GTPase Pull-down and Detection Kits

The Ras superfamily of small GTPases serve as molecular switches to control diverse eukaryotic cellular behaviors, including cell growth, differentiation and mobility. Consequently, small GTPases are involved in several disease states such as cancer and metabolic disorders. GTPases are active when bound to guanosine triphosphate (GTP) and inactive when the triphosphate is hydrolyzed to guanosine diphosphate (GDP). The Thermo Scientific Pierce Active GTPase Pull-down and Detection Kits enable GTPase activation studies by preferentially enriching their active form. These kits contain a GST-protein binding-domain (PBD or RBD) fusion that is selective for active Rho, Ras, Rac1, Cdc42, Rap1, Arf1 or Arf6 (Table 1).

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<th>GTPase</th>
<th>Downstream effector binding domain</th>
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<td>Filopodia, lamellipodia formation, and stress fibers</td>
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<td>Ras</td>
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<td>Rac1</td>
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This pull-down method is based on the affinity of known downstream effector proteins for the active forms of specific GTPases. The respective protein-binding domain (PBD) of these downstream effectors is expressed as a GST-fusion protein (Table 1). When immobilized on glutathione agarose resin, the PBD will bind active, GTP-bound GTPase from a cell lysate (Figure 1). The pulled-down active GTPase is detected via Western blotting. As a control, cell lysates can be treated with GTPγS, which is a non-hydrolyzable analog of GTP. This method traps all GTPases in the active form and results in high GTPase enrichment. As a negative control, cell lysates are treated with an excess of GDP to shift the majority of GTPases to the inactive state.

Highlights:
- Validated – functionally tested to ensure quality and performance
- Sensitive – optimized antibodies, reagents and Western blotting procedure accurately detect changes in GTPase activity levels
- Convenient – kits contain controls and all reagents needed to perform and detect 30 pull-downs
- Easy to use – conditions are optimized for immediate success in a two-hour assay
- Efficient – spin columns prevent sample loss

Figure 1. Thermo Scientific Pierce Active GTPase Pull-down and Detection Kit protocol summary.

Measure activation of small GTPases via their specific downstream effectors

Pull-down kits enrich active GTPases from cell or tissue lysates

Suzanne M. Smith, M.S.; Kay K. Opperman, Ph.D.; Rizwan Farooqui, Ph.D.; and Barbara J. Kaboord, Ph.D.; Thermo Fisher Scientific, Rockford, IL, USA

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Suzanne M. Smith, M.S.; Kay K. Opperman, Ph.D.; Rizwan Farooqui, Ph.D.; and Barbara J. Kaboord, Ph.D.; Thermo Fisher Scientific, Rockford, IL, USA
Figure 2. Specific detection of active Rho, Ras, Rac1, Cdc42, Rap1, Arf1 and Arf6 by Western blotting. NIH 3T3 cell lysate treated with GTPγS or GDP was incubated with the appropriate GST binding domain and immobilized glutathione resin. Eluted samples and a portion of the lysate were analyzed by Western blot using GTPase-specific antibodies.

To determine the specificity and function of the GTPase pull-down and detection kits, NIH 3T3 cell lysate was incubated with either GTPγS or GDP to activate or inactivate endogenous GTPases, respectively. The specific GST-PBD or -RBD was used to pull down active Rho, Ras, Rac1, Cdc42, Rap1, Arf1 or Arf6. A strong signal is detected in the GTPγS-treated lysate; however, minimal or no signal is detected in the GDP-treated lysate (Figure 2). These results illustrate the specificity of the PBD for active GTPases.

The pull-down of endogenous active small GTPases after growth factor or serum stimulations was highly effective in a variety of cell types derived from different species (Figure 3). Changes in the GTPase activities can be detected in time-course studies and differ with cell type and treatment. Because total GTPase levels in each lysate are constant, the amount of GTPase pulled down in each assay reflects activation rather than changes in GTPase expression levels. The activity profiles detected are similar to those reported in the literature. These results demonstrate the effectiveness of the GTPase pull-down and detection kits for monitoring sensitive changes in activity using time-dependent activity assays.

