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GTPase Protocols

The Ras Superfamily

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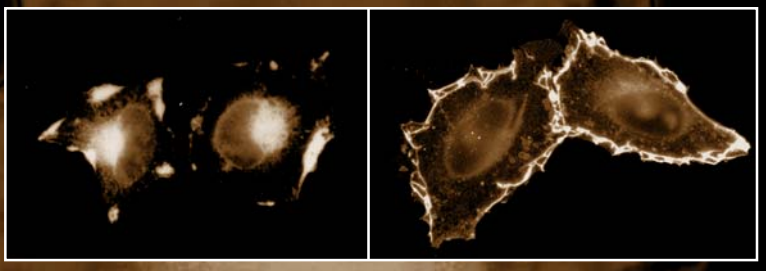

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Cell Motility and Invasion Assays

Jackie Banyard and Marc Symons

1. Introduction

Cell motility and invasion play an essential role in a wide range of biological functions, including many stages of development, wound healing, and immune function. Deregulated motile behavior is believed to contribute to pathological processes such as metastasis, tumor angiogenesis, and atherosclerosis ***(1–3)***.

Rho family members control a multitude of functions that have been implicated in the regulation of cell migration. Most notable among these are the organization and dynamics of the actin cytoskeleton, intercellular and cell-substrate adhesion, vesicle trafficking, and lipid metabolism ***(4)***. Recently, Rac has been shown to stimulate the transcription of collagenase-1, a metalloproteinase involved in the remodeling of extracellular matrix ***(5)***.

Therefore, it is not surprising that several Rho family proteins have been implicated in the control of cell motility and invasion. Rac, Cdc42, and Rho have been shown to play essential roles in growth factor- and cytokine-stimulated chemotaxis in fibroblasts, macrophages, and neutrophils ***(6–9)***. There is also substantial evidence that activation of Rac, Cdc42, and Rho is necessary for the invasive behavior of carcinoma cells and fibroblasts ***(7,10–12)***. There is still much uncertainty however regarding the signaling pathways activated by Rho proteins to regulate motile behavior.

The identification of the biological functions of Rho proteins has mostly relied on the specific inhibitory effects of dominant-negative mutants of these GTPases, and on the stimulation of signaling pathways by constitutively active versions. To introduce these mutants into cells, researchers have employed microinjection of plasmids or recombinant proteins ***(8,13,14)***, transient transfections, or the establishment of stable cell lines ***(7)***. More recently, cell-permeable peptides corresponding to the Cdc42/Rac-interacting-binding

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(CRIB) motifs have also been successfully used to interfere with Cdc42- and Rac-mediated functions ***(15)***.

We have focused on the use of stable cell lines for our studies on the role of Rho GTPases in cell motility for several reasons. Foremost among these is the possibility of using the same lines to study the roles of these GTPases in other functions that are relevant to cell motility. In addition, the characterization of these and other parameters provides a large number of internal controls for the specificity of action of the dominant-negative and constitutively active GTPase mutants. Thus, we have established a panel of Rat1 fibroblasts expressing either dominant-negative or constitutively active versions of Rac1, Cdc42, and RhoA ***(16–18)***. The motility parameters that we have determined for this panel of cell lines include chemotaxis toward platelet-derived growth factor (PDGF) and soluble fibronectin, random motility stimulated by Lysophosphatidic acid (LPA), and PDGF-stimulated invasion into three-dimensional collagen gels ***(6,7)***. We have also used these lines to study the role of Rho GTPases in adhesion signaling, cell proliferation, and lipid metabolism ***(16–21)***.

The use of stable cell lines also avoids the pitfall of employing exceedingly high expression levels of mutant GTPases that may lead to nonspecific cross-communication and/or cell toxicity. Cell lines that express mutant GTPases from an inducible promoter have several additional advantages. Control of the level of inducer allows careful titration of GTPase expression levels. Inducible expression also largely eliminates the possibility of long-term adaptation of the cells to the presence of recombinant proteins.

