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[53] Morphological and Biochemical Analysis of Rac1 in Three-Dimensional Epithelial Cell Cultures

By LUCY ERIN O'BRIEN, WEI YU, KITTY TANG, TZUU-SHUH JOU, MIRJAM M. P. ZEGERS, and KEITH E. MOSTOV

Abstract

Rho GTPases are critical regulators of epithelial morphogenesis. A powerful means to investigate their function is three-dimensional (3D) cell culture, which mimics the architecture of epithelia *in vivo*. However, the nature of 3D culture requires specialized techniques for morphological and biochemical analyses. Here, we describe protocols for 3D culture studies with Madin-Darby Canine Kidney (MDCK) epithelial cells: establishing cultures, immunostaining, and expressing, detecting, and assaying Rho proteins. These protocols enable the regulation of epithelial morphogenesis to be explored at a detailed molecular level.

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Introduction

Epithelial tissues are coherent sheets of cells that form a barrier between the body interior and the outside world. These sheets line internal organs such as the kidney, lung, and mammary gland. They are commonly one cell thick and enclose a central lumen, thereby creating the spherical cysts and cylindrical tubules that are the architectural hallmarks of internal epithelial organs. The formation of cysts and tubules during embryogenesis requires coordinated regulation of cellular behaviors such as migration, differentiation, proliferation, and polarization. Studies with epithelial cells grown as monolayers on Petri dishes or permeable filter supports have established critical roles for Rho GTPases in these processes, and studies *in vivo* have demonstrated the necessity of these factors during epithelial organogenesis (Burridge and Wennerberg, 2004; Van Aelst and Symons, 2002). Between these two experimental approaches, a mechanistic understanding of how Rho GTPases function to coordinate cell behavior during morphogenesis of multicellular tissue architecture is largely lacking.

Three-dimensional cell culture is a powerful tool for bridging this gap (Debnath and Brugge, 2005; O'Brien *et al.*, 2002). When embedded inside a 3D extracellular matrix (ECM), many epithelial cell lines reproduce a tissue-like architecture (Walpita and Hay, 2002). For instance, when grown in a matrix of collagen I, MDCK cells form structures that resemble epithelial organ rudiments. Furthermore, MDCK cysts develop branching tubules after treatment with mesenchymally derived hepatocyte growth factor (HGF), a response reminiscent of the epithelial–mesenchymal interactions that induce tubulogenesis *in vivo* (Montesano *et al.*, 1991a,b).

The relative simplicity and tractability of 3D cultures enables detailed exploration of how Rho GTPases specify the organization of multicellular epithelia. Here, we delineate the methods we have used to investigate the role of the Rac1 GTPase during MDCK development in 3D. The methods are divided into techniques for morphological analysis and biochemical analysis. Combining these methods can shed significant insight on the mechanisms that regulate epithelial morphogenesis.

Morphological Analysis of 3D MDCK Cultures

Background

We have found *in situ* confocal analysis of immunostained, whole-mounted 3D cultures to be a powerful tool for understanding MDCK cyst and tubule morphogenesis (O'Brien *et al.*, 2001; Pollack *et al.*, 1998). Staining and imaging intact gels involves considerably less time than cutting

sections, enables a large number of structures to be examined without changing slides, and allows 3D reconstruction with imaging software. Phalloidin staining of F-actin is sufficient to reveal detail at the cellular and, in some cases, subcellular level. This resolution allows the morphogenesis of these 3D structures to be examined in a depth not possible with bright-field microscopy.

Growing MDCK Cysts for Immunofluorescence

Reagents (All Sterile)

1. L-Glutamine stock, 29.2 mg/ml in H_2O.
2. $NaHCO_3$ stock, 2.35 g/100 ml in H_2O.
3. 10× MEM without glutamine (Invitrogen 11430-030).
4. Purified bovine collagen I (Vitrogen 100, 3 mg/ml, Angiotech Biomaterials FXP-019).
5. Nunc Anapore membrane inserts, 0.2-μm pore, 10-mm diameter (Nalge Nunc 136935).