Figure 3. Specific, induced changes in the level of endogenously activated GTPases from a variety of cell types are easily monitored by the pull-down assay. In each panel, the top Western blot shows the level of active GTPase isolated by pull-down assay; the lower Western blot shows the total amount of expressed GTPase in the lysate. Densitometry was performed on the Western blots and plotted graphically for each system. Panel A: Rho activity in HeLa (human) cells stimulated with EGF. Panel B: Ras activity in NIH 3T3 (murine) cells stimulated with PDGF. Panel C: Rac1 activity in NS1 (rodent) cells stimulated with NGF. Panel D: Arf1 activity in MDCK (canine) cells stimulated with HGF. Panel E: Arf6 activity in C2C12 (murine) cells stimulated with serum.
Methods

Cell culture and treatments

HeLa cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to ~70% confluency and then starved in 1% FBS medium for 24 hours before stimulation with 100μL/mL of epidermal growth factor (EGF) for the indicated times. NIH 3T3 cells were grown in DMEM supplemented with 10% FBS to ~70% confluency and starved in 0.1% FBS medium for 24 hours. Platelet-derived growth factor (PDGF) was added at 50ng/mL for the indicated times. NS1 cells were grown in RPMI supplemented with 10% FBS to ~70% confluency and nerve growth factor (NGF, 50ng/mL) was added for the indicated times. MDCK cells were grown in EMEM supplemented with 10% FBS to ~70% confluency and starved in serum-free medium for 48 hours before stimulation with 50ng/mL of hepatocyte growth factor (HGF) at indicated times. C2C12 cells were grown in DMEM supplemented with 10% FBS to ~70% confluency and were starved in serum-free medium for 48 hours before adding 10% serum at the indicated times.

Active GTPase pull-down and detection

NIH 3T3 cells were lysed on the culture plate with 1mL lysis/bind-wash buffer. The clarified cell lysate (500μg) was treated with either GTPγS (positive control) or GDP (negative control). The treated lysates (or 1mg of the endogenous time-course lysates) were incubated with 400μg GST-Rhotekin-RBD (for active Rho), 80μg GST-Raf1-RBD (for active Ras), 20μg GST-Pak1-PBD (for active Rac1 or Cdc42), 20μg GST-RalGDS-RBD (for active Rap1) or 100μg GST-GGA3-PBD (for active Arf1 or Arf6). Half of each elution was analyzed by SDS-PAGE and detected by Western blot using the specific GTPase primary antibody.

FREE Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate

Order any Thermo Scientific Pierce Active GTPase Pull-down and Detection Kit between March 1 – August 31, 2011, and receive a FREE 100mL package of Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate for Western blotting.

References


Ordering Information

Product # Description Pkg. Size Unit Price
16116BNDL Active Rho Pull-Down and Detection Kit 30-rxn kit $670
16117BNDL Active Ras Pull-Down and Detection Kit 30-rxn kit $670
16118BNDL Active Rac1 Pull-Down and Detection Kit 30-rxn kit $670
16119BNDL Active Cdc42 Pull-Down and Detection Kit 30-rxn kit $670
16120BNDL Active Rap1 Pull-Down and Detection Kit 30-rxn kit $670
16121BNDL Active Arf1 Pull-Down and Detection Kit 30-rxn kit $670
16122BNDL Active Arf6 Pull-Down and Detection Kit 30-rxn kit $670

Kit Contents

FREE SuperSignal West Pico Chemiluminescent Substrate 100mL
GST Fusion Protein of Specific Binding Domain 1 vial
Glutathione Agarose Resin 3mL
GTPγS (100X) 50μL
GDP (100X) 50μL
Lysis/Binding/Wash Buffer 100mL
GTPase-Specific Primary Antibody 1 vial
SDS-PAGE Sample Loading Buffer (2X) 1.5mL
Spin Cups 30 cups
Collection Tubes 90 tubes
Detection and localization of active GTPases in neuronal cell differentiation

