Microtubule involvement in NIH 3T3 Golgi and MTOC polarity establishment

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Summary
Scratch-wound assays are commonly used to study the ability of cells to polarize and migrate. In a previous study we showed that Golgi reorientation in response to a scratch wound is actin-dependent in NIH 3T3 cells but not in astrocytes.

In this investigation, to study cell polarity and motility further, we used the polarization of the Golgi and microtubule organizing center (MTOC), as well as the ability of NIH 3T3 cells to migrate, in a scratch-wound assay. Unlike Golgi polarization, MTOC polarization was not dependent on actin, the Arp2/3 complex or Wiskott-Aldrich syndrome protein (WASP)-family proteins. By contrast, disruption of microtubules inhibited MTOC polarity, but not Golgi polarity. Migration was found to be dependent both on actin and microtubules. Expression of the formin-homology 2 (FH2) region of mDia1 inhibited Golgi polarization and migration but not MTOC polarization. Similarly, ST638, a Src inhibitor, inhibited Golgi polarization and migration but not MTOC polarization, whereas expression of the actin regulator IRSp53 only inhibited cell migration. Interestingly, the inhibition of cell migration by the mDia1 FH2 domain could be overcome by addition of Y27632, an inhibitor of ROCK (Rho-associated kinase). In fact, in the presence of ROCK inhibitor, cell migration was accelerated but polarization of both the Golgi and MTOC were inhibited. These data show that, in NIH 3T3 cells, different aspects of cell polarization and migration occur by different mechanisms, and both actin and microtubule networks are required. In addition, this study indicates that MTOC and Golgi polarization events are separately controlled.

Key words: Golgi, MTOC, Polarization, mDia, FH, Actin, Microtubules, Wound healing, Cell migration

Introduction
Microtubules (MTs) are essential for the polarization of many cell types (Etienne-Manneville and Hall, 2001; Goldman, 1971; Gundersen and Cook, 1999; Vasiliev et al., 1970). Despite the central role of MTs in cell polarization, relatively little is known about the molecular mechanisms by which they exert their polarizing activity. One key event is probably the formation of stabilized MTs. The small GTPase Rho is necessary for selective stabilization of MTs (Cook et al., 1998; Nagasaki and Gundersen, 1996). Rho does not bind directly to MTs, but rather mDia2 was identified as a Rho effector involved in MT stabilization (Krebs et al., 2001; Palazzo et al., 2001a). In addition, there is a coordination of MT and actin cytoskeletons by mDia1 (Ishizaki et al., 2001). The mDia1 (Watanabe et al., 1997) and mDia2 (Alberts et al., 1998) proteins are the mammalian homologues of Drosophila Diaphanous, which is a member of the formin-homology (FH) family and important for cell polarity establishment in Drosophila (Emmons et al., 1995; Evangelista et al., 1997; Frazier and Field, 1997; Jansen et al., 1996; Petersen et al., 1995; Tanaka, 2000). The mDia protein contains two formin-homology domains: FH1 and FH2. MDial exerts its effects on actin and MTs through its FH1 and FH2 regions, respectively (Ishizaki et al., 2001). Interestingly, inhibitors of the Src tyrosine kinase inhibit mDia1 function and wound closure (Owens et al., 2000; Satoh and Tominaga, 2001; Yamada et al., 2000). Additionally, mDia works in association with ROCK for the formation of stress fibers and focal adhesions (Nakano et al., 1999).

Recently we showed that Golgi polarization is actin-dependent in NIH 3T3 cells but actin-independent in astrocytes (Magdalena et al., 2003). The important involvement of MTs in astrocytes has been previously reported (Etienne-Manneville and Hall, 2001). In the present study, we examined MT organizing center (MTOC) polarization in the NIH 3T3 wound model. We investigated the relationships of cell polarity with both actin and MT networks. We determined first, whether Arp2/3 delocalization and Wiskott-Aldrich syndrome protein (WASP)-family protein overexpression interferes with MTOC polarization and second, whether overexpression of the mDia1 FH1 and FH2 domains interferes with Golgi and MTOC polarization. We found that Golgi polarization and MTOC polarization are separately controlled. All together, our data show that, in NIH 3T3 fibroblasts, Golgi polarization is actin-dependent, MTOC polarization is actin-independent and MT are differently involved in both polarization processes. We also show the importance of ROCK activity for MTOC polarization and of mDia in association with ROCK for Golgi polarization and cell migration. Finally, Src activity is required for proper Golgi polarization and migration, whereas aberrant expression of IRSp53 interferes with cell migration. Therefore, our results suggest that NIH 3T3 fibroblasts co-ordinate their actin and...
MT cytoskeletons during cell migration and polarity establishment, under the control and involvement of several proteins (Fujiiwara et al., 2000; Nakano et al., 1999; Satoh and Tominaga, 2001).

Materials and Methods
Antibodies and reagents
Antibodies against pericentrin from Eurogentec (Herstal, Belgium), anti-tubulin from Sigma (St Louis, MO), anti-myc tag (9E10) (gift from Alan Hall) and anti-β-COP from ABR Affinity Bioreagents (Cambridge, UK) were used at dilutions 1:150, 1:250, 1:200 and 1:750, respectively, in immunofluorescence microscopy. Secondary antibodies used in immunofluorescence staining (Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse) were from Molecular Probes (Leiden, The Netherlands) and used at a dilution of 1:750. Texas Red-conjugated phalloidin and Alexa fluor 594-conjugated dextran (10,000 MW) were purchased from Molecular Probes.

Cell culture and wounding
NIH 3T3 cells were grown and passaged in DMEM supplemented with 5% DCS (donor calf serum, Calbiochem, Nottingham, UK) and penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively), and incubated at 37°C and 10% CO2. For the wound-healing assays, cells were seeded on coverslips and grown to confluence; the wound was made by scraping the cell monolayer across the coverslip with a microinjection needle. As recently described (Magdalena et al., 2003), the dimensions of the wound were approximately 6-8 cells across (300-700 µm) and about 300-400 cells long (about 7-8 mm). In our assays, the wound was usually closed in 10-11 hours.