2. Materials

2.1. Cell Culture

1. Rat1 fibroblasts are maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 m*M* glutamine, 4.5 g/L glucose, 100 U penicillin, and 2 µg/mL streptomycin.
2. Cdc42-expressing stable cell lines stably transfected with the pCMVneo*Myc* vector are maintained in the same media supplemented with 400 µg/mL G418. Rac- and Rho-expressing stable cell lines, containing a tetracycline-sensitive promotor, as detailed in **Subheading 2.1.1.**, are maintained in the same media supplemented with 400 µg/mL G418, 2.5 µg/mL puromycin, and 2 µg/mL tetracycline. Expression of Rac or Rho is induced by withdrawal of tetracycline 2 d before experimentation. Cell culture reagents were obtained from Life Technologies, MD.

2.1.1. Stable Lines

1. The tetracycline-regulatable expression system that we have used to derive stable lines expressing mutant Rho proteins was developed by Gossen and Bujard

(22,23). This system is based on two regulatory elements that are derived from the *Escherichia coli* (*E. coli*) tetracycline-resistance operon, the *tet* repressor protein, and the *tet* operator DNA sequence. The tet repressor protein has been fused to the activation domain of virion protein 16 of herpes simplex virus, to make the tetracycline-controlled transactivator (tTA). This transactivator in turn stimulates transcription from a tetracycline-response element (TRE) containing a minimal promoter sequence derived from the human cytomegalovirus (CMV) promoter, combined with seven repeats of the *tet* operator sequence.

2. The establishment and characterization of the stable cell lines was carried out in two steps, as previously described in detail ***(16,22)***. Briefly, in the first step a Rat1 fibroblast line that expresses the tTA transactivator was established. To test for expression and inducibility, approx 75 clones were isolated and transfected with a luciferase reporter that is controlled by a tTA-dependent promoter. Several clones were selected that showed 5–10-fold repression by tetracycline. In the second step, the tTA-expressing Rat1 line was transfected with plasmids containing the respective cDNAs for the epitope-tagged mutant GTPases controlled by the TRE. From the experience shared by many laboratories, it appears that the selection of a highly inducible tTA-expressing clone is the most tedious part of the cell-line construction. Often, a large number of clones (>100) must be inspected to yield a satisfactory cell line. However, several tTA-expressing cell lines representing a variety of different tissues are now commercially available (Clontech).
3. A number of other inducible expression systems have been described in the literature ***(24–26)*** and several of these are commercially available.

2.1.2. Transient Transfections

We have transiently overexpressed mutants of the Rho family GTPases in Rat1 fibroblasts using pEXV, pCMV, pcDNA3 (Invitrogen, CA) and pIRES2-EGFP (Clonetech, CA) vectors. Transfection was achieved using FuGENE 6 reagent (Roche, IN) that results in high transfection levels (approx 35%) and low toxicity in Rat1 fibroblasts. Transfection was performed on cells at 50–70% confluence according to the supplier's protocol: 2 µg DNA is used per 60-mm tissue-culture dish. Sorting of the transfected cell population is a valuable step, allowing interpretation of results without contamination by the untransfected population. Transfection using a green fluorescent protein (GFP) vector enables fluorescence-activated cell sorting (FACS) to be used. We have found that FACS sorting did not affect Rat1 fibroblast migratory response under the conditions used ***(7)***.

2.2. Boyden-Chamber Migration Assay

The 48-well modified Boyden chamber used in our studies is obtained from Neuroprobe Inc. (Cabin John, MD). The 8.0-µm pore PVP-F polycarbon-

ate membranes are available from Nucleopore (Corning Separations, MA). Membranes are precoated with an extracellular matrix macromolecule before use, to enhance cell attachment. We routinely use gelatin, fibronectin, or collagen. Gelatin (Difco, MI) is prepared as a 1.5% solution, autoclaved, and stored at 4°C. The sterile gelatin solution is dissolved at 37°C before use. Fibronectin, collagen, and Matrigel are obtained from Becton Dickinson, MA. The assay solution used to dilute all test solutions and to resuspend cells is serum-free cell-culture media—i.e., DMEM supplemented with 0.1% bovine serum albumin (BSA), and filter-sterilized. Cells are stained using Gill's Hematoxylin #3 (Polysciences, PA). We use Permount mountant (Fisher Scientific, NJ), but other mountants may be used. We obtained PDGF-BB from R&D Systems, MN.

