Effects of constitutively active GTPases on fibroblast behavior

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Abstract

The GTP-binding proteins RhoA, Cdc42 and Rac1 cooperatively regulate the organization and turnover of the cytoskeleton and cell adhesion structures. An important function of these structural elements bridging cells to their support is to translate and counter-balance forces, external or generated within the cell, in order to maintain cell shape and homeostasis. However, cooperation between the Rho GTPases may vary according to fundamental cellular activities such as adhesion, migration and matrix remodeling. To investigate these issues, we have used a panel of functional analyses to compare side-by-side stable lines of human fibroblasts expressing constitutively active (CA) RhoA, Cdc42 or Rac1. There was no marked effect of any of the CA GTPases on cell adhesion to different extracellular matrix proteins, including collagen I, fibronectin, laminin 1 and laminin 5. The spreading of CA RhoA, Rac1 and Cdc42 fibroblasts was specific for each active Rho GTPase and independent of the extracellular matrix proteins to which cells adhere. CA Rac1 and Cdc42 stimulated cell migration as observed by time-lapse video-microscopy, while CA RhoA dramatically restricted cell movement. The ability of cells to contract collagen lattices was retarded by CA RhoA.

Key words: RhoA, Rac1, Cdc42, QL mutants, fibroblast, adhesion, contraction, migration, cytoskeleton

Introduction

The Rho subfamily of small GTPases, in particular the archetypal trio RhoA, Rac1 and Cdc42, governs the dynamics of the actin cytoskeleton [1]. Thereby, these proteins regulate multiple cellular functions including cell migration, polarisation, survival and proliferation as well as the activation of transacting factors and their translocation to the nucleus and the trafficking and positioning of organelles [2]. As other small GTPases, the Rho GTPases operate as binary molecular switches by cycling between active, GTP-bound and inactive GDP-bound conformations [1, 3]. When GTP-bound, the GTPases are targeted to the cell membrane where they activate defined sets of effectors specific for each GTPase [4,5]. The Rho GTPases are activated in response to different stimuli including soluble factors such as growth factors and cytokines, and integrin-mediated interactions with extracellular matrix proteins [1,5]. Integrins are cell surface receptors integrating the biological and mechanical information from the extracellular matrix at specific sites of the cell membrane, the cell-matrix adhesions. There, the transmembrane integrins provide a physical link between extracellular matrix proteins and the intracellular cytoskeleton, allowing the transmission of mechanical forces necessary for cell adhesion and movement and for the assembly and remodeling of the extracellular matrix [6,7]. By controling the organization of the cytoskeleton and of cell-matrix adhesions, the trio of small GTPases Cdc42, Rac1 and RhoA regulates these integrin-linked mechanical functions [1,8].

The functions of the Rho GTPases and their signaling pathways have been mainly identified in experiments using specific dominant negative (displaying a higher affinity for GDP) or CA (loss of the intrinsic GTPase activity) mutants generated by

amino acid substitution [9-14]. Microinjection of CA RhoA in quiescent Swiss 3T3 fibroblasts induced the assembly of actin filaments into stress fibres while CA Cdc42 and Rac1 triggered de novo actin polymerization and the formation of filopodia and lamellipodia, respectively. By using cell migration as a model of integrated mechanical functions, it was shown that although each of the three Rho GTPases has a distinct function, their cooperation is critical in determining the final pattern of cytoskeleton organization. Cell migration is a multi-step process including the extension of a leading edge protrusion or lamellipodium, the establishment of new adhesion sites at the front, cell body contraction and detachment of adhesive structures at the cell rear. Achievement of each of these steps requires proper spatiotemporal regulation and defined cooperation of the three Rho GTPases [15]. However, how Rho GTPases cooperate may depend on the task endowed by the cell and may vary according to fundamental cellular activities such as adhesion, migration and matrix remodeling. To address this issue, we have generated lines of fibroblasts stably transfected with CA forms (Cdc42-Q61L, Rac1-Q61L and RhoA-Q63L) of RhoA, Rac1 and Cdc42 and selected the most pertinent clones [16]. Using these clones we here compare side-by-side the role of RhoA, Rac1 and Cdc42 during cell adhesion, migration and matrix remodeling.

Materials and Methods

Plasmids and generation of stable transfectants

Wi-26 cells (SV40-transformed human lung fibroblasts) transfected with the cDNA coding for the CA forms of RhoA (RhoA-Q63L), Rac1 (Rac1-Q61L) and Cdc42 (Cdc42-Q61L) in pIRESpuro vector (Clontech, Palo Alto, CA, USA) were sub-cloned by limited dilution and amplified as reported [16]. The cloned cells (hereafter referred to as RhoA-QL, Rac1-QL and Cdc42-QL) and the parental line transfected with the empty vector (hereafter referred to as control) were 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, penicillin, puromycin) at 37°C under 5% CO_2 except otherwise stated. All products were from Seromed/Biochrom (Berlin, Germany).

GTPase pull-down assays

Confluent cells were serum-starved for 24 hours and lysed as previously described [16]. Active GTPases were pulled-down from the lysates (500 µl) using either the GST-PBD fusion protein with the Cdc42 and Rac1 binding region of PAK-1B, or the GST-RBD fusion protein with the RhoA binding region of rhotekin [17,18]. The total lysate (40 µl) 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, 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 signals were visualized using ECL (Amersham Biosciences Europe, Freiburg, Germany).

Immunofluorescence staining

Cells grown on glass coverslips for 24 hrs in complete medium were fixed with freshly prepared 2% paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS) for 15 min, permeabilized 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 hour. The cells were labeled with either affinity purified rabbit antiserum against FHL2 [19] or mouse monoclonal antibody F-VII against vinculin (a gift from Dr. M. Glukhova, Institut Curie, Paris, France), followed by Cy3-conjugated secondary antibodies against rabbit or mouse immunoglobulins (Jackson, distributed through Dianova). Fibrillar actin was visualised with FITC-conjugated phalloidin (Sigma-Aldrich, Deisenhofen, Germany). The coverslips were mounted on histoslides and the stainings were observed by LASER scanning confocal microscopy (Leica, Heidelberg, Germany) with single channel excitation. The confocal images were acquired and stored using the Leica confocal software and mounted using Adobe Photoshop software.

Cell adhesion and spreading assays

Multiwell tissue culture plates (96-wells; Costar, Bodenheim, Germany) were

coated with collagen I (20 µg/ml; Seromed-Biochrom), laminin 1 (20 µg/ml; kindly provided by Dr. R. Timpl, Max-Planck Institut für Biochemie, Martinsried, Germany), fibronectin (40 µg/ml; Chemicon) or laminin 5 (5 µg/ml; [20]. After saturation of the wells with 1% BSA, equal numbers of control, Cdc42-QL, Rac1-QL and RhoA-QL cells were seeded in triplicate for 15, 30, and 60 minutes in FCS-free DMEM. At the end of the experiments, adherent cells were fixed, stained with crystal violet, and extent of adhesion was quantified by colorimetry as previously reported [21]. To monitor cell spreading, photographs of the adherent cells in triplicate wells were taken with a phase contrast microscope (Axiovert S100TV, Zeiss) equipped with a monochromatic digital camera (PowerShot G5, Canon Inc., Tokyo, Japan). Round and spread cells were counted for a population of at least 100 cells on each photograph.