Drug treatments
NIH 3T3 cell monolayers were nocodazole treated (using final concentrations of 16 µM, 300 nM and 100 nM) and wounded simultaneously. To study the effect of the drug on cell polarity, 3 hours after treatment and wounding, cell monolayers were fixed and labeled for tubulin and Golgi or for tubulin and MTOC. To follow the drug treatment effect on Golgi morphology, cells were treated with nocodazole at a final concentration of 16 µM and wounded, then fixed 0.5, 1.5 and 3 hours after wounding and labeled for tubulin and Golgi. The wound-closure process of nocodazole-treated (using final concentrations of 16 µM, 300 nM and 100 nM) and non-drug-treated wounded monolayers (experiments performed in triplicate) was compared.

Cytochalasin D was used at final concentrations of 0.5, 2 and 10 µM. Confluent monolayers of NIH 3T3 cells were incubated with the drug and directly wounded. Three hours later, cells were fixed and labeled for actin and Golgi or for actin and MTOC. Similarly, latrunculin B was also used at concentrations of 20, 80 and 200 nM, and MTOC polarization of wound-edge cells was determined.

NIH 3T3 cells were also treated with ST638 (Calbiochem, UK) at a final concentration of 50 µM and concomitantly wounded. To study the effect of the drug on cell polarity, the cells were fixed 3 hours later, and labeled for actin and Golgi or for actin and MTOC. Wound closure was observed under ST638 treatment and compared with wound closure with non-drug-treated cells (experiments performed in triplicate).

Taxol (also called paclitaxel; Sigma) was used at a final concentration of 100 nM to treat monolayers of cells, which were directly wounded. Cells were fixed and labeled for tubulin and Golgi or for tubulin and MTOC. 3 hours after wounding (experiments were performed in triplicate). Wound closure was observed under taxol treatment and compared with wound closure with non-drug-treated cells (experiments were performed in triplicate).

Y27632 (Calbiochem) was used at a final concentration of 20 µM. In all cases, cell monolayers were pretreated with Y27632 1 hour before wounding. Under that drug treatment, we looked at wound-edge cell morphology, wound closure, Golgi polarization and MTOC polarization. Y27632-pretreated monolayers were wounded, fixed 15 minutes or 4 hours post wounding and labeled for actin (experiments performed four times). Y27632-pretreated monolayers were also wounded, fixed and labeled for tubulin and Golgi or for tubulin and MTOC, 3 hours after wounding (experiments were performed in triplicate). Wound closure was observed under Y27632 treatment and compared with wound closure with non-drug-treated cells (experiments were performed in triplicate).

DNA constructs
Green fluorescent protein (GFP)-tagged expression vectors encoding the WCA (aa 443-559) region of Scar1, as well as the deltaA constructs of Scar1, Scar2, Scar3 and N-WASP, were obtained as recently described (Magdalena et al., 2003). We also used a myc-tagged IKSp53 expression construct, which was a kind gift from Alan Hall and described previously (Krugmann et al., 2001). The GFP-tagged mDia1 expression constructs were a kind gift from Shuh Narumiya (Kyoto, Japan) and were previously described (Ishizaki et al., 2001; Watanabe et al., 1997) (see Fig. 4A).

Microinjection
Nuclear microinjection in the first row of the wound-edge NIH 3T3 cells were performed about 1 hour after wounding. Expression vectors were used at 100-200 µg/ml. When needed, Alexa fluor 594-conjugated dextran was used at a final concentration of 2 mg/ml as a marker of microinjection.

Immunofluorescence: Golgi and MTOC reorientation and motility assay
NIH 3T3 cells were stained and mounted on glass slides as previously described (Machesky and Hall, 1997). Briefly, cells were fixed with 4% paraformaldehyde in PBS, blocked in 50 mM NH4Cl in PBS, permeabilized in 0.1% Triton X-100 in PBS and stained with phalloidin or the appropriate antibodies. Between each incubation step, cells were washed several times in PBS. The expressed proteins from the microinjected vector were either GFP-labeled or myc-tagged. For cell polarity analysis, cells were fixed 2, 4 and 6 hours after microinjection and labeled for the Golgi apparatus with the anti-β-COP antibody or for MTOC with the anti-pericentrin antibody. The orientation of the Golgi and MTOC was assessed as described previously (Etienne-Manneville and Hall, 2001; Nobes and Hall, 1999). The significance of the inhibition (or lack of inhibition) of the Golgi polarization and the MTOC polarization was determined by the statistical t-test (P<0.025) for all the performed experiments. In each case, we compared treated and untreated cells at the same time point to determine significance. The number of Golgi and MTOC examined and the number of separate experiments performed are indicated in the results section. To test the motility of microinjected cells in the cell sheet, cells were fixed and labeled for actin 4 hours after microinjection. We scored how many microinjected cells were still present at the wound edge (migrated forward) or how many microinjected cells were left behind the advancing cell sheet margin (did not migrate). The percentage of the total number of microinjected cells that migrated with the wound edge was determined for each expression vector used. In general, to study wound closure, cell monolayers were fixed 9 hours post wounding and labeled for actin, except for Y27632 drug-treated cells, which were fixed and labeled 4 hours after wounding. The percentage of wound closure inhibition by drug treatments was determined by measuring the distances between the opposite margins of the wound,
along the wound, in drug-treated and non-drug treated wounded monolayers. Experiments were performed in triplicate and distances were measured at least at ten different points along the wound for each wound. Average distances were estimated for each wound and in the case of drug- (a) and non-drug-treated (b) monolayers. Inhibition of wound closure was calculated as the (a-b)/b ratio. To study wound closure, we also quantified the number of closure bridges formed between both margins of the wound along the entire wound. The closure bridges were defined as formed by cells from opposite sides of the wound meeting as a result of cell migration. We compared drug treated wounded monolayers with non-drug treated wounded monolayers. The cells were examined on a Zeiss microscope using 40× and 60× oil immersion lenses. Fluorescence images were recorded and processed using Openlab software (Improvis) with Hamamatsu C4880 camera.