2.3. 3D Collagen Invasion Assay

1. Collagen used to form gels in this assay was extracted from rat tail tendons ***(27)***. Rat tails are cut from young control rats at sacrifice, wiped with 70% ethanol, and stored at –20°C until use. Tails are thawed in 70% ethanol overnight. Under sterile conditions, a pair of serrated edge clamp forceps are positioned 2 cm apart from each other, toward the tip of the tail. The forceps are twisted, which snaps the tail, allowing the tendons to be pulled out. The tendons are cut using sterile scissors into cold 3% acetic acid (99%+ purity). Tendon extraction is repeated for each tail, proceeding toward the body end of the tail, giving 3–4 tendon pulls per tail. Drop tendons from 10 tails into 500 mL acetic-acid solution. A sterile stir bar is added to each bottle and collagen is extracted by stirring overnight at 4°C.
2. The extract is centrifuged at 2,500*g* for 2.5 h at 4°C. The collagen supernatant is poured into sterile dialysis tubing, using a sterile funnel. The dialysis tubing is pretreated by boiling in 0.07 *M* EDTA/0.05 *M* sodium bicarbonate solution before being thoroughly washed with distilled water and autoclaved. Dialyze the collagen extract against cold distilled water at 4°C for 2 d, changing water twice per day. The dialysis tubing is sprayed with ethanol, then cut and poured into sterile centrifuge tubes and recentrifuged at 2,500*g* for 2.5 h at 4°C, and supernatant is collected. Concentration is determined against a standard curve of Vitrogen 100 (Cohesion) at 230 nm and diluted to 2 mg/mL using sterile, cold distilled water. 1% penicillin/streptomycin is added, and the stock collagen solution is stored at 4°C.
3. Obviously, this bulk extraction protocol—although cost-efficient for a laboratory that frequently uses the 3D collagen-gel invasion assay—is somewhat laborious. Many commercial sources of collagen are unsuitable for this assay, but we have successfully used collagen purchased from Vitrogen, and other investigators have reported using rat-tail collagen from Collaborative Biomedical Products ***(28)***. Collagen is mixed with 10X Minimal Essential Medium (MEM) solution and 7.5% sodium bicarbonate solution (Life Technologies, MD) to form gels.

3. Methods

3.1. Boyden-Chamber Migration Assay

The Boyden chamber is a transmembrane assay in which cell migration across a membrane is measured in response to a chemoattractant. This is a useful method for examining the effect of Rho GTPase activity on the migration of stable cell lines. The use of transiently transfected cells in this assay is unlikely to provide clear results, as any effect of the Rho GTPases will be masked by the normal response of the untransfected cell population. This problem may be circumvented by sorting of transiently transfected cells. We have, for example, sorted transiently expressed GFP plasmids using FACS *(7)*. Another alternative may be to use retrovirus or adenovirus vectors, which typically result in much higher efficiencies of cell infection.

We use a 48-well Microchemotaxis chamber, which is a modified version of the Boyden chamber that allows multiple conditions and replicates to be run in one chamber. The chamber apparatus consists of lower and upper wells that are separated by the insertion of an extracellular matrix-coated porous membrane. Chemotactic stimulus is placed in the lower wells, and cells are plated in the upper wells. Cells migrate through the membrane pores to the lower side. The chamber is disassembled and the membrane is fixed and stained. Nonmigrating cells on the upper side of the membrane are removed, and the migrated cells on the lower side of the membrane are counted.

