Establishment of stable human fibroblast cell lines constitutively expressing active Rho-GTPases

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Summary

Small GTP-binding proteins of the Rho family (RhoA, Cdc42, Rac1) regulate the organisation and the turnover of the cell's cytoskeleton and adhesion structures. A significant function of these cellular structures is to translate and counter-balance forces applied to, or generated by cells in order to maintain homeostasis and control cell movement. We therefore hypothesised that Rho-GTPases are directly involved in cellular gravity perception and may participate in the alterations induced in microgravity. To define an adequate cellular model allowing to investigate this issue, we have established stable cell lines constitutively expressing active forms of either RhoA, Cdc42 or Rac1. The three cell lines differ by morphology and by their ability to form filopodias, lamellipodia and bundles of actin stress fibers. Over-expression of the active form of of either RhoA, Cdc42 or Rac1 is compatible cell viability and does not affect cell population doubling time. Thus, our series of mutant cells appear well-suited to gain further knowledge on the molecular mechanisms of cellular gravity perception.

Introduction

The small Rho-GTPases, RhoA, Rac1 and Cdc42, are GDP/GTP-regulated binary switches operating in the coordination between extracellular signals identified by integrins and growth factor receptors and intracellular effectors pathways. These molecules are key regulators for the organisation and turn-over of the cytoskeleton, formation of cell-matrix adhesions, transcriptional control of gene expression, cell survival and proliferation (Etienne-Manneville and Hall, 2002). In their inactive, GDP-bound forms, Rho-GTPases are sequestrated in the cytoplasm, while upon signalling elicited by external stimuli, they switch to their active, GTP-bound forms and translocate to the cell membrane (Hall, 1992). There, they activate distinct and specific effector molecules, which in turn regulate the organisation of the cytoskeleton and cell-matrix adhesions, thereby controling cellular activities such as adhesion (Chrzanowska-Wodnicka and Burridge, 1996). By interacting with different specific signalling pathways, including NFκB and MAP kinase pathways, the effector molecules also affect cell proliferation and expression of specific genes (Denhardt, 1996).

A wide array of external stimuli leads to activation of Rho-GTPases, including soluble receptor ligands coupled to G proteins, tyrosine kinases and cytokines (Kjoller and Hall, 1999) and insoluble extracellular matrix ligands for cell surface receptors of the integrin family (Chrzanowska-Wodnicka and Burridge, 1996). Among soluble ligands, lysophosphatidic acid activates RhoA, bradykinin induces Cdc42 activation and platelet-derived growth factor (PDGF) activates Rac1 (Nobes et al., 1995; Kozma et al., 1995; Moolenaar et al., 2004). Stimulation of Rho-GTPase

activity by extracellular matrix ligands has mainly been investigated for fibronectin, a ligand for the $\alpha5\beta1$ integrin. Cell adhesion to fibronectin induces first a transient activation of Rac1 and Cdc42, followed by a sustained activation of RhoA (Hotchin and Hall, 1995; Clark et al., 1998; Bourdoulous et al., 1998). The role of Rho-GTPases on the organisation of the cytoskeleton was previously characterised by microinjecting quiescent Swiss 3T3 mouse fibroblasts with specific dominant negative or constitutively active mutant Rho proteins. This model system was very instrumental to show that GTP-bound active forms of Cdc42, Rac1 and RhoA, generated by glutamine to leucine mutation (Cdc42-Q61L, Rac1-Q61L and RhoA-Q63L) causing a loss of the intrinsic GTPase activity, are involved in the formation of filopodia, lamellipodia and actin stress fibers, respectively (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996; Nobes and Hall, 1999).

An important function of the cytoskeleton and cell-matrix adhesions is to transduce and counterbalance forces applied to, or generated by cells, to maintain the mechanical and biological cell homeostasis (Mauch et al., 1989; Lambert et al., 1992;1998; Kessler et al., 2001). Our overall research objective is to investigate the role of the Rho-GTPases in functional alterations induced by microgravity in human connective tissue cells. We assume that loss of gravity as experienced during space flight will affect the control of cell architecture and the function of the Rho-GTPases. As a first step towards testing this hypothesis, we report here the generation of stable human fibroblast cell lines (Wi26) constitutively expressing active (QL) form of each of the three GTPases, RhoA, Rac1 and Cdc42. Over-expression of either

RhoA, Cdc42 or Rac1 typically modifies the actin cytoskeleton without affecting cell viability and proliferation.