Results

MTOC polarization in a NIH 3T3 wound model

In this study, we investigated the role of several proteins in the regulation of MTOC polarization, including regulators of both the actin and microtubule cytoskeletons. We used the scratch-wound model recently described (Magdalena et al., 2003).

The position of the MT organizing center (MTOC) can be visualized as the region from which most of the cytoplasmic MTs emanate when the cells are labeled with the anti-tubulin antibody as shown below (Fig. 1) and described earlier (Gotlieb et al., 1981). We determined whether the MTOC, visualized by tubulin labeling showing a ‘bright crescent moon-shaped structure’, orientated to face the wound or not. When the MTOC was present in the 120° sector facing the wound, it was scored positive. Three hours after wounding, about 55±8% of wound-edge cells were found to have an MTOC facing the wound border (n=102 and was performed in three separate experiments) (data not shown).

Alternatively, the MTOC was localized by immunolabeling using an anti-pericentrin antibody as described more recently (Etienne-Manneville and Hall, 2001; Palazzo et al., 2001b). MTOC that were within the 120° sector facing the wound were scored positive. Fig. 1 shows the tubulin and pericentrin co-labeling. For the following experiments, we selected the pericentrin rather than the tubulin labeling to determine the MTOC polarization. The pericentrin labeling of NIH 3T3 wound-edge cells allowed us to clearly differentiate polarized from non-polarized MTOC (Fig. 1B, Fig. 2B, Fig. 3B, Fig. 6B, Fig. 8C and Fig. 10D). At time zero, when no wound was made, MTOC polarization was close to random (Fig. 1). At 3, 5 and 7 hours after wounding, the wound-edge cells had a polarized MTOC (Fig. 1). These data were also included in Figs 2, 3, 6, 8 and 10, for the following experiments, as control of MTOC polarization in response to wounding.

Fig. 1. MTOC polarization over the time course of wound closure using NIH 3T3 fibroblasts.

(A) Co-labeling of wounded NIH 3T3 monolayers for tubulin and pericentrin. NIH 3T3 fibroblasts were fixed and stained for MTOC concomitantly with the anti-tubulin antibody (top panels) and with the anti-pericentrin antibody (bottom panels), 3 hours post wounding. Enlarged pictures of positively scored MTOC polarization are shown on the left panels and correspond to a region from the middle panel as indicated. Using white dotted lines, we indicate the 120° sector facing the wound. (B) The percentage of wound-edge cells with a MTOC orientated towards the wound was determined at 3, 5 and 7 hours post wounding. (C) Quantification data. The first number corresponds to the number of MTOC observed; the second, in parentheses, is the number of separate experiments. Bar, 40 μm.
Nocodazole and taxol interference with polarization of the Golgi and MTOC
To investigate the involvement of MTs in NIH 3T3 cell polarity, polarization of both Golgi and MTOC, as well as wound closure, were studied after nocodazole treatment in our wound-healing model. A range of concentrations of nocodazole was tested (16 μM, 300 nM and 100 nM). At low concentrations, nocodazole partially depolymerizes MTs, resulting in short MTs. Complete disruption of the MTs when cells were treated with 16 μM nocodazole was confirmed by immunostaining for tubulin (Fig. 2D).

Wound closure in NIH 3T3 monolayers treated with 16 μM nocodazole was inhibited by 86±4% and the number of migrating cells coming from both sides of the wound and meeting to close the wound was greatly reduced. For the non-drug treated wounded monolayers, the number of closure bridges (formed by cells from opposite sides of the wound meeting as a result of migration) increased from 14±2 at 8 hours post wounding to 22±4 at 9 hours post wounding. These values were close to zero in the case of nocodazole treated cells, there were no closure bridges seen at 8 hours post wounding and, on average, 0.6±0.6 closure bridges were visible at 9 hours post wounding. When high doses of nocodazole were used, β-COP Golgi labeling reorganized into spots dispersed throughout the cells as early as 30 minutes post nocodazole incubation (Fig. 2D). The NIH 3T3 cells at the edge of the wound made in a monolayer treated with nocodazole at high doses presented an inhibited MTOC polarization (Fig. 2). Thus, we can conclude that MTs are important for the cell migration that drives wound closure, for Golgi morphology and for MTOC polarization.

Nocodazole, 100 nM, did not visibly disrupt MTs but did inhibit wound closure by 61±8%. At 300 nM, nocodazole also inhibited wound closure by 75±5%, and the MTs directed towards the leading edge were fewer and shorter, whereas the cell body MT appeared normal (Fig. 2D). At 9 hours after wounding, the number of closure bridges was, on average, 5±3 and 0.7±1.2 at 100 and 300 nM, respectively. When these low nocodazole concentrations were used to treat the cells, the Golgi was not disrupted. At 100 and 300 nM, nocodazole inhibited wound-edge cell MTOC polarization but not

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Fig. 2. Nocodazole, cytochalasin D and taxol effect on Golgi and MTOC polarization in wound-edge NIH 3T3 cells. Percentage of Golgi (A) or MTOC (B) orientated towards the wound was determined at 3 hours post wounding in cell monolayers treated with nocodazole (blue bars), cytochalasin D (pink bars) or taxol (green bars). Cell polarity of wound-edge cells of non-drug treated monolayers was included as a control of Golgi polarization (grey bar) (A) or MTOC polarization (white bar) (B) (see also legend at the bottom of the graphs). (C) Quantification data. The first number corresponds to the number of Golgi (A) or MTOC (B) observed and the second, in parentheses, is the number of separate experiments. (D) Golgi polarization of wound-edge cells treated with nocodazole at 300 nM and 16 μM. Cells were fixed and stained for tubulin (top panels) and for the Golgi apparatus with anti-β-COP antibody (bottom panels).
Golgi polarization (Fig. 2). Therefore, at low nocodazole concentrations, wound closure and MTOC polarization were inhibited (Fig. 2B) but Golgi polarization was not prevented (Fig. 2A).