1. Membranes are precoated with extracellular matrix. A number of different matrices are suitable depending upon cell type. We usually use fibronectin at 7.5 µg/mL in PBS coated at room temperature for 30–60 min. Alternatives we have used are:
   a. Gelatin—1.5% solution, *see* **Subheading 2.2.**, immerse membrane for 1–2 h at room temperature
   b. Collagen—100 µg/mL solution in phosphate-buffered saline (PBS). Immerse membrane overnight at room temperature.
   c. Matrigel—150 µg/mL in PBS. Immerse membrane for 1–2 h at 37°C. After coating, wash membranes twice in PBS and dry on Whatman paper before use. It is helpful to mark the orientation of the membrane by cutting the upper left-hand corner.
2. Chamber assembly: lower wells are filled with chemoattractant to form a slight positive meniscus. Exact volume varies between different chambers, but is typically 28 µL. The coated membrane is placed over the lower wells, and must not be moved once positioned, or the media will mix. The membrane is overlaid with a silicone gasket, and then the upper-well chamber is added, using even pressure while the chamber screws are fastened. The chamber is then equilibrated in a 37°C, 5% CO_2-humidified incubator. We find it helpful to place the chamber

in a plastic box containing dampened kimwipes to ensure proper humidity, which is left ajar for gas exchange.

3. Cells are trypsinized according to regular protocol for the cell type in question, then pelleted and resuspended in a set volume of assay media—serum-free-culture media supplemented with 0.1% bovine serum albumin (BSA). An aliquot is removed for cell counting, and the remainder is pelleted to wash. The cell pellet is resuspended at 3×10^5 cells/mL in assay media.
4. 50 µL of cell suspension is plated in each upper well. To avoid air bubbles being trapped at the membrane, pipet by placing the tip close to the membrane (do not touch membrane, as this may damage the matrix coating) at a 45° angle and raising the pipet tip as the cell suspension is expelled.
5. The chamber is then incubated at 37°C in a 5% CO_2-humidified incubator for 4 h.
6. Disassemble the chamber by removing the screws and lifting off the upper-well section. The gasket and membrane will be removed with the upper section, leaving the migrated cells on the uppermost side. The cut corner should now be on the upper right-hand side.
7. The membrane is removed using forceps, and is gently immersed in PBS to remove any cells not attached to the membrane. The membrane is then fixed in 10% buffered formalin for 10 min, rinsed in PBS, and stained in Gill's hematoxylin overnight.
8. The membranes are washed in several changes of tap water. Nonmigrating cells are then scraped off the membrane. This can be done using a rubber squeegee apparatus provided with the Microchemotaxis chamber, but we find it more controllable to do this manually, as follows. The membrane is cut vertically into two sections, and the corner of the second section is cut to ensure correct orientation. The first membrane section is then picked up at an edge using forceps, and the nonmigrating cells are scraped off by wiping the membrane across damp Whatman paper wrapped on the outside of a beaker. The scraping is repeated several times until all nonmigrating cells are removed. Keeping the membrane wet will prevent tearing. The membrane is then placed on a glass slide. While the membrane is still wet, an imprint of the wells can be seen. It is helpful to mark the positions of the wells using a red marker pen so that any wells in which few cells have migrated can be easily located. After the membrane has completely dried, mountant is added and either a large cover slip or a second glass slide is overlaid.
9. Analysis: Cell migration from the upper to the lower side of the membrane is assessed microscopically. A grid eyepiece reticule is used to define a counting area. Magnification of ×100 or ×200 may be used, depending on the level of cell migration. Control-cell migration should be greater than 10 cells per field so that any effect on basal migration is detectable. Cell number in the defined reticule area is counted in the center of each well, with 3–4 replicate wells per experimental condition. The mean and standard error are then calculated.

3.2. 3D Collagen-Gel Invasion Assay

Movement of cells into a matrix of type I collagen fibers is measured by the 3D collagen-gel invasion assay *(28a)*. In this assay, cells are plated as a monolayer on the gel surface. Cells are stimulated to invade the gel, either by the addition of growth factor to the culture media or by incorporation into the gel. We have used this assay to determine the effect of Rho GTPase expression on cell invasion in response to PDGF-BB in stable cell lines *(7)*. In addition, transient overexpression of V12-Rac increased Rat1 fibroblast invasion into collagen gels *(7)*, using cells plated in the invasion assay 24 h after transfection using FuGENE reagent, as described in **Subheading 2.1.2.** As discussed for the Boyden chamber, transient transfection may not be adequately sensitive to detect an inhibitory effect of Rho GTPase expression on the cell population, as the response of untransfected cells may dilute any effect. However, we have observed an inhibitory effect of N17-Rac expression on Rat1 fibroblast invasion in response to PDGF-BB *(7)*.