Cell migration assays

Confluent cells were suspended by 0.05% trypsin and 0.02% EDTA in PBS, centrifuged and resuspended in FCS-containing DMEM at high cell density (10^6 cells/ml). Aliquots of the cell suspension (10µl) were deposited as colonies in the center of duplicate wells (one colony/well; 24-well tissue culture plates, Costar) and the cells were allowed to attach at 37°C in a humidified incubator. After one hour, the colonies were washed with PBS and the wells were filled with 400 µl of FCS-free medium. At this time point (T0), a first photograph of each colony was taken with an inverted phase contrast microscope (Zeiss Axiovert S100TV, Leipzig, Germany) equipped with a CCD camera (Xillix MicroImager, Richmond, British Columbia, Canada) and further photographs of the colonies were captured automatically every

10 min. Images were stored and processed using Openlab software (Improvision, Heidelberg, Germany). The sequences of images were converted to Quick Time movies to analyze cell migration tracks using Dynamic Image Analysis System software (Solltech Inc., Oakdale, USA). Extracted migration parameters included cell velocity (speed in µm/min) and processive index defined by the ratio between the linear and the absolute distances covered by a cell during the time of recording.

Collagen gel contraction

Cells were seeded in triplicate at a density of 1.5 x 10^5 cells/ml into 32 mm bacteriological dishes (2 ml/dish; Renner, Dannstatt, Germany) in DMEM supplemented with 10% FCS, Na-ascorbate (50 µg/ml), antibiotics and 0.3 mg/ml of newborn calf skin, acid extracted collagen I (IBFB-Pharma, Leipzig, Germany) as previously described [22]. Lattices were maintained for up to 48 hours in a humidified incubator at 37°C under 5% CO_2. Phase contrast micrographs were taken using an Olympus IX-81 microscope equipped with a monochromatic digital CCD camera.

Results

Basal Rho GTPase activities in RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts

Clonal stable lines of Wi-26 fibroblasts that express the CA forms of RhoA, Rac1 and Cdc42 (RhoA-QL, Rac1-QL, Cdc42-QL) generated by glutamine to leucine mutation have been established [16]. The mutation abolishes intrinsic GTPase activity and the protein should be locked in its GTP-bound, active conformation [3]. This was confirmed by pull-down assays demonstrating increased active forms of RhoA, Rac1 and Cdc42 in the selected clones [16] (Fig. 1A and B). Because cooperation between the three Rho GTPases may result in an activation cascade [5,23], such as Cdc42 activating Rac1, which in turn activates RhoA [10, 24], we measured the basal level of activity of each of the three GTPases in serum-starved mock-transfected parental cells (control) and selected QL clones (Fig. 1A and B). The basal level of GTP-bound RhoA was equally very low in the control, Cdc42-QL and Rac1-QL cells and as expected high in RhoA-QL cells. GTP-bound Cdc42 was also low in the parental, RhoA-QL and Rac1-QL cells, and significantly higher in the Cdc42-QL clone. The level of GTP-bound Rac1 in the RhoA-QL and Cdc42-QL clones was as low as in the parental mock-transfected parental cells and increased in the Rac1-QL clone.

Distribution of fibrillar actin and cellular adhesions in RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts

The effects of activated Rho GTPases on the organization of the actin

cytoskeleton and cellular adhesions have been extensively documented after transient transfection or micro-injection of active forms of the GTPases. Namely, active Cdc42 and Rac1 induce de novo actin polymerisation and cellular protrusions, i.e. filopodia and lamellipodia, respectively, while bundling of actin filaments into stress fibers occurs upon RhoA activation [9,10,14,24]. To determine whether these features are reproduced in the stable QL mutants that we have generated, we stained fibrillar actin and markers for nascent and mature adhesion complexes, FHL2 and vinculin, respectively. Observation of the staining by laser scanning confocal microscopy showed thin actin filaments in filopodia developed by Cdc42-QL cells as well as nascent and mature adhesion complexes at the cell periphery (Fig. 2). Cells expressing Rac1-QL had lamellipodia containing a layer of cortical actin and thin actin filaments while the distribution of FHL2 and vinculin was similar to that observed in Cdc42-QL cells (Fig. 2). In contrast, the RhoA-QL cells exhibited robust actin stress fibers and thick, strong vinculin-containing adhesion plaques, not only at the cell periphery but also over the entire ventral cell surface of the cells (Fig. 2). Thus each of the three different QL clones displayed specific cytoskeletal and cellular adhesion patterns as initially reported for Swiss 3T3 fibroblasts micro-injected with CA forms of RhoA, Rac1 and Cdc42 [9,10,24].

Adhesion and spreading of Cdc42-QL, Rac1-QL and RhoA-QL fibroblasts on extracellular matrix proteins

To examine whether the stable QL mutations affect integrin-mediated adhesion, the attachment of the three QL clones to collagen I, fibronectin, laminin 1 and laminin 5 was measured after different time points. These substrates were

chosen since they mediate cell adhesion by different integrins [25]. Furthermore, although there are many reports on the role of Rho GTPases in cellular interactions with fibronectin [26-28], much less information is available regarding cell adhesion to collagens and laminins. The Cdc42-QL cells showed higher adhesion efficiency to collagen I, fibronectin, laminin 1 and laminin 5 than the control, Rac1-QL and RhoA-QL cells (Fig. 3). The higher adhesion efficiency of the Cdc42-QL cells was observed already after 15 min of incubation on collagen I and fibronectin, and somewhat later on laminin 1 (30 min) and laminin 5 (60 min). The Rac1-QL cells had a lower adhesion efficiency to fibronectin, which was seen best after 30 and 60 min, and there was only little or no difference for their adhesion to the three other substrates when compared to control cells (Fig. 3). The adhesion profiles for RhoA-QL and control cells to collagen I, fibronectin and laminin 1 were similar, while a reduction in the adhesion to laminin 5 was observed after 60 min (Fig. 3). Next we examined the spreading developed by the cells on the different extracellular matrix proteins. A specific cell morphology started to show after 30 min and was seen best after 60 min (Fig. 4A-D). A distinct characteristic of the Cdc42-QL cells was that they formed clusters of round cells and had more cell-cell contacts than the two other QL mutants on all substrates. Digitally zoomed images clearly showed extended filopodia connecting adjacent Cdc42-QL cells (Fig. 4E). The Rac1-QL cells were polarized with a fan-shaped morphology and lamellipodia on all four substrates (Fig. 4E). Under all conditions tested, the RhoA-QL cells showed a well-spread, flat, unpolarized morphology, with the nucleus circumscribed by cytoplasm all around (Fig. 4E). Thus the spreading of RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts appears to be specific of each of the active Rho GTPase and independent of the extracellular matrix proteins to which cells adhere. In addition, a larger number of spread cells was

observed for the RhoA-QL and Rac1-QL than for the Cdc42-QL fibroblasts (Fig. 4F-I).

Migration of Cdc42-QL, Rac1-QL and RhoA-QL fibroblasts

Cell migration is critical for embryogenesis and development, and it continues to play an essential role in the adult organism, ranging from normal physiological activities, such as wound healing, to pathological situations as tumor invasion. Rho GTPases play a pivotal role in regulating the mechanical pathways most relevant to cell migration, with RhoA and Rac1 often exerting antagonistic effects by segregating actin filaments in different sub-compartments [15, 29]. To investigate the influence of CA RhoA, Rac1 and Cdc42 on migration, the different clones were monitored by time-lapse video microscopy for 1000 minutes. Frames recorded at 0, 400 and 800 min are shown in Fig. 5A and full recordings are available as supplementary material. Over 1000 min, Rac1-QL fibroblasts extended many lamellipodia (Fig. 5A and supplemental material) and had significantly moved away from their original position with a speed of 0.27 ± 0.06 μm/min and a processive index of 0.7 ± 0.1 (Fig. 5B,C), indicating persistant migration. The migratory behavior of Cdc42-QL cells was more complex, with single cells extending several filopodia quickly towards different directions and cell clusters migrating by extending protrusions at their leading edges (supplemental material). The migration parameters of Cdc42-QL cells were however not different from that of mock-transfected cells with a velocity of 0.15 ± 0.06 versus 0.15 ± 0.05 μm / min and a processive index of 0.4 ± 0.2 versus 0.5 ± 0.2 (Fig. 5B,C). In contrast, RhoA-QL fibroblasts were still very close to the original position after 1000 min, displaying a velocity of 0.06 ± 0.02 μm / min and a processive index of 0.2

± 0.1 (Fig. 5B,C).

