Regulation of mitochondria distribution by RhoA and formins

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Summary

The distribution of mitochondria is strictly controlled by the cell because of their vital role in energy supply, regulation of cytosolic Ca^{2+} concentration and apoptosis. We employed cultured mammalian CV-1 cells and *Drosophila* BG2-C2 neuronal cells with enhanced green fluorescent protein (EGFP)-tagged mitochondria to investigate the regulation of their movement and anchorage. We show here that lysophosphatidic acid (LPA) inhibits fast mitochondrial movements in CV-1 cells acting through the small GTPase RhoA. The action of RhoA is mediated by its downstream effectors: formin-homology family members mDia1 in mammalian cells and diaphanous in *Drosophila*. Overexpression of constitutively active mutant forms of formins leads to dramatic loss of mitochondrial motility and to their anchorage to actin microfilaments. Conversely, depletion of endogenous diaphanous protein in BG2-C2 cells by RNA interference (RNAi) stimulates the mitochondrial movement. These effects are not simply explained by increased cytoplasm viscosity resulting from an increased F-actin concentration since stimulators of Arp2/3-dependent actin polymerization and jasplakinolide do not cause inhibition. The observed effects are highly specific to mitochondria since perturbations of diaphanous or mDia1 have no effect on movement of other membrane organelles. Thus, mitochondrial movement is controlled by the small GTPase RhoA and this control is mediated by formins.

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Introduction

Mitochondria play a unique role in cellular physiology. They are responsible for energy production, regulation of Ca^{2+} concentration in the cytoplasm and programmed cell death. These important functions make the study of mitochondrial transport and localization an important topic in cell biology (Hollenbeck, 1996). Previous research has shown that mitochondria can move in cells along microtubules and actin filaments (Morris and Hollenbeck, 1995; Ligon and Steward, 2000) and several molecular motors that could be involved in this movement have been identified (Nangaku et al., 1994; Tanaka et al., 1998).

In many types of cultured cells, mitochondria are distributed throughout the cytoplasm and this distribution depends on the integrity of cytoplasmic microtubules (Ball and Singer, 1982). However, often a considerable fraction of these organelles reside in the perinuclear area, whereas at the periphery mitochondria are distributed more sparsely (Tanaka et al., 1998; Trinczek et al., 1999; Collins et al., 2002). The majority of mitochondria at the periphery remains immobile or moves slowly, and only a smaller fraction moves with relatively high speed (Trinczek et al., 1999; De Vos et al., 2003). The presence of immobile organelles is probably explained by their anchoring at sites in the cytoplasm where local ATP supply is required. On the basis of ultrastructural and biochemical studies, several mechanisms for mitochondria 'docking' to microtubules and intermediate filaments have been proposed (Leterrier et al., 1994; Wagner et al., 2003). Thus, the control of the mitochondrial motility should be considered not only in terms of regulation of motor protein activity but also as a switching between stationary and motile phases.

Substantial progress has been achieved in studies of mechanisms by which extracellular signals affect the cytoskeleton and change cell morphology. Members of the Rho family of small GTPases and their effectors were shown to regulate both actin cytoskeleton re-arrangements and microtubule dynamics (for reviews, see Bar-Sagi and Hall, 2000; Wittmann and Waterman-Storer, 2001). For example, Rac1 and Cdc42 induce formation of lamellipodia and filopodia, respectively, by activation of actin polymerization at the plasma membrane (Nobes and Hall, 1995). At the same time, Rac1 promotes microtubule growth into advancing lamellipodia of migrating cells (Wittmann et al., 2003), and Cdc42 is involved in the orientation of the microtubule-

organizing center towards the leading edge of migrating cells (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001b). Activation of RhoA induces the formation of focal adhesions and stress fibers (Ridley and Hall, 1992) and has been implicated in the stabilization of microtubules (Cook et al., 1998; Palazzo et al., 2001a).

The action of RhoA on the cytoskeleton is mediated by its downstream effectors. One of them, Rho-associated kinase (ROCK), phosphorylates the phosphatase of myosin light chains, thus inhibiting its activity and increasing myosin activity (Kimura et al., 1996). However, the enhanced myosin contractility induced by ROCK leads to the formation of normal stress fibers only when its action is coordinated with another RhoA effector, mDia1 (Watanabe et al., 1999). The molecular mechanism of this coordination remains to be elucidated, but the available data point to the involvement of microtubules in the activity of this protein.

mDia1 is a mammalian homolog of diaphanous, a protein first found in *Drosophila*, and is a member of the formin-homology family of proteins (Watanabe et al., 1997). Formins are highly conserved among eukaryotes and are implicated in many actin-based processes such as cytokinesis, cell polarization, spermatozoa acrosome formation, and others (reviewed by Zeller et al., 1999). The most important common feature of formin proteins is their two proline-rich forming-homology domains, FH1 and FH2 (Castrillon and Wasserman, 1994), that participate in control of actin polymerization (Evangelista et al., 1997; Evangelista et al., 2002; Sagot et al., 2002a; Sagot et al., 2002b; Watanabe et al., 1999). The FH1 domain binds to G-actin-binding protein profilin (Chang et al., 1997; Evangelista et al., 1997; Watanabe et al., 1997; Krebs et al., 2001; Kovar, 2004), which can deliver actin monomers to growing ends of actin filaments. Some other proteins such as non-receptor tyrosine kinase Src also associate with the FH1 domain of mammalian diaphanous mDia (Tominaga et al., 2000). The FH2 domain possesses a unique property to nucleate actin filaments in vitro (Pruyne et al., 2002; Sagot et al., 2002b; Li and Higgs, 2003) and maintains their assembly in cells (Copeland and Treisman, 2002; Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002a; Sagot et al., 2002b). mDia1, like other diaphanous-related formins (DRFs), also contains a Rho-GTP-binding domain (RBD) at the N-terminus (Evangelista et al., 1997; Watanabe et al., 1997) and a diaphanous autoregulatory domain (DAD) at the C-terminus (Castrillon and Wasserman, 1994; Watanabe et al., 1997; Alberts, 2001). An intramolecular inhibitory interaction occurs between the RBD and DAD regions of mDia in the absence of other ligands; RhoA-GTP binds to the RBD terminus, disrupting this interaction and activating mDia (Watanabe et al., 1997; Alberts, 2001). Thus, DRFs can translate different signals into Arp2/3-independent formation of actin filaments (reviewed by Zigmond, 2004).

In addition to their role in actin-based processes, mDia proteins were identified as RhoA effectors involved in microtubule stabilization (Palazzo et al., 2001a; Bershadsky et al., 2003). Furthermore, a constitutively active mutant of mDia1 induces the alignment of microtubules and bundles of actin filaments, and point mutations in its FH2 domain impede this alignment (Ishizaki et al., 2001).

Much less is known about the consequences of mDia-induced reorganizations of cytoskeleton on organelle transport. However, it was reported that mDia3 (hDia2C), activated by RhoD, had an inhibitory effect on the motility of early endosomes inducing their alignment with actin filaments (Gasman et al., 2003). Recently, another Rho GTPase, RhoB and its effector Dia1, were implicated in the regulation of endosome transport (Fernandez-Borja et al., 2005). In both cases, DRFs were recruited to endosomes by activated Rho GTPases and caused the formation of F-actin coat around these organelles.

An important contribution in studies of regulation of mitochondrial motility was provided by P. Hollenbeck's laboratory. They have demonstrated that the transport and distribution of mitochondria in neuronal cells is regulated concordantly with growth cone motility: the organelles are actively translocated to the sites of ATP consumption, whereas their supply to non-motile growth cones is severely restricted (Morris and Hollenbeck, 1993; Chada and Hollenbeck, 2003). In addition to an upregulation of mitochondrial transport to the active regions of the neurites, their selective retention at these sites was also observed (Chada and Hollenbeck, 2003).

Here, we show that a new regulatory pathway that includes the small GTPase RhoA and its downstream effector mDia1/diaphanous is responsible for regulation of motility of mitochondria in mammalian and *Drosophila* cells by inducing organelle binding to the actin cytoskeleton. Inhibition of fast mitochondrial movements was not simply a consequence of increased cytoplasm viscosity as a result of enhanced actin polymerization since other ligands that are known to induce actin polymerization had no effect on mitochondrial movement. Effects of mDia1/*diaphanous* were also highly specific to mitochondria, as no modulation of transport of such membrane organelles as lysosomes or peroxisomes was observed, which additionally argues against the viscosity model. Thus, mDia1 and diaphanous specifically regulate the movement of mitochondria in cells. This regulatory mechanism is conserved between *Drosophila* and mammals, suggesting its biological significance.

