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Dual function of rhoD in vesicular movement and cell motility

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The trafficking of intracellular membranes requires the coordination of membrane-cytoskeletal interactions. Rab proteins are key players in the regulation of vesicular transport, while Rho family members control actin-dependent cell functions. We have previously identified a rho protein, rhoD, which is localized to the plasma membrane and early endosomes. When overexpressed, rhoD alters the actin cytoskeleton and plays an important role in endosome organization. We found that a rhoD mutant exerts its effect on early endosome dynamics through an inhibition in organelle motility. In these studies, the effect of rhoD on endosome dynamics was evaluated in the presence of a constitutively active, GTPase-deficient mutant of rab5, rab5Q79L. As rab5Q79L itself stimulates endosome motility, rhoD might counteract this stimulation, without itself exerting any effect in the absence of rab5 activation. We have now addressed this issue by investigating the effect of rhoD in the absence of co-expressed rab5. We find that rhoDG26V alone alters vesicular dynamics.
Vesicular movement, in particular the endocytic/recycling circuit, is altered during processes such as cell motility. Due to the participation of vesicular motility and cytoskeletal rearrangements in cell movement and the involvement of rhoD in both, we have addressed the role of rhoD in this process and have found that rhoDG26V inhibits endothelial cell motility.

Abbreviations. BBCE cells Bovine brain capillary endothelial cells. – TLVM Time lapse video microscopy.

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Introduction

Cellular organelles have a characteristic intracellular distribution that depends on specific membrane-cytoskeleton interactions. In the early 1970s, calculations considering only Brownian motion failed to account for the rapid and long-range movements of cytoplasmic organelles (reviewed in (Rebhun, 1972)), implying that vesicular movement requires an active motive force. It is now well established that microfilaments and microtubules together with their associated motors are involved in governing the motility and morphology of cellular organelles as well as the movement of vesicular carriers shuttling between them (reviewed in (Cole and Lippencott-Schwartz, 1995; Goodson et al., 1997; Hirokawa, 1998)). Microtubules are thought to provide tracks for movement over long distances (>0.5 μm) while actin filaments preferentially control movement to local sites (Atkinson et al., 1992; Langford, 1995). However, there seems to be an extensive cross-talk between the two cytoskeletal systems (Kuznetsov et al., 1992). Vesicles have motors for both actin- and microtubule-mediated transport and a direct interaction between actin- and microtubule-dependent motors themselves has been found, suggesting the existence of a motor complex allowing a vesicle to move on both actin and microtubule tracks (Huang et al., 1999). Thus, a model is emerging whereby vesicular transport is elaborately coordinated by actin- and microtubule-dependent motors.

Besides moving cargo from one compartment to another, vesicular movement participates in the process of cell locomotion. The actin cytoskeleton plays an essential role in cell movement (Mitchison and Cramer, 1996; Waterman-Storer and Salmon, 1999). It appears, however, that both membrane dynamics and cytoskeletal rearrangements are coordinated during this process. As the cell is stimulated to move, exocytosis becomes targeted to the leading edge (Marcus, 1962; Bretscher, 1983; Hopkins, 1985; Hopkins et al., 1994), and it has been

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shown that the polarization of exocytosis during cell motility involves the activity of the small GTPase Rac (Bretscher and Aguado-Velasco, 1998). This targeted exocytosis serves the purpose of directing membrane, receptors and adhesion molecules to the leading edge of the cell (Fabbri et al., 1999). An attractive model system to study cell motility is that of endothelial cells. Endothelial cells, which line the vasculature, undergo extensive alterations in shape and motility during developmental processes and in response to injury. Similarly, during tumor angiogenesis endothelial cells undergo motile and proliferative responses to extracellular cues such as vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Brown et al., 1977; Klein et al., 1997). Therefore, coordinated changes in adhesion and cytoskeleton occur during the transition between quiescence and angiogenesis and this can be reconstituted in vitro.