Contraction of collagen lattices by Cdc42-QL, Rac1-QL and RhoA-QL cells

Cultivating fibroblasts within floating collagen lattices results in the contraction of the gels by the cells over time [30]. This model is best suited to study the mechanical properties of cells within a tridimensional network and to test the integrity of integrin-mediated interactions between the extracellular matrix and the cytoskeletal system [7]. The different clones of QL fibroblasts were suspended into lattices of native collagen I, and the extent of contraction quantified by measuring gel diameter over time. Compared to control cells, the contraction of the collagen gels was delayed for all three QL mutants (Fig. 6A). A delay of about 5 hours was observed before the Cdc42-QL and Rac1-QL mutants started to contract the gels. Thereafter contraction kinetics were similar for the two lines and, in spite of the delay, contraction nevertheless reached control values after 48 hours (Fig. 6A). In contrast, the RhoA-QL cells displayed negligible contraction for 24 hours and did not achieve at 48 hours the same extent of gel contraction as the controls and the two other QL cell lines (Fig. 6A). Within the contracting gels, the morphology of Cdc42-QL and Rac1-QL fibroblasts (Fig. 6C and D) was similar to that of the mock-transfected parental cells(Fig. 6B), with multiple inter-connected cellular extensions. In contrast, RhoA-QL fibroblasts embedded within the collagen lattices differed significantly from the other cells as they remained round and isolated (Fig. 6E).

Discussion

The role of the Rho GTPases Cdc42, Rac1 and RhoA in the organization of the actin cytoskeleton has been well established at a single cell level by transient overexpression or microinjection of recombinant dominant negative or CA forms in 3T3 fibroblasts [1]. In this report we have used lines of human fibroblasts stably transformed with CA forms of the three Rho GTPases to investigate by a panel of functional tests the behavior of clonal cell populations. This strategy has several advantages as compared to transient overexpression of genes encoding the GTPases or to microinjection of the recombinant proteins. The activity of the GTPases is similar to that observed in parental cells upon induction with the cognate agonists. Furthermore, besides the fact that all cells express the transgene, what is rarely the case in transient expression experiments, nearly unlimited number of identical cells can be obtained.

Locking Rho GTPases in the GTP-bound, active state can be achieved by mutating glycine in position 12 (Rac1, Cdc42) or 14 (RhoA) to valine (G12V or G14V), or alternatively by substituting glutamine 61 (Rac1,Cdc42) or 63 (RhoA) to leucine (Q61L or Q63L). These two mutated variants have mostly been used interchangeably, but distinct differences in cellular behavior have been described for the two forms of CA RhoA [31,32]. Both mutations, G14V and Q63L, are located in nucleotide-binding pockets and interfere with hydrolysis of the γ-phosphate of GTP, rendering the protein constitutively active. Although the two mutants have an overall high degree of structural similarity, the affinity for the inhibitory accessory protein RhoGDI is lower for the G14V than Q63L mutant, suggesting that RhoA-Q63L functions as a more active constitutive mutant than RhoA-G14V [33]. Consistent with

previous observations, CA RhoA-Q63L induced actin assembly into stress fibres and focal contacts while CA Cdc42 and Rac1 triggered the formation of filopodia and lamellipodia, respectively. The cytoskeletal modifications induced by stable expression of CA Rho-GTPases are compatible with fibroblast survival and proliferation [16]. Furthermore, as shown in this report, locking one of the three GTPases into its active GTP-bound conformation did not affect the basal level of activity of the two others. Together these observations demonstrate that the Cdc42-QL, Rac1-QL and RhoA-QL cell lines represent suitable models to compare side-by-side the role of the three GTPases in functional assays involving their mechanical properties.

Rho GTPases were shown to be important for cell adhesion, in particular Rac1 and RhoA enhance integrin clustering, thereby increasing adhesive strength, however without changing integrin affinity for extracellular matrix ligands [5,34]. In fibroblasts, distinct sets of integrins initiate adhesion to different extracellular matrix proteins, for example $\alpha 1\beta 1$, $\alpha 2\beta 1$ and $\alpha 11\beta 1$ for collagen I, $\alpha 2\beta 1$ and $\alpha 6\beta 1$ for laminin 1, $\alpha 3\beta 1$ for laminin 5 and $\alpha 5\beta 1$ for fibronectin. Overexpression of CA RhoA-QL and Rac1-QL had no marked effect on the kinetics and overall fibroblast adhesion these extracellular matrix proteins. In contrast, the number of Cdc42-QL adherent cells was distinctly increased on all substrates. Enhanced cell adhesion to extracellular matrix proteins has been proposed to result from increased receptor affinity or post-receptor events involving cell spreading and/or integrin clustering [35,36]. However, microscopic monitoring of the morphology of adherent cells revealed that a large proportion of Cdc42-QL fibroblasts were round and associated into clusters, which was not the case for RhoA-QL and Rac1-QL cells. As also observed in the migration assays, Cdc42-QL cells did not migrate as individual cells,

but as groups of cells. It strongly suggests that the enhanced number of Cdc42-QL adherent cells resuts from cell-cell interaction rather than increased adhesion to the substrates. This is consistent with the role of Cdc42 in promoting cell-cell adhesion by extending filopodia [37, 38] and in the regulation of E-cadherin-mediated cell-cell adhesion [39]. The spreading patterns of Cdc42-QL, Rac1-QL and RhoA-QL fibroblasts appeared to be specific for each of the active GTPase that act as downstream effectors of integrin-mediated adhesion independently of the extracellular matrix proteins to which cells adhere.

Adhesion to extracellular matrix proteins is an essential step for cell migration, a process required in many physiological and pathological conditions. We therefore examined how the alterations in fibroblast adhesive properties and focal adhesion distribution would impact on migration of the individual GTPase mutants. Cell migration is complex and needs the cooperation of all three Rho GTPases [15]. In particular, Cdc42 is required for cell polarity, Rac1 for the protrusion of lamellipodia at the cell leading edge and forward movement, while RhoA maintains cell adhesion during movement without the need of stress fibers and focal adhesion [14]. Our study shows that RhoA-QL fibroblasts cannot migrate, which agrees with a recent report [40]. On the contrary, cells with constitutive expression of active Rac1 move faster than the parental cells. Immunofluorescence analyses showed that RhoA-QL fibroblasts displayed very strong actin stress fibres and focal adhesions all over the ventral surface. It suggests that CA RhoA inhibits the turnover of focal adhesions, a process necessary for detachment of the rear of the cells to enable locomotion. Alternatively, the increased number in focal adhesions observed in RhoA-QL cells may not transmit adequate traction for cell movement. Indeed, it has been shown that small adhesions transmit strong propulsive tractions, whereas mature focal

adhesions exert weaker forces [41]. Moreover, the collagen gel contraction assays clearly demonstrated that RhoA-QL mutants are impaired in their contractile capacities in addition to altered focal adhesion architecture and failure to extend cellular protrusions inter-connecting adjacent cells. These properties are required for the transmission of mechanical signals from the extracellular environment through focal adhesions to the actin cytoskeleton. Lattice contraction further requires cell locomotion along collagen fibers, another feature, which we found impaired in RhoA-QL mutant fibroblasts. Apparently, our results are in contrast to the report by Chrzanowska-Wodnicka and Burridge [12], who described increased contractility following RhoA activation by introducing the G14V mutation into 3T3 fibroblasts. However, the models used here, a three-dimensional collagen network, and in their study, wrinkling of silicone rubber membranes are different. In our model, the tridimensional collagen network lacks mechanical loading, while the other is a gold-coated, planar geometry, which confers different mechanical cues to the cells. This interpretation is supported by findings that contraction of floating collagen lattices in the presence of serum does not require Rho kinase activity, while that of cells in a more constrained mechanical environment does [42]. Moreover, recent studies showed that Rho activity is regulated in a feedback manner by mechanical forces with contraction of floating lattices being paralleled by a decrease in active Rho [43]. It suggests that Rho activity during collagen lattice contraction is not an on/off response, but rather requires tight temporal and spatial regulation. We think that in our RhoA-QL fibroblasts, the critical balance between inactive and active RhoA and regulation of the activity of the associated factors is no longer maintained, resulting in particular in a non-dendritic shape, locomotive defect and the inability to compact and remodel collagen fibers proximal to the cell surface.