Material and Methods

Plasmids and cell transfection

The pIRESpuro vector (Clontech, Palo Alto, CA, USA) containing an EcoRI site upstream of a BamHI site in its multiple cloning sequence was restricted with EcoRI and BamHI. A linker with internal BamHI and EcoRI restriction sites in opposite directions and containing overhangs compatible to the linearised vector was ligated to the precut plasmid in order to provide an upstream BamHI and a downstream EcoRI sites (Fig. 1, pIRESpuro2). The cDNA coding for the constitutively active forms of RhoA (RhoL63), Rac1 (RacL61) and Cdc42 (CdcL61), cloned between BamHI (upstream) and EcoRI (downstream) restriction sites of pcDNA3 (kindly provided by Dr. V. Bours, Liège, Belgium) were excised, purified on agarose gel and cloned in pIRESpuro2 to generate thereafter collectively named pGTPase-IRESpuro (Fig. 1). Inserts and junctions were verified to be correct by sequencing. EGFP cDNA was directly cloned between the EcoRI and BamHI sites in pIRESpuro (pEGFP-IRESpuro). Sub-confluent Wi26 cells (SV40-transformed human lung fibroblasts) were transfected with the various pGTPase-IRESpuro and pEGFP-IRESpuro vectors by electroporation (300 V, 960 µFd). After two days the transfected cells were selected by addition of puromycine at a final concentration of 0.5 µg/ml and grown. Cells were sub-cloned by limited dilution and amplified. Wi26 cells and derivatives were further cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 mM glutamine and a mixture of antibiotics (streptomycin and penicillin with puromycin) at

37°C under 5% CO2 except otherwise stated. All products were from Seromed/Biochrom (Berlin, Germany).

GTPase pull-down assays

The pull-down assays were performed as previously described (Deroanne et al., 2005). Briefly, cells were lysed in ice-cold buffer containing 1% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 4 µg/ml aprotinin, 1 µg /ml leupeptin and 1 µg/ml pepstatin. Lysates were cleared by centrifugation for 10 min. at 13000g. An aliquot of each supernatant was kept to measure total Rho GTPase content by immunoblotting. Other aliquots of the supernatant were transferred quickly into tubes containing either the GST-PBD fusion protein with Cdc42 and Rac1 binding region of PAK-1B, or the GST-RBD fusion protein with RhoA binding region of rhotekin, affinity-linked to glutathione-Sepharose beads (Sander et al., 1999; Ren and Schwartz, 2000). The beads were washed 4 times in lysis buffer. The total and pull-down fractions were separated by SDS-PAGE on 15% acrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose membranes and immunodetected with mouse monoclonal primary antibodies against either RhoA (Sc-418; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rac1 (clone 05-389; Upstate biotechnology, Lake Placid, NY, USA) or Cdc42 (clone 610928; Transduction Laboratories, San Diego, CA, USA), followed by secondary horseradish peroxidase-conjugated antibodies (DAKO, Glostrup, Denmark). The bands were visualised by enhanced chemoluminescence using ECL (Amersham, Little Chalfont, England). For some experiments, cells were serum-starved for 24 hrs

and the medium was replaced by DMEM containing either FCS (10%) or PDGF (20 ng/ml) for 5 minutes before processing cells for pull-down assays.

Staining of fibrillar actin

Cells were grown on glass coverslips for 24 hrs, fixed with freshly prepared 2% paraformaldehyde in PBS for 15 min, permeabilised with ice-cold 0.2% Triton X-100 in PBS for 1 min and incubated with 3% bovine serum albumin (BSA, Fraction V, Serva, Heidelberg, Germany) for one hr. Fibrillar actin was stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma-Aldrich, Deisenhofen, Germany). The coverslips were mounted on histoslides and observed by laser scanning confocal microscopy (Leica, Heidelberg, Germany). Images were acquired and stored using Leica software.

Analysis of vinculin and actin in detergent-soluble and -insoluble fractions

Cells grown overnight in DMEM containing 10% FCS were placed on ice, washed with ice-cold PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) and lysed in 10mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 4.7, containing 0.1% Nonidet P-40 as previously described (Deroanne et al., 1996). The soluble and insoluble fractions were mixed with Laemmli buffer containing 50 mM DTT. Proteins were separated by SDS-PAGE on 7.5 and 12.5% acrylamide gels, transferred to nitrocellulose membranes and immunodetected with mouse monoclonal primary antibodies against vinculin (Sigma, dilution 1:5000) or actin (A7400, Sigma, dilution 1:500) followed by horseradish peroxidase-conjugated secondary antibodies against mouse immunoglobulins (Dako, dilution 1:2000).

Signals were revealed using enhanced chemoluminescence and quantified using a Fluor-S Imager (BioRad).

DNA measurements

Cells were grown in triplicate in tissue culture 24-well plates for various periods of time (4 hrs to three days) during their exponential growth phase. At the end of the culture period, cells were harvested in phosphate-buffered saline using a rubber policeman and lysed by sonication. The amount of DNA was measured by fluorimetry on a SpectraMAX GeminiXS fluorimeter (Molecular Devices, Wokingham, UK) at wavelengths of 355 nm for excitation and 460 nm for emission using bis-benzimide dye and salmon sperm DNA to establish a standard curve.

