RhoB regulates endosome transport by promoting actin assembly on endosomal membranes through Dia1

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Summary

Rho GTPases are crucial regulators of the actin cytoskeleton and they play a role in the control of membrane trafficking. In contrast to the close family members RhoA and RhoC, RhoB localises to endosomes and delays epidermal growth factor receptor traffic. Here, we show that activated RhoB induces the peripheral distribution of endosomes, which align along subcortical actin stress fibres and are surrounded by an actin coat. The Diaphanous-related formin, Dia1, is recruited to endosomes by activated RhoB. Dia1 is required for the formation of the actin coat around endosomes downstream of RhoB, connecting membrane trafficking with the regulation of actin dynamics.

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Key words: RhoB, Dia1, Endocytosis, Transport

Introduction

Cell morphological changes, such as the extension of lamellipodia during migration, require the coordinated regulation of membrane trafficking, and actin and microtubule cytoskeletons. Long-range bidirectional transport of vesicles between the plasma membrane and the juxtanuclear area occurs along microtubules. Endocytic vesicles are transported towards the microtubule minus-end located at the perinuclear microtubule-organizing centre (MTOC) where they fuse with the sorting/recycling endosomes that reside in this region (Aniento et al., 1993). However, before they reach microtubules, endocytic vesicles must cross the cortical actin cytoskeleton underlying the plasma membrane (Aschenbrenner et al., 2004). Actin polymerisation has been shown to play a role in endocytosis (Lamaze et al., 1997; Fujimoto et al., 2000; reviewed in Merrifield, 2004), but its role in post-endocytic transport is less clear. Endocytic vesicles could be transported on subcortical actin microfilaments by a myosin motor until they reach the microtubular network. An additional mechanism implicates the phosphatidylinositol-4 phosphate 5 kinase-stimulated polymerisation of a Listeria-like actin comet on raft-enriched vesicles by the WASP-Atlast2 pathway (Rozelle et al., 2000). This actin tail is suggested to contribute to endocytic transport by propelling newly formed pinosomes towards the cell interior (Merrifield et al., 1999).

Rho GTPases are critical regulators of actin dynamics and have been involved in the control of endocytosis, although the connection between these two activities is not completely understood. Activated RhoA, Rac and Cdc42 localize to the plasma membrane and consistently have been implicated in the regulation of receptor internalization (reviewed in Symons and Rusk, 2003). Correspondingly, RhoB localises to the cytoplasmic face of endosomal membranes (Adamson et al., 1992; Robertson et al., 1995) and has been suggested to regulate endosome traffic. Specifically, activated RhoB has been shown to delay the transport of internalized epidermal growth factor (EGF)-receptor to lysosomes (Gampel et al., 1999) by an as yet unknown mechanism. RhoB is highly homologous to the well-studied Rho family member RhoA (83% identity). Due to this high homology, both isoforms exhibit a similar ability to bind specific effector proteins in vitro. However, it is unclear whether this also applies in vivo. For example, the serine/threonine protein kinase PRK1 was identified as a downstream effector of RhoA (Amano et al., 1996), but recent work shows that PRK1 is recruited to endosomes by RhoB (Mellor et al., 1998). Similarly, the Rho-binding domain of the mammalian Diaphanous-related formin (DRF), Dia1, interacts with recombinant RhoA, B and C in vitro (Watanabe et al., 1999). Dia1 contains an N-terminal Rho-binding domain and two C-terminally conserved FH domains responsible for actin filament growth (Watanabe et al., 1997; Wasserman, 1998) as well as for Arp2/3-independent actin assembly (Pruyne et al., 2002; Evangelista et al., 2003). Interestingly, Dia1 has been localised to endosomes in HeLa cells (Tominaga et al., 2000), which suggests that it may regulate endocytic traffic downstream of RhoB. Notably, another endosomal Rho GTPase, RhoD, was shown to regulate endosome transport through hDia2C, a novel splice variant of the human DFR2 (Murphy et al., 1996).

Here, we have investigated the effect of activated RhoB on endocytosis and the actin cytoskeleton. Activated RhoB...
promotes the polymerisation of an actin coat around endosomes and the association of these vesicles to subcortical actin cables, effectively inhibiting further endosomal transport. The Rho effector protein Dia1 is recruited to endosomes by activated RhoB and is critically involved in the assembly of actin on the vesicle membrane. This exemplifies a mechanism of control of endocytosis by a Rho GTPase through the regulation of actin polymerisation on membranes by a DRF protein.