Preparation of Cells. There are numerous clones of MDCK cells, which can differ in their ability to form cysts and tubules in 3D culture. We use MDCK strain II cells, derived from a clone isolated by Louvard (Louvard, 1980). These cells are maintained in MEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified 37° incubator with 5% CO_2. MDCK cells optimally form cysts if they are actively proliferating at the time they are plated in collagen I. An easy way of assuring that the cells are rapidly dividing is by splitting a confluent 10-cm Petri dish of cells 1:10 1 day before plating in collagen I.

Plating in Collagen I

1. Prepare the collagen I solution by combining the following: 750 μl glutamine stock, 625 μl $NaHCO_3$ stock, 625 μl 10× MEM without glutamine, 125 μl 1 *M* Hepes (pH 7.6), and 4.13 ml Vitrogen. The final concentration of collagen I in this mixture is about 2 mg/ml. Slowly pipet up and down to mix, avoiding bubbles. Store this solution at 4° while preparing the cells to avoid premature polymerization.
2. Place the Nunc filter inserts individually in the wells of a standard 24-well tissue culture plate.
3. Trypsinize cells and resuspend in about 10 ml of standard culture media.
4. Pellet cells, remove the supernatant, and resuspend in 2–3 ml standard culture media. Triturate cells thoroughly to attain a single cell suspension.

This step is critical, because single cells tend to form spherical cysts with single lumens, whereas clumps of cells tend to form irregular cysts with multiple lumens. Determine the number of cells/ml using a hemocytometer.

5. Add cells to the collagen I solution for a final concentration between 2×10^4 and 5×10^4 cells/ml, typically 50–200 μl of resuspended cells. If the volume of the cell solution exceeds roughly 10% of the collagen I solution, the ability of the collagen I to polymerize will be diminished. Gently pipet to mix, avoiding bubbles.

6. Add 150 μl of the cell–collagen I mixture to each filter insert. Gently tap the tray to spread the mixture over the entire surface of each filter.

7. Place tray in a 37° oven (not a CO_2 incubator) for 10–20 min to harden. Because the collagen I solution is bicarbonate-buffered, it has a higher pH in room air than in 5% CO_2, and the collagen I sets faster and more reliably at this higher pH. The collagen I is polymerized when gentle taps generate no ripples.

8. Once the collagen I has solidified, add 0.5 ml standard culture media to the top of filter and 1 ml to the bottom. Place in a 37° CO_2 incubator for development.

Plating in Other ECMs. MDCK cells are competent to form cysts in other types of ECMs, and comparing cyst morphogenesis in different matrices can be revealing. One alternative matrix is Matrigel™ (BD Biosciences 356234), reconstituted basement membrane purified from the Engelbreth-Holm-Swarm mouse sarcomaline. Plating cysts in Matrigel™ is largely similar to plating cysts in collagen I. The differences reflect the fact that Matrigel™ solidifies very rapidly above 4°; thus, all plastic ware that contacts the liquid Matrigel™ must be diligently cooled to 4° before use, and once plated the cell/Matrigel™ mixture polymerizes within 5 min. Matrigel™-grown MDCK cysts do not tubulate in response to HGF (Santos and Nigam, 1993).

MDCK cells also form cysts in collagen I mixed with ECM components such as laminin-1, collagen IV, and fibronectin (BD Biosciences). These other components are unable to form a solid gel alone, so mixing them with collagen I provides structural support. To enable polymerization, care must be taken not to dilute the collagen I below a final concentration of 1 mg/ml. Purified laminin may be added directly to the collagen I solution. Purified collagen IV and fibronectin are stored in acid; to remove the acid, we dialyze these components overnight against PBS^+ at 4° before adding the collagen I solution.

Maintaining Cyst Cultures. Maintain the growing cysts by replacing media every 2–3 days. In our hands, lumens are visible by bright-field microscopy after 4–5 days, and cysts are terminally developed by 10–12 days.