Results

Selection of clones stably expressing constitutively active GTPases

Fibroblasts of the established cell line Wi26 were transformed with either EGFP or constitutively active form of RhoA, Rac1 and Cdc42 (RhoA-Q63L, Rac1-Q61L, Cdc42-Q61L, collectively named QL mutants thereafter) cloned in pIRESpuro vector. This vector was chosen because almost all cells resistant to puromycin should express the protein of interest due to expression of a bicistronic RNA. Indeed almost all cells, if not all, transformed with pEGFP-IRESpuro actually expressed EGFP as visualised by epifluorescence microscopy (not shown). Cells from mixed populations transformed by the pGTPase-IRESpuro plasmids were cloned by limited dilution and expanded. Seven to twenty-four clones were obtained in individual experiments. The basal activity of GTPases in these clones was compared to that in mock-transfected (control) cells, untreated or treated with a known inducer of the GTPases. The basal level of active RhoA in serum-starved mock transfected Wi26 fibroblasts was low, and was largely and maximally increased after treatment with FCS as expected (Fig. 2). One out of nine serum-starved RhoA transfectants tested (RhoA-QL 13H8) expressed active RhoA at a level much higher than serum-starved mock-transfected cells, i.e. at a level similar to that of serum-primed mock-transfected cells (Fig. 2). RhoA activity in the mixed population and in other tested clones did not reproducibly exceeded that in parental cells (not shown). One out of five tested clones transfected with pCdc42-IRESpuro (Cdc42-QL 18E7) expressed active Cdc42 at a distinctly higher level than starved mock-transfected cells (Fig. 2). The basal level of active Rac1 in untreated mock-transfected cells was already high

but was nevertheless distinctly increased upon treatment with PDGF (Fig. 2). One out of seven tested clones transfected with pRac1-IRESpuro (Rac1-QL 15H10) had an higher level of active Rac1 than untreated mock-transfected cells (Fig. 2). Thus for each constitutively active GTPases, at least one highly expressing stable clone was selected.

Effect of constitutively active Rho GTPases on actin cytoskeleton

To examine the actin organisation patterns of fibroblasts stably expressing constitutively active Rho GTPases, fibrillar actin was stained with FITC-conjugated phalloidin. Observation of the staining by laser scanning confocal microscopy showed actin-containing filopodia and few thin actin fibers in Cdc42-QL mutants (Fig. 3). Cells expressing Rac1-QL characteristically formed lamellipodia containing thin actin filaments (Fig. 3). In contrast, RhoA-QL cells exhibited robust actin stress fibers (Fig. 3). We further analysed by immunoblotting the levels of actin and vinculin in the detergent-soluble and -insoluble fractions of the different clones. The detergent-insoluble fractions of vinculin were similarly low in cells expressing active Rac1 and Cdc42 and, by contrast, significantly increased in cells expressing active RhoA (Fig. 4A,B). The detergent-insoluble fraction of actin was rather high (~50%) in cells expressing active RhoA and Cdc42 and much lower in cells expressing active Rac1 (Fig. 4A,B).

Population doubling time of QL mutant cells

To examine whether stable expression of active forms of RhoA, Rac1, and Cdc42 has an effect on fibroblast proliferation, the multiplication potential of the three clones was determined by DNA measurement at various time points (from 4 hrs to three days) after seeding the cells. Analysis of the growth curves (Fig. 5) indicated a population doubling time of 34 hrs for RhoA-QL, 32 hrs for Rac1-QL and 37 hrs for Cdc42-QL. Thus, there was no marked difference in the proliferation of fibroblasts stably expressing constitutively active forms of RhoA, Rac1 and Cdc42.

Discussion

The cytoskeleton plays a major role in the determination of cell shape and function and in the transmission of forces applied to cells (Ingber, 1997a). It has therefore been postulated that the cytoskeleton might allow cells to sense microgravity (Ingber, 1997b;1999). The cytoskeleton dynamics is regulated by small GTP-binding proteins of the Rho family (RhoA, Cdc42, Rac1) which control actin polymerisation. Specifically, activation of Rac1 and Cdc42 trigger de novo actin polymerisation in lamellipodia and filopodia, respectively, while activation of RhoA induces bundling of actin filaments into robust stress fibers (Nobes and Hall, 1995; Machesky and Hall, 1997). We therefore postulate that Rho GTPases might be crucial in cellular response to changes in gravity. Testing this hypothesis requires submitting live cells to extreme conditions such as those during space fligth. To this end, we have stably transfected SV40-transformed Wi26 fibroblasts with cDNAs for Cdc42, Rac1 and RhoA containing a glutamine to leucine mutation (Cdc42-Q61L, Rac1-Q61L and RhoA-Q63L). By abolishing intrinsic GTPase activity, these mutations impair the switch from GTP-bound to GDP-bound conformations and the mutant protein is maintained in a constitutively active form (Krengel et al., 1990). Several clones were obtained and the most pertinent ones were selected by pull-down assays of the active forms of the Rho-GTPases. These clones have now been serially propagated over several months, indicating that over-expression of GTP-bound active forms of Cdc42, Rac1 and RhoA does not alter long term cell viability. Moreover, analysis of

the proliferation potential of cloned cells indicates a similar cell doubling time for each of the three different clones.