Materials and Methods
Cell lines and treatments
The human melanoma cell line Mel JuSo and HeLa cells were cultured in Iscoves medium with 10% fetal calf serum. A Mel JuSo cell line stably expressing GFP-actin was generated by calcium phosphate transfection with an expression plasmid containing Myc-GFP-human β actin cDNA (kindly provided by P. Stein, Harvard Medical School, Boston, MA). A cell line stably expressing GFP-FYVE (EEA1Δ1-1256) was generated by calcium phosphate transfection of Mel JuSo cells with the corresponding expression plasmid (kindly provided by H. Stenmark, The Norwegian Radium Hospital, Oslo, Norway). Expressing cells were selected in Iscoves medium supplemented with 2 mg ml–1 G418 (Gibco). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. pcDNA3-myc-RhoAG14V was kindly provided by H. Stenmark, The Norwegian Radium Hospital, Oslo, Norway. pcDNA3-myc-RhoB wild type (RhoBwt) was generated by site-directed mutagenesis using QuickChange (Stratagene) following the manufacturer’s instructions. pcDNA3-myc-RhoB wild type (RhoBwt) was generated by site-directed mutagenesis using QuickChange (Stratagene) following the manufacturer’s instructions. pcDNA3-myc-RhoAΔG14V was kindly provided by W. Moolenaar (The Netherlands Cancer Institute, Amsterdam, The Netherlands). pEGFP-mDia1 full length and the RhoBG14V plasmid (Chardin et al., 1988), kindly provided by A. Hall (ICRF, London, UK), into the serum starved for 1 hour prior to the incubation with 50 µM Y-27632 (Sigma) for 1 hour before fixation.

Fluid-phase and receptor mediated endocytosis
To follow receptor-mediated endocytosis of transferrin, cells were serum starved for 1 hour prior to the incubation with 50 µg ml–1 Texas Red labelled human transferrin (Molecular Probes) for 15 minutes. Fluid phase endocytosis was analysed by using sulforhodamine 101 (SR101, Molecular Probes) as a fluid-phase endocytosis marker (Wubbolts et al., 1996). Cells were incubated with SR101 for 15 minutes, washed to remove non-internalised marker, and chased for up to 60 minutes prior to fixation with 3.7% formaldehyde.

Expression plasmids
Myc-RhoBΔG14V cDNA was transferred from the pEXV-myc-RhoBΔG14V plasmid (Chardin et al., 1988), kindly provided by A. Hall (ICRF, London, UK), into the EcoRI site of pcDNA3 (Invitrogen). pcDNA3-myc-RhoB wild type (RhoBwt) was generated by site-directed mutagenesis using QuickChange (Stratagene) following the manufacturer’s instructions. pcDNA3-myc-RhoAΔG14V was kindly provided by W. Moolenaar (The Netherlands Cancer Institute, Amsterdam, The Netherlands). pEGFP-mDia1 full length and the AN3 and N1 deletion mutants were kindly provided by S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan) and are described elsewhere (Watanabe et al., 1999). pcDNA3-myc-Rab5ζ (Stenmark et al., 1994) and pEGFP-FYVE were kindly provided by H. Stenmark (Norwegian Radium Hospital, Oslo, Norway).

Antibodies
Myc- and HA-tagged proteins were detected with the mouse 9E10 and 12CA5 monoclonal antibodies, respectively. Rabbit polyclonal antibody sc-119 anti-RhoB was purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-EEA1 antibodies were kindly provided by H. Stenmark (Norwegian Radium Hospital, Oslo, Norway) (Simonsen et al., 1998). Mouse monoclonal antibody 4F11 anti-Rab5 (Stein et al., 2003) was generously provided by A. Wandinger-Ness (University of New Mexico, Albuquerque, NM). Rabbit polyclonal anti-myosin IIA heavy chain antibody was purchased from Biomedical Technologies Inc. Mouse monoclonal antibody anti-EGF receptor was from BD-Transduction Labs. Secondary antibodies labelled with FITC (Dako), Texas Red (Molecular Probes) or Cy5 (Jackson Labs) were used. F-actin was detected with Alexa 568-labelled phalloidin (Molecular Probes).

