Targeting of p0071 to the midbody depends on KIF3

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Summary

P0071 (plakophilin-4) is a member of the p120ctn subfamily of armadillo proteins that are essential for cell contact formation. Additionally, p0071 plays a role in cytokinesis, in which it regulates local activation of RhoA together with Ect2. Because spatiotemporal regulation is required for progression through cytokinesis, we analyzed when and how p0071 is targeted to the midbody to induce RhoA activation. We show that Ect2 precedes p0071 accumulation at the midbody and that targeting is mediated by different motor proteins. p0071 interacted with the kinesin-II family member KIF3b, and knockdown of KIF3b interfered with p0071 midbody recruitment whereas Ect2 or RhoA localization was not affected in these cells. Moreover, knockdown of KIF3b induced a similar phenotype as the p0071 knockdown, with reduced actin and phospho-myosin-light-chain accumulation at the midbody and decreased levels of active RhoA during cytokinesis. The lack of RhoA activation in KIF3b-deficient cells was not rescued by overexpression of wild-type p0071 but was substantially ameliorated by a p0071–MKLP1-motor-domain fusion protein that was targeted to the furrow independently of KIF3. These data indicate that p0071 and Ect2 are transported via distinct motors and identify a novel pathway implicating KIF3 in the regulation of actin organization during cytokinesis.

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Key words: p0071, p120ctn, Cytokinesis, Kinesin

Introduction

P0071 (plakophilin-4) is a member of a subfamily of cell contact-associated proteins of the armadillo superfamily. P120ctn is the prototype of this subfamily that comprises p0071, δ-catenin, ARVCF and the more distantly related plakophilins 1-3 (Hatzfeld, 2005). P0071, like p120ctn, ARVCF and δ-catenin localizes to adherens junctions by binding to E-cadherin in a region that is distinct from the β-catenin binding site. In contrast to β-catenin, the p120ctn-related proteins apparently are not involved in anchoring the E-cadherin complex to the cytoskeleton but rather function to stabilize E-cadherin at the membrane by cluster formation (Calkins et al., 2003; Davis et al., 2003; Hatzfeld et al., 2003). Besides this structural function in cell contact formation, p0071 has a role in the regulation of Rho signaling during cytokinesis (Keil et al., 2007; Wolf et al., 2006). In anaphase, contractile ring assembly starts around the central spindle. Assembly and ingression of the contractile ring involves activation of myosin II and actin polymerization, which are triggered by the GTPase RhoA (Piekny et al., 2005). The Rho-GEF Ect2 has been reported to be required for cortical localization of RhoA and contractile ring assembly (Yuce et al., 2005). P0071 interacts with RhoA and with Ect2 and together with Ect2 it stimulates RhoA-activity to promote contractile ring formation and contraction. Accordingly, knockdown of p0071 has been shown to interfere with completion of cytokinesis by downregulation of Rho signaling and by preventing actin accumulation at the central spindle (Wolf et al., 2006).

As cytokinesis critically depends on the spatiotemporal regulation of actin remodeling and membrane insertion, transport to the midbody by molecular motors plays an essential role in this process and many kinesins have been reported to function during cytokinesis. In a screen for motor proteins involved in mitosis and cytokinesis, Zhu et al. (Zhu et al., 2005) identified a total of 12 kinesins involved in mitosis. MKLP1, MKLP2 (members of the kinesin-6 family) and KIF4 were essential for cytokinesis. Of these, MKLP1 is a component of the centralspindlin complex that targets Ect2 to the central spindle (Yuce et al., 2005). The centralspindlin complex consists of a kinesin-6, MKLP1 (Pavarotti in Drosophila and ZEN4 in Caenorhabditis elegans) and a Rho-GTPase-activating protein MgcRacGAP (CYK-4 in C. elegans), and is required for the formation of stable microtubule bundles at the spindle midzone (Mishima et al., 2002). The dynactin complex contributes to cytokinesis by promoting targeting of the centralspindlin complex to microtubule plus ends at anaphase onset (Delcros et al., 2006). Depletion of the centralspindlin component MKLP1 prevents central spindle localization of Ect2; however, RhoA, F-actin and myosin have been shown to accumulate on the equatorial cell cortex indicating that their localization is regulated independently (Yuce et al., 2005). MKLP2 associates with the mitotic kinesins Aurora-B and Plk1 (Gruneberg et al., 2004). KIF4 translocates PRC1, a spindle midzone-associated cyclin-dependent kinase substrate protein, to spindle microtubules during the metaphase-to-anaphase transition (Kurasawa et al., 2004; Zhu and Jiang, 2005).