The stable over-expression of active RhoA results in the formation of bundles of actin stress fibers as shown by immunofluorescence staining of fibrillar actin. This is underscored by our immunoblotting results indicating high levels of detergent-insoluble actin and vinculin in RhoA-QL cells. These results are in good agreement with previous reports showing that micro-injection of constitutively active RhoA or transient transfection with the corresponding cDNA induce the formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992; Chrzanowska-Wodnika and Burridge, 1996). By contrast, overexpression of active Rac1 is associated with lower amounts of detergent-insoluble actin and vinculin. Together with the fact that Rac1-QL cells have a poorly developed cytoskeleton and are rich in lamellipodia, it suggests that cells with high levels of active Rac1 are more deformable. Interestingly, Cdc42-QL cells have high amount of fibrillar actin and a low proportion of detergent-insoluble vinculin. It suggests that de novo polymerisation of actin reported to be associated with active Cdc42 leads to actin polymers which are more stable, hence detergent-insoluble, than those induced by active Rac1. In both cases, however, the low amounts of detergent-insoluble vinculin very likely reflect the immature stage of focal adhesions associated with Rac1 activation reported previously (Nobes and Hall, 1995).

By controling the organisation of the cytoskeleton and of cell-matrix adhesions, Cdc42, Rac1 and RhoA are thought to regulate the cell's mechanical functions, in particular the transmission of forces necessary for cell adhesion and movement and for the assembly and remodeling of the extracellular matrix (Chrzanowska-Wodnicka

and Burridge, 1996). We already know that manipulating mechanical load alters cell homeostasis (Mauch et al., 1989; Lambert et al., 1992;1998; Kessler et al., 2001). Thus, stable cell lines characterised by constitutive expression of active RhoA, Cdc42 or Rac1, represent suitable tools to monitor a rescue or an exacerbation of the microgravity-induced phenotype by mutant Rho GTPases. In particular, we plan to test the adhesion and migration properties of the cell lines generated in this study in future space experiments.

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References

Bourdoulous S, Orend G, MacKenna DA, Pasqualini R, Ruoslahti E (1998) Fibronectin matrix regulates activation of RHO and CDC42 GTPases and cell cycle progression. J Cell Biol 143: 267-276

Chrzanowska-Wodnicka M, Burridge K (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 133: 1403-1415

Clark EA, King WG, Brugge JS, Symons M, Hynes RO (1998) Integrin-mediated signals regulated by members of the rho family of GTPases. J Cell Biol 142: 573-586

Denhardt DT (1996) Oncogene-initiated aberrant signaling engenders the metastatic phenotype: synergistic transcription factor interactions are targets for cancer therapy. Crit Rev Oncog 7: 261-291

Deroanne CF, Colige AC, Nusgens BV, Lapiere CM (1996) Modulation of expression and assembly of vinculin during in vitro fibrillar collagen-induced angiogenesis and its reversal. Exp Cell Res 224: 215-223

Deroanne CF, Hamelryckx D, Ho TT, Lambert CA, Catroux P, Lapière CM, Nusgens BV (2005) Cdc42 downregulates MMP-1 expression by inhibiting the ERK1/2 pathway. J Cell Sci 118: 1173-1183

Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420: 629-635

Hall A (1992) Ras-related GTPases and the cytoskeleton. Mol Biol Cell 3: 475-479

Hotchin NA, Hall A (1995) The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. J Cell Biol 131: 1857-1865

Ingber DE (1997a) Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol 59: 575-599

Ingber DE (1997b) Integrins, tensegrity, and mechanotransduction. Gravit Space Biol Bull 10: 49-55

Ingber D (1999) How cells (might) sense microgravity. FASEB J 13: S3-15

Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, Eckes B (2001) Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. J Biol Chem 276: 36575-36585

Kjoller L, Hall A (1999) Signaling to Rho GTPases. Exp Cell Res 253: 166-179

Kozma R, Ahmed S, Best A, Lim L (1995) The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 15: 1942-1952

Krengel U, Schlichting L, Scherer A, Schumann R, Frech M, John J, Kabsch W, Pai EF, Wittinghofer A (1990) Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. Cell 62 :539-548

Lambert CA, Soudant EP, Nusgens BV, Lapiere CM (1992) Pretranslational regulation of extracellular matrix macromolecules and collagenase expression in fibroblasts by mechanical forces. Lab Invest 66: 444-451

Lambert ChA, Nusgens BV, Lapiere ChM (1998) Mechano-sensing and mechano-reaction of soft connective tissue cells. Adv Space Res 21: 1081-1091

Machesky LM, Hall A (1997) Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. J Cell Biol 138: 913-926

Mauch C, Hatamochi A, Scharffetter K, Krieg T (1988) Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. Exp Cell Res 178: 493-503