Nocodazole depolymerizes MTs, whereas taxol binds to the N-terminal region of β-tubulin and promotes the formation of highly stable MTs that resist depolymerization (Wang et al., 1999). Thus, taxol was used to treat the NIH 3T3 cell monolayers so that we could look at the Golgi and MTOC polarization in wound-edge cells composed of stabilized, non-dynamic MTs. We found that the wound-edge cells of a taxol-treated NIH 3T3 monolayer exhibited Golgi but not MTOC polarization (Fig. 2). Tubulin labeling of wound-edge cells under taxol treatment revealed long MTs and a pattern that was clearly different from non-drug treated cells mostly at the leading edge of the cell: MTs were pointing out towards the wound margin but failed to show any tubulin-rich labeling along the cell border. In addition, the wound closure was inhibited by 67±8%, in contrast to non-drug treated wounded monolayers. At 9 hours after wounding, the number of closure bridges was 4±1, about five time less than obtained with non-drug treated wounded monolayers (see above). Thus, when MTs were not dynamic, wound closure, as well as MTOC polarity, was inhibited, but Golgi polarization still occurred (Fig. 2).

In conclusion, although nocodazole-shortened or taxol-stabilized MTs were not sufficient for wound-edge cell MTOC polarization and migration, they were sufficient for wound-edge cell Golgi polarization.

**Arp2/3 and WASP-family proteins are not required for MTOC polarization**

Cellular proteins essential for actin polymerization include the Arp2/3 complex as an actin nucleator and members from the WASP family (including WASP/N-WASP and Scar/WAVE proteins) as activators of the Arp2/3 complex (Machesky and Gould, 1999; Machesky and Insall, 1998; Machesky and Insall, 1999; Millard and Machesky, 2001).

As we showed that the actin nucleator Arp2/3 complex is essential for Golgi polarization in wound-edge NIH 3T3 cells (Magdalena et al., 2003) and that MTOC polarization also occurs in response to wounding, we tested whether Arp2/3 was also necessary for MTOC polarization. This raises the question of whether Arp2/3 delocalization affects MT distribution in cells. We recently showed that in NIH 3T3 cells, overexpression of Scar1-WCA, which binds to and delocalizes Arp2/3 (Machesky and Insall, 1998), caused inhibition of Golgi polarization of wound-edge cells. By contrast, overexpression of Scar1-deltaA, which lacks the Arp2/3 binding region, did not prevent Golgi polarization in wound-edge NIH 3T3 cells. In the present study, we observed no alteration in MTOC polarization when the wound-edge cells were microinjected with either expression vector (Fig. 3). Thus, Arp2/3 does not appear to be essential for MTOC polarization in NIH 3T3 cells.

As we found that Scar2 is necessary for Golgi polarization in wound-edge NIH 3T3 cells (Magdalena et al., 2003), we investigated whether WASP-family proteins are also important for MTOC polarization. In our previous study, we found that

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**Fig. 3.** Arp2/3 and WASP-family proteins are not involved in MTOC polarization of wound-edge NIH 3T3 cells. MTOC polarity, at the edge of the wound, of cells expressing Scar1-WCA, Scar1-2-3-deltaA and N-WASP-deltaA: MTOC reorientation to face the wound was evaluated when wound-edge NIH 3T3 cells were microinjected with the five expression vectors encoding for the WASP-family deletion constructs described earlier (Magdalena et al., 2003). (A) The microinjected cells expressing the GFP-fusion protein are shown (top panels, from left to right): Scar1-deltaA, Scar2-deltaA and Scar3-deltaA. Cells were fixed and stained for the MTOC with the anti-pericentrin antibody (bottom panels). (B) The percentage of MTOC orientated towards the wound was determined at 3 hours post wounding for cells microinjected with the expression vectors encoding for the WASP-family deletion constructs. MTOC polarization of non-microinjected wound-edge cells was included as a control (white bar) (see legend on the right side of the graph; n is the number of MTOC observed and the second number, after the slash, indicates the number of separate experiments). Bar, 40 μm.
overexpression of Scar2-deltaA caused inhibition of Golgi polarization in NIH 3T3 cells, whereas expression of the deltaA constructs of Scar1, Scar3 or N-WASP did not (Magdalena et al., 2003). Of note, the deltaA constructs, which have no acidic domain, are unable to interact with or delocalize Arp2/3 (Machesky and Insall, 1998; Nakagawa et al., 2001; Takenawa and Miki, 2001). Therefore, if deltaA constructs have an effect on polarization in the microinjected cells, this could be attributed to the interaction of WASP-family proteins with partners other than Arp2/3. In this present study, the expression vectors encoding Scar2-deltaA, Scar3-deltaA and N-WASP-deltaA were microinjected in wound-edge cells and MTOC polarization was quantified. No change in MTOC polarization was observed in wound-edge cells expressing any of the deltaA constructs relative to non-microinjected wound-edge cells (Fig. 3). Thus, WASP-family proteins do not appear to be involved in MTOC polarization in NIH 3T3 cells.

We previously showed that cytochalasin D inhibited Golgi polarization in wound-edge NIH 3T3 cells (Fig. 2A) (Magdalena et al., 2003). We therefore looked at the actin dynamics in MTOC polarization of wound-edge cells in our NIH 3T3 wound healing model and found that cytochalasin D has no effect on MTOC polarization (Fig. 2B). The result obtained with cytochalasin D was confirmed using latrunculin B, which disrupts actin through monomer sequestration. We titrated the latrunculin B and found that 80 nM was the highest dose that left the cells intact, but it also caused significant disruption of the actin network. At 80 nM of latrunculin, 72±7% of wound-edge cells exhibited MTOC polarization (n=168, in four separate experiments). Thus, although we cannot rule out some minimal requirement for actin filaments needed to keep the cells intact and adherent to the dish, we did not find any effect of disruption of the actin cytoskeleton by either of these drugs on the polarization of the MTOC.