In addition to these kinesins, the kinesin-6-family protein KIF12 has been reported to localize to the central spindle and the cleavage furrow during cytokinesis and to be essential for INCENP (inner centromere protein) localization at the cleavage furrow (Chen et al., 2007). Kinesin-13 proteins KIF2a, KIF2b and MCAK all localize to the spindle midzone in late anaphase and telophase but defects in cytokinesis have only been observed in KIF2b-deficient cells (Manning et al., 2007). Kinesin-13 proteins are nonmotile and induce microtubule depolymerization by disassembling tubulin subunits from the polymer end (Desai et al., 1999). Thus, KIF2b may be needed to disassemble the microtubule bundle linking daughter cells or may disrupt cytokinesis indirectly by impairing the targeting of critical midzone components that have not yet been characterized. KIF14 accumulates with spindle poles, spindle microtubules and the midbody...
in mitotic cells, and silencing of KIF14 was observed to induce cytokinesis failure (Carleton et al., 2006). Citron kinase but not other components of the central spindle and cleavage furrow failed to localize in KIF14 knockdown cells thus identifying citron as a target transported by KIF14 (Gruneberg et al., 2006).

Finally, KIF3, a complex of kinesin-II family proteins, has been implicated in cytokinesis. KIF3 is composed of two closely related motor subunits, KIF3a and KIF3b, and a kinesin-associated protein known as KAP3, which has been reported to link KIF3a and KIF3b to its cargo (Takeda et al., 2000; Yamazaki et al., 1995). It is one of the most widely expressed kinesins and has been implicated in the intracellular transport of membrane-bound organelles and protein complexes in various tissues. KIF3b has been shown to localize to the central spindle and the midbody during cytokinesis and dominant negative mutants were seen to interfere with normal progression through mitosis (Fan and Beck, 2004; Haraguchi et al., 2006). KIF3b binds to the spectrin family member Syne-1 (synaptic nuclear envelope protein-1, also known as Nesprin and Enap1), the localization of which apparently depends on KIF3b (Fan and Beck, 2004). It has been proposed that Syne-1 and KIF3b function together in cytokinesis by facilitating the accumulation of membrane vesicles at the spindle midzone.

Here, we show that p0071, which acts together with Ect2 to promote RhoA activation at the cleavage furrow during cytokinesis, is transported independently of Ect2 and the centralspindlin complex. Instead, KIF3b appears essential for p0071 midbody association. P0071 interacts with KIF3b and this interaction is direct and not dependent on KAP3, which had been reported to link the KIF3a-KIF3b heterodimer to its cargo. Moreover, we show that the knockdown of KIF3b interferes with cytokinesis by preventing p0071 midbody targeting. Accordingly, KIF3b knockdown interferes with actin accumulation and myosin light chain (MLC)-activation at the region of furrow ingression as well as with RhoA activation during cytokinesis, thus mimicking the effect of the p0071 knockdown. A p0071–MKLP1-motor-domain fusion protein that is targeted to the midbody independently of KIF3b is able to rescue the KIF3b-induced reduction in RhoA activity supporting our conclusion that the KIF3b contribution to cytokinesis is partially dependent on p0071.

**Results**

P0071 and Ect2 are targeted independently to the microtubule midzone in cytokinesis

As p0071 and Ect2 colocalize at the midbody and act together to promote RhoA activation and contractile ring formation during cytokinesis, we asked when and where these two proteins interact to mediate cytokinesis. Both proteins have been found to colocalize in the pericentrosomal region (Wolff et al., 2006), suggesting that they could form a complex that is co-transported to the midbody in the centralspindlin complex. To analyze this, we performed colocalization studies for endogenous Ect2 and p0071 from anaphase to telophase and during cytokinesis in HeLa cells. Ect2 associated with the central microtubule bouquet at anaphase when furrow ingression begins (Fig. 1A). At this time point, p0071 was essentially absent from the microtubule midzone. When furrow ingression proceeded during telophase, p0071 was recruited to the region of furrow ingression (Fig. 1B) where it accumulated together with Ect2 at the midbody during cytokinesis (Fig. 1C). This indicates that Ect2 precedes p0071 targeting to the microtubule midzone and strongly suggests that p0071 is not transported together with Ect2 in the centralspindlin complex.