Conditionally Expressing Rho-family GTPases in Cyst Culture

Background. Rho GTPases are critical regulators of epithelial polarity and morphology. Conditional expression of dominant-negative and dominant-active Rho GTPases enables detailed investigation of their function during cyst morphogenesis. We have extensively studied the role of Rac1 during cyst development with MDCK cell lines that inducibly express the dominant negative N17 or dominant active V12 alleles of Rac1 under the tetracycline-repressible system (Jou and Nelson, 1998; Jou *et al.*, 1998; O'Brien *et al.*, 2001). Inducible expression of these mutant proteins confers the ability to modulate expression levels, control for clonal variation, and determine whether effects of mutant protein expression are reversible.

In our hands, expression of exogenous Rho GTPases is induced efficiently only when cells are actively proliferating. We believe this characteristic may be intrinsic to the parental tet-off MDCK cell line (Clonetech 630913). Inducing exogenous Rac1 expression in mature cysts is thus difficult, because cell proliferation diminishes to replacement levels once cysts complete development. Because tubules are derived from mature cysts and Rac1 is required for cyst formation, this barrier has stymied our efforts to use the tet-off system during tubulogenesis.

Expressing Mutant Rac1 GTPases During Cyst Development

1. To ensure uniform expression and minimize cell line drift, we freeze a large number of vials of low-passage cells and thaw a fresh vial for each experiment. Plate the thawed cells in a 10-mm Petri dish in the presence of 20 ng/ml doxycycline to suppress expression completely.
2. The next day, wash cells thrice with PBS^{-} to remove doxycycline and induce expression. Cells can then be plated in collagen I in the complete absence of doxycycline. For the N17Rac1 cell line we use, this results in expression levels approximately sixfold above endogenous Rac1. Expression can be modulated by varying doxycycline concentrations; with 200 pg/ml doxycycline results in N17Rac1 expression comparable to endogenous levels.

Inducing Tubulogenesis with HGF

Background. During embryogenesis, epithelial tubulogenesis is often initiated in response to signals from mesenchymal growth factors (Hogan and Kolodziej, 2002). Tubulogenesis of MDCK cysts in response to mesenchymal HGF is a cell-culture analogy of this paradigm. Montesano *et al.* (Montesano *et al.*, 1991a,b) detailed three ways in which MDCK cells could

be grown for assaying tubulogenesis: as a monolayer on top of a cell-free collagen I layer, as a single-cell suspension in collagen I, and as a culture of mature cysts. We have had best results with this last approach. With mature cysts, tubulogenesis proceeds with relative synchrony and completes within 3–4 days. When starting with a single cell suspension, tubule development is highly asynchronous and substantially slower (10–14 days).

MDCK Strain tubulogenesis involves two distinct stages (O'Brien *et al.*, 2004), although other MOCK lines may tubulate via a slightly different process (Williams and Clark, 2003). The initial stage is a partial epithelial–mesenchymal transition (p-EMT). The extensions that mark the first manifestation of p-EMT are visible at 6 h. These extensions develop into single-file chains of cells that are apparent by 24 h. Cells in the chains have lost apicobasolateral polarity and are migratory and spindle shaped. The second stage of tubule development is epithelial redifferentiation. At approximately 48 h, cells in chains cease migration, repolarize, and become cuboidal, forming multilayered cords in the process. Nascent lumens develop between these cell layers. By 72–96 h, tubules reach maturity. Repolarization is complete, and lumens are continuous (Pollack *et al.*, 1998). With 20× phase magnification, we are able to identify extensions and chains and distinguish each of these from redifferentiating tubules. We require confocal analysis to distinguish cords from mature tubules.

Whenever possible, we use recombinant, purified HGF. Media conditioned by fibroblasts such as MRC-5 cells contain HGF and can be used to induce tubulogenesis at a dilution ranging from 1:2–1:8 with standard MDCK culture medium. However, it is important to confirm results obtained with conditioned media using purified HGF.