Results

Lysophosphatidic acid (LPA) inhibits the motility of mitochondria through RhoA

Mitochondria at the cellular periphery are characterized by two distinct types of behavior – stationary state and motile state. In the stationary state, they are probably anchored to the cytoskeleton; by contrast, in the high-motility state, they are transported along microtubules. To investigate the mechanisms of transition between these two states, we studied the behavior of fluorescently tagged individual mitochondria in live cells. To accomplish this goal, we selected two cell lines, mammalian CV-1 cells and *Drosophila* BG2-C2 neuronal cells stably transfected with the plasmids pEYFP-Mito and pAc-EGFP-Mito, respectively. These plasmids encode fluorescent protein probes targeted to mitochondria. Both probes displayed clear mitochondrial localization, as was demonstrated earlier for EYFP-Mito (Rizzuto et al., 1995), and completely colocalized with fluorescent mitochondrial dyes (MitoTracker-Red and Rhodamine 123) (data not shown). Expression of fluorescently tagged mitochondrial markers had no effect on cellular morphology and behavior. A typical distribution of fluorescent mitochondria in a polarized CV-1 cell and in BG2-C2 cells with pronounced neurite-like processes is shown in Fig. 1.

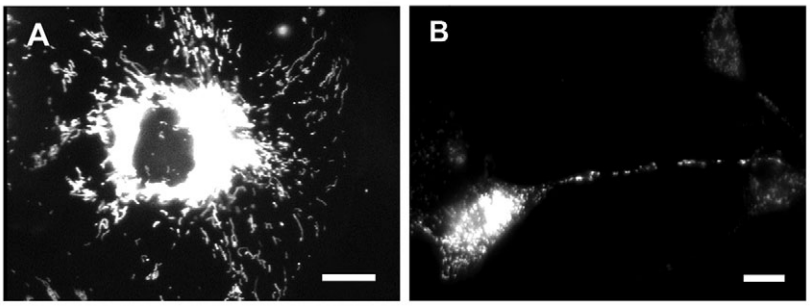

Fig. 1. Distribution of fluorescently labeled mitochondria in CV-1 and BG2-C2 cells. Fluorescent images showing (A) a CV-1 cell transfected with the plasmid pEYFP-Mito and (B) *Drosophila* BG2-C2 cells transfected with pAc-EGFP-Mito plasmid. See Movies 1 and 2 in supplementary material. Bars, 10 μm.

The morphology of mitochondria in two cell types was different: those in mammalian CV-1 cells had an elongated form (Fig. 1A), whereas those in *Drosophila* BG2-C2 cells were much smaller but still elongated (Fig. 1B). However, when analyzed by time-lapse microscopy, the motility of mitochondria in both cell types was similar. First, only a small fraction of mitochondria moved relatively long distances, whereas the majority of organelles were stationary (Fig. 2A and supplementary material Movies 1 and 2). Second, in both cell lines, the movement was saltatory and equal amounts of organelles were transported towards the cell center and towards the periphery in each given time interval (data not shown). Finally, stationary mitochondria at the periphery often formed aggregates. Quantitative analysis of the movements of individual mitochondria in CV-1 cells showed that only 7±1.5% of all displacements exceeded the threshold of 0.2 μm/second and 60±4% of mitochondria did not noticeably move during an interval of 3 minutes. The fast movements ceased in cells treated with the microtubule antagonist nocodazole, indicating that they move along microtubules, as was shown earlier by others (Morris and Hollenbeck, 1995; Varadi et al., 2004). We did not observe any movements of mitochondria faster than 0.2 μm/second after microtubule depolymerization. Analysis of mitochondrial motility in BG2-C2 cells gave similar results (see below).

Our previous work showed that distribution and transport of mitochondria depend on the presence of serum in the tissue culture medium (Kulik et al., 2003). To investigate which serum components affect mitochondrial motility, we tested several agents and found that LPA, a prominent serum growth factor (Moolenaar, 1999), has a dramatic effect on the behavior of mitochondria in CV-1 cells. Even in the presence of serum in the culture medium, this growth factor greatly decreased the motility of these organelles almost immediately. As seen in Fig. 2C, after addition of LPA, mitochondria formed clusters or groups in lamellae and some looked swollen (compare with Fig. 1A and Fig. 2A). In some cells, LPA treatment led to the formation of elongated and branched mitochondria (data not shown). When analyzed by time-lapse microscopy, very few mitochondria in these cells displayed motile behavior. As shown in Fig. 2D, the tracks of the moving mitochondria in the presence of LPA were very short and most organelles did not change their position during the recording time. The results of quantitative analysis of mitochondrial motility in cells treated

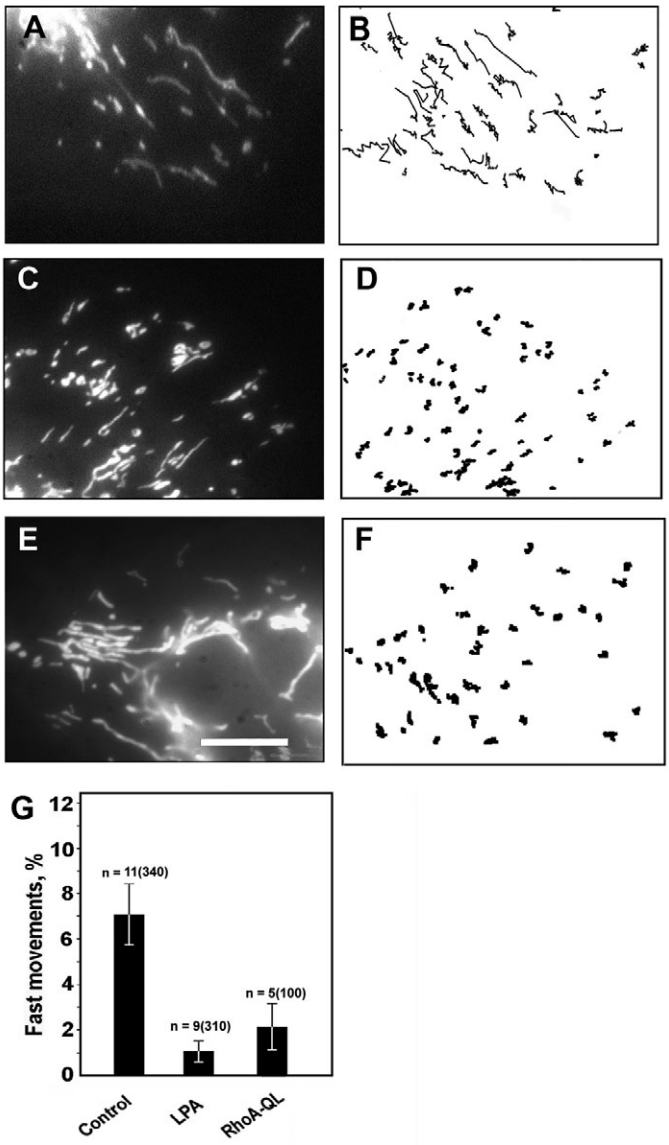

Fig. 2. LPA inhibits the motility of mitochondria in CV-1 cells through RhoA. (A,C,E) The first frame of the image sequence recorded, and (B,D,F) the plotted tracks of individual mitochondria in the same cells. (A,B) Control cells; (C,D) cells were treated with 5 μM LPA for 5 minutes; (E,F) cells were transfected with plasmid pcDNA3-EGFP-RhoA(Q63L). See also Movie 3 in supplementary material. Bar, 10 μm. (G) Quantification of mitochondrial motility in control cells, cells treated with 5 μM LPA or cells expressing EGFP-RhoA(Q63L) (RhoA-QL). Values are the mean percentage of movements exceeding 0.2 μm/second from all movements ± s.e.m.; $P<0.05$; n = number of cells and, in brackets, number of organelle movements.

with LPA are shown in Fig. 2G. These results indicate that LPA stops movement of mitochondria.