Moolenaar WH, van Meeteren LA, Giepmans BN (2004) The ins and outs of lysophosphatidic acid signaling. Bioessays 26: 870-881

Nobes CD, Hall A (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81: 53-62

Nobes CD, Hall A (1999) Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol 144: 1235-1244

Nobes CD, Hawkins P, Stephens L, Hall A (1995) Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J Cell Sci 108: 225-233

Ren XD, Schwartz MA (2000) Determination of GTP loading on Rho. Methods Enzymol 325: 264-272

Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389-399

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70: 401-410

Sander EE, ten Klooster JP, van Delft S, van der Kammen RA, Collard JG, 1999. Rac downregulates Rho activity: reciprocal balance between both GTPases determines cellular morphology and migratory behavior. J Cell Biol 147: 1009-1022

Legends to figures

Figure 1

Cloning strategy

The EcoRI and BamHI restriction sites within the multiple cloning sequence of the pIRESpuro vector (Clontech) were reversed using a linker with internal BamHI and EcoRI sites as indicated for pIRESpuro2. The cDNA of constitutively active GTPases (QL mutants of RhoA, Rac1 or Cdc42) cloned in pcDNA3 was ligated to the new BamHI and EcoRI restriction sites introduced by the linker to generate the pGTPase-IRESpuro plasmids.

Figure 2

Characterisation of QL mutant cells by pull-down of active GTPases.

Cells transfected with empty pIRESpuro vector (Control) and with the different pGTPase-IRESpuro vectors resulting in constitutive active QL mutation (QL) were serum-starved for 24 hrs and left untreated (Control and QL) or treated with 10% FCS for 5 minutes (Control + FCS) or with 20 ng/ml PDGF (Control + PDGF, QL + PDGF). After cell lysis, active forms of RhoA, Rac1 and Cdc42 were pulled-down using GST-PBD fusion protein with the Cdc42 and Rac1 binding region of PAK-1B or the GST-RBD fusion protein with RhoA binding region of rhotekin. The pulled-down active (upper blots) and total (lower blots) proteins were fractionated by SDS-PAGE and detected by immunoblotting with antibodies against RhoA, Rac1 and Cdc42 as indicated. The following clones are shown: 13H8 for RhoA-QL, 18E7 for Cdc42-QL and 15H10 for Rac1-QL.

Figure 3

Staining of fibrillar actin in QL mutant cells.

Cells transfected with empty pIRESpuro vector (Control) and the different pGTPase-IRESpuro vectors resulting in constitutive active QL mutation (RhoA-QL, clone 13H8; Rac1-QL, clone 15H10; Cdc42-QL, clone 18E7) were grown for 24 hrs on glass coverslips. After fixation, cells were incubated with FITC-conjugated phalloidin to visualise fibrillar actin. The stainings were observed and recorded by laser scanning confocal microscopy. Arrows indicate filopodia in Cdc42-QL, lamellipodia in Rac1-QL and bundles of actin stress fibers in RhoA-QL, respectively.

Figure 4

Subcellular distribution of actin and vinculin in QL mutant cells.

Cells expressing constitutive active QL mutation (RhoA-QL, clone 13H8; Rac1-QL, clone 15H10; Cdc42-QL, clone 18E7) were grown in the presence of FCS-containing medium. After overnight culture, the cells were lysed and fractionated with 0.1% Nonidet P-40. The content of vinculin and actin was measured in the detergent-soluble and -insoluble fractions by immunoblotting. A: immunoblot analysis of one representative experiment. B: ratio of the levels of soluble vinculin and actin compared to total amount of the proteins (detergent-soluble and -insoluble fractions). Columns represent mean + S.D. of two independent experiments.

Figure 5

Proliferation of QL mutant cells.

RhoA-QL, Rac1-QL and Cdc42-QL clones were plated in triplicate in 24-well plates and DNA content was measured in cell lysates after the indicated periods of time. Each assay point represents the mean average of triplicate wells and the DNA content is expressed in logarithmic arbitrary units.