The integrity of the cellular actin and microtubule cytoskeletal networks is essential for vesicular transport and, consequently, proteins that regulate the organization of the cytoskeleton may also participate in vesicular trafficking. Indeed, members of the Rho family of GTPases, key regulatory molecules of the actin cytoskeleton, have been increasingly implicated in intracellular trafficking (Ellis and Mellor, 2000). For example, RhoA and Rac1 have been shown to regulate transferrin receptor uptake (Lamaze et al., 1996). RhoB, a protein localised to the endocytic pathway, regulates epidermal growth factor receptor trafficking (Gampel et al., 1999), while Cdc42 controls basolateral plasma membrane transport in polarised epithelial cells (Kroschewski et al., 1999). Whether the above affects of the rho GTPases on vesicular transport are due to actin rearrangements remains to be elucidated.

We have previously identified a Rho family member, rhoD, and explored its functional properties. Expression of rhoD in BHK cells caused the disappearance of actin stress fibers from the cell body, the formation of plasma membrane protrusions containing F-actin, and a concomitant loss of focal adhesion-associated proteins (Murphy et al., 1996). RhoD was localized to transferrin receptor-positive early endosomes and its overexpression resulted in the inhibition of early endosome fusion by blocking the movement of this organelle. In this specific case, the effect of rhoD on endosome dynamics was evaluated in the presence of a constitutively active, GTPase-deficient mutant of rab5, rab5Q79L, which enhances both endosome fusion (Stenmark et al., 1994) as well as the motility of early endosomes along microtubules (Nielsen et al., 1999). Thus, rhoD counteracted the stimulatory effects of rab5 on endosome dynamics. In this study, we have investigated the effect of rhoD in the absence of co-expressed rab5 in order to uncouple the function of rhoD from the stimulatory activity of rab5 in endosome trafficking and have found that rhoD alone alters endosome dynamics. Moreover, due to the participation of vesicular motility and cytoskeletal rearrangements in cell movement, we have here explored the role of rhoD in endothelial cell motility and have found a decrease in cell motility upon expression of the protein.

Materials and methods

Cell culture

Baby hamster kidney-21 (BHK-21) cells were cultured in Glasgow MEM supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 10 μg/ml streptomycin, 10% tryptose phosphate, and 2 mM glutamine. All media and reagents for cell culture were purchased from Gibco Life Technologies (Gaithersburg, MD, USA).

Bovine brain capillary endothelial (BBCE) cells were handled as described in (Schweigerer et al., 1987). bFGF (2.5 ng/ml) was added directly to the medium to stimulate cell motility.

Expression constructs

Myc-rhoDG26V was cloned into the HindIII-XbaI sites of expression vector pHSVPUC to generate construct: pHSV-myc-rhoDG26V. Myc-rhoDG26V cDNA was cloned in frame into the EcoRI-BamHI sites of expression vector pEGFP-C2 (Clontech, Palo Alto, California, USA) to generate a green fluorescent protein (GFP) fusion: GFP-myc-rhoDG26V.

Transient transfection of rhoDG26V and human transferrin receptor

BHK-21 cells were trypsinized 24 h prior to transfection and were seeded onto 11-mm glass coverslips. Cells were infected with T7 RNA polymerase recombinant vaccinia virus (Bucci et al., 1992) and transfected with either T7-myc-rhoDG26V and T7-human transferrin receptor (T7-hTR) or T7-hTR alone (Murphy et al., 1996) using DOTAP (Boehringer Mannheim, Mannheim, Germany). The cell number and also the amount of DNA and lipid were constant. The final concentration of DNA per coverslip was 1 μg.

Following transfection, the cells were incubated at 37 °C, 5% CO_2, for 2 h, cycloheximide was then added for 1 h. Cells were washed 8 times for 1 min in preheated medium, and rhodamine transferrin (50 μg/ml) was uptaken in a preheated humidified chamber at 37 °C, 5% CO_2, for 20 min. Coverslips were washed in preheated medium and then mounted into chambers for video microscopy (Bradke and Dotti, 1998) as described below. Time lapse video microscopy (TLVM) was carried out immediately. Hydroxyurea was present at all times to prevent late viral gene expression.

Transient transfection of T7-rhoDG26V and T7-gfp

To monitor lysosomal motility in the presence and absence of rhoDG26V we made use of a green fluorescent protein-expressing vector cloned into pGEM1 harboring the T7 promoter. Co-transfection of this plasmid with T7-myc-rhoDG26V allowed us to identify the transfected cells for video microscopy. Handling of the cells was as outlined above with the exception that they were incubated in the presence of 50 nM Lysotracker (Molecular Probes, Leiden, The Netherlands) for 15 min at 37 °C, 5% CO_2 to label the lysosomes, and TLVM was carried out as outlined below.