LPA has multiple effects on the cytoskeleton. When added to culture medium, it stimulates the formation of stress fibers and focal adhesions in different cell types, and induces the formation of stable microtubules. LPA acts through a small GTPase RhoA (Ridley and Hall, 1992; Cook et al., 1998) and its downstream effectors, including Rho-kinase (ROCK) (Matsui et al., 1996; Ishizaki et al., 1997) and mDia (Watanabe et al., 1997; Ishizaki et al., 2001; Palazzo et al., 2001a). We set out to verify whether RhoA is involved in the regulation of mitochondrial distribution by LPA. We transfected CV-1 cells with a constitutively active mutant form of RhoA. To visualize cells that co-expressed an enhanced yellow fluorescent protein (EYFP)-labeled mitochondria probe and an enhanced green fluorescent protein (EGFP) fusion of RhoA(Q63L), we utilized the fact that EYFP fluorescence that can be detected both in green and red channels.

Expression of a constitutively active mutant of RhoA, EGFP-RhoA(Q63L), in CV-1 cells resulted in a gross alteration of mitochondria morphology (Fig. 2E). Some mitochondria merged to form long-branched organelles and fused to form the extensive networks filling the entire cytoplasm (data not shown). Neither the large organelles nor those of smaller size moved significant distances when analyzed by time-lapse microscopy (see the tracks in Fig. 2E and supplementary material Movie 3). The overall motility of mitochondria in these cells was considerably suppressed (Fig. 2G). We microinjected CV-1 cells with C3 transferase, a potent inhibitor of RhoA (Ridley and Hall, 1992), to confirm its involvement in the regulation of mitochondrial distribution. This led to fragmentation of mitochondria and their collapse to the perinuclear region (supplementary material Fig. S1A,B). Thus, our data suggest that RhoA controls not only the anchoring of mitochondria to the cytoskeleton but also their morphology.

Diaphanous formins are responsible for mitochondrial anchoring

Since perturbations of the cytoskeleton induced by RhoA, such as a formation of stress fibers, are mediated by its downstream effectors ROCK (Ishizaki et al., 1997) and mDia (Watanabe et al., 1999), we next investigated their possible involvement in the regulation of mitochondrial movement. Towards this goal, we expressed mutant forms of mDia1 in CV-1 cells. A constitutively active mutant of mDia1, EGFP-mDia1-ΔN3, containing both FH1 and FH2 domains but lacking the Rho-GTP-binding RBD region, induced the formation of parallel bundles of actin filaments that were co-aligned with microtubules, in agreement with previously published data (Fig. 3A,B) (Ishizaki et al., 2001). The majority of microtubules were stabilized, as revealed by immunostaining with antibodies against Glu-tubulin (Fig. 3B). Mitochondria in these cells formed a pattern parallel to the direction of the cytoskeletal structures (Fig. 4A). Fast movements of organelles in cells expressing EGFP-mDia1-ΔN3 were strongly inhibited (Fig. 4C and supplementary material Movie 4). Thus, activated mDia1 not only induced rearrangement of microtubules and the actin cytoskeleton, but also caused the anchoring of mitochondria along cytoskeletal structures.

Our data suggest that mitochondrial motility could be regulated by LPA through the signaling cascade that involves

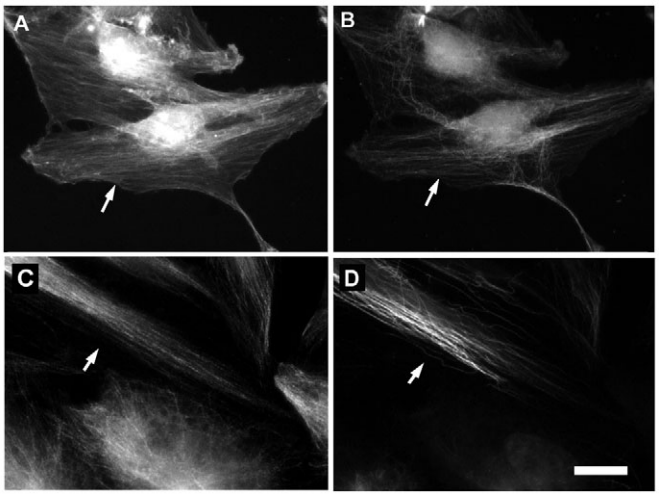

Fig. 3. Effects of a constitutively active mutant of mDia1 on the cytoskeleton of CV-1 cells. Cells were transfected with plasmids encoding EGFP-tagged mDia-ΔN3. After fixation in 4% formaldehyde with 1% Triton X-100, cells were stained with TRITC-phalloidin to visualize F-actin (A) or with antibodies against α-tubulin and TRITC-labeled secondary antibodies (C), and with antibodies against Glu-tubulin and FITC-labeled secondary antibodies to visualize stable microtubules (B,D). Transfected cells were found before fixation by EGFP fluorescence (shown by arrows). Bar, 10 μm.

RhoA and its downstream effector mDia1. If this is the case, expression of a constitutively active mutant of mDia1 should relieve the inhibitory effect of C3 toxin on mitochondria. In fact, microinjection of C3 toxin did not affect either distribution or morphology of mitochondria in cells transfected with pEGFP-mDia1-ΔN3 (supplementary material Fig. S1C,D).

Since DRFs could be activated not only by RhoA but also by other Rho-family GTPases such as Cdc42 (Peng et al., 2003), we sought to examine the possibility that mitochondrial motility was inhibited through the Cdc42-mDia pathway. We expressed a constitutively active mutant of Cdc42 in CV-1 cells and analyzed its effect on mitochondria movements. In contrast to the action of an activated mutant of RhoA, Cdc42(Q61L) slightly enhanced motility (Fig. 4C). Thus, our data argue against the involvement of Cdc42 in mDia-dependent inhibition of mitochondria movements.

mDia1-ΔN3 is a potent activator of actin polymerization (Ishizaki et al., 2001; Krebs et al., 2001), and therefore suppression of mitochondria transport could potentially be explained by trapping of organelles in a network of actin filaments formed in cells expressing it. If this explanation is correct, then any stimulator of actin polymerization should have a similar inhibiting effect on movement of mitochondria. To test this possibility, we expressed an unrelated stimulator of actin polymerization, C-terminal WA domain of Wiskott-Aldrich syndrome protein (N-WASP) (Moreau et al., 2000). As expected, the expression of this protein caused induction of actin polymerization (Fig. 4D); however the fast movements of mitochondria remained at the same level as in control cells (Fig. 4C). We also tested the effect of a constitutively active mutant of a small GTPase Rac1 on mitochondrial motility (Fig.