Microinjection and immunofluorescence
Cells were microinjected on a heated xy-stage of an inverted Olympus IX70 microscope equipped with an Eppendorf Micromanipulator InjectMan device. Expression plasmids were injected in the nuclei of 100-150 cells at a concentration of 0.1 mg ml–1 in microinjection buffer (120 mM potassium glutamate, 40 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.2 mM Ca Cl2, 10 mM Hepes, 40 mM mannitol, pH 7.2). For image recordings of living cells, Texas Red labelled dextrane 70 kDa (Molecular Probes) was added as microinjection marker. After microinjection, cells were incubated for 4 to 5 hours to allow protein expression and were subsequently fixed with 3.7% formaldehyde in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 and immunostained. Primary and secondary antibodies and phalloidin were diluted in PBS containing 0.5% bovine serum albumin. Confocal images were obtained with a Leica TCS SP confocal laser-scanning microscope.

Fluorescence live imaging microscopy
Time-lapse recordings of GFP-FYVE expressing cells were done by collecting 36 images with a time interval of 15 seconds on a Leica TCS SP CLSM. The images recorded were maximally projected and encoded green. This projection image was then combined with a red-coloured image of the cells at t=0 (initial position of vesicles) as previously described (Wubbolts et al., 1996).

Results
Activated RhoB induces endosome dispersion and cell elongation
Previous studies have shown that RhoB localises to the endosomal compartment (Adamson et al., 1992; Robertson et al., 1995) and causes the dispersion of EGF receptor-containing endosomes (Gampel et al., 1999). Consistently, we found that the constitutively active GTPase-deficient mutant RhoBG14V largely co-localised with the early endocytic marker EEA1 on endosomes that were relocated from their usual juxtanuclear location to the cell periphery and appeared smaller than in non-injected cells (Fig. 1b, compare injected cell with neighbouring control non-injected cells). In addition, RhoBΔG14V induced F-actin polymerisation in thin parallel stress fibres running along the longitudinal axis of the cells (Fig. 1c). Endosomes aligned along the RhoB-induced actin cables (Fig. 1a-c, arrowheads). The wild-type form of RhoB (RhoBwt) also co-localised with EEA1 on vesicles, but had no effect on the action cytoskeleton or the distribution of endosomes, which localised to the juxtanuclear area as in control cells (Fig. 1d-f). We observed that (RhoBwt)/EEA1 positive endosomes had polymerized actin on discrete sites on their membrane (arrowheads in Fig. 1d-f and Fig. 3). These results suggest that RhoBΔG14V induces the association of endosomes to actin cables preventing their transport to the cell centre.

To test for the specificity of the effects induced by RhoBΔG14V, we expressed an activated mutant of RhoA...
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(RhoA<sup>G14V</sup>). RhoA is highly homologous to RhoB and their respective effector loops differ only in two residues. Unlike RhoB<sup>G14V</sup>, RhoA<sup>G14V</sup> localised to the cell cytoplasm (Fig. 1g) and did not alter endosome distribution (Fig. 1h). RhoA<sup>G14V</sup> induced actin polymerisation and the formation of parallel stress fibres (Fig. 1i), but it had markedly different effects on cell shape compared to RhoB<sup>G14V</sup> (compare Figs 1c and 1i, supplementary material Fig. S1). To analyse this, we measured the length of the perpendicular long and short axes of RhoB<sup>G14V</sup> and RhoA<sup>G14V</sup> expressing cells and compared them with control cells. The average lengths of the long and short axes in control cells were 47.32±7.61 µm and 25.70±5.93 µm (n=30), respectively. RhoB<sup>G14V</sup> expressing cells exhibited an elongated, spindle-like shape; the average lengths of the long and short axes were 62.95±8.89 µm and 18.81±3.05 µm (n=30), respectively. Notably, the length of the axes parallel to the stress fibres was reduced in RhoA<sup>G14V</sup> expressing cells, being 35.85±9.55 µm on average, while the axes perpendicular to the stress fibres was longer, 31.62±8.87 µm (n=25). The ratio between the lengths of the long and short axes in every condition was 3.43±0.59 for RhoB<sup>G14V</sup> expressing cells; 1.22±0.46 for RhoA<sup>G14V</sup> expressing cells; and 1.96±0.52 for control cells (Fig. 2). These results show that, despite the almost complete identity between the effector loops of the two GTPases, RhoB affects endosome distribution and cell morphology in a different manner than RhoA.