Immunofluorescence and confocal microscopy

To test the possible colocalisation of rhoDG26V and Lysotracker, 50 nM Lysotracker was uptaken by BHK cells expressing rhoDG26V. The cells were then fixed for 30 min in 3.7% paraformaldehyde at room temperature. Following fixation cells were permeabilised in saponin 0.5% in 5% FCS for 30 min. RhoDG26V was detected using the 9E10 anti-myc monoclonal antibody and FITC-labelled anti-mouse secondary antibody (DIANOVA). The cells were viewed using a Leica scanning confocal microscope and images were exported to Adobe Photoshop (Photoshop, Adobe, Mountain View, CA).

BBCE cell motility

To address the effect of rhoDG26V on cell motility, BBCE cells were trypsinised and plated at a density of 1500 cells per cm^2, 24 h later nuclear microinjection was carried out with a rhoDG26V expression construct at 50 μg/ml concentration, FITC-dextran was coinjected to

identify the injected cells. Approximately, 12 h following injection the cells were stimulated with bFGF (2.5 ng/ml added directly to the culture medium) and subjected to TLVM as described below.

Endothelial cell wounding experiments

BBCE cells were plated in 24-well dishes and stimulated with bFGF until confluent. The cells were then infected for 2 h with recombinant adenoviruses expressing green fluorescence protein alone (GFP) or GFP and rhoDG26V (the viruses will be described elsewhere) at an multiplicity of infection of 2. The medium was removed, cells were washed with PBS and were further incubated in full medium for 24 h to allow expression of the recombinant adenoviruses. The monolayer was then wounded using a plastic cell scraper and images were taken of the wounded area. bFGF (2.5 ng/ml) was added to stimulate cell motility across the wound and the cells were photographed again 8 h following addition of the bFGF. Cells crossing the wounded area were counted and expressed as number of cells per mm of wound. Four fields were counted in each experiment (approximately 300 cells were counted in each experiment in the case of GFP alone) and the experiment was repeated twice.

Time lapse video microscopy

The cells for TLVM were grown on coverslips and mounted into aluminium chambers as described in (Bradke and Dotti, 1998). Briefly, aluminium was cut into 7.5 cm × 2.5 cm × 2 mm rectangles, the dimensions are similar to a standard glass slide and fit onto the microscope stage. In the center a circular hole of 8 mm diameter was cut, and around it 4 mm area was milled from both sides. A glass coverslip was inserted onto one side of the chamber, sealed with a lubricant, medium was then added to fill the chamber, cells grown on coverslips were then inverted onto the other side of the metal slide and sealed with lubricant. TLVM was carried out as previously described (Murphy et al., 1996).

Image and statistical analysis of the data

A dedicated automatic program was developed to detect and track endosomes/lysosomes as they move. It runs on a SPARC station Ultra1 (SUN, Mountain View, CA) to which a Series 151/40 digital image processor (Imaging Technology, Bedford, MA) is connected. The detection of fluorescent spots corresponding to endosomes/lysosomes is performed automatically by a multi-resolution algorithm based upon selectively filtering an undecimated wavelet decomposition of the image through the use of wavelet coefficient thresholding and correlation (Olivo, 1996). At the end of this step, all endosomes/lysosomes in the sequence are characterized and their coordinates are determined and stored. A tracking algorithm is then used to establish valid trajectories. The algorithm uses a first-order Kalman filtering approach whereby at each frame and on the basis on the previous ones, predictions of the endosome/lysosome positions are established and compared with the computed ones (Nguyen Ngoc et al., 1997). The best matches are selected as trajectory points, and tracks are finally analyzed to compute the data that was used for generating values in Tables I and II. For each data set, a sequence of 30 images was analyzed. Speed values reported here are mean values of the speed of several endosomes/lysosomes and errors are standard deviations of these group estimates.