GTPase kits enable powerful signal transduction studies

Kay K. Opperman, Ph.D.; Suzanne M. Smith, M.S.; Hai Yan Wu, Ph.D.; Barbara J. Kaboord, Ph.D.; and Rizwan Farooqui, Ph.D.; Thermo Fisher Scientific, Rockford, IL, USA

The differentiation of neuronal cells requires signaling pathways that are responsive to the extracellular matrix as well as extensive remodeling of the cytoskeleton. GTPases are critical for these processes. The Ras GTPase family acts as membrane-associated signal transducers; the Rho GTPase family regulates actin and microtubule dynamics. The cellular location of the GTPases and their respective effector binding proteins contribute to cellular differentiation. In this study, Rho and Ras GTPases were assayed for activity and cellular localization using the components of the Thermo Scientific Active GTPase Pull-down and Detection Kits. In addition to using the kits for pull-down assays, the GTPase antibody and GST-tagged effector binding domain were used for immunofluorescent localization. Stimulation of NS-1 cells (neuronal cell line derivative of PC-12) with neuronal growth factor (NGF) resulted in a time- and location-dependent activation of the GTPase targets that were tested. These results could be correlated to staining patterns in primary rat cortical neurons. The kit enabled detection of active GTPase activity in neuronal cells and was an effective tool for studying cellular localization of active GTPase activity.

Introduction

Differentiated cells have highly specified roles guided by cascades of protein interactions. In these cascades, small GTPases help link cell surface receptors to the actin cytoskeleton, guide interaction with other cells and the extracellular matrix, and direct the delivery and internalization of lipids and proteins. In neurogenesis, cytoskeleton rearrangement and microtubule organization are critical for the initial disruption of cell shape and bud formation for neurite outgrowth and extension. To study neuronal outgrowth in vitro, undifferentiated neuronal cell lines are stimulated with neuronal growth factor (NGF) and monitored for a time period. NGF signaling occurs through the tyrosine kinase receptor (TrkA) and activates Ras GTPase at the membrane. Additional Ras and Rho GTPases are activated after signaling of Ras via PI3 kinase, resulting in active Rap1, RaIa and the Rho GTPases. The Ras, Rap1 and RaIa GTPases serve as upstream signal transducers; however, the Rho GTPases (Rac1, Cdc42, and RhoA) act antagonistically and affect the actin skeleton and microtubules, transcriptional activation, and membrane trafficking. Rac1 and Cdc42 promote neurite formation and RhoA inhibits neural differentiation. The intricate regulation of GTPases determines the neuronal cell differentiation fate (Figure 1).

Figure 1. Role of GTPases in neurogenesis. A complex balance of interacting signaling pathways controls neuronal cell differentiation. Green arrows: promotes changes in cell morphology; Red lines: abrogates changes in cell morphology.
Regulation of neural differentiation is dependent on both the signaling cascade and the spatial location of the GTPases in context to their respective effector binding proteins. After NGF stimulation, Rac1 is recruited to the membrane to form membrane ruffles and then localizes to the distal half of the neurites during differentiation. Cdc42 is present in the microspikes projecting from the tips. Both Rac1 and Cdc42 trigger neuronal differentiation through Pak1 kinase. If RhoA is activated, RhoA forms a thick ring-like structure at the cell periphery to prevent recruitment of Rac1 to the cell membrane, resulting in neurite retraction. The negative regulation of neurite extension by RhoA is dependent on Rho kinase (ROCK). After the initial signaling event that triggers differentiation, each GTPase may induce both positive and negative regulation of neurite growth and axonal signaling (Figure 2) to provide cellular fluidity for signaling and regulatory roles in polarization, extension, guidance and regeneration.

Figure 2. Stages of neuronal development. The GTPases that were assayed in this study are listed below the stages. Green: positive; Red: negative.