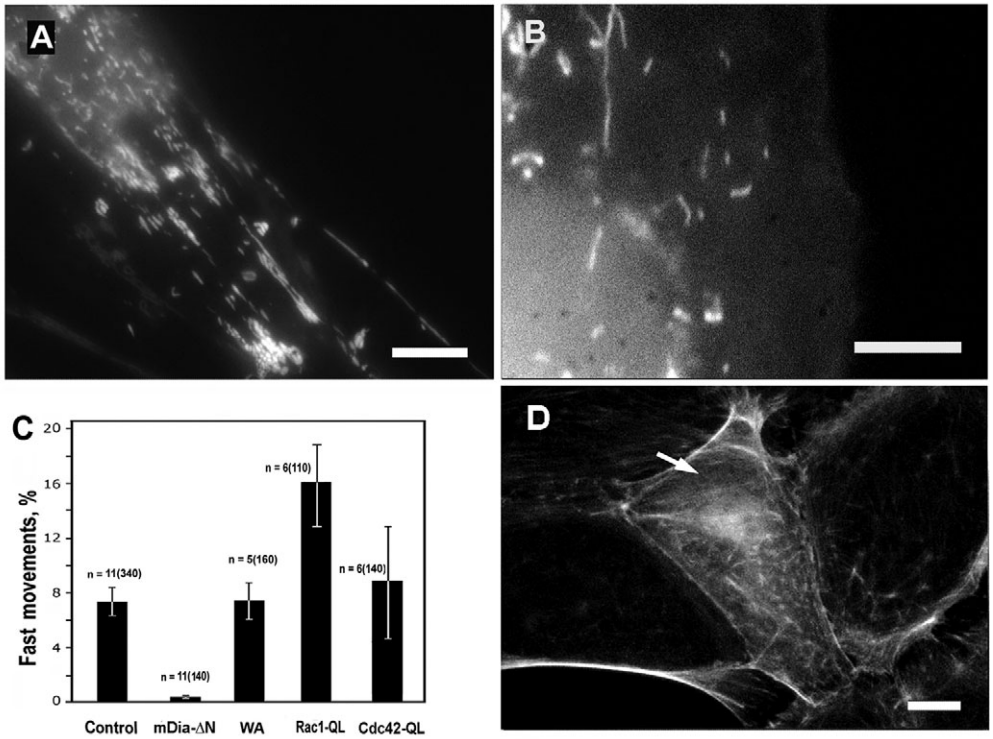

Fig. 4. A constitutively active mDia1 mutant, but not other proteins promoting actin polymerization, inhibits the motility of mitochondria. CV-1 cells were transfected with plasmids encoding EGFP-mDia-ΔN3, EGFP-Rac1(Q61L), EGFP-Cdc42(Q61L) or EGFP-WA, and the movement of fluorescently labeled mitochondria was recorded by time-lapse video microscopy. The first frames of the image sequences, showing different mitochondria distribution in cells expressing EGFP-mDia-ΔN3 (A), and EGFP-Rac1(Q61L) (B) (see Movies 4 and 5 in supplementary material). (D) The cell expressing EGFP-WA (shown with arrow) with increased level of F-actin visualized by TRITC-phalloidin staining. Bars, 10 μm. (C) Quantification of mitochondrial motility in cells expressing EGFP-mDia-ΔN (mDia-ΔN), EGFP-WA (WA), EGFP-Rac1(Q61L) (Rac1-QL) or EGFP-Cdc42(Q61L) (Cdc-QL). Values are the mean percentage of movements exceeding 0.2 μm/second from all movements ± s.e.m.; $P<0.05$; n = number of cells and, in brackets, number of organelle movements.

4B and supplementary material Movie 5). This mutant, Rac1(Q61L) activates polymerization of actin and lamellipodia formation (Ridley et al., 1992). However, mitochondria in cells expressing Rac1(Q61L) were even more motile than in control cells (Fig. 4C). In addition, we examined the effect on mitochondrial motility of jasplakinolide, a potent stabilizer of F-actin (Bubb et al., 2000). Although this drug caused a massive increase of F-actin in the treated cells (supplementary material Fig. S2D), the motility of mitochondria in these cells was not inhibited (supplementary material Fig. S3). Thus, our data demonstrate that induction of actin polymerization in cells using different modes has different consequences for mitochondrial motility. Whereas mDia-dependent F-actin induction inhibits movements of mitochondria, Arp2/3-dependent or jasplakinolide-induced F-actin increases do not affect it.

In order to confirm our finding implicating mDia in the regulation of mitochondrial anchoring, we used a different cell model, a *Drosophila* neuronal cell line BG2-C2. Experiments with BG2-C2 cells allow us to see if identical mechanisms regulate mitochondria movement in two evolutionarily distant species and were facilitated by the use of RNA interference (RNAi) technology to selectively knockdown components of the regulatory pathway to determine their role in regulating motility of mitochondria. Assuming that, similar to its mammalian counterpart, *Drosophila* diaphanous is inhibited by interaction of its RBD domain with the C-terminus, we generated a construct encoding EGFP fused with a truncated version of diaphanous lacking 425 N-terminal residues. This protein contains both FH1 and FH2 domains but lacks RBD. Overexpression of this C-terminal fragment of diaphanous, EGFP-C-Dia, in BG2-C2 cells resulted in substantial morphological changes. Transfected cells were round in shape and had no processes (Fig. 5). The amount of polymerized actin as detected by fluorescent phalloidin staining was strongly elevated (Fig. 5F), and microtubules had a characteristic rosette-like pattern (compare with a non-transfected cell in the upper right corner of Fig. 5C). Thus, our results show that the C-terminal fragment of diaphanous acts in *Drosophila* cells as a constitutively active mutant and induces gross cytoskeleton rearrangements. The morphology and distribution of mitochondria in transfected cells were also altered: organelles assumed an elongated shape and were localized along cellular radii (Fig. 5I). Such redistribution was accompanied by a complete loss of fast movements (Fig. 7E and supplementary material Movie 6). Thus, our data demonstrate that, like mDia1 in mammalian cells, diaphanous regulates motility of mitochondria in cultured *Drosophila* cells.

Using mutant forms of mDia1 in CV-1 cells and diaphanous protein in *Drosophila* BG2-C2 cells, we showed that these proteins are involved in the regulation of mitochondrial motility. However, it was not clear to what extent endogenous diaphanous affects the motility of these organelles. The *Drosophila* cell cultures offer an amenable model for knockdown proteins by RNAi (Rogers et al., 2003), and we used double-stranded (ds)RNA to deplete this protein. Western blot analysis (Fig. 6A) demonstrated that incubation of BG2-C2 cells in medium containing dsRNA complementary to diaphanous mRNA for 3 days caused the marked decrease of this protein and, after 7 days treatment, the level of diaphanous decreased to below 5% of untreated control. At the same time, the amount of kinesin heavy chain used as a control was not

changed (Fig. 6A). The depletion of diaphanous did not change the morphology of BG2-C2 cells: they remained attached to the substrate and had normal processes (Fig. 6B). However, in agreement with our data obtained in mammalian and *Drosophila* cells using mutant constructs, knockdown of diaphanous led to an increased motility of the mitochondria (Fig. 6C). Thus, our data indicate that diaphanous inhibits the motility of mitochondria.

Role of ROCK in mitochondrial anchoring

The second downstream effector of RhoA that acts on the actin cytoskeleton together with mDia is ROCK (Ishizaki et al., 1997). Activation of this kinase by RhoA is implicated in the formation of stress fibers and focal contacts (Amano et al., 1997). ROCK increases the phosphorylation of myosin light chains and thus increases acto-myosin-based contractility by directly phosphorylating myosin light chains and by negatively regulating myosin phosphatase (Kureishi et al., 1997) (reviewed by Fukata et al., 2001). In the experiments described above, we demonstrate that mDia1/diaphanous regulates mitochondrial motility. It is therefore important to understand whether movement of mitochondria is similarly regulated by the second RhoA effector (ROCK). We first examined the effect of a chemical inhibitor of ROCK, Y-27632 (Uehata et al., 1997), on the inhibition of mitochondrial movements by LPA. These experiments demonstrate that Y-27632 restores normal motility of mitochondria blocked by LPA (Fig. 7E). Thus, our data could argue for the participation of ROCK together with mDia1 in the regulation of mitochondrial motility. To test if the activity of ROCK is required for mitochondrial anchoring induced by an active mutant of mDia1, we treated cells expressing EGFP-mDia1-ΔN3 with Y-27632. The results of these experiments show that inhibition of Rho-kinase does not restore movement of mitochondria inhibited by constitutively active mDia1 (Fig. 7E).

To explore the role of ROCK further, we expressed dominant-negative (RB/PH) and constitutively active (CAT) forms of ROCK (Amano et al., 1998) in CV-1 cells. As was expected, expression of ROCK-CAT resulted in strongly developed actin stress fibers (Fig. 7B), whereas ROCK-RB/PH prevented their formation at the central part of the cells (Fig. 7D). However, the motility of mitochondria in these cells did not change (Fig. 7E). In addition, when ROCK-RB/PH was co-expressed with EGFP-mDia1-ΔN3, microfilament bundles became wavy and less straight compared with cells expressing EGFP-mDia1-ΔN3 alone, similar to the effect of Y-27632 on these cells (see supplementary material Fig. S2E,F). However, this co-expression did not suppress the inhibition of mitochondrial movements caused by the mDia1 mutant (Fig. 7E). Thus, activation of mDia1 was necessary and sufficient for mitochondrial anchoring at the cell periphery. We believe that modulation of LPA effects by Y-27632 could not be explained by inhibition of ROCK, but is probably mediated by its action on a different protein kinase; for a discussion of Y-27632 specificity, see Davies et al. (Davies et al., 2000)].