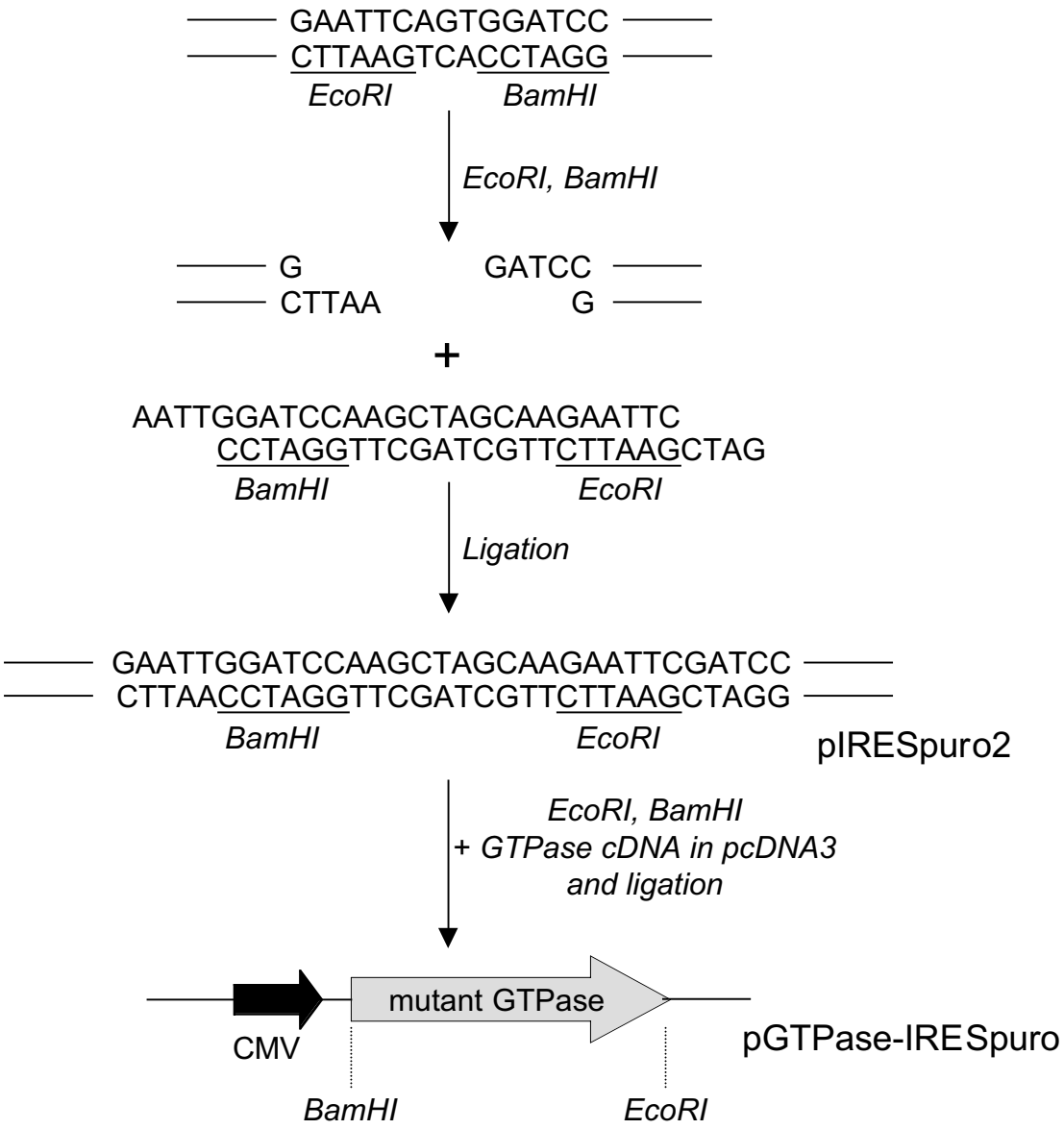
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Figure 1

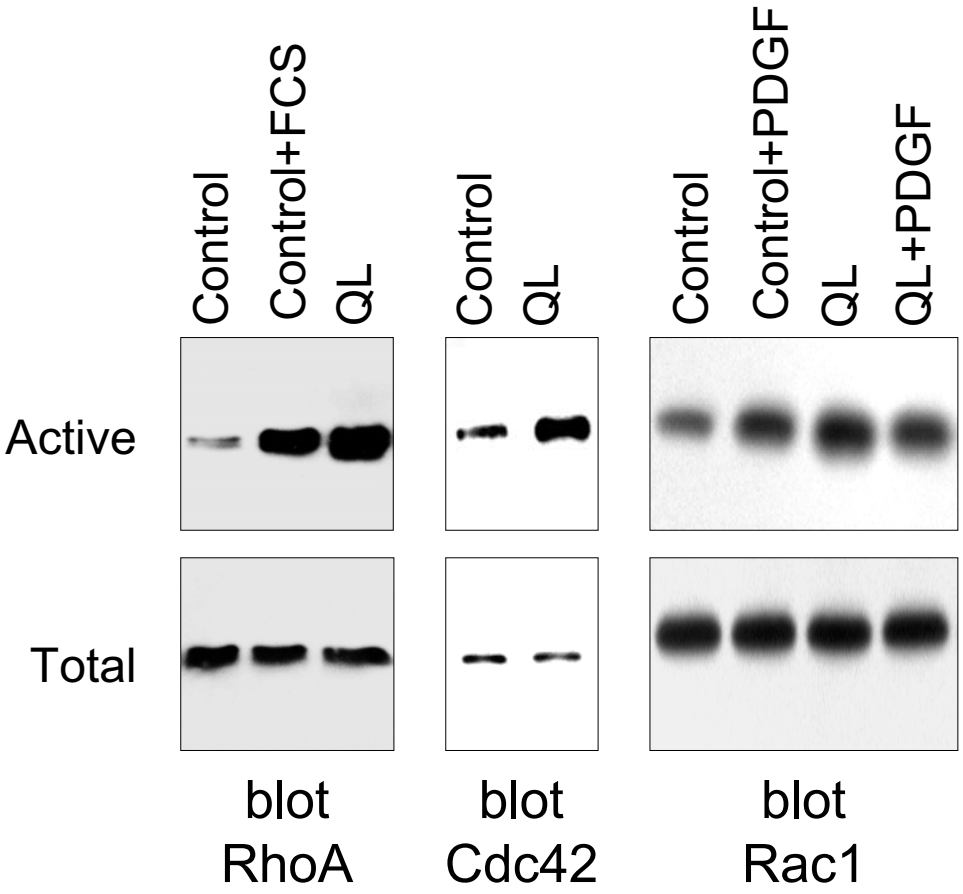
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Figure 2