3.2.1. Assay Set-Up

1. Collagen gels are assembled by mixing 10.2 mL 2 mg/mL collagen solution with 1.2 mL 10X MEM and 0.6 mL 7.5% sodium bicarbonate solution. Mix while cold, then immediately pipet 1 mL into each well of a 12–well tissue-culture plate. Allow to gel undisturbed at room temperature for 15 min, then incubate at 37°C in a 5% CO_2-humidified incubator for 2 h before use.
2. 0.5 mL of serum free cell-culture media is then plated on the surface of the gel, with or without the addition of PDGF-BB. This is allowed to equilibrate for 1–2 h.
3. Cells are trypsinized, collected by centrifugation, and resuspended at 3×10^5 cells/mL in assay media: DMEM supplemented with 4% fetal bovine serum (FBS) and glutamine, so that the final concentration of each is 1%, considering the total 2-mL volume of the gel culture. Then, 1 mL of cell suspension is plated onto the surface of the gel, swirled to cover, then incubated at 37°C in a 5% CO_2-humidified incubator for 96 h.
4. Analysis: Cell invasion into the collagen matrix is microscopically assessed at ×200 magnification. The cell number is counted on the surface of the collagen matrix (approx 70–100 cells/field with a confluent-cell monolayer), and the number migrated into the matrix is counted in 10 random fields per well in duplicate gel cultures. A cell is defined as having invaded the gel when the cell surface is no longer focused. Data is expressed as percentage of cell invasion, calculated as follows:

$$[\text{cells in gel}/(\text{total cells: in gel + on gel})] \times 100$$

Consideration of the total number of cells allows any change in proliferation to be monitored, although we generally observe negligible proliferation of the

contact-inhibited monolayer, as growth and saturation density are suppressed on collagen matrices ***(29)***. We routinely count collagen-gel cultures using phase optics, but the ease of counting can be enhanced by fixing the gel cultures in 10% buffered formalin for 15 min, gently rinsing the gels 2× in water, then staining the cells with Toluidine blue for 15 min before rinsing off the stain with water. Fixed gel cultures can be stored at 4°C for several days before counting.

4. Notes

1. The Rho family GTPases play a critical role in cell migration and invasion. We have demonstrated this using two methods: the modified Boyden chamber, which measures cell migration across a membrane, and the 3D collagen-gel-invasion assay, which measures cell movement into a matrix. A number of other methods have been used to study the effects of the Rho family GTPases on cell motility, and a brief overview of some of these techniques follows.
   a. In-vitro wound-healing assay: The in-vitro "wound healing" assay analyzes cell migration away from the edge of a wounded monolayer into the denuded space. Migration of cells from the front of the wound edge is assessed microscopically. Cells on the wound edge can also be microinjected to determine the effect of protein expression on cell migration into the wound ***(14)***. A possible caveat is that cell migration may be confounded by the fact that cells at the wound edge are essentially released from contact inhibition, since they have been grown to a quiescent monolayer. However, the assay set-up is very simple, and can readily be used as a first line of investigation.
   b. The Dunn chamber: Cell movement across a planar surface in a gradient of chemoattractant can be measured in the Dunn chamber (Weber Scientific International Ltd., Teddington, U.K.). This chamber is a modified Helber cell-counting slide. The slide consists of two concentric wells separated by a bridge marked with orientation points. Cells are plated onto a cover slip that is inverted onto the chamber and sealed to leave a 20-µm gap between the bridge and the cover slip. The outer well is drained and replaced with a chemotactic factor, forming a concentration gradient between the inner and outer wells. Cell movement in a demarcated area of the cover slip is tracked by time-lapse video microscopy over several hours. The directionality and speed of the cell-migration response is determined by analysis of the trajectories of individual cells. This assay has been used in combination with microinjection to investigate the role of Rho GTPases in macrophage chemotaxis toward CSF-1 ***(8)***. This assay may be limited to motile cell types, for which cell translocation is sufficient to allow quantitative evaluation in the time frame in which the concentration gradient persists.
   c. Cell tracking of random motility: A number of motility parameters (including speed and directional persistence) can be obtained by computer-aided cell tracking of cells in combination with video microscopy. This approach has been used to study the role of PAK, an effector of Cdc42 and Rac, in directed cell migration ***(30)***. Similarly, in the phagokinetic track assay, cells move

across a planar surface that has been coated with colloidal gold particles. The particles are phagocytosed, leaving tracks to indicate where the cells have moved, which can be analyzed in terms of both length and directionality. A detailed methodology for this assay has been described elsewhere ***(31)***.