We analyzed further whether p0071 targeting depended on Ect2 or MKLP1, the motor component of the centralspindlin complex. Knockdown of both proteins was verified by western blot analysis (Ect2 knockdown: 36% ± 5%; MKLP1 knockdown: 48% ± 7%) (Fig. 2A) and severely interfered with cytokinesis. In both cases, only very few of the transfected cells (~10%) showed furrow ingression (Fig. 2B). In those Ect2 knockdown cells that underwent furrow ingression, p0071 was strongly reduced but not completely absent from the midbody and sometimes revealed a more dispersed localization compared with control transfected cells, indicating that depletion of Ect2 did not completely prevent p0071 midbody association (Fig. 2B). In MKLP1 knockdown cells, Ect2 was essentially absent from the midbody (not shown) whereas some p0071 accumulated at the midzone (Fig. 2D). As MKLP1 and Ect2 localize to the midzone from anaphase onset onwards but p0071 associates with the midbody only at late telophase (see Fig. 1 for p0071+Ect2), we exclude the centralspindlin complex as the primary motor responsible for transporting the major fraction of p0071 to the midbody. As MKLP1 and the centralspindlin complex are required for formation of stable microtubule bundles (Mishima et al., 2002), the MKLP1 knockdown may interfere indirectly with p0071 targeting by destabilizing the central microtubules. Nevertheless, Ect2 and MKLP1 could play a role in supporting or stabilizing p0071 midbody association.

P0071 interacts with the KIF3b motor protein

To further analyze how p0071 is targeted to the midbody we analyzed the function of other motor proteins. Among the motor
proteins with a well-documented role in cytokinesis, we identified KIF3b in a yeast 2-hybrid screen as a putative interaction partner of p0071. Therefore, we analyzed whether KIF3b and p0071 colocalized during mitosis. In interphase, both proteins colocalized at the centrosome (Fig. 3A). During telophase and cytokinesis, KIF3b associated with the central microtubules whereas p0071 accumulated at the midbody (Fig. 3B). These data suggest that KIF3b could be involved in transporting p0071 during telophase and cytokinesis. We next analyzed whether p0071 directly binds to KIF3b. We used the yeast 2-hybrid system to validate and map p0071 binding sites in KIF3b. KIF3b consists of an N-terminal motor domain, a central α-helical coiled coil domain responsible for dimerization and a C-terminal cargo-binding domain. The p0071 armadillo repeat domain interacted with both wild-type KIF3b and the C-terminal cargo-binding domain, but not the N-terminal motor domain, as revealed by activation of the HIS3 and the lacZ reporter genes in the double transformants (Fig. 4A). Mapping of the KIF3b binding site in p0071 revealed that the arm repeats 1-3 were sufficient to mediate this interaction. Deletion of the first arm repeat of p0071, however, interfered with the p0071-KIF3b interaction (Fig. 4A), indicating that this sequence was required for KIF3b to bind to p0071. An interaction between p0071 and KIF3b was confirmed in a GST pulldown assay. The p0071 armadillo repeat domain was fused to GST and the fusion protein coupled to glutathione beads. These co-precipitated overexpressed KIF3b as well as endogenous KIF3b from total cell lysates (Fig. 4B,C). Co-immunoprecipitation from HEK293 cells expressing FLAG-tagged KIF3b and HA-tagged p0071 confirmed that the two proteins formed a complex in the cell (Fig. 4D). In order to exclude that the interaction between p0071 and KIF3b was mediated by a linker protein present in the cell lysates used in the GST pulldown as well as in the co-immunoprecipitation approach, we also used in vitro translated p0071 and KIF3b proteins for co-immunoprecipitation. This approach confirmed an interaction (Fig. 4E), strongly
suggested that the p0071-KIF3b association did not depend on the KAP3-linker protein. Taken together, these data suggest that p0071 and KIF3b associated directly and that this interaction was mediated by the p0071 armadillo repeats 1-3 and the KIF3b C-terminal cargo-binding domain. We therefore concluded that KIF3b might be responsible for transporting p0071 to the microtubule midzone during telophase and cytokinesis.

**Fig. 4.** KIF3b interacts with p0071 in vitro and in vivo. (A) The interaction between p0071 and KIF3b was examined in the yeast 2-hybrid system. YRG2 yeast cells were co-transformed with the p0071 repeat domain or truncated variants of the p0071 repeat domain and wild-type KIF3b, KIF3b N-terminal (NT) and KIF3b C-terminal (CT) domains as indicated and plated on selection plates without tryptophane, leucine and histidine (–WLH). Colonies grown on selection plates (–WL) were probed for β-galactosidase activity (lacZ). (B,C) Overexpressed and endogenous KIF3b interact with the p0071 repeat domain in a GST pulldown experiment. The glutathione-agarose immobilized recombinant GST-p0071/-repeat domain or truncated KIF3b from wild-type KIF3b-HA overexpressing (B) and nontransfected HEK293 cell lysates (C). Binding of KIF3b was determined by western blotting using an HA-specific (B) or a KIF3b-specific (C) antibody (PD: pull down). (D) A FLAG-IP approach revealed an association between p0071 and KIF3b in vivo. After overexpression of FLAG-tagged wild-type KIF3b and/or HA-tagged wild-type p0071 in HEK293 cells, KIF3b was precipitated from total lysates using anti-FLAG-agarose beads. Eluates were analyzed for p0071 binding using a polyclonal HA-antibody. (E) A KIF3b-binding-deficient p0071 mutant does not localize at the cleavage furrow during cytokinesis.