HGF Treatment

1. Add 10 ng/ml HGF or diluted, conditioned medium to a culture of 10-day MDCK cysts in collagen I.
2. Replenish HGF-containing media daily until samples are harvested.

Immunostaining 3D MDCK Cultures In Situ

Reagents

1. Collagenase-1 (Type CVII, Sigma C-2799). Use of high-purity collagenase is critical. We use aliquots of a stock of 1000 U/ml in PBS^+, flash frozen and stored at 80°. Thaw and dilute aliquots 1:10 in PBS^+ before use.
2. Paraformaldehyde (PFA), 4%.
3. Quenching solution. Made fresh for each experiment from two stock solutions, 1 *M* NH_4Cl and 1 *M* glycine (pH 8). Combine 1.5 ml

NH_4Cl stock and 0.4 ml glycine stock, then add PBS^+ to a final volume of 20 ml.

4. Saponin stock solution. 10% saponin (Calbiochem 558255) in H_2O, filter sterilized, and stored in aliquots at 4° because prone to contamination.

5. Permeabilization solution. Made fresh for each experiment from 3.5 g fish skin gelatin (Sigma G-7765) and 1.25 ml saponin stock brought up to 500 ml with PBS^+. Triton X-100 at a final concentration of 0.5% can be substituted for saponin and is preferable for some antigens. The permeabilization solution can be stored at 4° over the course of a single experiment with the addition of 0.02% NaN_3.

6. RNase A (if nuclei are to be stained). Made fresh for each experiment by diluting RNase A stock solution (10 mg/ml in H_2O) 1:100 with permeabilization solution.

7. ProLong mounting media (Molecular Probes P-7481), prepared according to manufacturer's directions.

Collagenase Treatment. Brief treatment of 3D cultures with collagenase before fixation significantly increases the ability of antibodies to permeate the collagen I gel without detectably compromising extracellular antigens.

1. Leaving the gels in the filters, quickly wash the filters three times with room-temperature PBS^+.
2. Add 1× collagenase solution to the filters (approximately 0.25 ml on top, 0.5 ml on bottom) and incubate 8–10 min at 37°.

Fixation and Quenching

1. Wash three times quickly with room-temperature PBS^+.
2. Add 4% PFA and shake samples slowly 30 min.
3. Wash five times with PBS^+, three quick washes and two washes shaking slowly for 5 min each.
4. Samples may be stored up to 2 weeks in 0.1% PFA at 4°.

Permeabilization and Antibody Treatment

1. Incubate with permeabilization solution 30 min at room temperature or overnight at 4°, shaking slowly.

2. If nuclei are to be stained, incubate with 1× RNase A solution 60 min at 37°.

3. Dilute primary antibodies in permeabilization solution, 0.5 ml per sample. Add 0.25 ml of diluted antibodies to top of filter and 0.25 ml below filter. Incubate 2–3 h at room temperature or overnight at 4°, shaking slowly.

4. Wash filters with permeabilization solution, once quickly and three times for 10 min each, shaking slowly at room temperature.

5. Dilute fluorophore-conjugated secondary antibodies in permeabilization solution, 0.5 ml/filter. Because of the thickness of the gel, best results are obtained with bright fluorophores such as the Alexa dyes from Molecular Probes, which we use at a dilution of 1:400. Add 0.25 ml to the top and bottom of filter. Incubate 2–3 h at room temperature or overnight at 4°, shaking slowly.

6. Wash with permeabilization solution, once quickly and three times for 10 min each time, shaking slowly at room temperature.

7. Wash twice with PBS^+ for 5 min each.

Postfixation, Nuclear Staining, and Mounting

1. Add 4% PFA to samples and incubate 30 min at room temperature, shaking slowly.

2. Wash five times with PBS^+, three times quickly and twice for 5 min each.

3. If nuclear staining is desired, the far red nucleic acid dye TO-PRO3 (Molecular Probes T-3605) works well. Dilute TO-PRO3 1:100 in PBS^+, add to samples, and incubate 60 min at 37°. Do not wash samples before mounting; unbound dye does not fluoresce, and washing significantly diminishes the nuclear signal. Hoechst 33342 at a dilution of 1:1,000 can also be used, but include the Hoechst stain with the secondary antibody incubation, not after postfixation.