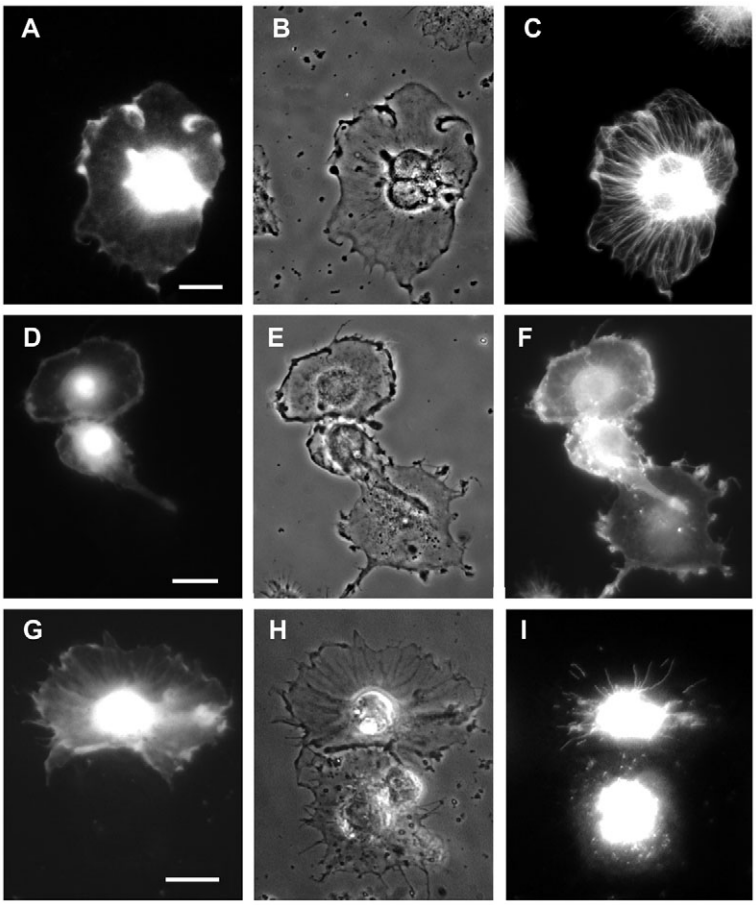

Fig. 5. The constitutively active mutant of diaphanous induces mitochondrial anchoring in *Drosophila* BG2-C2 cells. BG2-C2 cells were transfected with a plasmid encoding EGFP-C-Dia. Cells were either fixed for cytoskeleton staining (A-F) or incubated with 100 nM Texas Red Mitotracker for the analysis of mitochondrial motility (G-I). (A,D,G) Fluorescent images that show the transfected cells. (C) Cells were stained with anti-tubulin antibody and TRITC-labeled secondary antibody. Microtubules in transfected cells form the characteristic rosette-like pattern. (F) Cells were stained with TRITC-phalloidin; the higher level of F-actin could be seen in transfected cells. (B,E,H) Phase-contrast images of cells shown in A,D,G, and (I) the first frame in the video sequence (Movie 6 in supplementary material) showing the distribution of elongated mitochondria in the transfected cell shown in G. Bars, 10 μm.

The motility of lysosomes and peroxisomes is not affected by diaphanous

We next asked if the affects of diaphanous are specific for mitochondria or if it affects the transport of other organelles as well. To address this question, we selected stably transfected BG2-C2 cell lines expressing fluorescently labeled lysosomes or peroxisomes. The movements of both types of organelles could be easily observed in the cell body as well as in the processes (data not shown). Similar to mitochondria, both peroxisomes and lysosomes can be in either motile or stationary states, although lysosomes showed a much higher level of motility than mitochondria (Fig. 6D). However, depletion of diaphanous in these cell lines had no effect on the movement of lysosomes (Fig. 6D). The motility of

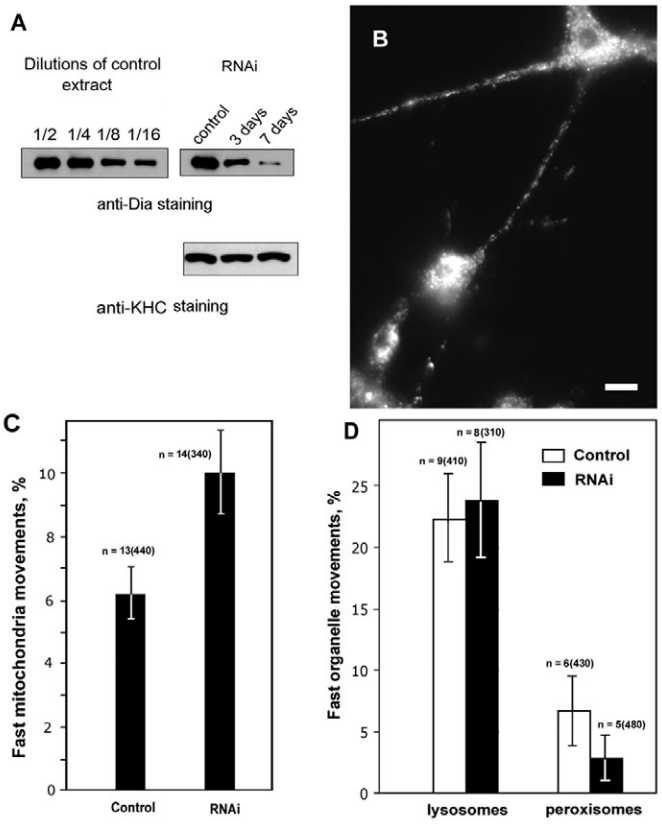

Fig. 6. RNAi-mediated depletion of diaphanous increases the motility of mitochondria in BG2-C2 cells. Cell lines with fluorescently labeled mitochondria, peroxisomes and lysosomes were incubated in culture medium containing 10 μg per ml of dsRNA complementary to diaphanous mRNA. The content of target protein was estimated before treatment and after 3 and 7 days of incubation. The organelle motility in these cells was analyzed after 7 days of incubation. (A) Western blot analysis of diaphanous before and after incubation of cells with dsRNA for 3 and 7 days, respectively. Left panel shows results of serial dilutions of control cell extract used for calibration of the western blot. KHC, kinesin heavy chain. (B) Fluorescent image of cells with labeled mitochondria after 7 days of incubation with dsRNA. Bars, 10 μm. (C) Quantification of mitochondrial motility in cells after RNAi. The control cells were treated the same way as those exposed to RNAi with the only exception that dsRNA was omitted. Values are the mean percentage of movements exceeding 0.2 μm/second from all movements ± s.e.m.; $P<0.05$; n = number of cells and, in brackets, number of organelle movements. (D) The motility of lysosomes and peroxisomes labeled with EGFP-tagged probes (see Materials and Methods) was analyzed as described for mitochondria. Average travel distances and velocities were measured as described in the Materials and Methods, and fast movements were determined as in Fig. 2. Values are the mean percentage ± s.e.m.; $P<0.05$; n, number of cells and, in brackets, number of organelle movements.