To further test our hypothesis that the p0071-KIF3b interaction mediates p0071 midbody targeting, we constructed a p0071 mutant that lacked arm repeat 1 and therefore was deficient in KIF3b binding (see Fig. 4). This mutant was still able to localize at the plasma membrane but was not able to associate with the central microtubule bundle in telophase and did not accumulate at the midbody in cytokinesis (Fig. 7A,B). These results strongly suggest that KIF3b is directly or indirectly involved in the transport of p0071 during telophase and cytokinesis but not in the targeting of Ect2 or RhoA.

**Knockdown of p0071 or KIF3b interferes with normal progression through cytokinesis.** KIF3b has been shown to play a role in cytokinesis as expression of a truncated form of KIF3b that contained the C-terminal cargo.
binding plus the coiled coil domain but that lacked the N-terminal motor domain gave rise to an increased frequency of bi- and multinucleated cells (Fan and Beck, 2004). However, the severity of this phenotype was not determined because the number of multinucleated cells was not quantified. We used siRNA-mediated knockdown of KIF3b in addition to mutant protein expression to determine its role in mitosis. Knockdown of KIF3b resulted in formation of multinucleated cells at 72 hours post transfection (Fig. 8A). In order to compare this with the effect of p0071 downregulation, we quantified the number of multinucleated cells after KIF3b or p0071 knockdown. In both cases we found between 20% and 25% of multinucleated cells compared with 3% in control siRNA-transfected cells, indicating that both proteins are required for normal progression through mitosis (Fig. 8B). A double knockdown of p0071 plus KIF3b did not further increase the number of multinucleated cells indicating that the defects were not additive. This is consistent with the idea that p0071 could be an essential target and downstream mediator of KIF3b. To further characterize the phenotype after KIF3b knockdown, we compared the process of furrowing in KIF3b and p0071 knockdown cells. Depletion of both proteins resulted in either delayed or totally disturbed furrowing.
with similar frequencies. Again the effect was not further increased in the double knockdown cells (Fig. 8C,D).

The defect induced by KIF3b silencing strongly resembles the defect in p0071 knockdown cells

Our results on the role of KIF3b in targeting p0071 to the midbody suggest that the cytokinesis defect observed after the KIF3b knockdown might result from the depletion of p0071 from the midbody in these cells. If this was the case, the KIF3b knockdown should interfere with actin reorganization and contractile ring formation as observed after the p0071 knockdown. To test this, we analyzed actin organization in the region of furrow ingression in KIF3b knockdown cells compared with p0071 knockdown cells. The p0071 as well as the KIF3b knockdown correlated with a considerable reduction of actin accumulation at the central microtubule bundle compared with control siRNA-transfected cells (Fig. 9A).

Contractile ring formation depends on the activation of RhoA, which is stimulated by p0071. Effectors of RhoA are formin family proteins that induce actin polymerization and Rho-kinases that lead to myosin activation as revealed by myosin light chain (MLC) phosphorylation (Murthy and Wadsworth, 2005). By using phospho-MLC-specific antibodies we show that p0071 as well as KIF3b knockdown led to a considerable reduction in phospho-MLC accumulation during cytokinesis, indicating that Rho signaling was affected by the KIF3b knockdown (Fig. 9A). To further validate these findings we used semi-quantitative western blotting to demonstrate that the phospho-MLC content was decreased to ~40% during cytokinesis in the p0071 and the KIF3b knockdown cells (Fig. 9B). To directly show that the effect was mediated by RhoA activation, we used synchronized cells at cytokinesis to measure RhoA activity after p0071 and KIF3b siRNA transfection compared with control siRNA-treated cells (Fig. 9C). This experiment revealed a similar reduction in RhoA activity to ~60% in both p0071 and KIF3b knockdown cells. Based on this direct correlation of phospho-MLC content and RhoA activity, we conclude that the phospho-MLC content is a suitable measure for RhoA activity. Taken together, these results indicate that the KIF3b knockdown interfered with normal Rho-signaling and strongly suggest that the function of KIF3b during cytokinesis is partially mediated by p0071.