Taken together, the QL mutants of RhoA, Rac1 and Cdc42 used in this study displayed the expected cytoskeletal changes characteristic of each GTPase. The side-by-side comparison of all three mutants revealed that there was no influence of one onto the expression of the other two GTPases in the fibroblast transformants. Distinct alterations were observed with respect to spreading morphology, migration behavior and collagen lattice remodelling. These were specific for each of the three GTPases with most severe differences from controls seen in the RhoA-QL mutants.

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Legends to figures

Figure 1

Basal levels of active GTPases in RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts

Cells were serum-starved for 24 hours and the activity of RhoA, Rac1 and Cdc42 was measured by pull-down assays in parental mock-transfected cells (control) and in the RhoA-QL, Rac1-QL and Cdc42-QL clones as indicated in A. Signals obtained for the pull-down samples correspond to 800 µl of lysate for the blots with anti-RhoA and anti-Cdc42 and 500 µl of lysate for the blot with anti-Rac1. For the crude lysates, signals correspond to 40 µl. B, Band intensity in the blots shown in A were determined by densitometry and the levels of pull-down active GTPases were normalized to the total amount of GTPase in the crude lysate.

Figure 2

Fibrillar actin and focal adhesions in RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts.

Mock-transfected parental cells (control), RhoA-QL, Rac1-QL and Cdc42-QL cells were seeded on glass coverslips and grown for 24 hours in complete medium. Nascent and mature adhesion complexes were stained with antibodies against FHL2 (panel A, a-d, red) and vinculin (panel B, a-d, red), respectively. Fibrillar actin was visualized with phalloidin-FITC (panel A and B, green, a'-d'). Arrows indicate filopodia (b', Cdc42-QL), lamellipodia (c', Rac1-QL) and actin stress fibers (d', RhoA-QL).

Figure 3

Adhesion of RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts to extracellular matrix proteins.

Equal numbers of control (white columns), Cdc42-QL (black columns), Rac1-QL (hatched columns), and RhoA-QL (grey columns) cells were seeded in triplicate wells coated with optimal concentrations of collagen I (A; 20 g/ml), fibronectin (B, 40 µg/ml), laminin 1 (C; 20 µg/ml) and laminin 5 (D; 5 µg/ml). The extent of cell adhesion to the different substrates was measured after 15, 30 and 60 minutes by crystal violet staining and colorimeric measurement. The mean absorbance of triplicate wells and standard deviations are shown. The numbers of Cdc42-QL mutant cells adhering on the different substrates were higher than either the control or the two other QL mutants. After 60 minutes, the adhesion of Rac1-QL cells to fibronectin and of RhoA-QL cells to laminin 5 was decreased in comparison to the other clones.

Figure 4

Spreading of RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts on extracellular matrix proteins.

After 15, 30 and 60 minutes of adhesion to collagen I (A), fibronectin (B), laminin 1 (C) and laminin 5 (D), Cdc42-QL, Rac1-QL and RhoA-QL cells were stained with cristal violet and photographed under phase contrast microscopy at an original magnification of 200 X. Details of the specific morphology of the QL mutant cells are shown at higher magnification in E. Spreading of control (white columns), Cdc42-QL (black columns), Rac1-QL (hatched columns), and RhoA-QL (grey columns) on collagen I (F), fibronectin (G), laminin 1 (H) and laminin 5 (I), was quantified by counting the number of round and spread cells on photographs. At least 100 cells /

photograph were counted and each column represents the mean average of counts and standard deviation determined on three photographs for each condition.

Figure 5

Migration of RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts.

Small colonies (10 µl) of Cdc42-QL, Rac1-QL and RhoA-QL cells were seeded in serum-containing medium in the center of tissue culture wells and allowed to attach at 37˚C. After one hour, the cells were washed several time with serum-free medium and the wells were filled with serum-free medium. At this point, cell migration was recorded at the border of the colonies by time-lapse videomicroscopy for 1000 minutes, with one picture every 10 minutes. A. Frames onset of the recording (T0, a-c) and after 400 (T400, a'-c') and 800 (T800, a''-c'') minutes are shown. Black arrows indicate the initial position of selected cells and white lines the movement and final position of those cells at T400 and T800. Single cell trackings was used to determine cell velocity (B) and processive index (C) of the different clones as described in Materials and methods.

Figure 6

Contraction of collagen gels by RhoA-QL, Rac1-QL and Cdc42-QL fibroblasts.

A. Time course of collagen gel contraction. Equal numbers of control (empty square), Cdc42-QL (empty circles), Rac1-QL (black square), and RhoA-QL (black circles) cells were seeded within triplicate gels of collagen I. The time course of collagen gel contraction was monitored by photographing the gels at successive time intervals as indicated in the figure. The gel diameters were measured and plotted as a function of time. Each point represents the average of three independent experiments. Gel

contraction is delayed for the three QL clones, the largest delay being observed for the RhoA-QL cells. B. Morphology of mock-transfected parental (B), Cdc42-QL (C), Rac1-QL (D) and RhoA-QL (E) cells embedded within the gel of collagen I.

Supplementary material (Quick Time movies)

Time lapse videomicroscopy recording of cell movement for RhoA-QL (RhoA movie), Rac1-QL (Rac1 movie) and Cdc42-QL (Cdc42 movie) clones. Sequential images were stored using Openlab software and used to create Quick Time movies.