**RhoB induces the assembly of an actin coat on endosomes**

We next investigated in more detail the apparent association of RhoB<sup>G14V</sup>-positive endosomes with actin cables. High magnification microscopic analysis of RhoB<sup>G14V</sup> expressing cells revealed that RhoB<sup>G14V</sup>/EEA1 positive endosomes were coated with F-actin and apparently associated to F-actin filaments (Fig. 3d-f, arrows). In contrast, juxtanuclear EEA1 positive endosomes of control cells (non-injected cells in Fig. 1) and RhoBwt/EEA1-positive endosomes showed F-actin associated to a discrete site on their membrane (Fig. 3a-c, arrows) as in control cells and were never encapsulated by actin. Treatment of the cells with nocodazole induced the dispersion of these endosomes, indicating that their perinuclear location depends on microtubule association (not shown). We also observed the formation of circular actin structures apparently associated to actin fibres in live GFP-actin Mel JuSo cells expressing RhoB<sup>G14V</sup>. Immunodetection of RhoB in these cells showed that these actin structures surrounded RhoB<sup>G14V</sup>-positive vesicles (Fig. 3b, arrowheads), confirming the results obtained after phalloidin staining for F-actin. Together, these results suggest that endogenous RhoB activity controls endosomal interactions with actin, which transiently occur during endocytosis.

**RhoB causes reduced motility of endosomes**

In Mel JuSo cells the transferrin receptor-positive recycling compartment and the lysosomes reside in the juxtanuclear area (Wubbolts et al., 1996). Our data suggest that the association of endosomes to subcortical actin fibres in RhoB<sup>G14V</sup>-expressing cells prevents their transport to the juxtanuclear area. We examined the fate of molecules internalised either by receptor-mediated or fluid phase endocytosis. Receptor-mediated endocytosis was assessed by incubating cells with...
transferrin-Texas Red. The ligand was endocytosed and reached the endosomes labelled for RhoB, while it concentrated in juxtanuclear vesicles in control non-injected cells, where the recycling compartment resides (Fig. 4a).

Subsequently, fluid-phase endocytosis was studied by feeding cells with the fluid-phase marker sulforhodamine 101 (SR101) for 15 minutes, followed by a 60 minutes chase period, in which SR101 reaches the lysosomal compartment (Wubbolts et al., 1996). SR101 was internalised and accumulated in vesicles that remained in the periphery in RhoBG14V expressing cells, while in control non-injected cells SR101-containing vesicles concentrated in the juxtanuclear area (Fig. 4b). These results suggest that RhoB is implicated in the regulation of an endocytic step posterior to the internalization at the plasma membrane and prior to the transport of endocytic vesicles to the cell centre.

Since long-distance transport of endosomes is known to occur along microtubules, our data imply that association of endosomes to actin fibres may prevent their transfer to microtubules and thus impair endosome motility. To confirm this, we examined endosome dynamics in a stable cell line expressing the EEA1 domain responsible for endosome targeting tagged with GFP (GFP-FYVE). RhoB<sup>G14V</sup> cDNA was introduced in GFP-FYVE expressing cells together with a 70 kDa Texas Red-labelled dextrane to mark injected cells. Vesicle movement was followed by fluorescence live imaging confocal microscopy. The images recorded during the time-lapse experiment (36 images with a time interval of 15 seconds) were maximally projected and encoded green. This image was then combined with a red-coloured image of the cells at t=0 (initial position of vesicles) (Fig. 4c; see also supplementary material Movie 1). Vesicle movement appears as green tracks on the projection (Fig. 4c, arrowheads). The average length of the tracks formed by vesicles in control cells during approximately 9 minutes was of 3.6±0.8 µm, ranging from 2.6 to 4.5 µm. GFP-FYVE-positive vesicles in RhoBG14V-injected cells exhibited Brownian-like motion (Fig. 4c, arrows). Occasionally, short tracks were observed with an average length of 1.3±0.3 µm, ranging from 0.8 to 1.6 µm. These results demonstrate that RhoB<sup>G14V</sup> reduces endosomal motility. Again, the peripheral location of endosomes marks the RhoBG14V-injected cell, and contrasts the juxtanuclear location in control cells (Fig. 4c). These results suggest that RhoB<sup>G14V</sup> inhibits microtubule-based endosome motility and transport to the juxtanuclear area as a result of endosome association to actin fibres.