Results

Expression of rhoDG26V decreases the speed, displacement and range of transferrin positive vesicular structures

To investigate the role of rhoD in endosome dynamics we expressed an activated mutant, rhoDG26V, in BHK cells and examined the effect of this protein on the motility of endocytic organelles by TLVM. We conducted single cell TLVM analysis. To this end, the early endocytic/recycling compartment was visualized by internalization of rhodamine-labeled transferrin for 40 min. Given that rhoD does not significantly perturb the transferrin cycle (Murphy et al., 1996) this method allowed a direct comparison between mock-transfected cells and cells expressing the rhoDG26V mutant protein. To facilitate the phenotypic analysis we applied a computer program (described in Materials and methods) which tracks the vesicular structures in each frame of the video sequence. Using this program, individual vesicular structures were detected and grouped into tracks that are characterised by three distinct parameters: *displacement, speed, range and search radius* (expressed in pixels). *Displacement* is defined as the sum of the absolute value of all elementary movements (irrespective of direction) effected by the vesicles during the sequence. *Speed*, is the displacement over time (in pixels per second) and represents the average vesicle velocity along a track during the complete sequence. *Range* is defined as the distance between the starting point and the point the furthest apart reached from the initial position. *Search radius* is the maximum distance from the actual position over which the search window can be moved to track the new position of a vesicle in between two successive video frames. Due to the high density of transferrin receptor-positive vesicular structures the tracking of individual structures moving long distances was difficult to follow, therefore the threshold of the search radius was fixed at a value of 7 pixels. Consequently, we refer here as short-range vesicular movements those which are tracked with a search radius value of 7 pixels. Long-range vesicular movements, which require search radius values higher than 7 pixels, needed to be tracked and quantified manually.

Twelve video sequences of control cells (in the absence of rhoDG26V expression) were analyzed (see Table I, M-1 to M-12). Throughout these videos, the transferrin-labeled vesicular compartment was highly motile, exhibiting both oscillatory short-range movements and some long-range rapid directional movements. In some cases both the short- and long-range vesicular movements were bi-directional. In contrast, expression of rhoDG26V significantly reduced both short- as well as long-range endosome motility. Figure 1b shows that, under these conditions, the vesicular structures display a decreased range compared to those in control cells (Fig. 1a). To further illustrate this point we plotted some examples of the tracks in Figure 1, c–f. It is clear that in the cells expressing rhoDG26V the vesicles are moving within a much smaller area then in control cells (compare Fig. 1 panels c, d with e, f). Quantitations of the parameters of the short-range motility (see Table I) showed that in this transfected cell (M-27) there was a 58% reduction in displacement, 58% in speed and 52% in range when compared to the mean of the control videos. Naturally, cells displayed different degrees of responsiveness to the rho protein, which reflect cell variability and variations in expression levels. Nevertheless, statistically significant decrease in vesicular displacement (21.8%, $p = 0.003$), speed (22.7%, $p = 0.002$) and range (23.7%, $p = 0.007$) were observed even if the parameters of 16 independent video sequences were pooled and quantified, to provide an average estimate of vesicular motility in a cell population.

The long-range displacements were calculated by counting all the long-range movements in the videos manually and, because of that, their speed and displacement were not estimated. We calculated the number of long-range displacements in the presence and absence of rhoDG26V expression. An example is shown in Figure 2, panel a (representing data from M-1, Table I). Here are shown 3 consecutive frames (taken at 1 frame/sec) from the video showing the movement of a vesicle (indicated by the white arrow). The results of the

Tab. I. Endosomal displacement, speed and range are presented in cells in the absence (control videos) or presence of rhoDG26V expression.

Video	Speed	Displacement	Range
Controls			
M-1	1.82	25.47	9.37
M-2	1.95	27.29	8.76
M-3	1.93	26.99	9.85
M-4	1.87	26.21	9.21
M-5	1.74	24.43	10.39
M-6	1.77	24.76	8.68
M-7	1.53	21.45	6.97
M-8	1.06	14.89	4.08
M-9	1.50	21.00	8.43
M-10	2.00	27.99	9.31
M-11	1.63	22.84	7.28
M-12	1.23	17.18	6.57
Mean	1.67	23.38	8.24
SD	0.29	4.10	1.75
rhoDG26V			
M-13	1.20	16.79	5.61
M-14	1.00	13.97	4.12
M-15	0.78	10.95	3.64
M-16	1.60	22.40	6.78
M-17	1.62	22.67	8.69
M-18	1.21	17.00	5.37
M-19	1.18	16.59	4.79
M-20	1.85	25.95	8.58
M-21	1.43	20.06	6.65
M-22	1.64	25.74	9.02
M-23	1.41	19.77	7.22
M-24	0.95	13.25	4.49
M-25	1.69	23.73	7.45
M-26	1.20	16.82	8.96
M-27	0.70	9.78	3.94
M-28	1.22	17.03	5.38
Mean	1.29	18.28	6.29
SD	0.33	4.90	1.88
% Decrease	22.7	21.8	23.7
Significance	0.002	0.003	0.007