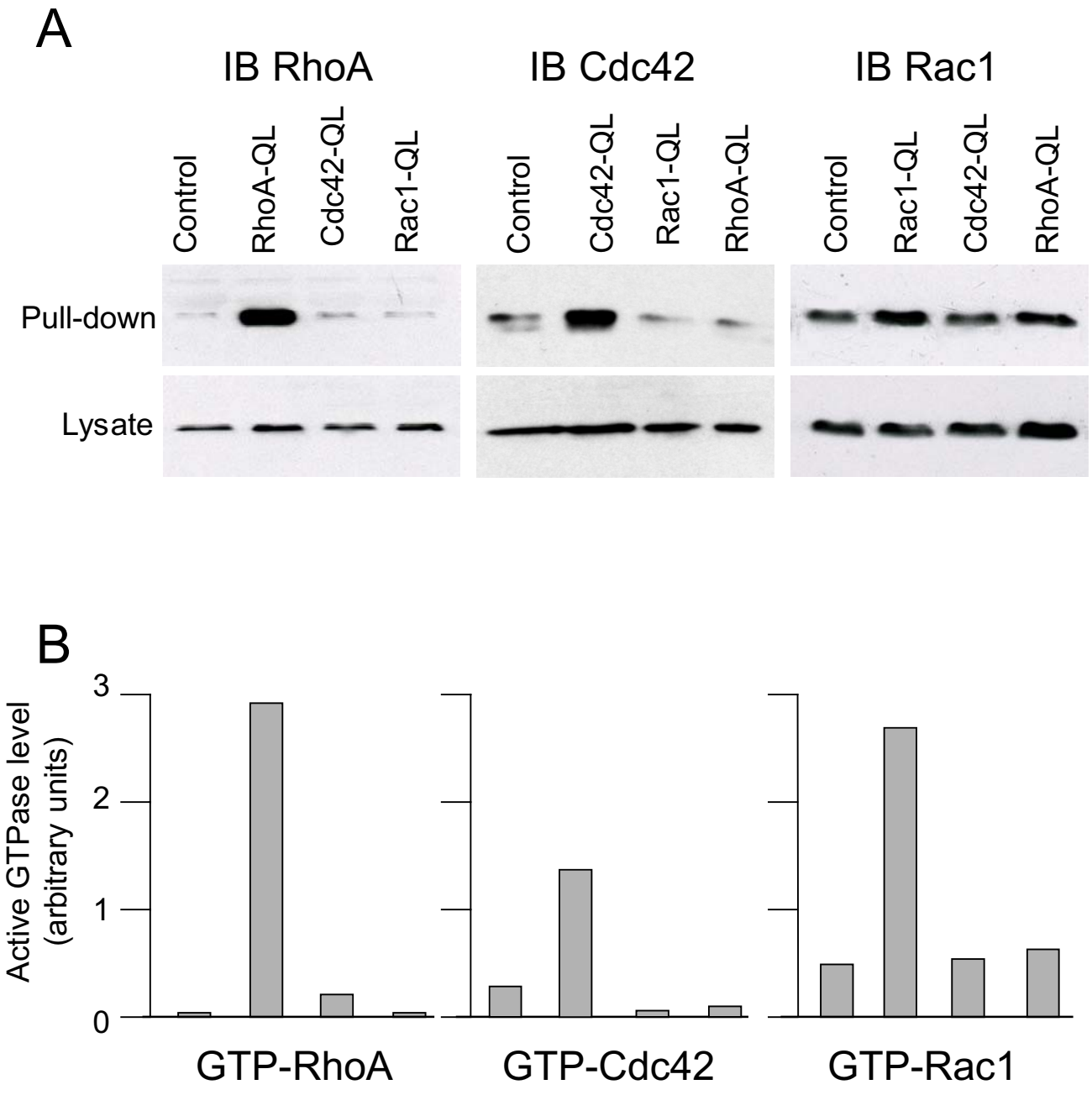
A
IB RhoA
IB Cdc42
IB Rac1
Control
RhoA-QL
Cdc42-QL
Rac1-QL
Control
Cdc42-QL
Rac1-QL
RhoA-QL
Control
Rac1-QL
Cdc42-QL
RhoA-QL
Pull-down
Lysate
B
Active GTPase level
(arbitrary units)
3
2
1
0
GTP-RhoA
GTP-Cdc42
GTP-Rac1

Figure 1

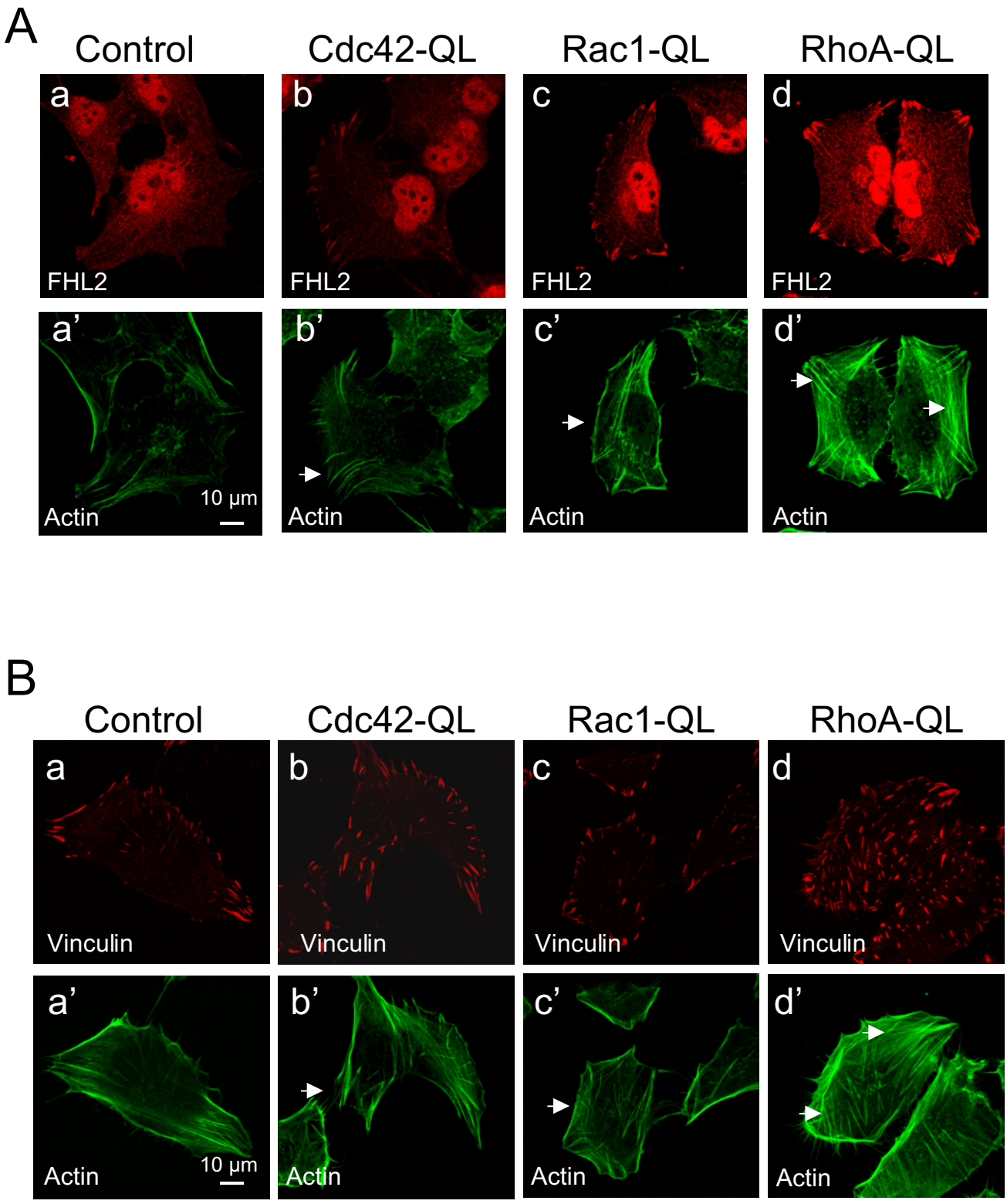
A
Control
Cdc42-QL
Rac1-QL
RhoA-QL
a
b
c
d
FHL2
FHL2
FHL2
FHL2
a'
b'
c'
d'
10 μm
Actin
Actin
Actin
Actin
B
Control
Cdc42-QL
Rac1-QL
RhoA-QL
a
b
c
d
Vinculin
Vinculin
Vinculin
Vinculin
a'
b'
c'
d'
10 μm
Actin
Actin
Actin
Actin