A p0071–MKLP1-motor-domain fusion protein is targeted to the microtubule midzone independently of KIF3b and rescues the KIF3b knockdown-induced defect in Rho signaling

If p0071 is a major mediator of the KIF3b-dependent function in cytokinesis, then a p0071 mutant that localizes independently of KIF3b should be able to rescue the KIF3b knockdown-induced defect. In order to test this hypothesis we constructed a p0071–MKLP1-motor-domain fusion protein. Targeting of this fusion protein to the central microtubule bundle was verified by immunofluorescence microscopy. As expected, this fusion protein associated with the central microtubules in anaphase and telophase reflecting the features of the MKLP1 motor (supplementary material Fig. S3) (Mishima et al., 2002). Overexpression of this fusion protein in KIF3b knockdown cells rescued Rho signaling as demonstrated by quantification of phospho-MLC (Fig. 10A). By contrast, overexpression of wild-type p0071 in KIF3b knockdown cells led only to a slight increase in phospho-MLC content that remained below the level of untreated control cells. This is in agreement with the finding that wild-type p0071 did not localize normally in KIF3b knockdown cells (supplementary material Fig. S3). We suppose that this partial rescue was mediated by residual KIF3b in the knockdown cells (see Fig. 10A for efficiency of the knockdown). The KIF3b-binding-deficient p0071 mutant (p0071Δrep1) revealed no rescue effect at all in agreement with the total lack of KIF3b association and midbody localization of this mutant. Taken together, these results demonstrate that p0071, when targeted independently of KIF3b to the region of furrow ingression, can rescue the KIF3b siRNA-induced defect in Rho signaling, strongly supporting our conclusion that p0071 is an essential mediator of KIF3b during cytokinesis.

To further explore whether p0071 is the primary mediator of the KIF3b function in cytokinesis we examined whether the p0071–MKLP1-motor fusion protein rescues the cytokinesis defect to a similar extent as it rescues Rho signaling in the KIF3b knockdown cells (Fig. 10B). Multinucleation was only slightly
reduced by the p0071–MKLP1-motor fusion protein, thus indicating that KIF3b mediates additional functions in cytokinesis. Therefore, we conclude that p0071 is not the only cargo protein of KIF3b that functions in cytokinesis.

Discussion

Our results identify p0071 as a new cargo protein transported by KIF3b to the microtubule midzone during anaphase and cytokinesis, and show an essential function of KIF3b in the regulation of local activation of RhoA during cytokinesis. The human genome contains 41 kinesins grouped into 14 families (Miki et al., 2005), and it is well known that multiple kinesins and microtubule-associated proteins act in concert to direct cytokinesis (Glotzer, 2005). For many kinesins, however, target proteins transported to the microtubule midzone at anaphase and telophase have not been determined and their interplay is not well understood. Two kinesins have been reported with essential functions in the transport of components of the Rho signaling pathway during cytokinesis: MKLP1, a member of the kinesin-6 family, transports Ect2 and MgcRacGAP in the centralspindlin complex to the microtubule midzone where Ect2 has an essential function in stimulating RhoA activity by promoting the exchange of GDP for GTP (Nishimura

Fig. 8. Knockdown of p0071 and KIF3b induces multinucleation. (A) HEK293 cells were transfected with unspecific control siRNA, p0071 or KIF3b-specific siRNAs, respectively, p0071+KIF3b siRNAs and GFP-tagged Histone2b to identify transfected cells. Subsequently, cells were synchronized by a double thymidine block, released for 12 hours, then fixed and stained with Alexa-Fluor-594-conjugated phalloidin. At this point, many cells were bi- or multinucleated in response to the knockdown of p0071 or KIF3b. Scale bars: 10 μm. (B) The number of multinucleated cells was quantified. Mean values ± s.d. of three independent experiments counting >100 cells each are shown. *** indicates P≤0.005. (C) HeLa cells were synchronized by successive thymidine and nocodazole treatment. At 30 minutes after nocodazole release cells were imaged for progression through cytokinesis. Cells transfected with control siRNA furrowed normally at 60 minutes after nocodazole release (1800 seconds, upper panel). P0071 knockdown cells exhibited strongly delayed and disturbed furrowing, resulting in failure of cytokinesis (lower panel). Scale bars: 10 μm. (D) HeLa cells were transfected with siRNAs as indicated, then synchronized, fixed at 90 minutes after nocodazole release and counted for normal and disturbed furrowing. Mean values ± s.d. of three independent experiments counting >100 cells each are shown. * indicates P≤0.01.

Fig. 9. The phenotype of the KIF3b knockdown strongly resembles the phenotype of the p0071 knockdown. (A) HeLa cells were transfected with control siRNA, p0071-specific siRNA and KIF3b-specific siRNA as indicated, then synchronized and stained for actin (green) and phospho-MLC (p-MLC, red). Knockdown of p0071 and KIF3b considerably reduced the accumulation of actin and phosphorylated myosin light chain at the region of furrow ingression. Scale bars: 10 μm; enlargements 5 μm. (B) The level of p-MLC was reduced to a similar level in p0071 and KIF3b siRNA-transfected cells, respectively (~40%). The graph displays the relative p-MLC amounts normalized to α-tubulin + s.d. for three independent experiments. * indicates P≤0.01. (C) KIF3b and p0071 knockdown interfered with RhoA activation in telophase and cytokinesis. Cells were synchronized and probed for active RhoA at 90 minutes after nocodazole release. Active RhoA was precipitated by the GST-ROCK-Rho-binding-domain coupled to glutathione-agarose. The graph displays the relative amounts of active RhoA normalized to total RhoA + s.s.d. for three independent experiments. * indicates P≤0.01.
Targeting of p0071 to the midbody by KIF3