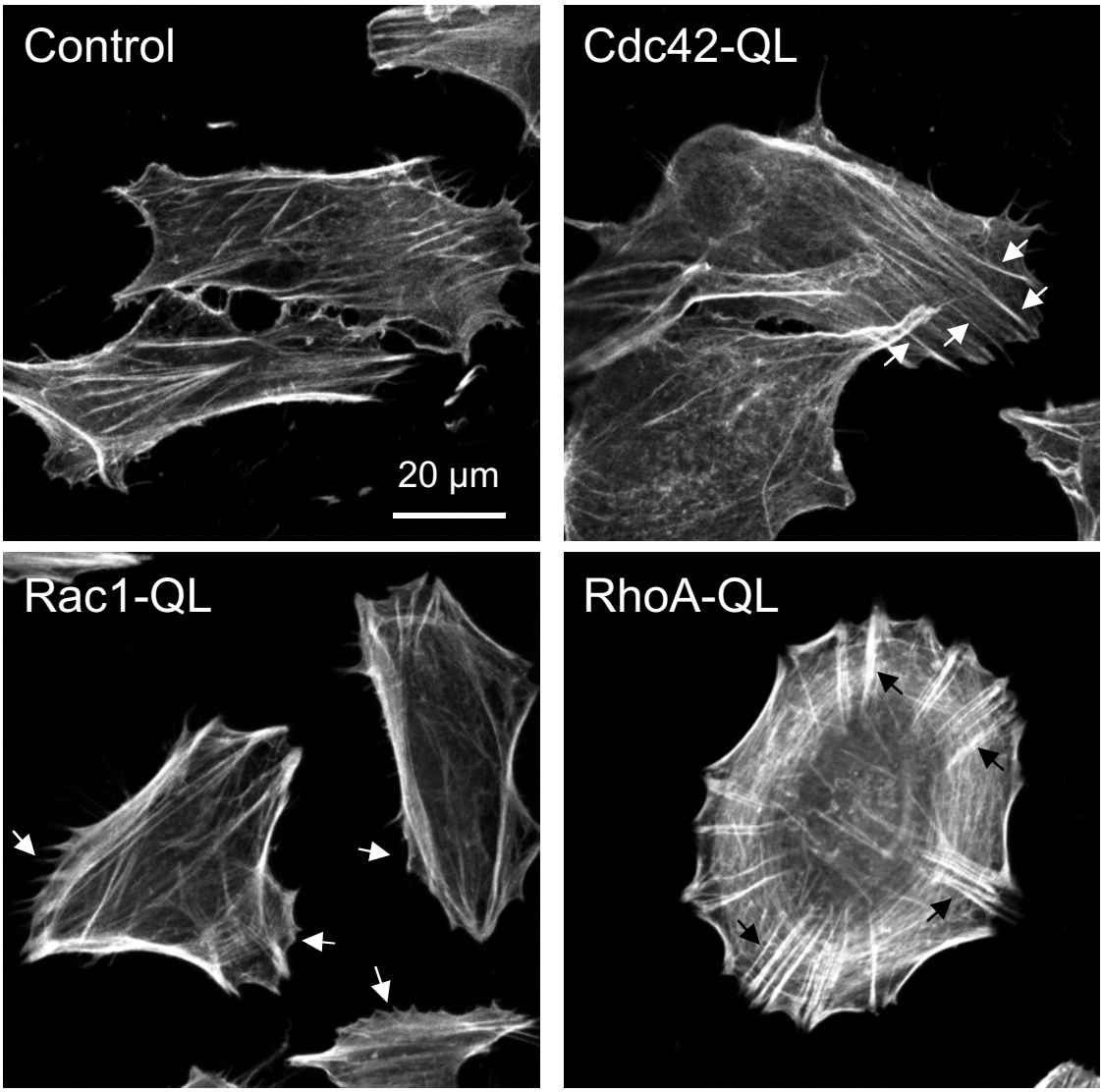

Figure 3

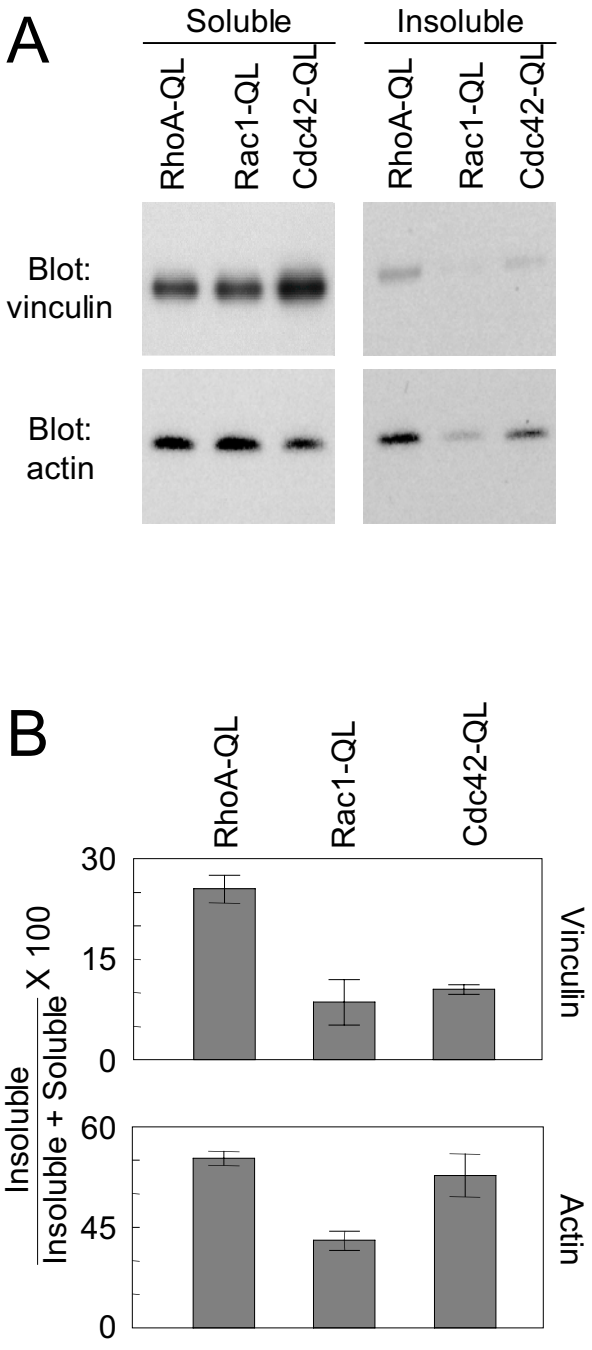