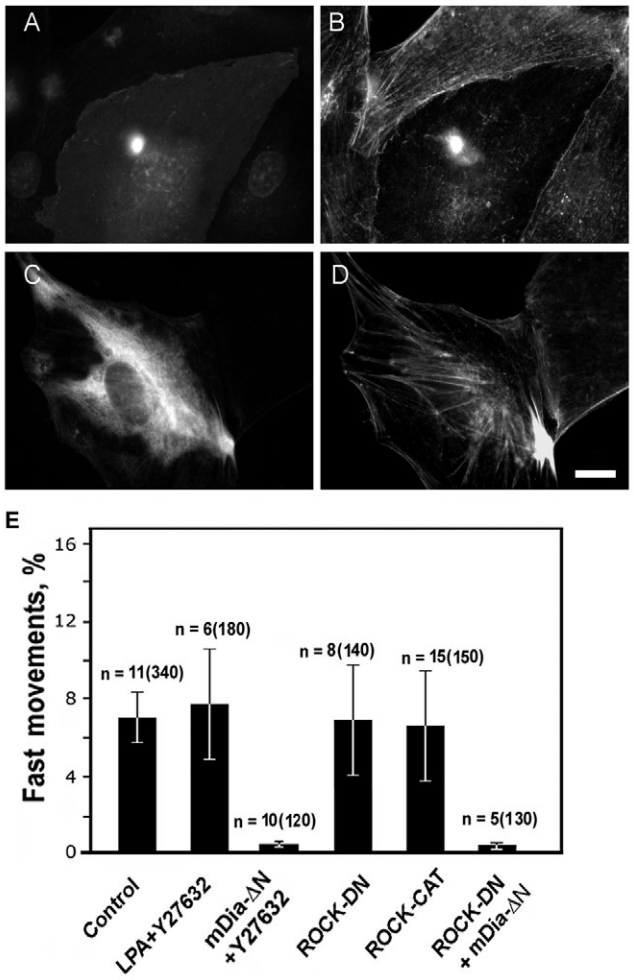

Fig. 7. Analysis of the role of ROCK in anchorage of mitochondria. CV-1 cells were transfected with plasmids encoding Myc-ROCK-RB/PH(TT) (ROCK-DN) (A,B) or Myc-ROCK-CAT (C,D). Cells were fixed; transfected cells were visualized by staining with anti-Myc antibody, and F-actin was visualized by TRITC-phalloidin. Bar, 10 μm. (E) Quantification of mitochondrial motility in cells expressing EYFP-Mito alone or EYFP-Mito and indicated mutant proteins in microinjected cells before or after treatment with 30 μM Y-27632 (Y27632) for 30 minutes or with 5.0 μM LPA for 5 minutes. Live cells expressing ROCK mutants were recognized by the presence of labeled mitochondria. Values are the mean percentage of fast movements ± s.e.m.; $P<0.05$; n, number of cells and, in brackets, number of organelle movements.

peroxisomes in such treated cells was slightly lower, although the difference with control cells was insignificant (Fig. 6D). Therefore, the increase of motility caused by the depletion of diaphanous was specific to mitochondria.

To determine if a constitutively active mutant of diaphanous could alter the motility of lysosomes or peroxisomes, we expressed EGFP-C-Dia in a BG2-C2 cell line expressing a red fluorescent marker for peroxisomes or in wild-type BG2-C2 cells followed by lysosome staining with Lyso-Tracker. It could be seen that individual organelles still maintained their motility (data not shown). Similar results were obtained using a stably transfected CV-1 cell line expressing fluorescently labeled peroxisomes: neither expression of EGFP-mDia1-ΔN3 nor their treatment with LPA inhibited movements of peroxisomes (data not shown). Thus, regulation of motility by mDia1/diaphanous is specific to mitochondria.

Cytoskeletal structures responsible for mitochondrial anchoring

Our next question pertained to how different cytoskeletal

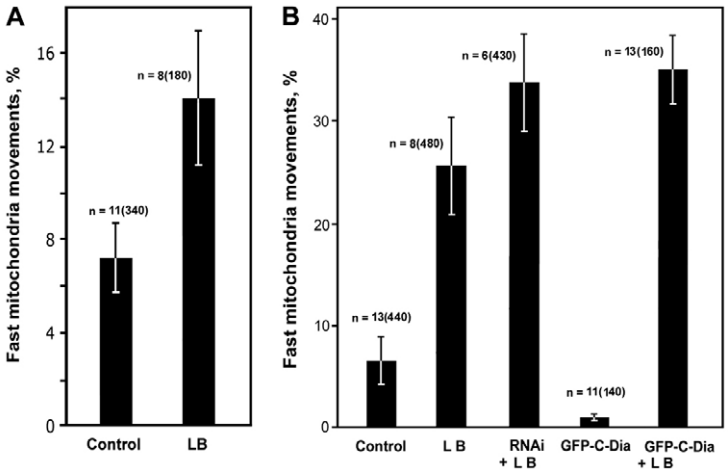

Fig. 8. Disruption of F-actin by latrunculin B increases the motility of mitochondria. The movements of fluorescently labeled mitochondria in CV-1 (A) and BG2-C2 (B) cells were recorded using time-lapse video microscopy before or after incubation with either 0.2 μM latrunculin B (LB) for 20 minutes (A) or with 10 μM latrunculin B for 1 hour (B) and analyzed, as described in the Materials and Methods. For depletion of diaphanous by RNAi, BG2-C2 cells were incubated for 7 days with dsRNA, as in Fig. 7. For the analysis of mitochondrial motility in BG2-C2 cells transfected with EGFP-C-Dia (GFP-C-Dia), they were incubated with 100 nM Texas Red Mitotracker for 40 minutes, and mitochondrial movements were recorded by time-lapse video microscopy using red fluorescence filter set (see also Movie 7 in supplementary material). Values are the mean percentage of fast mitochondria movements ± s.e.m.; $P<0.05$; n, number of cells and, in brackets, number of organelle movements.

components contributed to mitochondrial anchoring. Our data show that diaphanous, which was earlier implicated in the induction of actin polymerization (Evangelista et al., 1997; Evangelista et al., 2002; Sagot et al., 2002a; Sagot et al., 2002b; Pruyne et al., 2002; Li and Higgs, 2003), plays a regulatory role in the movement of mitochondria, suggesting the involvement of actin filaments. To investigate the role of the actin cytoskeleton in anchoring of mitochondria, we examined the consequences of F-actin depolymerization on the behavior of these organelles. We treated CV-1 cells with 0.2 μM latrunculin B for 20 minutes or BG2-C2 cells with 10 μM latrunculin B for 1 hour. Under such conditions, all stress fibers in these cells were removed and only traces of F-actin, as detected by fluorescent phalloidin staining, occasionally remained at the cell periphery (data not shown). Depolymerization of actin increased the percentage of fast mitochondrial movements in CV-1 cells by twofold (Fig. 8A) and to an even greater extent in *Drosophila* cells (Fig. 8B). Furthermore, comparison of control cells and cells expressing constitutively active diaphanous (EGFP-C-Dia) in the presence of latrunculin B shows that inhibitory action of C-Dia on mitochondrial motility is mediated by the actin cytoskeleton (Fig. 8B and supplementary material Movie 7). The treatment of CV-1 cells expressing EGFP-mDia1-ΔN3 with latrunculin B gave similar results (supplementary material Fig. S2B and Fig. S3). Cytochalasin D, another F-actin antagonist that we used to disrupt actin cytoskeleton in cells transfected with pEGFP-mDia1-ΔN3 (supplementary material Fig. S2C), also restored mitochondrial motility (supplementary material Fig. S3). Analysis of mitochondrial behavior in cells treated with latrunculin B shows that, in the absence of actin filaments, depletion of diaphanous causes only subtle activation of mitochondrial movements (Fig. 8B). This could be explained by either the presence of traces of F-actin after latrunculin B treatment or by some weak secondary effect of diaphanous that is independent of F-actin. To test if actin stress fibers directly interact with mitochondria, and whether this interaction causes an inhibition of fast movements of these organelles, we checked their possible colocalization in CV-1 cells. Stationary organelles had no obvious colocalization either with stress fibers or with vinculin-containing focal contacts (data not shown). Thus, the anchoring of mitochondria depends on intact actin cytoskeleton although they are not directly bound to stress fibers.

To investigate whether microtubules play a role in the regulation of mitochondrial movement by diaphanous and mDia1, we performed a reverse experiment, depolymerizing microtubules in CV-1 cells with nocodazole. In agreement with published results (Ball and Singer, 1982; Morris and Hollenbeck, 1995), nocodazole treatment of control cells induces collapse of mitochondria to the cell center. At the same time, in cells expressing EGFP-mDia1-ΔN3, the characteristic distribution of mitochondria was greatly altered. However, instead of collapse to the perinuclear region observed in non-transfected cells (Fig. 9A, left cell), they accumulated at the tips of processes of elongated cells (Fig. 9A, right cell). Transfection with EGFP-mDia1-ΔN3 induces formation of numerous bundles of actin filaments parallel to the long axis of the cell (Fig. 3A and Fig. 9B, arrows) as opposed to the diagonal system of stress fibers in radially symmetrical non-transfected CV-1 cells (Fig. 3C and Fig. 9B, right cell). Although depolymerization of microtubules results in the

