Cdc42, dynein, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization

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In migrating adherent cells such as fibroblasts and endothelial cells, the microtubule-organizing center (MTOC) reorients toward the leading edge [1–3]. MTOC reorientation repositions the Golgi toward the front of the cell [1] and contributes to directional migration [4]. The mechanism of MTOC reorientation and its relation to the formation of stabilized microtubules (MTs) in the leading edge, which occurs concomitantly with MTOC reorientation [3], is unknown. We show that serum and the serum lipid, lysophosphatidic acid (LPA), increased Cdc42 GTP levels and triggered MTOC reorientation in serum-starved wounded monolayers of 3T3 fibroblasts. Cdc42, but not Rho or Rac, was both sufficient and necessary for LPA-stimulated MTOC reorientation. MTOC reorientation was independent of Cdc42-induced changes in actin and was not blocked by cytochalasin D. Inhibition of dynein or dynactin blocked LPA- and Cdc42-stimulated MTOC reorientation. LPA also stimulates a Rho/mDia pathway that selectively stabilizes MTs in the leading edge [5, 6]; however, activators and inhibitors of MTOC reorientation and MT stabilization showed that each response was regulated independently. These results establish an LPA/Cdc42 signaling pathway that regulates MTOC reorientation in a dynein-dependent manner. MTOC reorientation and MT stabilization both act to polarize the MT array in migrating cells, yet these processes act independently and are regulated by separate Rho family GTPase-signaling pathways.

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Results and discussion

Serum and LPA stimulate MTOC reorientation

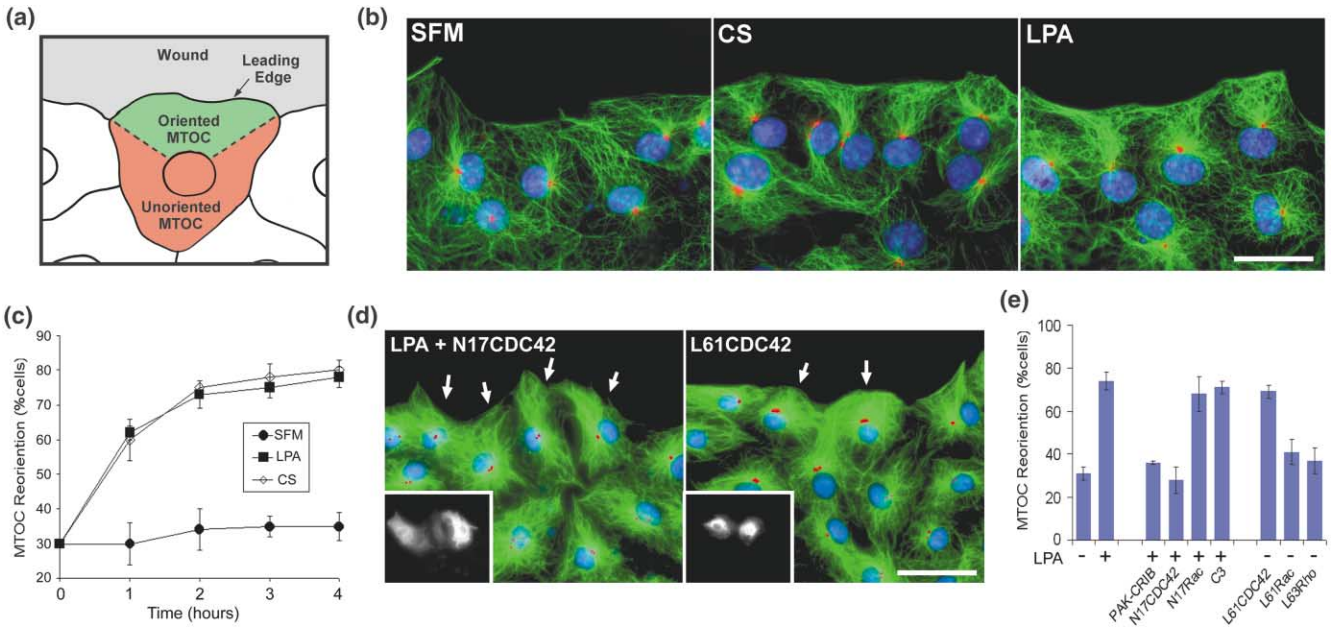
We tested whether soluble factors were required for MTOC reorientation by using wounded monolayers of serum-starved NIH 3T3 fibroblasts. With a similar approach, we showed previously that Rho and the Rho effector, mDia, were involved in selectively stabilizing MTs in the leading edge [5–7]. MTOC reorientation was assessed in fixed preparations stained for the centrosomal marker pericentrin, MTs, and nuclei. The MTOC was considered reoriented if it was located between the nucleus and the wound edge as shown in Figure 1a. This area represents about one-third of the cell, so the MTOC will be "reoriented" by chance in about 33% of the cells.