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Figure 4

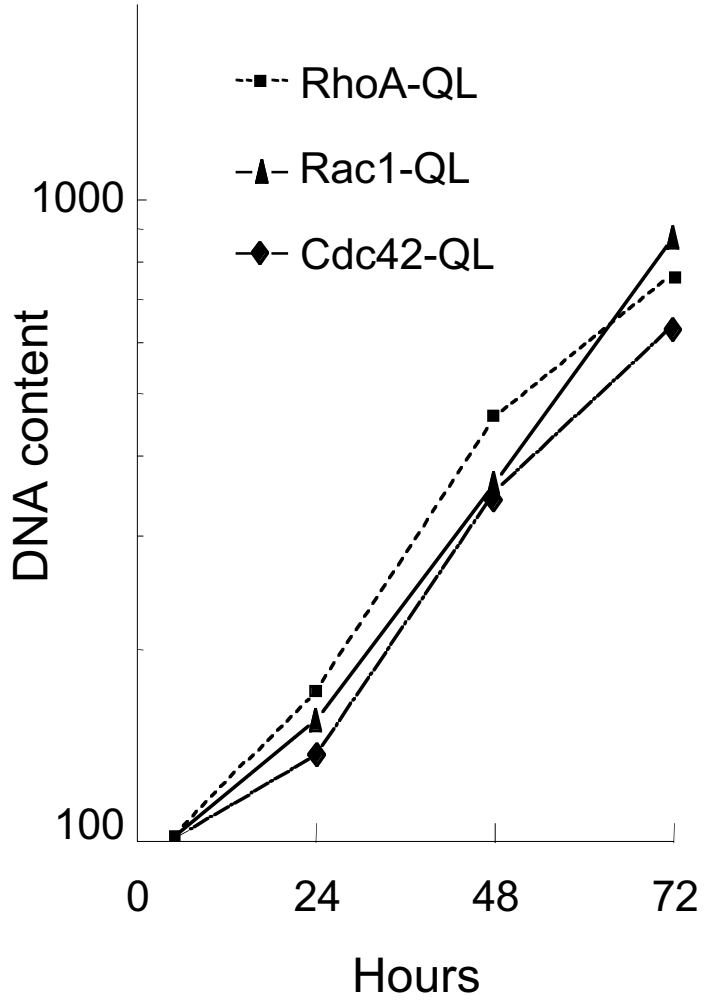

Figure 5