To further confirm the inhibition of microtubule-based endosome motility by RhoB<sup>G14V</sup>, we studied homotypic fusion between endosomes. Homotypic fusion of early endosomes requires endosome movement along microtubules and is regulated by the small GTPase Rab5 (Aniento et al., 1993; Stenmark et al., 1994; Nielsen et al., 1999). Expression of a constitutively active Rab5 (Rab5<sup>Q79L</sup>) causes massive homotypic fusion resulting in the formation of large endosomes (Stenmark et al., 1994). The association of endosomes to subcortical actin cables in RhoBG14V-expressing cells may thus prevent microtubule-based transport and inhibit endosome homotypic fusion. To test this we co-expressed Rab5<sup>Q79L</sup> and RhoB<sup>G14V</sup>. When expressed alone, Rab5<sup>Q79L</sup> localised to
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Dia1 is a downstream effector of RhoB

Next, we sought to identify the effector molecule implicated in the regulation of endosome transport downstream of RhoB. Due to the high homology between the effector-binding regions of RhoA and RhoB, both GTPases may be able to interact with the same effector proteins in solution. However, RhoA is largely cytosolic while RhoB is targeted to the endosomal compartment. Therefore, the intracellular distribution of these GTPases may determine their respective roles by the specific activation of similar pathways on different intracellular compartments.

ROCK proteins mediate stress fibre formation downstream from RhoA. We investigated whether ROCK was involved in the effects induced by RhoB<sup>G14V</sup> by incubating cells with the ROCK inhibitor Y-27632. This drug did not inhibit the effects of RhoB<sup>G14V</sup> on endosome distribution (Fig. 6a). Furthermore, diffuse thin F-actin fibres remained after Y-27632 treatment of cells expressing RhoB<sup>G14V</sup> (Fig. 6b). Finally, a constitutively active mutant, ROCK<sub>Δ</sub>3, did not cause endosome dispersion or actin assembly on endosomes (not shown). The presence of diffuse F-actin fibres after Y-27632 incubation has been previously described in cells expressing a constitutively active mutant of the Diaphanous-related formin Dia1 (Watanabe et al., 1999). Therefore, we investigated the possibility that Dia1 mediated the RhoB effects by expressing GFP-tagged full-length Dia1 or the GFP-tagged truncation mutants Dia1-N1 (N-terminal Rho-binding domains; dominant negative form) and Dia1-ΔN3 (C-terminal FH1 and FH2 domains; constitutively active form) (Watanabe et al., 1999). RhoB<sup>G14V</sup> was able to recruit full-length Dia1 to endosomes (Fig. 7b). Notably, Dia1-N1 partially co-localised with RhoB and effectively blocked the RhoB<sup>G14V</sup> effects on the distribution of endosomes, which now located to a juxtanuclear position (Fig. 6c,d and Fig. 8f). In the presence of Dia1-N1, RhoB<sup>G14V</sup>-positive vesicles were

**Fig. 4.** Activated RhoB inhibits transport of receptor-mediated and fluid-phase endocytosed cargo to the juxtanuclear area and impairs endosome motility. The effect of RhoB<sup>G14V</sup> on receptor-mediated endocytosis was studied by incubating cells with 50 µg ml<sup>-1</sup> Texas Red-labelled human transferrin for 15 minutes. Myc-RhoB<sup>G14V</sup> was detected with 9E10 and FITC-labelled secondary antibodies (a). Fluid-phase endocytosis was analysed by 15 minutes incubation with the fluid-phase endocytic marker sulforhodamine 101 (SR101), followed by a 60 minutes chase (b). The effect of RhoB<sup>G14V</sup> on endosome motility was analysed on live cells transfected with pEGFP-FYVE (c). Myc-RhoB<sup>G14V</sup> cDNA was microinjected together with a 70 kDa Texas-Red-dextrane to mark the injected cells. Endosome dynamics was observed by time-lapse fluorescence confocal microscopy and analysed by projection of the time-lapse images (36 images with a time interval of 15 seconds) where vesicle movement is seen as green tracks. The projected image was then combined with a red-coloured image corresponding to t=0. In the RhoB<sup>G14V</sup> expressing cell (asterisk) vesicles form much shorter tracks (c, arrows) than in control non-injected cells (c, arrowheads). Nuclei of cells microinjected with myc-RhoB<sup>G14V</sup> are marked with an asterisk. Nuclei of control non-injected cells are marked with a circle. Bars: 10 µm.