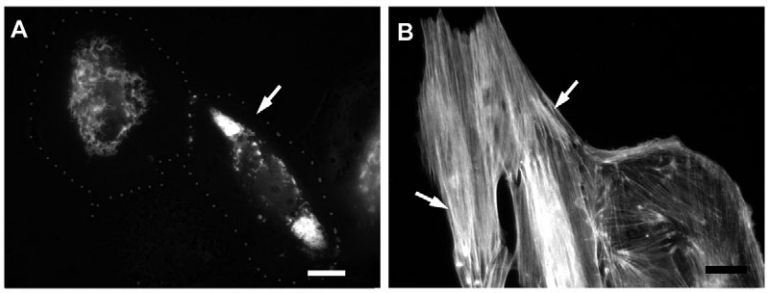

Fig. 9. Expression of pEGFP-mDia-ΔN3 in CV-1 cells affects the distribution of mitochondria caused by disruption of microtubules. Distribution of mitochondria (A) or F-actin (B) in cells that were either transfected with pEGFP-mDia-ΔN3 (B) or co-transfected with pEYFP-Mito and pEGFP-mDia-ΔN3 (A). After depolymerization of microtubules with nocodazole, cells were fixed in 4% formaldehyde. F-actin was stained with TRITC-phalloidin. Cell margins in A are indicated by dots. Transfected cells are shown by arrows. Bars, 10 μm.

absence of fast mitochondrial movement both in control and transfected cells (values of relative motility were equal to zero), the difference in the final distribution of mitochondria suggests a primary role of actin cytoskeleton in the mediation of mDia1 effects on mitochondrial distribution.

Several studies have implicated intermediate filaments, the third type of cytoskeletal structures, in mitochondrial 'docking' in mammalian cells (Leterrier et al., 1994; Wagner et al., 2003). However, we observed diaphanous-dependent docking of mitochondria in *Drosophila* cells that do not contain intermediate filaments (Goldstein and Gunawardena, 2000). Thus, the initial mechanism of mitochondrial anchoring in response to specific signals could be carried out without intermediate filaments and their role in this process in higher organisms might be auxiliary.

Discussion

The control of mitochondrial distribution might be realized both at the level of motor regulation (Morris and Hollenbeck, 1995; De Vos et al., 2000; De Vos et al., 2003) and modulation of their stationary interactions with cytoskeleton (Morris and Hollenbeck, 1993; Leterrier et al., 1994; Wagner et al., 2003). We present here results that suggest a new mechanism for regulation of mitochondrial motility by controlling their anchoring to the cytoskeleton. Our data indicate that a regulatory pathway including RhoA and its effector mDia1, which is responsible for the coordinated re-arrangements of actin cytoskeleton and microtubules (Watanabe et al., 1999; Ishizaki et al., 2001), is also involved in mitochondrial anchoring at the cell periphery. The activation of mDia1 is necessary and sufficient to induce such anchoring. Using cell lines from evolutionarily distant organisms, we have demonstrated that the regulation of mitochondria distribution by formin-related proteins, diaphanous in *Drosophila* and mDia1 in mammals is a well-conserved mechanism.

It was recently demonstrated that nerve growth factor (NGF) covalently conjugated to latex beads caused an accumulation of mitochondria at the region of the axon near the site of stimulation (Chada and Hollenbeck, 2003; Chada and Hollenbeck, 2004). The accumulation was specific for mitochondria and was completely eliminated after the inhibition of phosphoinositide 3-kinase (PI 3-kinase), one of the signaling components downstream of the NGF receptor. These data showed not only that the transport of mitochondria to a particular cytoplasmic domain could be stimulated by extracellular signals, but also that the retention of the organelles at this site was under regulatory control. On the basis of our data, this phenomenon could be explained by the assumption that, through guanine nucleotide exchange factors (GEFs), PI 3-kinase activates small GTPases Rac1 and RhoA (Arrieumerlou et al., 1998; Reif et al., 1996) and RhoA stimulates mDia1-dependent mitochondrial anchoring.

What are the cytoskeletal structures to which mitochondria are anchored? Increased motility of mitochondria in control cells and relief of inhibition of their fast movements in cells expressing EGFP-C-Dia or EGFP-mDia1-ΔN3 by latrunculin B indicated the key role of actin microfilaments in anchoring. However, several experiments argue against the trivial possibility of trapping mitochondria in the dense network of actin filaments induced by diaphanous/mDia. First, none of the other stimulators of actin polymerization that were tested in this work, such as constitutively active mutants of N-WASP and Rac1, and jasplakinolide, had inhibitory effects on the movement of mitochondria, even though all these ligands considerably increased the amount of F-actin in the cytoplasm. It could be speculated that only F-actin generated by an mDia-dependent mechanism is involved in mitochondrial anchoring. Furthermore, if non-specific trapping were responsible for inhibition of mitochondrial movement, it would affect not only mitochondria but also other membrane organelles. However, in our experiments, neither expression of constitutively active mutants of mDia1/diaphanous nor depletion of this protein in *Drosophila* cells affect motility of peroxisomes and lysosomes. Therefore, the observed effects are highly specific for mDia and mitochondria, are not caused by other modulators of actin, and do not affect other membrane organelles. Although two cellular components – mitochondria and actin filaments – are required for the observed effects, at present we do not know if the downstream target of mDia is associated with mitochondria or with the actin cytoskeleton. Nevertheless, the specificity of mDia/diaphanous and its evolutionary conservation suggest the importance of this regulatory pathway.

It was reported that the transport of endosomes could also be regulated by the Rho GTPases and DRFs (Gasman et al., 2003; Fernandez-Borja et al., 2005). These studies demonstrate that formins could be recruited to the surface of endosomes and induce the formation of an F-actin coat around them. However, we did not observe an F-actin coat on the mitochondrial surface nor any staining of these organelles with anti-mDia1 antibody (not shown) that could indicate the similarity in the regulatory mechanisms of their motility with that of endosomes. Although further research is needed to understand the molecular mechanisms of mitochondria interaction with the actin cytoskeleton, it seems most probable that the transport of mitochondria and endosomes is regulated in different ways.

Recent reports by Del Pozo et al. (Del Pozo et al., 2004) and Palazzo et al. (Palazzo et al., 2004) have implicated lipid rafts in signal transduction initiated by cell adhesion to the extracellular matrix. These new data indicate that lipid rafts recruited by integrins target Rho and Rac GTPases to specific plasma membrane domains and control their coupling to downstream effector molecules. When applied to our results, these data give new insight into the understanding of how spatial regulation of mitochondrial anchoring could be achieved. It could be speculated that the sites of organelle anchoring are determined by the same mechanism as the sites of microtubule stabilization (Palazzo et al., 2004), i.e. by the targeting of RhoA to certain regions of plasma membrane.

The interaction of mitochondria with different components of the cytoskeleton is important not only for their distribution but also for their integrity. We show here that immobilization of mitochondria on cytoskeletal structures is accompanied by their elongation. Since inhibition of RhoA by C3 transferase led to the fragmentation of mitochondria, and activation of this small GTPase caused the formation of extensive organelle networks, we proposed that regulation of mitochondrial fusion and their anchoring to the cytoskeleton might be interconnected. It would be interesting to test whether signaling factors involved in mitochondrial anchoring affect the components implicated in regulation of fusion and/or fission of these organelles.

The observation that mitochondria localized to the cell

periphery and interacting with the cytoskeleton have a higher transmembrane potential than those residing at the perinuclear region (Collins et al., 2002) indicate the possible interdependence of their anchoring with multiple properties of these organelles. It will be interesting to examine whether the energization of mitochondria could be regulated by interactions with the cytoskeleton.

Materials and Methods

Cell culture, treatments and transfections

CV-1 cells stably transfected with pEYFP-Mito or with pEYFP-PEX3 were selected using G-418 (Sigma) and maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, without G-418. Transfections of CV-1 cells were performed using Unifectin M reagent (kindly provided by A. Surovoy, Institute of Bioorganic Chemistry, Moscow, Russia) using 1 μg of plasmid DNA per 35 mm plate in serum-containing medium. For cotransfection with several plasmids, CV-1 cells were plated on cover slips with photoetched grids and microinjected into nuclei with plasmid solutions in 50 mM glutamate buffer (pH 7.4) as described previously (Minin, 1997). For microscopy, cells were plated at least 18 hours before experiments. To depolymerize microtubules, cells were treated with 10 μM nocodazole (Calbiochem-Behring) for 1-3 hours. F-actin in CV-1 cells was depolymerized by incubation with 0.2 μM latrunculin B (Calbiochem-Behring) for 20 minutes. Treatments of cells with lysophosphatidic acid (LPA; Sigma) and Y-27632 (Calbiochem-Behring) were performed as indicated in the figure legends.