Thus, in fibroblasts, unlike Golgi polarization, wound-edge cell MTOC polarization is not actin dependent and does not require Arp2/3 and WASP-family proteins for it to be established (Figs 2, 3).

The mDia1 FH2 domain interferes with Golgi but not MTOC polarization

Formin-homology (FH) family proteins are cytoskeletal organizers that play essential roles in cell polarity establishment in yeast and Drosophila (Frazier and Field, 1997; Wasserman, 1998). mDia is the mammalian homologue of the Saccharomyces cerevisiae protein Bni1p and Bnr1p and Drosophila protein Diaphanous (Alberts et al., 1998; Lynch et al., 1997; Watanabe et al., 1999). Two isoforms of mDia exist, mDia1 and mDia2. The FH2 domain of mDia orients MTs and coordinates them with the actin cytoskeleton (Ishizaki et al., 2001). The mDia FH1 interacts indirectly with actin by binding to the actin monomer-binding protein profilin and it also binds to Src (Ishizaki et al., 2001; Watanabe et al., 1997).

Because mDia has functional links with both MTs and actin, we were interested to study the effect of its expression on mammalian cell polarity, looking both at Golgi and MTOC polarizations. NIH 3T3 cells express mDia1 but not mDia2 (Alberts, 2001). In this study, we used four different mDia1 constructs: full-length mDia1, mDia-deltaN3, mDia F2 and mDia-deltaN3KA3 (Fig. 4A). Full-length mDia1 was shown to be folded and auto-inhibited (Alberts, 2001; Takaishi et al., 2000; Watanabe et al., 1999), and mDia-deltaN3KA3 carries mutations in the MT-interacting FH2 domain (Ishizaki et al., 2001). Therefore, both full-length mDia1 and mDia-deltaN3KA3 are unable to interact with MTs, whereas mDia-deltaN3 and mDia F2 possess an exposed MT-interacting FH2 domain (Ishizaki et al., 2001; Watanabe et al., 1999).

Wound-edge cells were microinjected with the expression vectors encoding full-length mDia1, mDia-deltaN3, mDia F2 and mDia-deltaN3KA3 (Fig. 4). Wound-edge cells expressing full-length mDia1 and mDia-deltaN3KA3 exhibited Golgi
polarization (Fig. 5). By contrast, a significant reduced percentage of both mDia-deltaN3 and mDia F2-expressing wound-edge cells exhibited Golgi reorientation towards the wound margin (Fig. 5). This reduction in percentage was persistent over the time course of wound healing (Fig. 5). Thus, mDia1 FH2 domain, exposed in both mDia-deltaN3 and mDia F2 but not in the other two constructs, might be involved in Golgi polarization during wound healing.

Similarly, we assessed whether MTOC polarization was affected by overexpression of the mDia1 FH1 and FH2 domains in wound-edge cells. The mDia-deltaN3-, mDia F2- and mDia-deltaN3KA3-expressing cells exhibited MTOC polarization, comparable to MTOC polarization in non-microinjected wound-edge cells (Fig. 6). Thus, when overexpressing the FH2 domain of mDia1 in NIH 3T3 wound-edge cells, Golgi polarization was prevented, whereas MTOC polarization was not (Figs 5, 6).

Nocodazole does not inhibit Golgi polarization (Fig. 3) and overexpression of the mDia1 FH2 domain inhibits Golgi polarization (Fig. 5). However, the mDia1 FH2 domain has been shown to induce the formation of stable MTs and to localize together with MTs (Kato et al., 2001; Palazzo et al., 2001b). To concomitantly investigate the FH2 domain interference with the Golgi polarization and the FH2 interaction with MTs, we treated mDia F2-expressing wound-edge cells with nocodazole (300 nM). We found that as little as 44±6% of these wound-edge cells had a polarized Golgi (n=210, three separate experiments). Thus, as there was still inhibition of the Golgi polarization under nocodazole treatment, FH2 interaction with short MTs must be sufficient to inhibit Golgi polarization. Alternatively, FH2 could also interact with a partner other than MT to inhibit Golgi polarization.

In conclusion, wound-edge NIH 3T3 cells overexpressing FH2-containing mDia1 constructs exhibited MTOC polarity but not Golgi polarity (Figs 5, 6). In addition, mDia1 FH2...
Fig. 6. Overexpression of the mDia1 domains does not interfere with MTOC polarization in wound-edge NIH 3T3 cells. MTOC polarity, at the edge of the wound, of mDia-deltaN3-, mDia F2- and mDia-deltaN3KA3-expressing cells: MTOC reorientation to face the wound was evaluated when wound-edge NIH 3T3 cells were microinjected with the three corresponding pEGFP-mDia1 expression constructs (see Fig. 4). (A) The microinjected cells expressing the GFP-fusion protein are shown (upper panels): mDia-deltaN3 (left), mDia F2 (middle) and mDia-deltaN3KA3 (right). Cells were fixed and stained for the MTOC with the anti-pericentrin antibody (lower panels). (B) The percentage of MTOC orientated towards the wound was determined at 3 hours post wounding for cells microinjected with the three mDia1 expression vectors. MTOC polarization of non-microinjected wound-edge cells was included as a control (see legend on the right side of the graph; n is the number of MTOC observed and the second number, after the slash, indicates the number of separate experiments). Bar, 40 μm.