d. Invasion assays: Transwell invasion assays operate on a principle similar to the Boyden chamber. Cells migrate across a porous membrane toward a chemoattractant in a tissue-culture well. However, the upper-chamber unit containing the membrane is coated with a layer of extracellular matrix (rather than a thin adhesive coating as in the Boyden-chamber assay), through which the cells must invade. The most commonly used matrix is Matrigel, although this invasion assay is also adaptable for use with other gel matrices, such as collagen gels. V12-Cdc42, Rac1, or L61-Rac overexpression increased epithelial-cell invasion across 2-mm collagen gels in the transwell system ***(10)***.

An adaptation of this assay makes it possible to simultaneously evaluate both cell motility and invasion properties ***(32)***. In this "inverse invasion" assay, cells are first attached to the underside of a Transwell™, and subsequently allowed to move through the pores into Matrigel. The number and position of cells is determined by confocal microscopy. Cells at or above 20 µm from the bottom of the filter are considered to have invaded the matrix, whereas cells that migrate through the pores, but are located below 20 µm from the bottom of the filter, are used to quantify chemotaxis.

Collagen-gel cultures can also be used to examine other invasive characteristics. For example, Madin-Darby canine kidney (MDCK)-epithelial cells seeded throughout collagen disperse and invade the collagen matrix. V12-Rac expression reduces this invasive phenotype, resulting in more compact colonies ***(33)***.

Cell-monolayer invasion assays represent an alternative axis through which to study effects on cell migration, looking at cell–cell interactions as compared to cell-matrix-based invasion assays, as discussed in **Subheadings 3.1.** and **3.2.** The invasive capacity of cells is determined by plating them onto a confluent-cell monolayer. Invasive cells infiltrate the monolayer and form foci. Overexpression of V12-Rac1 or V12-Cdc42 in T-lymphoma cells, or V14-Rho in hepatoma cells, increases invasion of a cell monolayer ***(12,34,35)***.

2. The 48-well modified Boyden chamber is a somewhat finicky assay that requires at least one practice-run setup. The chamber needs to be assembled fairly quickly, or the media in the bottom wells will begin to evaporate. If this happens, placing of the membrane on top often results in the trapping of air bubbles. Conversely, if the base wells are overfilled, then placing of the membrane will cause spillover between wells. If different stimuli are being used in the base wells, it is crucial that this contamination does not occur, and is especially critical for negative and positive controls. One precaution is to separate the different stimuli with empty wells.
3. Cells to be used in the Boyden-chamber assay should not have a fresh media change the day before the assay, in order to maximize growth-factor response.
4. We note that alternative Boyden-chamber protocols often remove nonmigrating cells from the upper side of the membrane before fixation and staining. However,

we find that the mechanical scraping easily removes nonmigrating cells even when fixed, and staining the membrane before scraping provides a very valuable source of information. We have found that treating the cells with various factors before assessing their migratory response can affect their adhesion to the membrane. A decrease in the number of cells that adhere reduces the final number of migrating cells. Using this protocol allows this change to be detected, avoiding the incorrect conclusion that cell migration is inhibited, when in fact this could be a secondary effect to that on cell adhesion.

5. Cell-migration analysis in the Boyden chamber can be semi-automated by using a florescent microscope and image-capture software. We have found that positive results can be achieved by staining the membrane with 4,6-diamindino-2-phenylindole (DAPI), as cell nuclei can easily be distinguished using image-analysis software. We have had accurate counts using Adobe Photoshop to capture microscopic fields, followed by the use of IPLab software to quantify cell number.

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