**Fig. 5.** Activated RhoB prevents Rab5-dependent endosome fusion. Cells were microinjected with the cDNA for the activated mutant of Rab5 (myc-Rab5<sup>Q79L</sup>) alone (a) or in combination with myc-RhoB<sup>G14V</sup> (b,b′). Rab5 was detected with the mouse 4F11 monoclonal antibody and RhoB with the rabbit sc-119 polyclonal antibody. Expression of myc-Rab5<sup>Q79L</sup> induced the formation of large endosomes that localised to the juxtanuclear area (a). Co-expressed myc-Rab5<sup>Q79L</sup> and myc-RhoB<sup>G14V</sup> co-localised in smaller endosomes scattered over the cell (b,b′, arrows). Bars: 10 µm.
no longer encapsulated by an actin coat but showed F-actin at a discrete site on their membranes, like endosomes in control cells (Fig. 8h,i). This strongly suggests that Dia1 is required for RhoB-induced actin assembly on endosomes and thus crucial for RhoB regulation of endosome traffic.

To test whether Dia1 was sufficient to mediate RhoB effects on endosomes, a constitutive active mutant of Dia1 (Dia1-ΔN3) was expressed. Dia1-ΔN3 was recruited to vesicles scattered over the cell that were coated with F-actin (Fig. 9c,d and high magnification insets). Dia1-ΔN3 also induced the dispersion of EEA1-positive endosomes (Fig. 9a,b). Similar to RhoBG14V, Dia1-ΔN3 induced cell elongation and the formation of parallel thin actin cables (Fig. 9b) (Watanabe et al., 1999).

RhoB has been previously implicated in endocytosis of the EGF receptor in HeLa cells (Gampel et al., 1999). To test whether Dia1 was sufficient to mediate RhoB effects on endosomes, a constitutive active mutant of Dia1 (Dia1-ΔN3) was expressed. Dia1-ΔN3 was recruited to vesicles scattered over the cell that were coated with F-actin (Fig. 9c,d and high magnification insets). Dia1-ΔN3 also induced the dispersion of EEA1-positive endosomes (Fig. 9a,b). Similar to RhoBG14V, Dia1-ΔN3 induced cell elongation and the formation of parallel thin actin cables (Fig. 9b) (Watanabe et al., 1999).

RhoB has been previously implicated in endocytosis of the EGF receptor in HeLa cells (Gampel et al., 1999). To test whether Dia1 is involved in the regulation of this pathway, HeLa cells were transfected with RhoBΔ14V, Dia1-N1 or Dia1-ΔN3 or with both RhoBΔ14V and Dia1-N1. Transfection of RhoBΔ14V or the constitutively active Dia1-ΔN3 resulted in the accumulation of non-degraded EGF-receptor, which was reversed by co-transfection of Dia1-N1 (supplementary material Fig. S4).

Together, these results indicate that the formin protein Dia1 functions downstream of activated RhoB to promote actin polymerization on endosomes and, therefore, is critically involved in the regulation of endosomal cargo transport.

**Discussion**

Rho GTPases are essential regulators of filamentous actin reorganisation and have been implicated in the control of endocytosis. However, the link between these two functions of Rho GTPases remains unclear. The results presented in this study show that an activated form of the endosomal GTPase RhoB promotes the polymerisation of an actin coat around early endosomes and induces their association to subcortical actin fibres. Activated RhoB does not inhibit internalization from the plasma membrane, in contrast to other RhoGTPases (Symons and Rusk, 2003). However, endosomes containing the internalised cargo remain in the cell periphery, whereas they reach the juxtanuclear area in control cells. Dispersed endosomes show reduced motility and appear to be retained on actin filaments. This might be due to the crosslinking of the F-actin around endosomes with the actin cytoskeleton by myosin II. Indeed, myosin II redistributes and co-localises with actin-coated RhoBΔ14V-positive vesicles (supplementary material Fig. S2) although we have no direct evidence for a role of myosin II in this process. Importantly, the overexpression of wild type RhoB had no effect on endosome transport, suggesting that endogenous RhoB transiently inhibits endocytosis under the regulation of the GTPase cycle.