Fig. 7. Motility of the microinjected wound-edge NIH 3T3 cells within the cell sheet during wound closure. Cells were microinjected with pEGFP-expression vectors encoding mDia-deltaN3 (left panels), mDia F2 (middle panels) and mDia-deltaN3KA3 (right panels). Cells were fixed 4 hours after microinjection and labeled for actin. (A) GFP-mDia-deltaN3-, GFP-mDia F2- and GFP-mDia-deltaN3KA3-expressing cells are shown in the top panels (left to right panels, respectively, as indicated). Actin labeling is shown under the corresponding top panel and merges of the two top panels are shown at the bottom of the corresponding column. Cells expressing mDia-deltaN3 and mDia F2 were left behind the wound edge (left and middle columns). Cells expressing mDia-deltaN3KA3 were motile as they persisted at the margin of the closing wound (right column). (B) The table includes the percentage of migrating cells for each expression construct microinjected in wound-edge cells and the number (n) of microinjected cells observed. Quantification was performed from three separate experiments for each construct. Bar, 40 μm.
domain overexpression in wound-edge cells inhibited Golgi polarization, even in a population of nocodazole-resistant stable MTs. But the overexpression of the mDia1 FH1 domain had no effect on the polarity of the wound-edge cells.

Overexpression of the mDia1 FH2 domain inhibits NIH 3T3 cell motility

Given that the FH2 domain of mDia1 interfered with Golgi polarization, we investigated whether migration of the mDia1 FH2 domain-overexpressing cells within a sheet was possible. Cells expressing mDia-deltaN3KA3, with the mutated FH2 domain, mostly migrated (up to 84±4%) with the edge of the wound (Fig. 4A and Fig. 7). However, cells microinjected with expression vectors encoding mDia-deltaN3 and mDia F2, having an exposed FH2 domain, were mostly left behind the wound edge; only 20±11% and 30±17%, respectively, were seen at the wound margin (Fig. 7). As a positive control, wound-edge cells were microinjected with Alexa fluor 594-conjugated dextran, and 93±6% of microinjected cells persisted at the wound border.

Taken together, our data show that NIH 3T3 cells overexpressing FH2-containing mDia1 constructs were non-motile, as they were left behind the advancing wound border and had inhibited Golgi polarization, whereas MTOC polarization was not prevented (Figs 5-7).

A Src tyrosine kinase inhibitor blocks Golgi but not MTOC polarization

Interfering with Src activity by microinjection of anti-Src antibodies block activated Dia function (Alberts, 2001; Roche et al., 1995). Additionally, inhibitors of Src, including ST638, inhibit wound closure in a keratinocyte tissue culture wound model (Owens et al., 2000; Yamada et al., 2000). It was concluded that wounds activate c-Src, which results in Src association with MTs (Yamada et al., 2000), and that Src is required to induce the disruption of established cell-cell contacts, which is necessary for cell repair (Owens et al., 2000). We first confirmed that wound closure in NIH 3T3 monolayers treated with ST638 was inhibited. The formation of closure bridges resulting from migrating cells coming from opposite sides of the wound and meeting to close the wound was totally prevented. For the non-drug treated cells, the number of closure bridges increased from 6±1 at 7 hours post wounding to 11±1 at 8 hours post wounding. These values were equal to zero in the case of ST638 treated cells at 7 and 8 hours post wounding (Fig. 8A). Next, we examined NIH 3T3 cell polarity establishment in ST638 treated cells. The ST638 drug inhibited Golgi polarization but not MTOC polarization (Fig. 8B,C).

Interestingly, mDia1 directly binds to an SH3 domain-containing protein IRSp53 (Fujiwara et al., 2000) and Src has been proposed as a possible candidate to regulate the formation of the GTP-RhoA-mDia1-IRSp53 ternary complex (Fujiwara et al., 2000). We therefore looked at the Golgi polarization in wound-edge cells overexpressing IRSp53 and

Fig. 8. ST638 inhibits wound closure as well as Golgi polarization but does not prevent the MTOC polarization of NIH 3T3 cells.

(A) Wounded NIH 3T3 monolayers not treated (left panel) or treated with ST638 (right panel) were fixed and stained for actin 9 hours post wounding. (B) Percentage of Golgi polarization and (C) percentage of MTOC polarization: Golgi and MTOC orientated towards the wound were determined at 3 hours following wounding for cells treated with ST638 (pink bars). For non-drug treated wound-edge cells were included as a control: grey bars (B) and white bars (C), respectively (see also legend on the right side of the graph; n is the number of Golgi (B) or MTOC (C) observed and the second number, after the slash, indicates the number of separate experiments). Bar, 80 μm.
found that 74±8% of them had a polarized Golgi (n=142, from three separate experiments). We also found that only 37±5% of these cells migrated (n=120, in three separate experiments) (Fig. 9).

In conclusion, in NIH 3T3 fibroblasts, by inhibiting Src – acting downstream of mDia1 and binding MTs – wound-edge cell Golgi polarization and migration were blocked, but not MTOC polarization (Fig. 8). However, IRSp53 overexpression failed to inhibit Golgi polarization of wound-edge cells, but interfered with cell migration (Fig. 9).

An inhibitor of ROCK blocks Golgi and MTOC polarizations but enhances cell migration

ROCK and mDia cooperatively act as downstream target molecules of Rho in the Rho-induced reorganization of the actin cytoskeleton (Nakano et al., 1999). Both ROCK and mDia are necessary for the formation of stress fibers and focal adhesions (Nakano et al., 1999). Y27632, an inhibitor of ROCK, induces membrane ruffles (Tsujii et al., 2002). Nobes and Hall (Nobes and Hall, 1999) also found that Y27632 treatment accelerated the migration of the cells. In addition, the requirement of ROCK for centrosome positioning during cell division was recently reported (Chevrier et al., 2002).

Therefore, to understand whether mDia functions in association with ROCK for Golgi polarization establishment, and to study whether ROCK is required for MTOC polarization in the NIH 3T3 wound model, we investigated both the Golgi and MTOC polarizations in wound-edge cells in Y27632-treated monolayers.