Although KIF3 has been implicated in cytokinesis (Fan and Beck, 2004; Haraguchi et al., 2006) the molecular mechanism of its action and target proteins remained essentially unknown and a link to Rho signaling has not been reported. Moreover, these data relied solely on the expression of dominant negative KIF3b mutant proteins, which can give rise to various side effects. Whereas the C-terminal KIF3b fragment would be expected to bind the cargo protein and thereby prevent its transport via endogenous KIF3, the N-terminal motor domain could interfere by blocking microtubules and thereby inhibit transport via endogenous KIF3 or other motor proteins. To show the function of KIF3 more directly, we have used a siRNA-mediated knockdown approach. Here, we showed that KIF3b silencing interfered with normal progression through cytokinesis in agreement with the earlier observation based on the dominant negative approach. Moreover, we showed that the phenotype of the KIF3b knockdown correlated with the reduced accumulation of actin at the midbody, decreased phospho-MLC and a reduced level of active RhoA indicative of a defect in Rho signaling. This mimics the defect that we observed after the p0071 knockdown (Wolf et al., 2006). Together with the finding of an interaction of KIF3b with p0071, this strongly suggests that KIF3b is essential for transporting p0071 to the region of furrow ingression to allow for local stimulation of Rho signaling. As localization of Ect2, the other known stimulator of RhoA during cytokinesis, and of RhoA itself were not affected in KIF3b knockdown cells, we conclude that the defect in contractile ring formation after KIF3b knockdown is most likely caused by the mislocalization of the p0071 cargo protein. In full agreement, expression of a p0071–MKLP1-motor fusion protein rescued the defect in MLC activation of KIF3b knockdown cells whereas wild-type p0071 did not, again supporting our conclusion that KIF3b affects Rho-signaling in cytokinesis through targeting of p0071.

Previous reports on the role of KIF3b in mitosis have shown that the overexpression of the N-terminus induced “an unusual number of centrosomes and abnormal spindle formation” and a higher frequency of chromosomal aneuploidy (Haraguchi et al., 2006). However, whether supernumerary centrosomes were the primary defect or rather a consequence of multinucleation resulting from a cytokinesis defect was not analyzed. Our data suggest that the primary defect is the failure of cytokinesis. As in the above-mentioned study, KIF3b fragments were expressed after retroviral transfection and selection for 3 days – the cells had undergone more than one cell cycle when analyzed. After the first cell cycle, multinucleated cells present as a consequence of a cytokinesis defect start mitosis again with an extra set of centrosomes. This results in an increased number of spindle aberrations as we have reported for the p0071 knockdown. However, at earlier time points such aberrant spindles have not been observed after either p0071 knockdown (Wolf et al., 2006) or KIF3b knockdown. Moreover, dominant negative mutants such as those used in the Haraguchi study can give rise to side effects, for example due to protein interactions and mislocalization of binding partners.

One other cargo protein of KIF3 reported to play a role in cytokinesis is Syne-1 (synaptic nuclear envelope protein-1, also referred to as Nesprin-1 or Enaptin), a spectrin family member with potential roles in inner nuclear envelope function, nuclear positioning and the maintenance of the Golgi structure. It has been proposed that KIF3b and/or Syne-1 could be involved in targeting membrane vesicles to the mitotic spindle during cytokinesis because midbody accumulation of vesicles containing the t-SNARE syntaxin was reduced upon expression of the KIF3b tail domain (Fan and Beck, 2004). Vesicle fusion is essential for cytokinesis and several constituents of the membrane trafficking apparatus have been implicated in cytokinesis (Straight and Field, 2000). As membrane trafficking is required after contractile ring assembly, a defect in vesicle delivery or fusion may be either an indirect consequence of disturbed actin organization or an independent phenotype. Using our p0071–MKLP1-motor fusion protein we showed that targeting of p0071 rescues the defect in Rho signaling. However, this fusion protein reduced cytokinesis failure only to a minor extent, suggesting that a second process contributing to cytokinesis is disturbed in the KIF3b knockdown cells. The reported role of KIF3b in targeting Syne-1- and syntaxin-containing vesicles to the region of furrow ingression (Fan and Beck, 2004) suggests that KIF3b also contributes to vesicle trafficking and membrane expansion during cytokinesis. Thus, KIF3 could coordinate the tight spatiotemporal regulation of actin reorganization and contraction through targeting of p0071 with the process of vesicle fusion through targeting of
Syne-1 to allow for membrane expansion in the region of furrow ingress.