4. Aspirate PBS^+ or $TO\text{-}PRO_3$ and add a drop of ProLong mounting media to the top of the filter.

5. Remove the gel from the filter. This can be done with a filter punchout tool (Nalge Nunc 139586) or by gently pinching opposite sides of the gel with fine forceps and lifting the gel from the filter.

6. Place a generous drop of ProLong on a microscope slide and place the gel on the drop. To facilitate confocal analysis, make sure that the top of the gel is facing up. Gently put a 25-mm diameter circular coverslip on top of the gel. The gel is less prone to slip out with a circular coverslip.

7. Wait approximately 5 min to allow the ProLong to spread underneath the entire surface of the coverslip. Do not press down on the coverslip during this time.

8. Carefully aspirate any excess ProLong from the edges of the coverslip.

9. Place slides in a dark area at room temperature and allow the ProLong to harden overnight.

10. Slides can be stored at 4° or room temperature. They remain suitable for confocal analysis for 3–6 months.

Biochemical Analysis of 3D MDCK Cultures

Background

Analyzing protein function directly in 3D cultures, rather than in monolayers, is imperative if at all possible. Changing the extracellular environment can substantially alter gene expression and signaling (O'Brien *et al.*, 2001, 2004; Wang *et al.*, 1998; Weaver *et al.*, 2002). Thus, extrapolating biochemical results obtained with monolayer cultures to cyst developmental phenotypes can be tenuous. However, biochemical analysis is inherently more difficult in 3D than in 2D. Because cells are embedded in ECM, accessibility to the basolateral surface is reduced, and accessibility to the apical surface is extremely limited. Furthermore, the density of cells in 3D culture is about two orders of magnitude less than in 2D, making biochemical analysis of low-abundance proteins problematic.

We have developed several strategies for analyzing proteins in 3D, including two methods for generating cyst and tubule lysates. The first of these involves direct and rapid solubilization of 3D cultures *in situ*. Note that for secreted and transmembrane proteins, the solubilized lysate contains intracellular, membrane-bound, and/or secreted populations. Because of its rapidity, we use this technique to analyze the activation state of signaling molecules such as Rac1 and extracellular-regulated kinase (ERK). The disadvantage of solubilization is that the resulting lysates are substantially diluted. Immunoprecipitation of the protein of interest can sometimes overcome this difficulty. The second strategy involves isolating intact cysts and tubules by digesting the collagen I gel. This technique allows populations of intracellular, membrane-bound, and soluble proteins to be distinguished. It also enables the cells to be lysed in a small volume, resulting in a concentrated lysate. The major disadvantage of isolation is the substantial time needed to proteolyze the gel. During this period, cyst and tubule architecture begins to disintegrate, and thus analysis of the activation state of dynamic signaling molecules is not advisable.

Growing MDCK Cysts for Biochemical Analysis

Two modifications of the cyst plating protocol described previously facilitate biochemical analysis of 3D MDCK cultures. The first involves cell density, whereas an initial plating density of 2×10^4 cells/ml produces a convenient density of cysts for morphological analysis, it can be difficult to obtain sufficient cellular material for biochemical analysis. We have grown cysts at an initial plating density as high as 2×10^5 cells/ml to generate

lysates. This higher density does not apparently alter cyst morphogenesis. However, an important caveat is that cysts at this concentration do not tubulate robustly; thus, we use the standard 2×10^4 density for tubulogenesis studies.