Figure 2

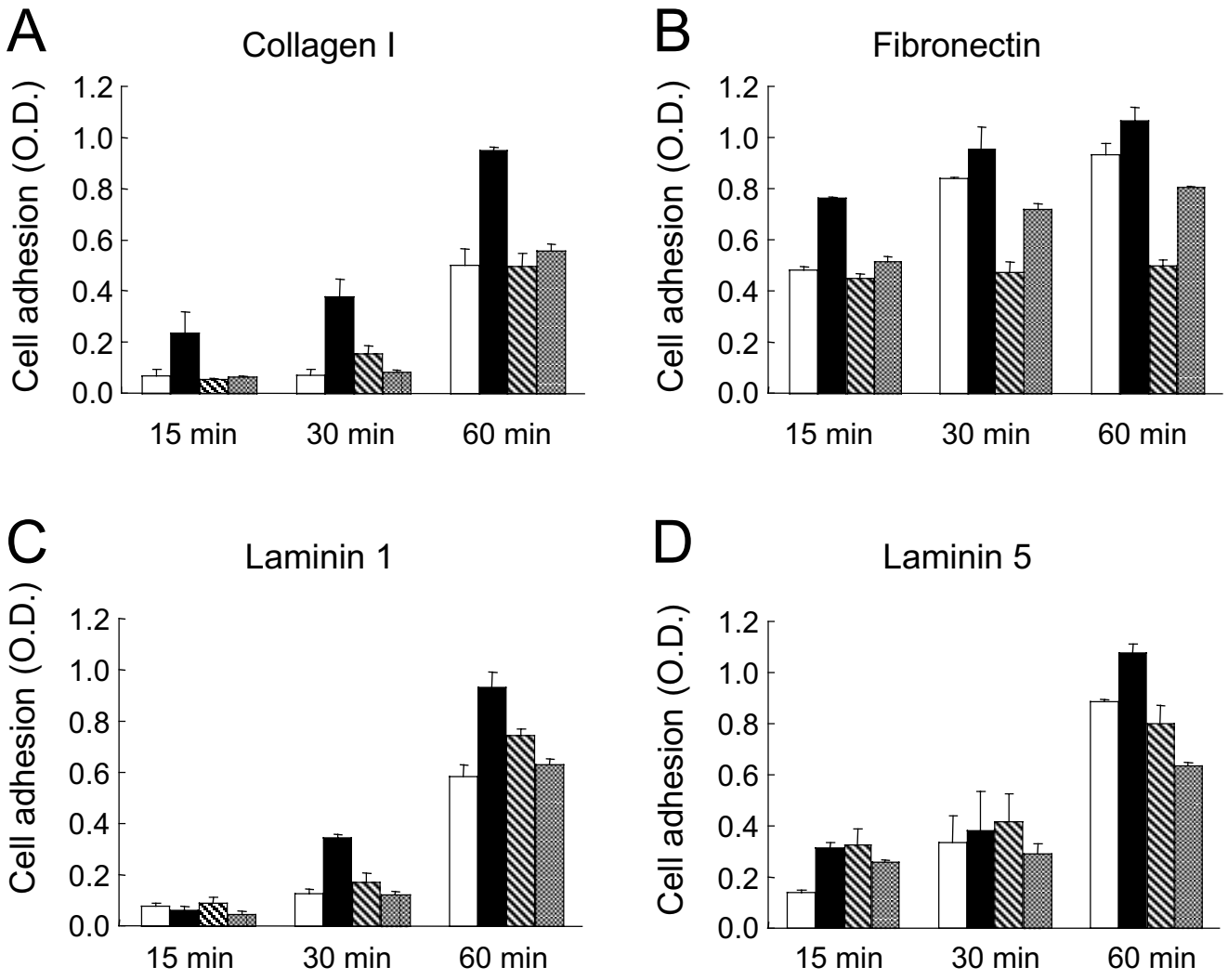
A
Collagen I
Cell adhesion (O.D.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15 min
30 min
60 min
B
Fibronectin
Cell adhesion (O.D.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15 min
30 min
60 min
C
Laminin 1
Cell adhesion (O.D.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15 min
30 min
60 min
D
Laminin 5
Cell adhesion (O.D.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15 min
30 min
60 min

Figure 3

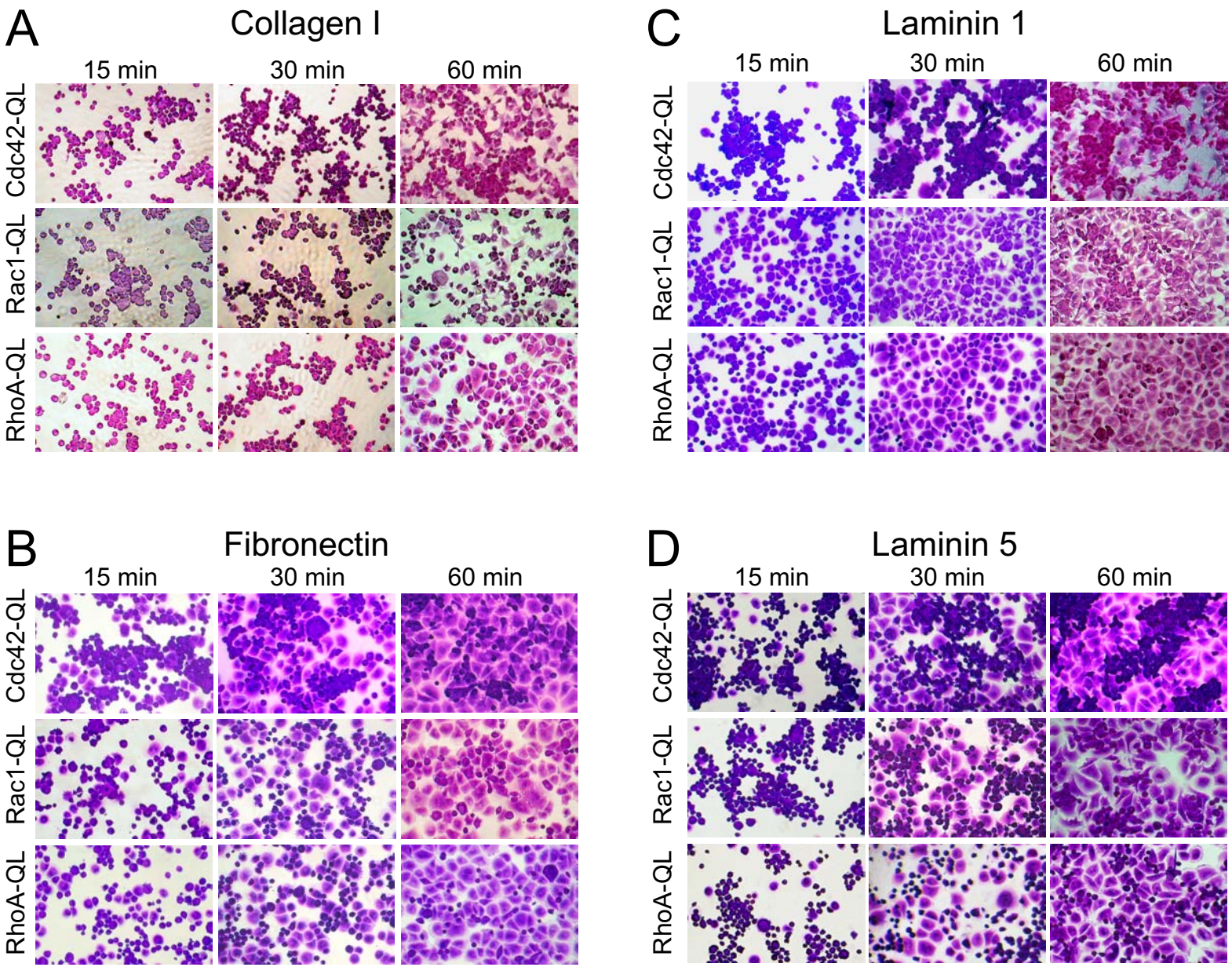
A
Collagen I
15 min
30 min
60 min
Cdc42-QL
Rac1-QL
RhoA-QL
C
Laminin 1
15 min
30 min
60 min
Cdc42-QL
Rac1-QL
RhoA-QL
B
Fibronectin
15 min
30 min
60 min
Cdc42-QL
Rac1-QL
RhoA-QL
D
Laminin 5
15 min
30 min
60 min
Cdc42-QL
Rac1-QL
RhoA-QL