We stimulated neuronal NS-1 cells with NGF and studied Rho and Ras family GTPase activity using the Active GTPase Pull-down and Detection Kits. Active GTPase activity was assessed by a functional pull-down assay using a GST fusion of the downstream effector protein that binds only the active form of the GTPase. The spatial distribution of active GTPases was determined by immunofluorescent staining using the GST-PBD protein and anti-GTPase antibody supplied in the kit.

Results and Discussion

Based on the functional pull-down results, GTPases are differentially regulated with time. Ras activity peaks at day 1 and 2, and Rac1 and Rho activity is present at early time points and diminishes with time. Activity of RafA showed no significant changes (Figure 3). Immunofluorescent staining revealed that Rac1 is present at the membrane ruffles and extends throughout the neurite extension. Colocalization with Pak1 at the periphery and in the neurite tips suggests “active” Rac1 in these regions (Figure 4). Similar staining patterns of Rac1 and Pak1 PBD were obtained with primary differentiated rat cortical neurons (Figure 5). These results loosely correlate with what is reported in earlier studies. Rho, however, is localized as a thick ring around the cell body and in the perinuclear region. After stimulation, colocalization with rhotekin is in the perinuclear region and does not extend into the neurite extensions, which is consistent with previous results. Ras is also present in the perinuclear region and at the cell periphery, consistent with its function in cell signaling from the membrane to the nucleus. Colocalization with its effector binding domain Raf1 suggests that active Ras is present in the nodes of the neurite extensions as well as in the cell body (Figure 4).

Figure 3. Assay of active GTPase activity by functional pull-down. Active GTPases were detected in NGF-stimulated NS-1 cells as described in Methods. Spot densitometry was performed on each scanned blot and normalized to scale. The graph summarizes the induction of Ras, Rac1, Rho, and RafA for a four day period.

Conclusion

Using the Active GTPase Pull-down and Detection Kits enabled visualization of GTPase activity during the course of differentiation and GTPase cellular localization. The GST-tagged GTPase effector binding domain should only stain “active GTPases”; however, some of the effectors bind multiple proteins and GTPases. Therefore, co-localization of the GTPase antibody with the respective binding domain suggests the cellular location of “active” GTPases. Co-localization studies provide a better understanding of spatial activity during differentiation; however, more optimization of the effector binding domains is necessary for better specificity and lower background.
Methods

Cell culture

NS-1 cells were cultured in RPMI media containing 15% FBS, pen/strep, and HEPES buffer on collagen IV-coated plates or on cell culture-treated eight-well chamber slides (BD Biosciences). At ~80% confluency, cells were stimulated with 50ng/mL NGF (EMD Biosciences) or untreated. For functional pull-down assays, cells were harvested at 1 hour and 1, 2, 3, and 4 days post-treatment using the lysis buffer supplied in the kit. Active GTPases were detected from fresh cell lysates (1mg total protein) by Western blot as per kit instructions. For immunofluorescent staining, media was gently removed and replaced with warmed 4% paraformaldehyde for 20-30 minutes at 37°C, to preserve neuronal structure. Slides were stored at 4°C until stained.

Immunofluorescent staining

After fixation, cells were permeabilized with 0.05% Triton® X-100 in phosphate-buffered saline (PBS) for 15 minutes, and blocked for 30 minutes in 5% fetal bovine serum (FBS) in PBS. Cells were incubated with the GST-effector binding domain fusion protein (Raf1, rhotekin, or Pak1) at 50-200μg/mL for 1 hour, rinsed and stained with anti-GTPase antibody (Ras, pan-Rho and Rac1, respectively) for 1 hour at room temperature (1:250-1:500 dilution). Cells were washed and stained with Thermo Scientific DyLight 549 Dye conjugated to goat anti-mouse or goat anti-rabbit IgG (1:500 dilution) and Hoechst 33342 (DNA stain) for 30 minutes at room temperature. Cells were washed and dehydrated using a 70, 80, 90, 100 ethanol series. Coverslips were mounted using VECTASHIELD® Mounting Media, and images were acquired using the Axio Observer (Carl-Zeiss, Inc.) inverted microscope (63X objective) and AxioVision Software Module.

References