The small GTPase Rab5 is known to regulate homotypic fusion of endosomes and a constitutively active Rab5 mutant was shown to drive massive endosome fusion in a microtubule-dependent manner resulting in the formation of unusually large endosomes (Stenmark et al., 1994). A consequence of reduced endosome motility would be the impairment of the Rab5-dependent endosome fusion. RhoBΔ14V-positive endosomes are smaller, suggesting that endosome fusion is inhibited. Corroborating this observation, we show that a constitutively active mutant of Rab5 co-localises with RhoB on endosomes but it is no longer able to drive endosome fusion. Altogether, these results suggest that activated RhoB induces retention of endosomes on actin fibres and prevents their transfer onto microtubules, inhibiting fusion and further transport of endosomal cargo to lysosomes. This might explain the previously reported delay of internalised EGF-receptor transport to lysosomes by activated RhoB (Mellor et al., 1998).

Despite the high homology between RhoA and RhoB, their cellular effects on endosome distribution and cell shape differ largely. This may be due to their different intracellular...
localisations: mainly cytosolic for RhoA and endosomal for RhoB. Thus, RhoA and RhoB may activate similar pathways, although of different subcellular locations, which may result in different biological outcomes. Alternatively, the subcellular location may determine the accessibility of the GTPase to certain effectors.

For example, ROCK is critically involved in RhoA-regulated polymerisation of actin filaments downstream of RhoA (Ishizaki et al., 1996; Kimura et al., 1996), however, we found that ROCK does not have a role in the effect of RhoB on endosome transport by treatment of RhoB<sup>G14V</sup> expressing cells with the specific ROCK inhibitor Y27632 (Uehata et al., 1997). Furthermore, cells expressing activated RhoB showed diffuse F-actin fibres after treatment with Y-27632. A similar effect has been described in cells expressing activated Dia1 (Watanabe et al., 1999), which is suggestive of an involvement of Dia1 in the RhoB-induced effects.

Dia1 belongs to the Diaphanous-related formin (DRF) subfamily characterized by the presence of an N-terminal Rho-binding domain (RBD) and a C-terminal Diaphanous autoinhibitory domain (DAD) (Alberts, 2001). Binding of activated Rho to the RBD causes the activation of DRF proteins by relieving the intramolecular interaction with the C-terminal DAD. This activation unmasks the two conserved C-terminal formin-homology (FH) domains that are implicated in the control of actin polymerisation (Evangelista et al., 2003). Dia1 has been shown to be a downstream effector of RhoA and to be required for RhoA-induced actin stress fibre formation, microtubule stabilization and SRF activation (Watanabe et al., 1997; Watanabe et al., 1999; Nakano et al., 1999; Tominaga et al., 2000; Geneste et al., 2002; Palazzo et al., 2004). Interestingly, Dia1 was shown to interact with RhoA as well as with RhoB in vitro (Watanabe et al., 1999) and to localise to endosomes in HeLa cells (Tominaga et al., 2000). Here we show that Dia1 is recruited to endosomes by activated RhoB and that a deletion mutant lacking the FH domains (Dia1-N1) suppresses RhoB effects on endosomes, strongly suggesting that Dia1 is a downstream effector of RhoB. Consistently, we show that activated RhoB induces cell elongation and the formation of parallel thin actin fibres, a phenotype also reported for constitutively active mDia1 (Ishizaki et al., 1996). The fact that Dia1-N1 inhibits actin coat assembly on endosomes and prevents their dispersion suggests that the
RhoB-induced actin coat is necessary for the association of endosomes with subcortical actin fibres and for the arrest of endosome motility. In our experiments expression of the Dia1-N1 mutant alone did not affect the F-actin content of cells or endosome distribution (Fig. 8a-c). This indicates that, under our experimental conditions, this mutant does not inhibit endogenous RhoA-dependent pathways. The discrepancy with previous studies that show the disappearance of actin stress fibres upon Dia1-N1 expression might be due to the shorter time lapse between cDNA transfection/microinjection and cell fixation in our experiments (4 to 5 hours) compared to others (Watanabe et al., 1999; Krebs et al., 2001). The role of Dia1 in endosome transport was further corroborated by the expression of Dia1-ΔN3, an active mutant lacking the RBD, which localised to endosomes and induced the same effects as activated RhoB. The fact that Dia1-ΔN3 localised to endosomes despite lacking the RBD suggests that the C-terminal part of Dia1 interacts with additional factors on endosomes (Olson, 2003). Interestingly, another endosomal Rho GTPase, RhoD, has been shown to regulate endosome transport through the DRF protein hDia2C, but not through mDia1 (Gasman et al., 2003). Similarly to RhoB, the expression of an activated mutant of RhoD (RhoD<sup>G26V</sup>) inhibits endosome motility and induces the association of endosomes with actin filaments. However, unlike RhoB<sup>G14V</sup>, RhoD<sup>G26V</sup> expression causes loss of stress fibres and only induces actin filaments when co-expressed with hDia2C (Murphy et al., 1999; Gasman et al., 2003). At any rate, both Gasman et al. and our study suggest that endosomal Rho GTPases (RhoB and RhoD) may stabilize endosome association with the actin cytoskeleton through the recruitment and activation of a DRF protein. In addition to this, our results provide a possible mechanism for the control of endosome traffic by RhoB-Dia1 through the assembly of actin on endosomes, in agreement with the established function of Rho GTPases as promoters of actin polymerisation. Furthermore, RhoB has a short half-life and its synthesis is upregulated by several growth factors and stress stimuli (Prendergast, 2001). Thus, although apparently both RhoB and RhoD have similar functions concerning the regulation of endosome traffic, the RhoB-Dia1 pathway may operate only under certain conditions while the RhoD-hDia2C pathway may act in a more constitutive manner.