Figure 4

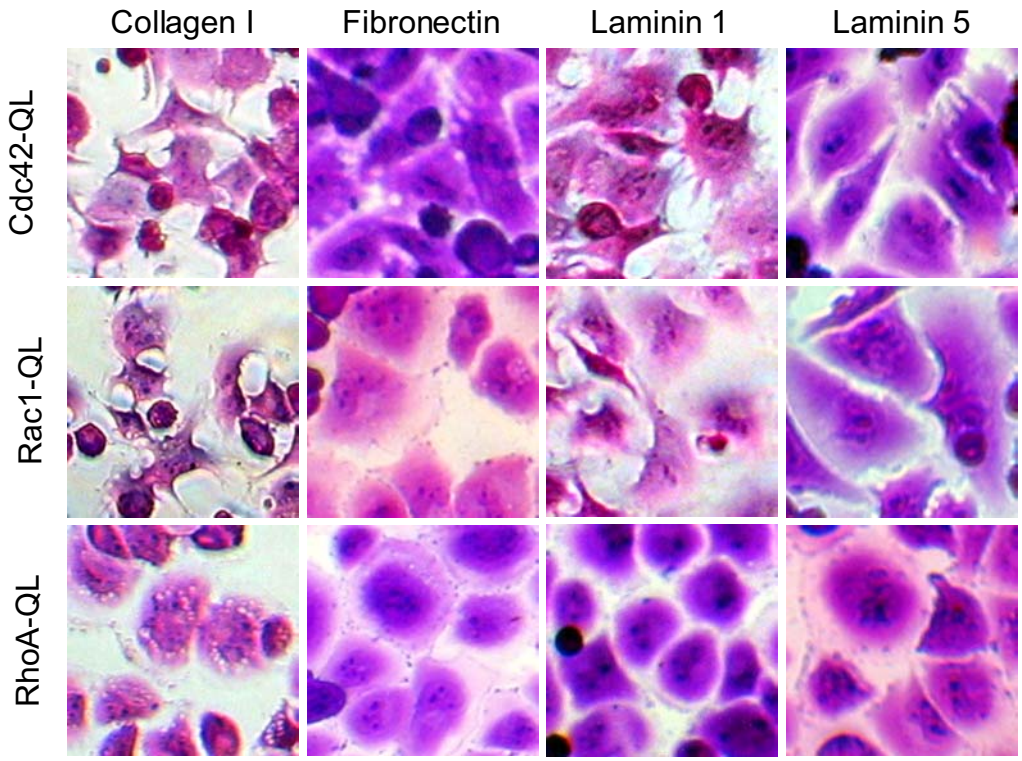
Collagen I
Fibronectin
Laminin 1
Laminin 5
Cdc42-QL
Rac1-QL
RhoA-QL

Figure 4E

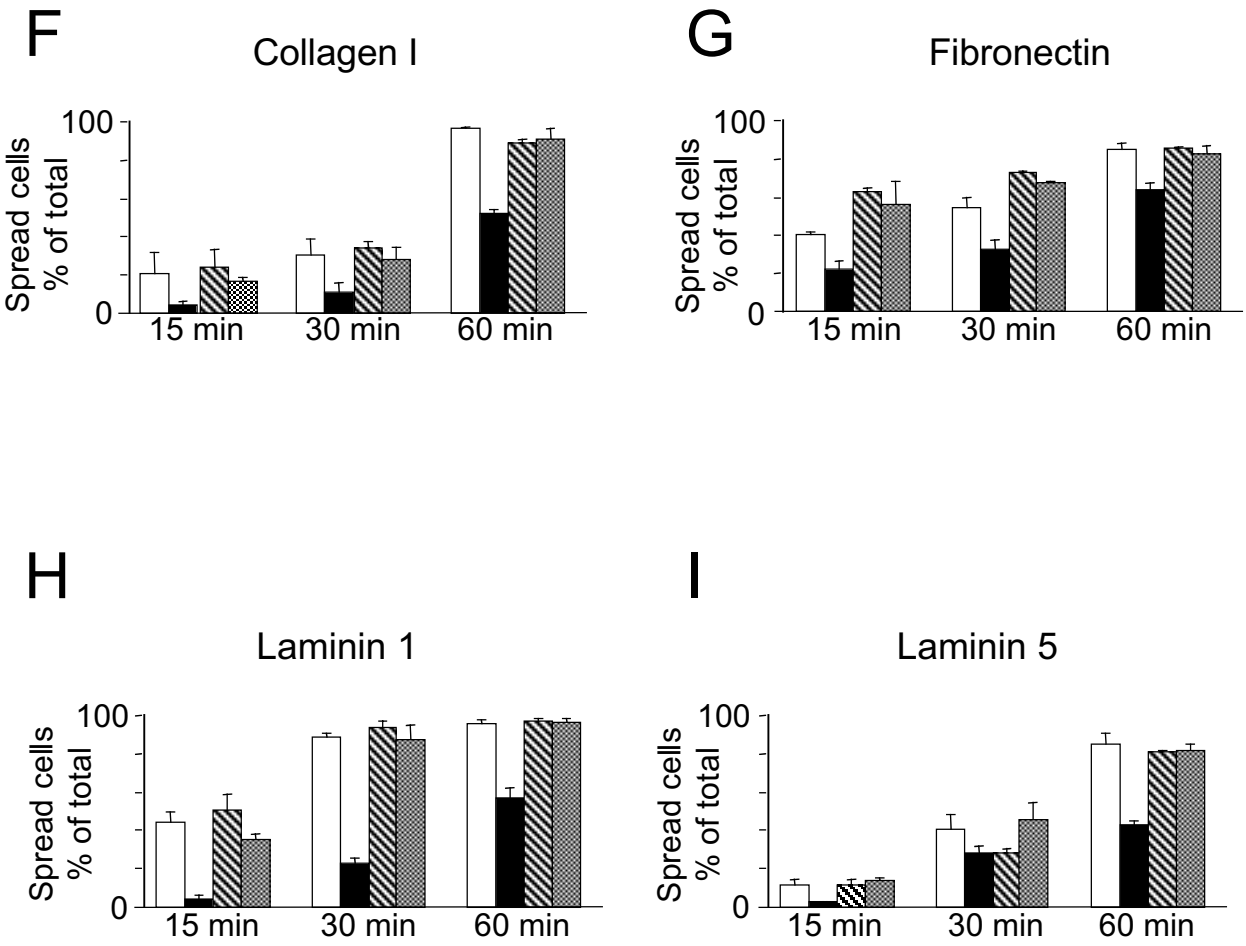
F
Collagen I
Spread cells
% of total
100
0
15 min
30 min
60 min
G
Fibronectin
Spread cells
% of total
100
0
15 min
30 min
60 min
H
Laminin 1
Spread cells
% of total
100
0
15 min
30 min
60 min
I
Laminin 5
Spread cells
% of total
100
0
15 min
30 min
60 min