Drosophila BG2-C2 cells were kindly provided by K. Ui-Tei (University of Tokyo, Japan) and maintained in Shields and Sang ME3 (Shields and Sang M3) insect medium (M3 medium) (Sigma) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin/streptomycin, and 50 μg/ml of insulin at 26°C as described previously (Ui et al., 1994). For microscopy, cells were plated on acid-washed coverslips that were coated with 0.5 mg/ml of concanavalin A (ConA) in medium conditioned for 24 hours by a dense culture of BG2-C2 cells. Under these conditions, many cells develop long processes that are convenient for microscopy. BG2-C2 cells were used for microscopy 16-48 hours after plating. F-actin in BG2-C2 cells was depolymerized with 10 μM latrunculin B for 1 hour.

BG2-C2 cells were transfected using Cellfectin (Invitrogen) in M3 medium containing 5% heat-inactivated serum for 5 hours and then the medium was changed to one with 10% serum. Stably transfected cell lines expressing EGFP-tagged probes for mitochondria, lysosomes and peroxisomes, and red fluorescent protein (RFP)-tagged probe for peroxisomes, were created by co-transfecting with selection vector pCoHygro (Invitrogen), and selecting with 300 μg/ml hygromycin.

For RNAi, BG2-C2 cells in a 35 mm plate were incubated for 1 hour in 1 ml of M3 medium containing 5% heat-inactivated fetal calf serum and 30 μg of dsRNA. Then 2 ml of M3 medium with 10% serum were added, and cells were cultured for 3 days. On the fourth day, a fresh portion of dsRNA was added and the cells were incubated for an additional 3 days.

Plasmids, RNAi and antibodies

Mitochondria in CV-1 cells were tagged using the pEYFP-Mito plasmid vector (Clontech). For labeling of mitochondria in *Drosophila* BG2-C2, cells were transfected with plasmid pAc-EGFP-Mito that was prepared by subcloning a sequence encoding mitochondria localization signal from pEYFP-Mito into pAc-EGFP vector (a gift of S.-C. Ling, Northwestern University, Chicago, IL). For peroxisome labeling, plasmids pAc-EGFP-SKL and pAc-RFP-SKL were made by attaching the Ser-Lys-Leu sequence to the C-terminus of EGFP and RFP. The tandem dimer2(12) version of mRFP was kindly provided by R. Tsien (UCSD, La Jolla, CA) (Campbell et al., 2002). Lysosomes were labeled using plasmid pAc-LAMP1-EGFP, which was made by fusion of putative *Drosophila* LAMP1 with EGFP. Peroxisomes in CV-1 cells were labeled with plasmid pEYFP-PEX3 kindly provided by A. Akhmanova (Erasmus University, Rotterdam, Netherlands). Plasmids pcDNA3-EGFP-Rac1(Q61L), pcDNA3-EGFP-Cdc42(Q61L) and pcDNA3-EGFP-RhoA(Q63L) were a gift from K. Hahn (University of North Carolina, Chapel Hill, NC). Plasmid encoding the mDia1 mutant pEGFP-mDia1-ΔN3 was obtained from S. Narumiya (Kyoto University, Kyoto, Japan). Plasmid encoding C3 transferase from *Clostridium botulinum* was a gift from A. Hall (University College London, London, UK). Plasmid CB6-EGFP-WA encoding the C-terminus of rat N-WASP was a gift from M. Way (London Research Institute, London, UK). Plasmids pEF-BOS-Myc-Rho-kinase-RB/PH(TT) and pEF-BOS-Myc-Rho-kinase-CAT encoding dominant-negative and dominant-active mutants of Rho-kinase were kindly provided by K. Kaibuchi (Nagoya University, Japan).

Plasmid pAc-EGFP-C-Dia, encoding the truncated version of *Drosophila* diaphanous (accession number NM-165341), containing only FH1 and FH2 domains but not the RBD domain, was prepared by inserting the 1297-3276 cDNA fragment into the pAc-EGFP vector between the *Bam*HI and *Age*I restriction sites. The fragment was amplified by PCR from a *Drosophila* embryonic phage cDNA library (gift of S. Dzitoeva, University of Illinois College of Medicine, Chicago, IL) using primers: 5′-CAAACCGGTCACAAGGGTTACTGTGATCCGA-3′ and 5′-CCGGATCCCTACGCGGAGCCTAGAACC-3′. By analogy with its mammalian counterparts, this truncation is assumed to encode a constitutively active version of diaphanous.

To prepare dsRNA complementary to diaphanous mRNA, a 4-1316 cDNA fragment was amplified by PCR from the same cDNA library using primers 5′-GAGCAACAACTAAATAAAATGTCTCGTCACGAG-3′ and 5′-GGATCACAG-TAACCCTTGTGGAAGAC-3′ and inserted into the TOPO-Vector (Invitrogen). Then, using primers 5′-TAATACGACTCACTATAGGGTCGTTCTGCATTGTC-TATGAGC-3′ and 5′-TAATACGACTCACTATAGGGGGATCACAGTAACCCTT-GTGGA-3′ as in (Rogers et al., 2003), the template containing promoters for T7-polymerase and the 584 bp fragment of diaphanous cDNA was prepared. In vitro transcription was carried out using a MEGAScript kit (Ambion).

A polyclonal antibody against Glu-tubulin was a gift of C. Bulinski (Columbia University, New York, NY); monoclonal antibody DM1α against α-tubulin was from Sigma; polyclonal rabbit antibodies against the motor domain of the kinesin heavy chain (HD) were prepared and characterized earlier (Rodionov et al., 1993); polyclonal rabbit antibodies against human mDia1 were purchased from Bethyl Laboratories; polyclonal rabbit antibodies against diaphanous (Afshar et al., 2000) was kindly provided by S. Wasserman (University of California, San Diego, CA). Monoclonal antibody against vinculin was from Y. M. Vasiliev (Moscow State University, Moscow). Secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase or fluorescent dyes were purchased from Jackson Immuno Research Laboratories.

Time-lapse fluorescent microscopy

Time-lapse epi-fluorescent microscopy of CV-1 cells was performed using Axiophot microscope (Zeiss) equipped with a Plan-Apochromat 63× 1.4 NA objective. Images were captured with Micromax 782Y cooled CCD camera (Roper Scientific) driven by WinView32 software. The frames were collected every 4 seconds with an exposure time of 0.5-1.0 second. For video microscopy of BG2-C2 cells a Nikon Diaphot 200 inverted microscope equipped with a Planapo 60× 1.4 NA objective was used. Images were collected with an Orca II cooled CCD camera (Hamamatsu Photonics) driven by Isee software (Isee Imaging Systems). To avoid phototoxic effects a 100 W halogen lamp was used for epi-fluorescent illumination.

SDS-PAGE and immunoblotting

Electrophoretic separation of proteins was performed using discontinuous 7.5% SDS-PAGE (Laemmli, 1970). For comparison of the content of diaphanous and KHC in cells treated with dsRNAs, the samples first were run on gels that were stained with Coomassie R250. The gels were scanned, and the load for western blots was normalized according to the scan data. KHC was detected using HD antibody at a dilution of 1:1000. Anti-diaphanous antibody was used at 1:5000. Blots were developed using SuperSignal (Pierce Chemical Co.). To determine an amount of residual diaphanous after RNAi we compared intensities of bands on blots with that obtained for serially diluted control cellular extract.

Quantitative analysis of mitochondrial motility

Image analysis was done with the open source image analysis software ImageJ using available plug-ins and macros, and with Photoshop (Adobe Systems). Because of the diverse morphology of mitochondria we plotted the coordinates of one end for longer organelles and the center of mass for shorter ones. Mitochondrial motility was expressed in terms of the displacement distances, and velocities. To define fast organelle movements we applied a threshold of 200 nm/second. The values were expressed as mean percentages of fast movements in all recorded displacements ± s.e.m. The significance of differences was estimated statistically by paired-sample Student's *t*-test. Variability of the values calculated for different cells in samples was analyzed by the same method and was insignificant.

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