Fig. 1. Short-range tracks of transferrin-positive vesicular structures in control cells (**a, c, d**) and in cells expressing rhoDG26V (**b, e, f**). (**a, b**) represent the total tracks summed over 30 video frames illustrating the distance over which the vesicles are moving in control cells (**a**) and in rhoDG26V-expressing cells (**b**). Each panel includes 3 different tracks and panel size is 4 microns. Two tracks from control cells (**c, d**) and rhoDG26V-expressing cells (**e, f**) are plotted to further illustrate the difference between vesicle movement in control and rhoDG26V-expressing cells.

quantitation are shown in Fig. 2b and represent the total long-range movements in all the videos combined. Altogether, these results demonstrate that expression of an activated rhoD mutant causes an inhibition of both short-range and long-range endosome motility independent of the stimulatory action of rab5. In some rhoDG26V-expressing cells, we also noticed that endosomes were distributed to the cell periphery. This phenotype is reminiscent of that observed when cells are treated with nocodazole to depolymerise microtubules, suggesting that under these conditions, endosomes may interact primarily with the cortical actin cytoskeleton (Nielsen et al., 1999). Furthermore, it was very striking that tubulation of the transferrin-containing compartment, clearly visible in the control cells, was absent upon expression of rhoDG26V as we have previously reported (Murphy et al., 1996).

Effect of rhoDG26V on lysosomal speed and displacement

To investigate whether the effect of rhoDG26V was restricted to the early endosome/recycling pathway or indeed affected also other compartments, we examined the effect of rhoDG26V on lysosomal speed and displacement. The lysosomal compartment was labeled with Lysotracker as described in Materials and methods. Lysotracker accumulates in acidic compartments and has been shown to colocalise to a significant degree with Lamp-1 and rab7 (Bucci et al., 2000), therefore indicating that the marker labels lysosomes and some late endocytic structures. Expression of rhoDG26V decreased the displacement and speed of lysosomal movements (24.18%, $p = 0.055$ and 24.66%, $p = 0.04$, respectively). The results are shown in Table II. The range of lysosomal movements was also decreased by 20% but did not reach statistical significance ($p = 0.102$) as a consequence of the higher standard deviation of the mean. We also quantitated the number of lysosomal long-range movements in the presence and absence of rhoDG26V expression manually and found that their number was decreased by 32%. The results are shown in Fig 2c and represent the results of all the videos combined.

Overall, it appears that lysosomal speed and long-range movements are decreased by rhoDG26V in BHK cells. The alteration of the dynamics in the late endocytic-lysosomal pathway was unexpected and suggests the possibility that the cytoskeleton-dependent motility of early and late endocytic organelles may

Fig. 2. An example of a long-range track of a transferrin-positive vesicular structure in control cells is shown in (**a**). The vesicle (indicated by a *white arrow*) is shown in 3 consecutive frames to move a distance of approximately 3 microns. Size bar is 4 microns. The number of long-range tracks (combined data from all the control and rhoDG26G videos) of transferrin-positive vesicles and lysosomes is shown in (**b, c**), respectively. *Black bars* represent the control cells and grey bars represent the situation in the presence of rhoDG26V expression.

Tab. II. Lysosomal displacement, speed and range are presented in cells in the absence (control videos) or presence of rhoDG26V expression.