The second modification involves a double layer of collagen I. During the time required for the collagen I mixture to polymerize, some cells sink to the bottom and adhere to the filter. These cells do not proliferate into cysts but rather form a pseudostratified layer on the filter interface. This layer does not interfere with morphological analysis, because one can simply adjust the microscope's plane of focus. However, it could arguably interfere with biochemical analysis, because cells in the pseudostratified layer would comprise a portion of the lysate. To avoid this scenario, we first add 100 μl of cell-free collagen I liquid to the filter and allow it to set. We then plate the cell/collagen I mixture on top of this cell-free collagen I layer. In this manner, cells that sink to the bottom are still completely surrounded by collagen I and form true 3D structures instead of pseudostratified layers. To generate cyst lysates, we harvest both layers as many cells adhere to the bottom layer when the two are separated.

Solubilizing 3D Cultures

Reagents

1. SDS lysis buffer. 0.5% SDS, 100 m*M* NaCl, 50 m*M* triethanolamine (TEA)-Cl, pH 8.1, 5 m*M* EDTA, pH 8.0, 0.2% NaN_3, 1× protease inhibitor cocktail (below).
2. Protease inhibitor cocktail, 100× stock. 5 mg/ml pepstatin, 10 mg/ml chymostatin, 5 mg/ml leupeptin, 50 mg/ml antipain, 500 m*M* benzamidine, 10 IU/ml aprotinin, and 1 m*M* PMSF.

Solubilization of 3D Cultures

1. Wash gels four times quickly in PBS^+.
2. Remove gels from the filters by pinching opposite edges of the gel together with forceps. Place gels in a Microfuge tube containing SDS lysis buffer. We generally combine two duplicate samples together in a single tube with 0.8 ml SDS lysis buffer.
3. Boil gels for 15 min. Shake tubes a couple of times during this incubation to facilitate matrix denaturation. The final volume of two solubilized gels in 0.8 ml lysis buffer is about 1 ml.
4. Add protease inhibitor cocktail to 1×.

Isolating Cysts from Collagen I

Reagents

1. MEM buffered with 20 m*M* HEPES (pH 7.6) and including 4% BSA and 1% antibiotics.

2. Collagenase-1 solution, 0.5 ml/filter. Low-purity collagenase contains significant quantities of contaminating proteases. Thus, use of high-purity collagenase (for instance, Sigma C-0773 or Worthington CLSPA) is crucial if the protein of interest is extracellular. Considerable lot-to-lot variability exists. Data on the purity of Worthington collagenase is available online (http://www.worthington-biochem.com/CLS/clssamp.html), and we often sample several lots before purchasing a large quantity. Protease inhibitor cocktail can also be included; these inhibitors do not affect collagenase-1 but do inhibit some common contaminants. Lower purity collagenase such as Worthington CLS-4 may be used for examining intracellular proteins. Prepare a solution of 4000 U collagenase/ml and 0.05 mg/ml DNase (Boehringer 104159) in MEM. The DNase prevents the DNA of any prematurely lysed cells from causing cell clumping.

3. Trypsin stock solution. To isolate the intracellular population of a secreted or transmembrane protein, trypsin (Worthington TRL3) can be included in the collagenase solution. Make a solution of 50 mg/ml trypsin in PBS^+.

4. Ultra Low Cluster 24-well plates (Corning 3473) to minimize cell adhesion.

5. TLCK (L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride) stock solution. Prepare a solution of 10 mg/ml TLCK (Boehringer 874485) in 1 m*M* HCl. Stock can be stored for a month at room temperature.

6. Protease inhibitor cocktail as described in the solubilization protocol previously.

Collagen I Digestion

1. Wash filters three times quickly with PBS^+.

2. Aliquot 0.5 ml collagenase solution into the wells of a 24-well low cluster plate, one well per gel. If digestion of extracellular proteins is desired, add 100 μl of trypsin stock to each well. The MEM will turn yellowish with trypsin addition. Prewarm the plate to 37°.

3. Remove gels from filters and place one gel in each well. Gently create four to five tears in the gel with forceps to facilitate digestion. Rotate at 37° 45 min or until gels are completely digested.

4. Add protease inhibitor cocktail to digested samples. If trypsin was included, add TLCK stock to 1×.

5. Transfer digested samples to Microfuge tubes. Even with low-cluster plates, we find that significant numbers of cells often adhere to the wells. It may be helpful to visually examine the wells at low magnification to assess whether additional washing to remove the cells is warranted. To wash, add 400 μl MEM to well and pipette up and down, then add the wash to the rest of the sample in the Microfuge tube.