Another cargo protein of KIF3 is the PAR-complex, which is transported by KIF3 in neuronal cells (Nishimura et al., 2004) and MDCK cells (Fan et al., 2004). The PAR-complex has been reported to localize at the central spindle and the midbody but its function in cytokinesis remains elusive and a role of KIF3 in PAR-complex targeting has not been demonstrated for this specific situation (Liu et al., 2006). While PAR-6 and PKCζ accumulated at the microtubule midzone and the midbody, phospho-PKCζ and PAR-3 appeared restricted to the microtubule midzone and were not concentrated at the midbody (Liu et al., 2006) suggesting that activated PKCζ was excluded from the midbody. A recent report has shown that Rho-kinase, an effector of RhoA, phosphorylated PAR-3 at Thr833 and thereby disrupted its interaction with αPKC and PAR-6 and dissociation of the complex resulted in Rac1 inactivation (Nakayama et al., 2008). As Rho-kinase and Citron-kinase become activated at the midbody once Ect2 and p0071 have stimulated RhoA, this mechanism might prevent Rac1 activation at the midbody, which often antagonizes RhoA. However, this hypothesis requires experimental confirmation. Based on the data presented here and published observations, we propose a model (supplementary material Fig. S4) that summarizes the function of the three kinesin motors involved in targeting components of the Rho-signaling pathway during cytokinesis.

Our results clearly indicate that Ect2 and p0071 are transported in different complexes that reach the microtubule midzone at different time points and that their transport depends on different motor proteins. After reaching the midbody, Ect2 could induce activation of RhoA. Once p0071 arrives at the midbody, a low level of activated RhoA could in turn trigger p0071 association with the complex and thus further RhoA activation leading to contraction ring formation and furrow ingress. Vesicle transport mediated by KIF3b could facilitate concomitant membrane expansion.

Materials and Methods

cDNA constructs

The pcCherry and the pHA vector were derived from pEGFP-C2 (Clontech) by replacing EGFP with pcCherry (AglI-BglII) or the HA-tag, respectively. Human KIF3b and its deletion mutants (KIF3b-NT aa1-345, KIF3b-CT aa346-747) were cloned into pcCherry, pGADT7 (Clontech), pHα and pcDNA3-FLAG. P0071 constructs have been described previously (Hatfield et al., 2003). Wild-type p0071 was subcloned into pcHA and pGADT7. P0071-Arepl was derived from wild-type p0071 by insertion of two EcoRV sites using site-directed mutagenesis. The EcoRV-fragment was removed and the vector religated to delete repeat 1 (aa510-551) of the p0071 armadillo domain. The p0071–MKLP1-motor-domain fusion construct was generated by removing and the vector religated to delete repeat 1 (aa510-551) of the p0071 armadillo domain. The p0071–MKLP1-motor-domain fusion construct was generated by removing the three kinesin motors involved in targeting components of the Rho-signaling pathway during cytokinesis.

Antibodies

Primary antibodies (Ab) were rabbit anti-p0071 (Hatfield and Nachtsheim, 1996), guinea pig anti-p0071, rabbit anti-Ect2 (C-20, Santa Cruz), rabbit anti-KIF3b (H60, Santa Cruz), anti-KIF3b mAb (clone 35, BD Transduction Laboratories), anti-α-tubulin mAb (DM1A, Sigma), rabbit anti γ-tubulin (Sigma), anti-FLAG mAb (M2; Sigma), anti-Myc mAb (9E10, Sigma), rabbit anti-HA (Rockland), anti-RhoA mAb (26C4), anti-MKLP1 mAb (BD Transduction) and rabbit anti-phospho-MLC2(Ser19) (Cell Signaling). Secondary Abs were donkey anti-rabbit, anti-mouse and anti-guinea pig conjugated to horseradish peroxidase (Dianova), donkey anti-mouse-Cy3 (Dianova), donkey anti-mouse-Alexa550, anti-guinea pig-Alexa-Fluor488, anti-rabbit-Alexa-Fluor488, anti-mouse-Alexa-Fluor488 and anti-rabbit-Alexa-Fluor594 (Molecular Probes / Invitrogen).

Cell culture, transfection and synchronization

MC7, HeLa and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Plasmids and siRNAs were transfected using Lipofectamine 2000 (Invitrogen). For synchronization in prometaphase, cells were switched to medium containing 5 mM thymidine (Sigma-Aldrich) and incubated overnight (16-20 hours). After a 4-hour release, mitotic cells were enriched in 100 nM nocodazole (Sigma-Aldrich) for 12 hours. At 90 minutes after nocodazole release, cells reached telophase and cytokinesis.