Video	Speed	Displacement	Range
Controls			
M-29	4.53	23.65	13.67
M-30	3.76	18.67	10.74
M-31	2.01	10.11	5.87
M-32	4.32	23.79	11.36
M-33	3.23	16.86	9.18
M-34	2.90	14.56	6.98
M-35	4.16	20.91	11.49
M-36	4.72	25.16	13.72
M-37	3.39	17.94	11.31
Mean	3.67	19.07	10.48
SD	0.88	4.88	2.70
rhoDG26V			
M-38	3.38	17.73	9.39
M-39	2.58	13.63	7.29
M-40	3.00	15.29	9.24
M-41	2.71	14.24	8.17
M-42	2.14	10.94	6.14
M-43	2.58	11.98	7.80
M-44	3.16	16.48	9.96
M-45	2.93	15.36	9.28
M-46	2.70	15.37	8.91
M-47	2.59	13.56	7.20
Mean	2.78	14.46	8.34
SD	0.35	2.03	1.22
% Decrease	24.66	24.18	20.42
Significance	0.040	0.055	0.102

be coordinated. We addressed the possibility that rhoDG26V may localise to some of the Lysotracker-positive vesicular structures. The results are shown in Figure 3. Even though there is some colocalisation of rhoDG26V and Lysotracker (indicated by arrows), the percentage of overlap is low.

RhoDG26V decreases endothelial cell motility

Given its impact on endosome motility we then investigated the effect of rhoDG26V on cell motility. We chose endothelial cells as a model system since *1*) these cells are characterised by high locomotive activity and *2*) this function is central to the maintenance of vascular plasticity (Shuster and Herman, 1998). We were particularly interested in molecules and mechanisms by which coordinated changes in adhesion and cytoskeleton occur during the transition between quiescence and angiogenesis and the manner in which these processes are linked to the transport functions of endothelial cells. The motility of endothelial cells in culture can be modulated by basic fibroblast growth factor (bFGF) (reviewed in (Klein et al., 1997)). Primary cultures of bovine brain endothelial cells were microinjected with an expression construct driving the expression of rhoDG26V. Representative examples of endothelial cells injected with rhoDG26V and control non-injected cells are shown in Figure 4. We observed that the rhoDG26V-injected cells (indicated with large arrowhead) were flattened, spread out and severely inhibited in motility. As shown in Figure 4, the cell injected with rhoDG26V failed to move throughout a video sequence lasting 460 min. In the same field, a control uninjected cell (indicated with thin white arrow) moves rapidly during the time course. We found that rhoDG26V decreased endothelial cell motility, both in the presence (Fig. 4a) and absence (data not shown) of bFGF. Strikingly, the endothelial cells were essentially motionless upon expression of the rhoDG26V protein, while other unrelated injected constructs (e.g. rab7) did not alter endothelial cell motility (data not shown). Therefore, in addition to its role in endosome motility these results strongly implicate rhoD in the control of cell locomotion.

To further investigate the decrease in cell motility apparent from the videos above we carried out wounding experiments using BBCE cells stimulated to move into a wounded area by bFGF. In this case we used recombinant adenoviruses expressing either green fluorescent protein (GFP) alone or expressing both GFP and rhoDG26V to infect the BBCE cells. The results presented in Figure 4b clearly show a decrease in the number of cells which move into the wounded area upon expression of rhoDG26V.

Discussion

Our results demonstrate that rhoD controls membrane dynamics in the early endocytic pathway even in the absence of over-expressed rab5. Furthermore, rhoDG26V also alters membrane dynamics in the late endocytic-lysosomal pathway. Due to the low percentage of colocalisation between Lysotracker-positive and rhoDG26V-positive structures, the effect of the rhoDG26V on the late endosomal pathway is likely to be indirect, suggesting the possibility that the cytoskeleton-dependent motility of early and late endocytic organelles may be coordinated. Short-range oscillatory vesicular movements are thought to be actin-associated, with actin filaments sustaining movements within confined regions of the cytoplasm (Langford, 1995). In Hep-2 cells, actin depolymerisation prevents the short, non-directional saltatory endosome movements (van Deurs et al., 1995). We find here that rhoDG26V expression decreases these short-range vesicular movements. Given the established role of rho GTPases in the regulation of

Fig. 3. Internalised Lysotracker (*red*) and rhoDG26V (*green*) in BHK cells. Images are shown from 2 cells expressing rhoDG26V (**A, D**) in which Lysotracker was internalised (**B, E**) and the overlays of both images are shown in (**C, F**). Size bar is 10 microns. *Arrows* indicate colocalisation of Lysotracker and rhoDG26V.