In the absence of serum, the MTOC did not reorient for up to 16 hr after wounding serum-starved monolayers (Figure 1b,c). However, if 10% calf serum (CS) was added after wounding, the MTOC reoriented to virtually the same extent as it did in unstarved cells (Figure 1b; see Table S1 in the Supplementary material available with this article online). Tests of growth factors and cytokines found in serum showed that only lysophosphatidic acid (LPA) triggered MTOC reorientation to a similar level as serum (Figure 1b; Table S1 in Supplementary material). The time course and extent of MTOC reorientation triggered by LPA was similar to that induced by serum (Figure 1c). LPA was active at concentrations greater than 10 nM and had an ED_{50} of ~80 nM (n = 2). The ED_{50} for CS was 1.0% (n = 3), and since the concentration of LPA in serum is 12–66 μM [8, 9], there is sufficient LPA in serum to account for its activity. LPA may be the major serum factor for triggering MTOC reorientation, since serum treated with trypsin to inactivate polypeptide growth factors still induced MTOC reorientation (data not shown).

Cdc42 mediates LPA-induced MTOC reorientation

In NIH 3T3 cells, LPA alone activates Rho GTP formation [10], and LPA plus adhesion activate Cdc42 GTP formation [11]. Using a PAK pull-down assay [12], we found that LPA and CS directly increased Cdc42 GTP

Figure 1



MTOC reorientation is triggered by serum, LPA, and active Cdc42 and inhibited by dominant-negative Cdc42. **(a)** Diagram showing criteria for assessing MTOC reorientation. The MTOC was identified as one or two closely spaced dots stained for pericentrin or as the focus of the MT array in cells stained for tubulin. **(b)** Representative fields of serum-starved wound-edge cells treated for 2 hr with serum-free media (SFM), 10% calf serum (CS), or 1 μM LPA (LPA) and then fixed and stained. The images are overlays of the distributions of pericentrin (red), DNA (blue), and tyrosinated MTs (Tyr MTs) (green). The bar equals 20 μm. **(c)** Time course of MTOC reorientation in serum-starved cells treated with serum-free media (SFM), 10% CS (CS), or 1 μM LPA (LPA). Data are from three independent experiments in which >100 cells were scored for each time point. Error bars are standard error of the mean. **(d)** Serum-starved wound-edge fibroblasts microinjected with N17Cdc42 and 30 min later treated with 1 μM LPA for 2 hr before fixation or microinjected with L61Cdc42 and fixed after 2 hr. Arrows indicate injected cells. Cells were immunostained for pericentrin (red), Tyr MTs (green), DNA (blue), and the injection marker, human IgG (shown in insets). The bar equals 50 μm. **(e)** Effect of Rho family GTPase microinjection on MTOC reorientation. Experiments were done as in (d). Cells were microinjected with the indicated protein and then 30 min later either treated with 1 μM LPA (LPA +) or no LPA (LPA −) for 2 hr and then fixed. Data were averaged from three or more experiments, each consisting of >50 cells. Error bars are standard error of the mean.

levels in serum-starved monolayers (see Supplementary material).