Figure 4

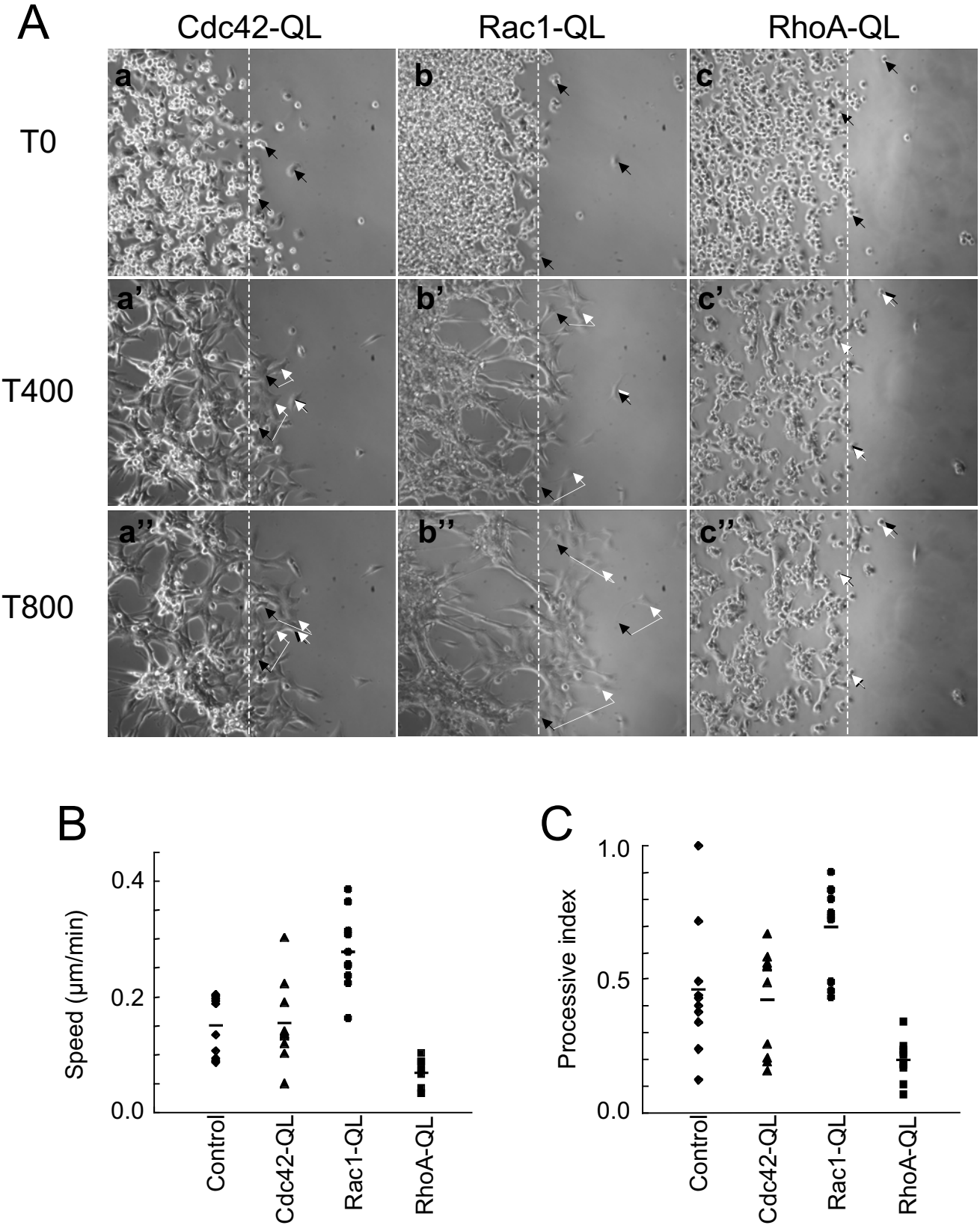
A
Cdc42-QL
Rac1-QL
RhoA-QL
T0
T400
T800
a
b
c
a'
b'
c'
a''
b''
c''
B
Speed (μm/min)
0.0
0.2
0.4
Control
Cdc42-QL
Rac1-QL
RhoA-QL
C
Processive index
0.0
0.5
1.0
Control
Cdc42-QL
Rac1-QL
RhoA-QL

Figure 5

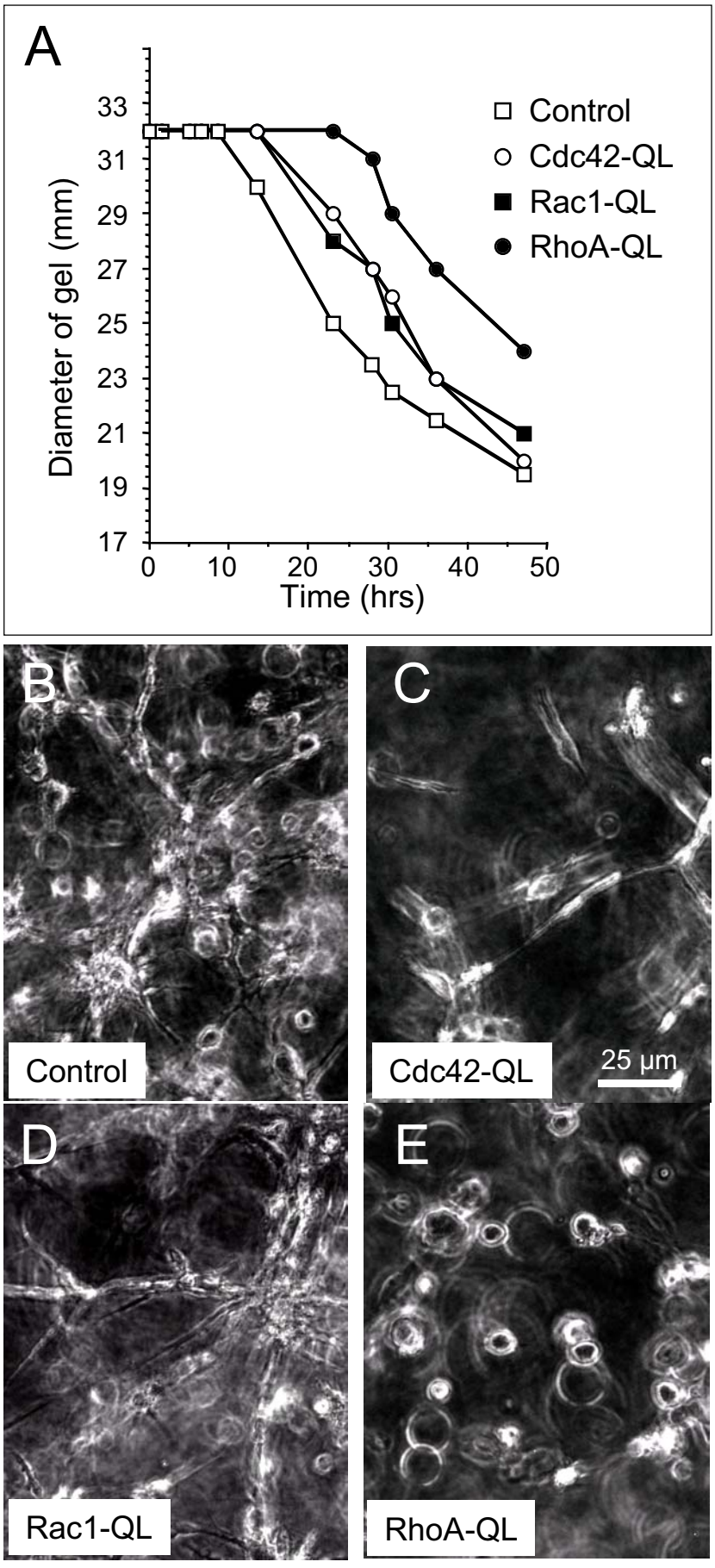
A
Diameter of gel (mm)
33
31
29
27
25
23
21
19
17
0
10
20
30
40
50
Time (hrs)
Control
Cdc42-QL
Rac1-QL
RhoA-QL
B
Control
C
Cdc42-QL
25 µm
D
Rac1-QL
E
RhoA-QL

Figure 6