6. Pellet cells by centrifuging 5 min at 3200*g*. A small pellet should be visible.

7. Remove the supernatant. This fraction contains digested matrix, as well as factors in the matrix that were not strongly cell associated. If desired, it can be set aside for immunoprecipitation of proteins of interest.

8. Wash pellets twice with ice-cold PBS^+, gently resuspending and then repelleting cells with each wash.

9. If sample normalization is desired, a small, concentrated fraction of the pellet should be set aside at this point. To do this, we resuspend each pellet in a volume of 50 μl (corresponding to one filter) and set aside 7.5 μl to measure for protein concentration using a bicinchoninic assay (BCA) (Sigma-Aldrich BCA-1). Because of the difficulty of repelleting the remaining 42.5 μl, we dilute the remaining suspension with about 1 ml PBS^+, then repellet the cells and remove the supernatant.

10. At this point, the cell pellet can be either lysed directly in sample buffer for SDS-PAGE or processed for immunoprecipitation (see the following).

Immunoprecipitation

Reagents

1. Triton dilution buffer. 5% Triton X-100, 100 m*M* TEA-Cl (pH 8.1), 100 m*M* NaCl, 5 m*M* EDTA, 1% Trasylol, 0.1% NaN_3.
2. SDS lysis buffer. Described previously in the solubilization protocol.
3. Superose 6 beads, ''prep grade'' (Amersham Pharmacia 17-0489-01).

Preparing Samples for Immunoprecipitation

1. If the starting material is heat-solubilized lysates already in SDS lysis buffer, add 500 μl Triton dilution buffer per 1 ml lysate.

2. If the starting material is a cell pellet, add 1 ml SDS lysis buffer containing 1$\times$ protease inhibitor cocktail, boil for 10 min, then add 500 μl Triton dilution buffer.

3. Preclear lysates by rotating with 20 μl Superose 6 beads at least 20 min at room temperature. Spin 5 min at 14,000*g* and retain the supernatant.

4. Add primary antibody for immunoprecipitation and continue as for conventional lysates (Lipschutz *et al.*, 2001).

Assaying Rac1 Activation

Background. The activation state of Rho GTPases in the context of 3D morphogenesis can be mechanistically revealing. In 2D culture, pull-down assays have been a common strategy to determine the extent of activated Rho proteins (Ren and Schwartz, 2000). Such assays take advantage of the ability of activated, GTP-bound Rho proteins to specifically interact with particular effector domains. For instance, the conserved cdc42/Rac1 interacting binding (CRIB) domain of Pak kinases 1–3 strongly binds only the GTP-forms of Rac1 and cdc42. Conjugating a recombinant glutathione S-transferase (GST) CRIB domain fusion protein to glutathione beads thus provides a means to uniquely isolate, or ''pull down'', the activated population of Rac1 or cdc42. This standard protocol requires that cells be lysed rapidly at 4° (to minimally perturb GTPase activation state) and without denaturation (to preserve the GTPases' CRIB-binding competence). The two methods of 3D culture lysis that were described here are, therefore, incompatible with the standard pull-down assay; collagenase isolation requires a lengthy incubation at 37°, and heat-mediated solubilization causes denaturation.

Recently, we have developed a lysis technique compatible with the requirements of the pull-down assay (Yu *et al.*, 2005). We have used this modified protocol to examine Rac1-GTP in 3D cultures. Because GTP-bound Rac1 is inherently unstable, the entire assay must be performed on ice within an hour of lysis. When performed with cyst and tubule lysates, the assay measures steady-state activation levels. We also describe an alternative 3D culture technique of collagen I overlay, reported by Schwimmer and Ojakian (Schwimmer and Ojakian, 1995), that can be used to determine the kinetics of β1-integrin–mediated Rac1 activation in response to cell adhesion to collagen.