We next tested whether Rho family GTPases were involved in MTOC reorientation. LPA-induced MTOC reorientation was blocked by microinjecting dominant-negative N17Cdc42 or PAK-CRIB, a Cdc42/Rac-specific inhibitor [12], but not by microinjecting dominant-negative N17Rac or botulinin C3 toxin to inhibit Rho (Figure 1d,e and Supplementary material). C3 toxin blocked LPA-induced stress fiber formation and stable MT formation as previously reported ([5]; see also Figure 4a,c). These results show that Cdc42, but not Rho or Rac, is necessary for LPA-induced MTOC reorientation.

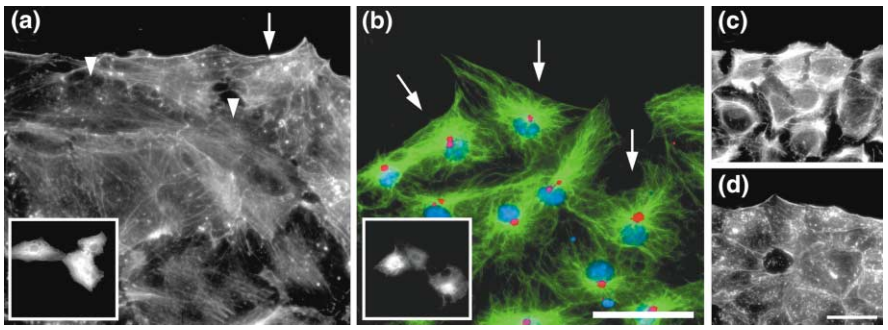
Wound-edge cells microinjected with constitutively active L61Cdc42 reoriented their MTOCs in 2 hr to the same extent as LPA (Figure 1d,e). In contrast, constitutively active L63RhoA and L61Rac did not induce MTOC reorientation in 2 hr (Figure 1e and Supplementary material). L63RhoA triggered stress fibers and stable MTs as expected ([5]; data not shown). These results show that Cdc42, but not Rho or Rac, is sufficient to induce MTOC reorientation.

MTOC reorientation occurs independent of changes in actin

Cdc42 activates the actin cytoskeleton in serum-starved Swiss 3T3 cells by inducing filopodia [13]. In serum-starved NIH 3T3 cells microinjected with L61Cdc42, we did not detect filopodia or other significant differences in actin distribution as revealed by phalloidin staining (Figure 2a). Variability in phalloidin staining was observed in the cells, but this was similar in injected and uninjected cells (Figure 2a).

We further tested actin involvement in MTOC reorientation by pretreating cells with 0.25 μM cytochalasin D (CD) and then inducing MTOC reorientation by microinjecting L61Cdc42. CD-pretreated cells reoriented their MTOCs to nearly the same extent as untreated, L61Cdc42-injected cells (61%, n = 172 compared to 70%, n = 200

Figure 2



MTOC reorientation occurs in the absence of Cdc42-stimulated changes in F-actin distribution and is not blocked by cytochalasin D. **(a)** Serum-starved cells were microinjected with L61Cdc42 and after 2 hr fixed and stained with rhodamine-phalloidin to reveal F-actin. Injected cells at the wound edge (arrow) or within the monolayer (arrowheads) do not show significant differences in F-actin distribution compared to uninjected cells. Inset shows human IgG marker in the injected cells. **(b)** Serum-starved cells were microinjected with L61Cdc42, treated with 0.25 μM CD, and after 2 hr were fixed and immunostained. Shown is an overlay image of the distributions of pericentrin (red), Tyr MTs (green), and DNA (blue). Injected cells (arrows) at the wound edge reorient their MTOCs. Inset shows human IgG marker in the injected cells. **(c,d)** Rhodamine phalloidin staining of LPA-treated serum-starved cells with (c) no additional treatment or (d) treated with 0.25 μM CD. The bars equal 50 μm.

in controls; Figure 2b). CD (0.25 μM) prevented LPA-induced stress fiber formation in starved 3T3 cells and blocked protrusive activity of the cells (Figure 2c,d), as reported earlier [14]. LPA-stimulated MTOC reorientation was also unaffected by CD (data not shown). These results are consistent with studies on the effect of CD on MTOC reorientation in endothelial cells and macrophages [15, 16] and suggest that stimulation of the actin cytoskeleton is not involved in Cdc42-induced MTOC reorientation. This does not completely preclude a requirement for actin, since CD treatment is unlikely to affect all actin filaments [17].

