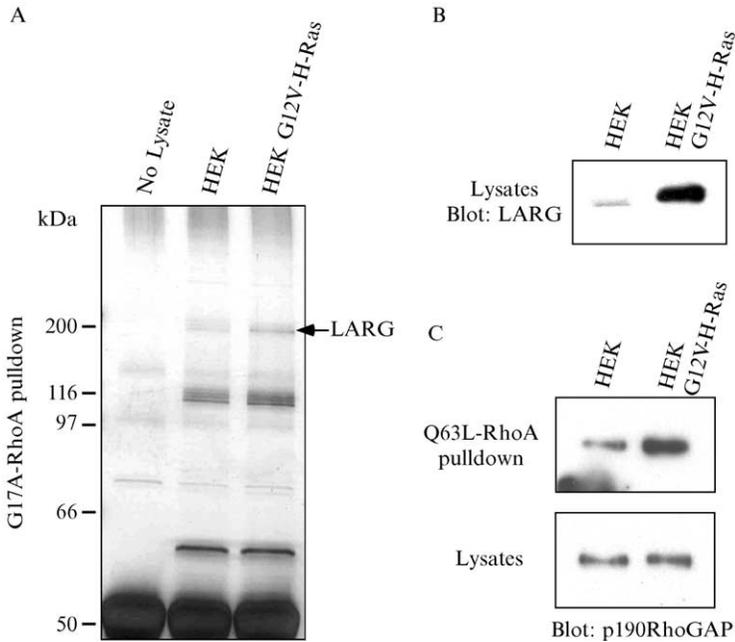


FIG. 4. Differential Rho regulatory activities in Ras-transformed cells. Immortalized human embryonic kidney cells (HEK) and Ras-transformed HEKs (HEK G12V-H-Ras) were tested for activities of RhoGEFs and RhoGAPs by pull downs as described in “[Methods](#).” (A) Precipitation of G17A-RhoA–interacting proteins from HEK and HEK G12V-H-Ras cell lysates. The precipitated material was run on an SDS-PAGE gel and Coomassie-stained. As a control, fusion protein alone was run in one lane (No Lysate). A band at approximately 200 kDa was pulled down in a larger amount from the HEK G12V-H-Ras HEK lysates (arrow). This band was identified as LARG by mass spectrometry. (B) Immunoblotting for LARG protein in HEK and HEK G12V-H-Ras lysates reveals that LARG is overexpressed in HEK G12V-H-Ras cells. (C) A pull down with GST-Q63L-RhoA indicates increased p190RhoGAP activity in HEK G12V-H-Ras cells while expression is unchanged.

where p190RhoGAP is located. Future studies will aim at determine whether this change in RhoA regulation contributes to cell transformation.

Troubleshooting

Stability of GST-Rho Proteins

Some of the GST-Rho mutants, in particular the empty nucleotide constructs, are not as stable as their wild-type counterparts when expressed in bacteria. Protein stability decreases even more when proteins are eluted

from beads. One way to deal with this problem is to prepare the GST proteins fresh when needed and to keep them at -20° with glycerol (see “Methods”) for no longer than 1 week.

Bacterial Contaminants

The method described here to purify the GST fusion proteins allows fast purification. However, depending on the expression level and the stability of each GST fusion protein, a significant amount of bacterial contaminant proteins can be nonspecifically purified with the GST. This is usually not a problem when precipitated proteins are analyzed by Western blot. However, it can be a major inconvenience if the goal of the experiment is to identify novel proteins by mass spectrometry analysis. A control lane should always be run with the GST fusion alone to distinguish contaminating bands from specific bands pulled down from the lysate. Alternatively, and depending on the stability of the protein, additional purification steps can be performed after elution of the GST fusion protein from glutathione-beads. It is not recommended to elute the free nucleotide forms from the beads, because they tend to degrade fast after elution, but it can be tried for the constitutively active mutants.

Even if contaminants are kept to a minimum, the range of molecular weights of GST-Rho proteins can still be a problem for mass spectrometry analysis. One way to overcome this problem is to cross-link the GST fusion proteins to the glutathione beads. There are very few GEFs and GAPs in the molecular weight range of GST-Rho proteins; however, some effectors are small and would be masked by the presence of the GST fusion protein.

Acknowledgments

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[32] Degradation of RhoA by Smurf1 Ubiquitin Ligase

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Abstract

The Rho family of small GTPases plays a key role in the dynamic regulation of the actin cytoskeleton that underlies various important cellular functions such as shape changes, migration, and polarity. We found that Smurf1, a HECT domain E3 ubiquitin ligase, could specifically target RhoA but not Cdc42 or Rac1 for degradation. Smurf1 interacts with the dominant inactive form of RhoA, RhoA N19, which binds constitutively to guanine nucleotide exchange factors (GEFs) *in vivo*. Smurf1 also interacts directly with either nucleotide-free or GDP-bound RhoA *in vitro*; however, loading with GTP γ S inhibits the interaction. RhoA is ubiquitinated by wild-type Smurf1 but not the catalytic mutant of Smurf1 (C699A) *in vivo* and *in vitro*, indicating that RhoA is a direct substrate of Smurf1. In this chapter, we summarize the systems and methods used in the analyses of Smurf1-regulated RhoA ubiquitination and degradation.

Introduction

The Rho family of small GTPases is a subset of the Ras superfamily and is an important regulator of the cytoskeletal dynamics that control cell shape, motility, and polarity (Bar-Sagi and Hall, 2000; Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002; Hall and Nobes, 2000; Van Aelst and Symons, 2002). The activity of Rho family members is regulated by their nucleotide-bound state, cycling between an active GTP-bound form and an inactive GDP-bound form. This cycling is tightly controlled by