Reagents

1. 24-mm transwell filters (Costar 3412) for collagen I overlay.

2. 2× lysis buffer containing 2% Triton X-100, 40 m*M* Tris (pH 8.0), 1 *M* NaCl, 20 m*M* $MgCl_2$, 30% glycerol, 1 m*M* DTT, and EDTA-free protease inhibitors (Roche).

3. GST-CRIB beads coupled to glutathione, prepared as described in Ren and Schwartz (2000) using a recombinant GST-CRIB fusion protein of Pak3 (Bagrodia *et al.*, 1995).

Overlaying with Collagen I and Lysing Cells. Overlaying filter-grown MDCK monolayers with collagen I initiates the formation of tubulocysts through a mechanism dependent on Rac1 and β1 integrin (Schwimmer and Ojakian, 1995; Yu *et al.*, 2005). During tubulocyst formation, cells in the monolayer first proliferate to form a stratified multilayer and then create lumens between the stratified layers. Many of the changes in cell shape and apicobasolateral polarity that occur during this process are reminiscent of cyst and tubule development, and in at least some of these cases similar signaling pathways are involved.

1. Trypsinize a confluent plate of MDCK cells. Split cells and plate onto 24-mm transwell filters, one filter per time point. Allow at least 4 days for cells to form a polarized monolayer. Remove old media and replace with fresh media every 2–3 days, including the day before the experiment.
2. Make collagen I solution as for cyst plating.
3. Wash filters once with PBS^- then once with 0.4 ml collagen I solution.
4. Remove PBS^- from both top and bottom chambers and wash the apical surface of the cells (top chamber) once quickly with 0.4 ml collagen I solution.
5. Add 0.4 ml collagen I solution on top of each filter and 2 ml growth media to the bottom chamber. Incubate at 37°. If doing a time course, stagger the overlay step as appropriate. Include a filter with cells but no collagen I overlay (as the zero time point) and a filter with collagen I but no cells (as a negative control).

Lysing Collagen I Cultures

1. Wash cyst cultures or the bottom chamber of overlaid filters once with ice-cold PBS^+.
2. Peel off the gel and place into a Microfuge tube containing ice-cold 2× lysis buffer. For overlay cultures, cut out the filter with a scalpel and add this to the tube. At time points earlier than 20 min, the collagen is likely not polymerized. In this case, transfer the collagen to the tube using a 1-ml pipette tip whose opening has been widened by cutting a few millimeters off the tip end. The volume of 2× lysis buffer should be such that the final concentration of the lysis buffer will be 1× after solubilization. This volume is 0.5 ml for collagen I–overlaid monolayers on a 24-mm filter. Therefore, to correct for the collagen present in the assay, we add 0.4 ml of gelled collagen solution to the tube containing the

filter that has not been overlaid with collagen. If starting with a different-sized filter or a different volume of collagen I, the amount of 2× lysis buffer to add for a final concentration of 1× can be estimated by weighing the filter and collagen I and using 1 ml of 2× lysis buffer per gram of weight.

3. Rotate the tubes at 4° for 30 min. After this incubation, over 95% of the collagen should be solubilized.

4. Spin samples at 12,000 rpm in a Microfuge at 4° for 5 min to remove traces of undissolved collagen gel.

5. Set aside 50 μl of the supernatant as a total Rac1 loading control. Add 50 μl sample buffer to the loading control and boil 5 min.

Pulling Down Rac1-GTP

1. Add 800 μl of the remaining supernatant to Microfuge tubes containing 20 μl CRIB beads and rotate at 4° for 30 min.

2. Spin at 2000 rpm for 2 min to pull down Rac1-GTP bound to the CRIB beads. Discard the supernatant.

3. Wash beads twice with 1× lysis buffer. Completely remove the last wash solution using a Hamilton syringe.

4. Add 12 μl sample buffer to beads. Boil 5 min. Pellet beads at 2000 rpm for 2 min. The supernatant contains the Rac1-GTP fraction and is ready for SDS-PAGE.

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