First, we looked at the morphology of the wound-edge cells in NIH 3T3 monolayers treated with Y27632. A early as 15 minutes after wounding, drug-treated wound-edge cells exhibited long extensions directed towards the empty space left by the wound, not seen in the case of non-drug treated wound-edge cells (Fig. 10A), revealed by both actin and tubulin labeling. Importantly, the MT network under Y27632 drug treatment revealed long MTs and was tubulin-rich along the cell borders. Wound closure was estimated to occur 64% faster for drug-treated cell monolayers than non-drug treated monolayers (Fig. 10B). As described earlier, we showed that the FH2 domain of mDia1 was able to interfere with motility of wound-edge cells (Fig. 7). As Y27632-treated cells moved faster, we therefore tested whether the wound-edge cells overexpressing the mDia1 FH2 domain would be able to move under Y27632 drug-treatment conditions. Wound-edge cells, from Y27632-treated monolayers, were microinjected with the expression vector coding for mDia F2, which has an exposed FH2 domain. Monolayers were fixed four hours post wounding and labeled for actin as described before and shown in Fig. 7. Cells expressing mDia F2 and treated with Y27632 mostly migrated – up to 82±5% of these cells were seen at the wound margin (n=110, four separate experiments). Therefore, the mDia1 FH2 domain did not inhibit motility under Y27632 treatment conditions. Thus, we observed that under Y27632 treatment, cells moved faster and even the FH2-containing mDia1 constructs expressing wound-edge cells migrated.

We also found that wound-edge cells from Y27632-treated monolayers exhibited neither Golgi polarization nor MTOC polarization (Fig. 10).

Therefore, these results show that inhibition of ROCK, with Y27632, enables wound-edge cells to move faster, but inhibits Golgi and MTOC polarizations (Fig. 10). Moreover, under Y27632 treatment, FH2-containing mDia1 constructs expressing wound-edge cells regain the ability to migrate.

Discussion

The polarized morphology of some types of migrating cells also involves the reorganization of the microtubular network (Etienne-Manneville and Hall, 2001; Gotlieb et al., 1981; Stowers et al., 1995). It was recently reported that wounding of astrocytes induces an actin-independent (cytochalasin D resistant) but MT-dependent (nocodazole sensitive) polarization of leading edge cells, characterized by the formation of a polarized, elongated morphology and by the reorientation of the MTOC and the Golgi in the direction of migration (Etienne-Manneville and Hall, 2001). We used a fibroblast model (NIH 3T3) to test the generality of the MTOC reorientation and MT requirement in wound healing.
As migrating wound-edge cells exhibited polarization of the Golgi and MTOC, we looked for a connection between actin, Arp2/3, WASP-family proteins and MTs in polarity and migration. We did not observe any change in MTOC polarization when the wound-edge cells were microinjected with an expression vector encoding Scar1-WCA, to delocalize the endogenous Arp2/3. In addition, Scar1-deltaA, Scar2-deltaA, Scar3-deltaA and N-WASP-deltaA overexpression did not affect the MTOC polarization of wound-edge cells. Similarly, treatment with cytochalasin D or latrunculin B failed to inhibit MTOC polarization. Thus, there is MTOC polarization in response to wounding, but the Arp2/3 complex, the WASP-family proteins and actin filaments do not appear to be involved in MTOC polarization, in contrast to Golgi polarization.

Formin-homology family proteins play roles in yeast and Drosophila cell polarity (Frazier and Field, 1997; Wasserman, 1998). Therefore, the mDia1 protein, a mammalian FH family member, might be involved in Golgi and MTOC polarization in fibroblasts. We show that the NIH 3T3 cells that overexpressed mDia1 deletion constructs containing the FH2 domain, which is able to interact with MTs, inhibited Golgi polarization and inhibited cell motility, whereas MTOC polarization was not affected. Our results suggest that indirect or direct interaction with MTs are needed to establish Golgi polarization. Furthermore, interaction(s) with some MT-interacting protein domains (including FH2), which are not essential for MTOC polarization, are necessary for proper Golgi polarization. Very recently, the FH2 domain of S. cerevisiae Bn1p was reported to be an actin nucleator (Pruyne et al., 2002). But the FH2 of Bn1p has a different defined length of 500 amino acid residues in the Pruyme report and of only about 100 amino acid residues in previous studies (Ishizaki et al., 2001). It will be interesting to determine whether mammalian Dia proteins also have actin nucleating FH2 domains. Taken together, our data indicate that mDia1 FH2, but not mDia1 FH1, is involved in Golgi polarization and migration of wound-edge cells.

Identically, we found that the ST638 drug, a Src inhibitor, prevented Golgi polarization and migration but not MTOC polarization. Our results are in agreement with a previous report showing that Src is required for directional cell migration (Timpson et al., 2001). Src binds to the FH1 domain of mDia1 and might therefore be an effector of mDia (Ishizaki et al., 2001). Although mDia is not the only protein upstream of Src, it is interesting that we obtained the same effect on Golgi polarization both with the mDia1 FH2 domain and the Src inhibitor. We found, to our surprise, that overexpression of the...
mDia1 FH1 domain did not interfere with Golgi and MTOC polarizations nor with migration of the wound-edge cells. Thus, the interaction between Src and FH1 might not be necessary or essential for Golgi polarization and migration of wound-edge cells.

mDia1 binds to IRSp53 and Src has been proposed as a possible candidate to regulate the formation of the GTP-RhoA-mDia1-IRSp53 ternary complex (Fujiwara et al., 2000). We found that IRSp53-expressing wound-edge cells exhibited Golgi polarization as well as motility. IRSp53 has been implicated in actin assembly (Miki et al., 2000). So far, the overexpression of the mDia1 FH2 domain prevented cell polarizations nor with migration of the wound-edge cells. Our results indicate that the mDia1 FH2 domain might interact with different partners, each leading specifically and independently to Golgi polarization or migration, or both. Our data agree with a possible role for Src in conjunction with mDia1 in the polarization of the Golgi and IRSp53 in conjunction with mDia1 and Src in cell migration. In addition, our data suggest that these proteins – namely mDia1, IRSp53 and Src – are not involved in MTOC polarization.