Inhibition of dynein or dynactin blocks MTOC reorientation

The mechanism of MTOC reorientation during cell migration has not been explored; however, the repositioning suggests that a motor may be involved. Cytoplasmic dynein and its accessory protein, dynactin, have been implicated in spindle positioning during cell division in a number of systems [18–22], but their role in MTOC reorientation in migrating cells has not been studied.

We microinjected monoclonal antibody (mAb) 74.1 to dynein intermediate chain, which blocks cytoplasmic dynein function in cells [23], and then stimulated MTOC reorientation. Injected mAb 74.1 completely blocked LPA-induced or L61Cdc42-induced MTOC reorientation (Figure 3 a–d; Figure 4b for quantification). Injected mAb 74.1 did not affect the tight focus of the MTs at the MTOC or the close apposition of the MTOC to the nucleus (Figure 3 a–d).

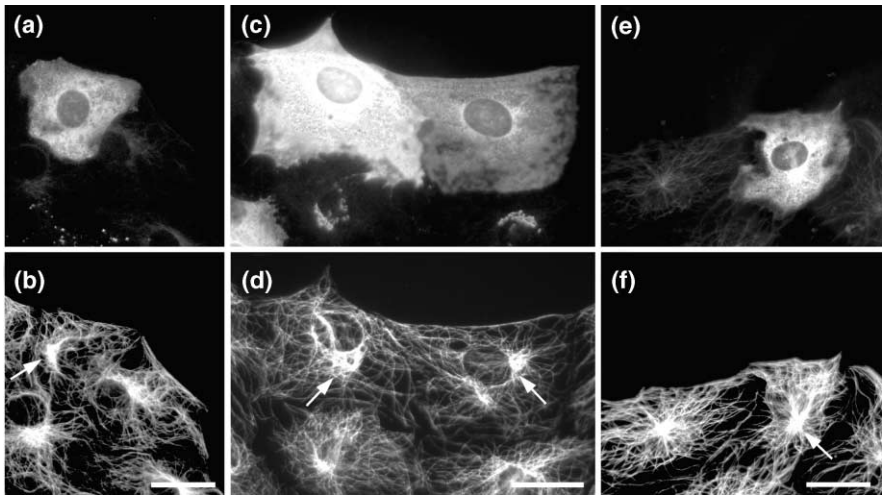
Overexpression of the dynamitin subunit of dynactin also blocks dynein function in cells [24, 25]. Overexpressed GFP-dynamitin inhibited MTOC reorientation and, like the injected mAb 74.1, did not interfere detectably with the focus of the MTs at the MTOC (Figures 3e,3f and 4). Interfering with dynein function disrupts the Golgi apparatus [24, 25]; however, we found that only ~50% of the cells injected with mAb 74.1 and none of the cells overexpressing dynamitin had dispersed Golgi (see Supplementary material). This suggests that Golgi disruption by dynein reagents is less sensitive than MTOC reorientation and is not responsible for the inhibition of MTOC reorientation.