Overexpression of RhoB was shown to cause the accumulation of endocytosed EGF in peripheral vesicles and to delay EGF-receptor traffic (Gampel et al., 1999). Furthermore, PRK1 was found to mediate the effects of RhoB on EGF-receptor traffic. Our results show that RhoB acts through Dia1 to induce actin polymerization on endosomes and their association to actin cables. In addition, dominant negative Dia1 reverses the inhibition of EGF-receptor degradation caused by activated RhoB in HeLa cells. Thus, it is possible that Dia1 and PRK1 co-operate in the regulation of endosome traffic downstream of RhoB, Dia1 through the polymerisation of actin on endosomes and PRK1 through the phosphorylation of a yet unknown target.

RhoB has been proven to be a negative regulator of cellular transformation (Liu et al., 2001; Jiang et al., 2004a; Jiang et al., 2004b) and a crucial target for the anti-tumour drugs farnesyl-transferase inhibitors (FTIs) (Prendergast, 2001). FTIs deplete cells from the farnesylated form of RhoB with the subsequent enrichment of the geranylgeranylated form, which reduces proliferation and survival of transformed cells (Du et al., 1999; Du and Prendergast, 1999; Liu et al., 2000). Notably, in HeLa cells geranylgeranylated-RhoB localises to endosomes and inhibits EGF receptor traffic in a greater extent when constitutively activated (Wherlock et al., 2004). Interestingly, inactive RhoB is localised to the plasma membrane and cytoplasm (supplementary material Fig. S3) and it is activated on endosomes by Vav2 (Gampel and Mellor, 2002). Furthermore, wild type RhoB localises to the plasma membrane and to juxtanuclear endosomes (supplementary material Fig. S3), suggesting that endogenous RhoB cycles between these two compartments. Taken altogether, a model for the biological function of RhoB emerges (Fig. 10) in which RhoB is internalised from the plasma membrane and activated on endosomes by the Vav2 exchange factor. Subsequently, activated RhoB recruits and activates Dia1 at endosomes, exposing the Dia C-terminal domain which then interacts with other endosomal proteins (such as actin or actin-polymerisation factors). Dia1 induces the assembly/elongation of actin filaments on endosomes and stabilises the association of endosomes with actin filaments. F-actin is a dynamic polymer, which length is determined by the respective rates of polymerisation and depolymerisation. In our model,
inactivation of RhoB stops actin polymerisation while depolymerisation continues, resulting in the removal of the actin coat from endosome membranes. Finally, endosomes bind to microtubules via the minus-end directed motor protein dynein for further transport toward the centre of the cell (Hirokawa, 1998).

In conclusion, our results suggest that the RhoB-Dia1 pathway induces the transient association of endosomes with actin fibres and delays endosome maturation into later compartments, further establishing the role of DRFs in the regulation of endocytosis. The tandem RhoB-Dia1 exemplifies a novel pathway linking membrane trafficking with the regulation of actin dynamics.

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References


RhoB$^{G14V}$

(a) Myosin IIA