ROCK and mDia cooperatively act as downstream target molecules of Rho in the Rho-induced reorganization of the actin cytoskeleton (Nakano et al., 1999). Both ROCK and mDia are necessary for the formation of stress fibers and focal adhesions (Nakano et al., 1999). The ROCK inhibitor Y27632 induces membrane ruffles (Tsui et al., 2002). Nobes and Hall (Nobes and Hall, 1999) also found that Y27632 treatment accelerated the migration of the cells. Interestingly, the requirement of ROCK for centrosome positioning during cell division was recently reported (Chevrier et al., 2002). In our hands, inhibition of ROCK caused wound-edge cells to move faster, allowed FH2 expressing wound-edge cells to migrate and inhibited Golgi and MTOC polarization. Therefore, ROCK has a negative effect on the cell-migration process, including the migration of cells expressing FH2-containing mDia1 constructs. In addition, ROCK is needed for cell polarity – Golgi as well as MTOC polarization – in the NIH 3T3 wound model. Of note, our data suggest, in contrast to mDia1, IRSp53 and Src, that ROCK controls MTOC polarization.

Euteneuer and Schliwa (Euteneuer and Schliwa, 1992) concluded that initiation of cell polarization is independent of MTOC repositioning. We asked the following question: is Golgi polarization independent of MTOC repositioning? Euteneuer and Schliwa (Euteneuer and Schliwa, 1992) postulated that it should be possible to block lamellipodia assembly without affecting MTOC polarization. Here, together with previous data from a recent study (Magdalena et al., 2003), we have shown that it is possible, by delocalizing Arp2/3, to block Golgi polarization without inhibiting MTOC polarization (Fig. 11). The same results were obtained with overexpression of the mDia1 FH2 domain and under ST638 treatment (Fig. 11). However, nocodazole blocks MTOC polarization [(Etienne-Manneville and Hall, 2001; Euteneuer and Schliwa, 1992) and our present study]. Euteneuer and Schliwa (Euteneuer and Schliwa, 1992) showed that short MTs are not sufficient to mediate MTOC repositioning in response to wounding. But they also

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**Fig. 11.** Involvement of microtubules in Golgi and MTOC polarity establishment in wound-edge NIH 3T3 cells. (A) Summary of results obtained in this study: the effect on Golgi polarization, MTOC polarization and cell migration of different drugs (nocodazole, taxol, cytochalasin D, Y27632, ST638) and/or different expression constructs (WASP family WCA, several deletion constructs of mDia1 and IRSp53). (B) Proteins involved in MTOC polarity, Golgi polarity and/or cell motility. (C) Rho, ROCK, mDia, IRSp53 and Src proteins are shown inside rectangles, and distributed as shown on the basis of the pathway proposed and described in the literature. It is shown at which level of the pathway and which proteins are important for MTOC polarity, for Golgi polarity and for cell motility.
showed that nocodazole does not prevent polarity initiation. In another study, it was also shown that nocodazole (low dose) has no effect on Golgi polarization (Nobes and Hall, 1999). Here, we have shown, by treating cells with low nocodazole doses, that MTOC polarization can be blocked without inhibiting Golgi polarization (Fig. 11). The same results were obtained with taxol (Fig. 11). Thus, with several experiments, we have clearly shown the dissociation between MTOC and Golgi polarization, although they both depend on MTs.

In conclusion, we have shown that MTOC and Golgi polarization are separately controlled and do not depend on each other to take place [as suggested by Euteneuer and Schiwa (Euteneuer and Schiwa, 1992)]. This is also in accordance with recently reported data by Chabin-Brion and co-workers (Chabin-Brion et al., 2001). This team showed that Golgi membranes undergo an intimate relationship with MTs, and especially with a population of nocodazole-resistant, stable MTs, and concluded that the Golgi apparatus is a MT-organizing organelle acting independently from the centrosome. Golgi membranes might assemble and perhaps stabilize the appropriate MTs instead of depending on centrosomally attached MTs for the vectorization of membrane carriers. This would enable the cell to define priority tracks to use efficient vesicular transport (Chabin-Brion et al., 2001). Interestingly, in 2002, Takatsuki et al. reported that MTs play a vital role in the organization and partitioning of the Golgi apparatus, but an additional mechanism, separate from MTs, has a role in Golgi localization (Takatsuki et al., 2002). This appears to agree with a recent study showing that GMAP-210, a large coiled-coil protein, links the cis-Golgi to the minus ends of centrosomally nucleated microtubules (Infante et al., 1999). Our study suggests that, although the Golgi might depend on centrosomally nucleated microtubules for stability and for trafficking, it does not necessarily use these microtubules for positioning.

Rho, ROCK, mDia, IRSp53 and Src are already described in the literature as forming part of the pathway as shown in Fig. 11 (Fujiiwara et al., 2000; Nakano et al., 1999; Satoh and Tominaga, 2001). Here, we attribute to these proteins their involvement in wound-edge cell Golgi polarization, MT OC polarization and migration (Fig. 11).

Recently, we showed that in NIH 3T3 cells, Golgi polarization is actin dependent, and needs Arp2/3 and Scar2 for establishment. Here, we show that Golgi polarity appears to be also dependent on mDia FH2, Src and ROCK (Fig. 11). By contrast, MTOC polarization is actin independent and does not need Arp2/3 or any of the WASP-family members, but requires dynamic and long MTs, as well as ROCK activity (Fig. 11).

Arp2/3 controls actin dynamic and cell motility as well as Golgi polarization. In association with Arp2/3, mDia also has a role to play in cell motility and Golgi polarization, and the mDia1 FH2 domain is necessary in both cases. In addition, mDia and ROCK controlled stable actin-bundle formation and MTs, and take part in Golgi polarization and cell motility. By contrast, ROCK controls the MT network and the MTOC polarity. There is a balance between dynamic actin, stable bundles of actin and MT networks, and they individually have an impact on polarity and motility.

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