MTOC reorientation and MT stabilization are regulated independently

In fibroblasts, there is evidence for crosstalk between Rho family GTPases [13]. Also, MT stabilization and MTOC reorientation occur with similar time courses after wounding [3], and it is not known whether MTOC reorientation depends on MT stabilization or vice versa. We addressed these issues by first examining whether factors that altered MTOC reorientation affected the formation of stable MTs. Stabilized MTs were detected with an antibody to detyrosinated tubulin (termed Glu tubulin after the Glu residue exposed by detyrosination [26]), which is a reliable marker of stabilized MTs [3, 5–7]. Inhibition of MTOC reorientation by overexpressing GFP-dynamitin (Figure 4a,b) or by microinjecting N17Cdc42, PAK-CRIB, or mAb 74.1 (Figure 4b) did not inhibit LPA induction of stable Glu MTs oriented toward the leading edge of cells at the wound. Conversely, activation of MTOC reorientation by microinjection of active L61Cdc42 did not induce the formation of stable Glu MTs (Figure 4b) or MTs resistant to nocodazole treatment (1 μM for 30 min) (data not shown), an alternative assay for MT stability [3, 5–7]. These results show that MTOC reorientation itself or the factors involved in MTOC reorientation (Cdc42/dynein/dynactin) are not involved in MT stabilization.

Next, we examined whether factors that affect stable Glu MT formation affected MTOC reorientation. Inhibition of LPA-induced stable Glu MTs by C3 toxin did not

Figure 3



Inhibition of dynein or dynactin blocks LPA- and Cdc42-induced MTOC reorientation. Serum-starved cells were **(a,b)** microinjected with mAb 74.1 and after 30 min treated with 1 μM LPA for 2hr, **(c,d)** comicroinjected with both mAb 74.1 and L61Cdc42 and then incubated for 2 hr, or **(e,f)** microinjected with GFP-dynamitin DNA and, after allowing for expression (2–4 hr), treated with 1 μM LPA for 2 hr. Cells were fixed and immunostained for (a,c) injected antibody and (b,d,f) Tyr MTs. (e) GFP-dynamitin-expressing cells were detected by GFP fluorescence. Arrows in (b), (d), and (f) show positions of MTOC. The bar equals 20 μm. Quantification of mAb 74.1 and GFP-dynamitin inhibition of LPA-stimulated MTOC reorientation is in Figure 4b. mAb 74.1 inhibited L61Cdc42-stimulated MTOC reorientation (37%, n = 162) compared to controls injected with L61Cdc42 alone (70%, n = 200).

inhibit MTOC reorientation (Figure 4a,c). Conversely, activation of MT stabilization with L63Rho, the mDia autoinhibitory domain (DAD) [6, 27], or constitutively active ΔGBDmDia2 [6] did not stimulate MTOC reorientation (Figure 4c). These results show that MT stabilization itself or the factors involved in MT stabilization (Rho/mDia) are not involved in MTOC reorientation.

An LPA/Cdc42 signaling pathway regulates MTOC reorientation

In this study, we have shown for the first time that there is a specific signal transduction pathway involved in regulating MTOC reorientation. We identified LPA as the soluble external trigger and Cdc42 as the cytoplasmic regulator of MTOC reorientation. We found that dynein and dynactin are required for MTOC reorientation, although more work is needed to determine whether these components are regulated directly by Cdc42.