Fig. 4. Endothelial cell motility was monitored over a period of 8 h. Six frames at 0, 100, 190, 280, 370 and 460 min are shown. An *arrowhead* indicates the rhoDG26V-injected cell while an *arrow* indicates the control, non-injected cell (**a**). Endothelial cell motility quantitated in BBCE wounding experiments is shown in (**b**).

the actin cytoskeleton, our results suggest that rhoD may regulate interactions between actin filaments and vesicular structures. We propose that over-expression of the activated rhoD protein would interfere with this regulation by stabilizing this interaction resulting in a "freezing" of the vesicles.

Long-range vesicular motility is also inhibited by rhoDG26V. This type of vesicular movement is considered to be mediated by microtubules, and is inhibited by nocodazole (van Deurs et al., 1995). It has been shown that organelles can shift from actin to microtubules in the squid axon (Kuznetsov et al., 1992). Cytochalasin D and nocodazole exert opposite effects on the intracellular distribution of rab5-positive endosomes (Nielsen et al., 1999). In the absence of microtubules, endosomes accumulate in the cortical region of the cytoplasm underneath the plasma membrane, whereas disruption of the actin network causes the endosomes to accumulate in the perinuclear region towards the microtubule-organizing center. Therefore, these observations argue that actin filaments and microtubules act in concert to regulate the intracellular position of endosomes. In the light of these data, rhoD may enhance vesicle-actin interactions, which would prevent the vesicle from switching to the microtubule machinery, thereby causing an inhibition of long-range microtubule mediated motility. This possibility is also consistent with the re-distribution of endosomes to the cell periphery observed in some cells.

There is a clear involvement of rho family members in cell motility, as might be predicted from their effects on the cytoskeleton. Activated cdc42 and rac increase cell motility when stably transfected into T47D cells through a PI(3)K-dependent mechanism (Keely et al., 1997), and rhoE enhances the effect of hepatocyte growth factor-induced cell migration in MDCK cells (Guasch et al., 1988). Mig2, a member of the rho family, has been implicated in cell migration in *C. elegans*, activated and null alleles all inhibit cell migration (Zipkin et al., 1997). RhoA has also been implicated in cell migration (Yoshioka et al., 1998). The underlying mechanisms controlling cell migration are not fully understood, even though many of the players are known. Upon expression of rhoDG26V the cells extend filopodia and vesicular dynamics is altered. Here we have found that expression of rhoDG26V drastically impairs the motility of endothelial cells. A similar effect of rhoD on 10TI/2 cells has recently been reported (Tsubakimoto et al., 1999), arguing that rhoD may decrease cell motility also in these cells. It is likely that this effect is due to a combination of alterations of the actin cytoskeleton and endocytic membrane dynamics. Membrane flow and the cytoskeleton cooperate in cell motility (Bretscher, 1996). Polarized exocytosis at the leading edge of motile cells occurs (Hopkins et al., 1994) and may assist a cell in moving forward. Furthermore, integrins have been shown to have a polarised distribution in migrating cells maintained by cycles of endocytosis and recycling to the leading edge (Lawson and Maxfield, 1995). Therefore, it seems likely that a polarised membrane trafficking system is important as cells move.

Cell tension and shape are determined by cell-extracellular matrix contacts and the integrity of the cytoskeleton. It has been shown that mechanical forces can modulate many cellular functions including cell growth, motility (reviewed in (Chicurel et al., 1998)) and gene expression in endothelial cells (Chen et al., 1997). Recently it has been shown that cell spreading and lamellipodial extension are increased by a reduction in membrane tension (Raucher and Sheetz, 2000). It is interesting to note that cells flatten in response to rhoDG26V expression. This raises the possibility that plasma membrane tension may be decreased in these cells as a consequence of the disassembly of stress fibers and focal adhesions, leading to cell spreading. In conclusion, our results lend further support to the view that rhoD is a regulator of membrane dynamics, both at the level of intracellular motility of endosomes and at the level of cell movement. Future studies are necessary to unravel the mode of function of this protein, and the identification of rhoD effectors will be a necessary step towards this end.

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