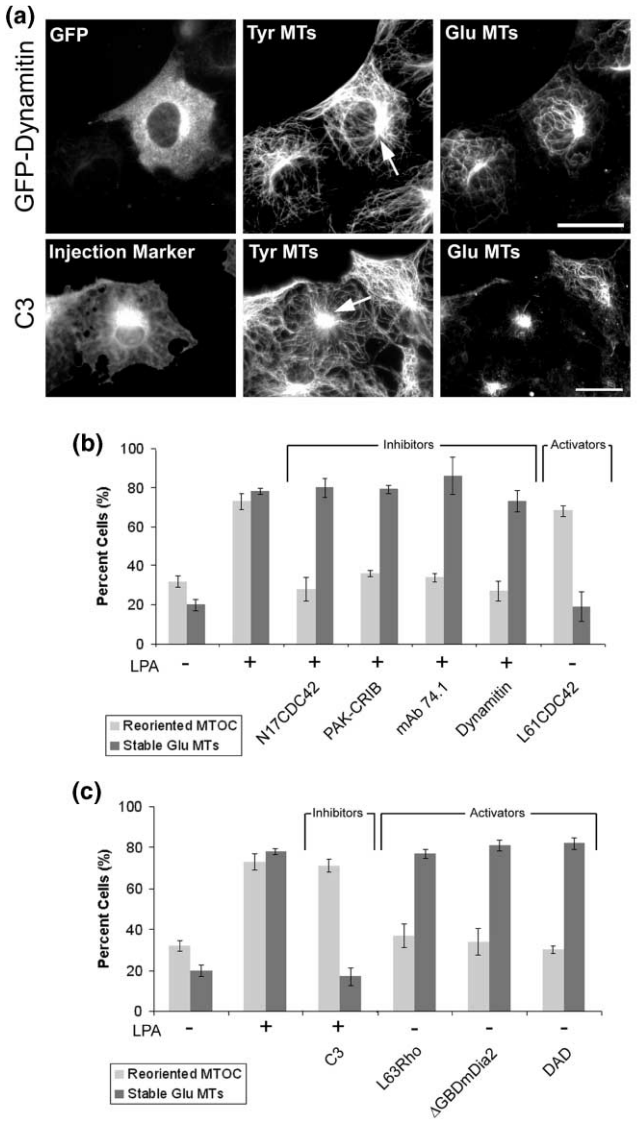
To our knowledge, the wounded fibroblast system is the first system in which it has been possible to activate MTOC reorientation by introducing intracellular factors (e.g., L61Cdc42). This allowed us to show for the first time that active Cdc42 is sufficient to trigger MTOC reorientation. Previously in unstarved fibroblasts, active V12Cdc42 was shown to have no effect on Golgi reorientation, which usually reflects MTOC reorientation, although N17Cdc42 partially inhibited Golgi reorientation [28]. In T cells, both active and dominant-negative Cdc42 inhibit MTOC reorientation [29]. This may reflect differences in cell type or alternatively, that MTOC reorientation requires cycling Cdc42 in T cells but not in fibroblasts, which may use other mechanisms to maintain MTOC position once it has reoriented.

Our study is the first to implicate dynein/dynactin in MTOC reorientation in migrating cells (see Note added in proof). There are a number of possible models for how dynein/dynactin may mediate MTOC reorientation, but the most straightforward one is that dynein in the cell cortex at or near the leading edge could exert a pulling force on MTs. Movement of cortically anchored dynein toward MT minus ends would pull MTs and the attached MTOC toward the leading edge. With such a model, dynein activity would have to be regulated locally, and this could be achieved directly or indirectly by Cdc42. Consistent with a cortical dynein pulling model is evidence that dynein/dynactin are localized in the cortex of mammalian cells [21, 30] and that they are involved in MT sliding in the yeast cortex [18, 19]. Dynactin has been localized on the ends of growing MTs [31] where it is poised to activate dynein in the cortex.

Our study clearly establishes that MTOC reorientation and MT stabilization are independent processes regulated by separate Rho family GTPases. This is analogous to the regulation of independent actin assemblies by separate Rho family GTPases. And yet, the effects of Rho GTPase regulation on each cytoskeletal system are distinct; for actin, Rho GTPases regulate the de novo formation of discrete actin assemblies, whereas for MTs, Rho GTPases modify a single array but polarize it in distinct ways.

Do stable MTs and reoriented MTOCs perform unique or overlapping functions? Both processes contribute to an increase in the number of MTs directed to the leading edge. MTOC reorientation does so by positioning the MTOC so that the nucleus does not come between it and the leading edge; stable MTs, which are capped and not dynamic [32], do so by adding additional MTs that

Figure 4



Cdc42-induced MTOC reorientation is independent of Rho-induced MT stabilization. **(a)** Serum-starved cells were either microinjected with GFP-dynamitin DNA and after expression (2–4 hr) treated with 1 μM LPA for 2 hr or were microinjected with C3 toxin and treated with 1 μM LPA for 2 hr. Cells were fixed and immunostained for human IgG (Injection Marker) dynamic MTs (Tyr MTs) and stable MTs (Glu MTs). GFP-dynamitin-overexpressing cells were identified by GFP fluorescence (GFP). The bar equals 20 μm. GFP-dynamitin inhibited LPA-stimulated MTOC reorientation but not LPA-stimulated MT stabilization (or the orientation of the stable MTs toward the wound edge). C3 microinjection inhibited LPA-stimulated MT stabilization but not LPA-stimulated MTOC reorientation. **(b)** Inhibitors or activators of Cdc42-induced MTOC reorientation do not affect formation of stable Glu MTs in serum-starved cells. The percentage of cells with reoriented MTOCs and stable Glu MTs is shown for four inhibitors of LPA-stimulated MTOC reorientation and for one activator of MTOC reorientation. **(c)** Inhibitors or activators of the Rho-induced formation of stable Glu MTs do not affect MTOC reorientation in serum-starved cells. The percentage of cells with reoriented MTOCs and stable Glu MTs is shown for one inhibitor of LPA-stimulated Glu MT formation and for three activators of Glu MT formation. "DAD" is the mDia autoinhibitory domain. For each experiment, cells were either microinjected with protein and incubated 2 hr in the absence of LPA (LPA −); microinjected with protein and then (30 min later) treated with LPA for 2 hr (LPA +); or, for GFP-dynamitin, ΔGBDmDia, and DAD, the cells were microinjected with DNA and then (2–4 hr later) either fixed (LPA −) or treated with LPA for 2 hr (LPA +). Data were averaged from three or more experiments, each consisting of >50 cells. Error bars are standard error of the mean.

are not governed by factors that restrict the number of dynamic MTs a cell can maintain. Increased numbers of MTs directed toward the leading edge would bias vesicle transport toward the leading edge, and this has been observed in wound-edge cells [33]. Stable MTs may also increase delivery or recycling of components to the leading edge by virtue of their posttranslational detyrosination, since stable Glu MTs exhibit enhanced binding to kinesin [34] and have been shown to be the preferred path for endocytic recycling (Lin et al., submitted).

Materials and methods

Chemicals and DNA constructs

LPA, L-α-lysophosphatidic acid (1-oleoyl), was from Avanti Polar Lipids (Alabaster, Alabama, USA). Unless noted otherwise, all other chemicals were from Sigma. mDia DNA constructs were prepared as described [27]. GFP-dynamitin was prepared in the pEGFP-N1 vector (Clontech) between the EcoR1 and BamH1 sites.

Cell culture and microinjection

NIH 3T3 cells were cultured, serum-starved, and wounded as previously described [5–7]. DNAs were microinjected into nuclei as previously described [6]. C3 toxin (1 μg/ml) and GST-tagged Cdc42, Rac, and Rho proteins (1 mg/ml) (Cytoskeleton, Denver, Colorado, USA) were microinjected with human IgG (1 mg/ml) as previously described [5]. GST-PAK-CRIB (residues 56–272 of PAK1B) was prepared as previously described [12], concentrated by vacuum dialysis against H-KCl (10 mM Hepes, 140 mM KCL [pH. 7.4]), and microinjected at 3 mg/ml. mAb 74.1 to dynein intermediate chain [35] was purified from ascites, concentrated by vacuum dialysis against H-KCl, and microinjected at 4 mg/ml.

Indirect immunofluorescence and fluorescence microscopy

Methanol fixation and staining were performed as described previously [3, 6, 26]. DAPI was used at 1 μg/ml to stain nuclei. Polyclonal antibody against pericentrin (Covance, Princeton, New Jersey, USA) was used at 1:200 dilution to stain the MTOC. Rhodamine-phalloidin (Cytoskeleton) staining of paraformaldehyde-fixed cells was as described [6]. Microscopy and imaging were performed as previously described [6].

Supplementary material

Supplementary material including three figures is available at http://images.cellpress.com/supmat/supmatin.htm.

Note added in proof

As this paper was being prepared for publication, S. Etienne-Manneville and A. Hall (*Cell* 2001, **106**:489–498) reported that dynactin overexpression blocked MTOC reorientation in wounded monolayers of astrocytes. They also found that MTOC reorientation was blocked by dominant-negative Cdc42.

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