RNA interference

Small interfering RNAs (siRNAs; MWG) for p0071 (Wolf et al., 2006), Ect2 (Kim et al., 2005) and MKLP1 (Yuce et al., 2005) have been described previously. Two siRNAs were used in combination for knockdown of KIF3b (KIF3bs1: 5'-CCCAAUCUAAUUCGGUGAATGT-3', KIF3bs2: 5'-GAAGAUCUGUG-UAGAUUGAATGT-3'). A nonspecific siRNA was used as a control (5'-UCCGCUGAGCCUCUGGUUGCUCUdTdT-3'). Knockdown efficiencies were determined by semi-quantitative western blotting.

Immunofluorescence

Transfected and synchronized HeLa cells were collected by mitotic shake off, seeded on coverslips coated with poly-L-lysine (Sigma) and incubated for 90 minutes at room temperature. For indirect immunofluorescence of p0071 and Ect2, cells were fixed in 10% trichloroacetic acid (TCA) (15 minutes, on ice), permeabilized in 0.5% Triton/PBS for 5 minutes and stained with the appropriate antibodies. For staining of filamentous actin and phospho-myosin light chain (MLC), cells were fixed for 10 minutes in 3.7% paraformaldehyde (PFA) at room temperature and permeabilized in 0.5% Triton-PBS for 15 minutes. Filamentous actin was labeled using Phalloidin-Alexa-Fluor-588 or Phalloidin-Alexa-Fluor-594. All images were taken at a Nikon Eclipse 600 microscope equipped with a CCD camera (CCD-1300QLN, VDS Vosskühler), NIS Elements AR 2.30 software (Nikon), and a Nikon CFI Apo TIRF 60×/1.49 objective. Adobe Photoshop (v7.0) and ImageJ were used for image processing (adjustment of brightness and contrast). Quantification of images was done using ImageJ wand tracing tool to measure fluorescence intensity of the whole cell. A circular selection (2.65 μm²) was used to determine fluorescence intensity of the midbody region.

Yeast two-hybrid analysis

YRG2 yeast cells were co-transformed with the p0071 repeat domain and its truncated variants in pGBK7 and wild-type KIF3b, KIF3b-NT or KIF3b-CT in pGADT7. Double transformants were grown on plates lacking leucine and tryptophane. The His reporter gene was analyzed on plates lacking histidine in addition to leucine and tryptophane. β-galactosidase activity was probed in a colony-lift filter assay according to the yeast protocols handbook (BD Clontech).

GST pulldown

The p0071 repeat domain (aa510-988) in pGEX-5X1 (Amersham) was expressed in Escherichia coli BL21DE3. Bacteria were harvested by centrifugation at 10,000 g, resuspended in lysis buffer [20 mM HEPES; pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Pefabloc and 1 mM diethofoxreitol (DTT)] and sonicated. Triton X-100 was added to a final concentration of 1% and the lysates were incubated for 30 minutes at 4°C. The cleared lysates were incubated with glutathione agarose (Pierce Biotechnology) for 60 minutes at 4°C. The concentration of bound glutathione S-transferase (GST)-p0071 repeat domain was determined using Coomassie Plus protein reagent (Pierce) and adjusted to 1 mg/ml. For a GST-pulldown approach 30 μg of GST or GST-p0071 repeat domain agarose respectively, was incubated with HEK293-lysates for 2 hours at 4°C. Bound protein was eluted in SDS-PAGE loading buffer, separated on 10% SDS gels and analyzed by western blotting.

Co-immunoprecipitation of in vitro translated proteins

In vitro transcription and translation was performed using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer’s protocol. 1 μg of wild-type p0071-pGADT7 (HA-tag) or wild-type KIF3b-pGBK7 (Myc-tag) was used for the coupled transcription-translation reaction. Lysates were mixed for 30 minutes at room temperature. 1 μg of anti-Myc mAb or 1 μg of the appropriate isotype control (Mouse IgG1, M1:45; Abcam) was added and incubated at room temperature for 1 hour. Antibodies were immobilized on protein A agarose for another hour and beads were washed five times in PBS, resuspended in 20 μl of SDS-PAGE loading buffer, and samples probed for co-precipitation of HA-tagged wild-type p0071 by western blotting.

FLAG-immunoprecipitation

HEK293 cells expressing wild-type KIF3b-FLAG and wild-type p0071-HA were lysed in buffer containing 50 mM Tris/HC1, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Pefabloc, 1 mM NaF, 1 mM NaVO₄. Lysates were cleared by centrifugation for 15 minutes at 4°C and subsequently incubated with anti-FLAG M2 affinity gel (Sigma). Bound proteins were solubilized in SDS-PAGE loading buffer, separated on 10% SDS gels and analyzed by western blotting.
Rho activity assay
Rho activity was determined as described previously (Wolf et al., 2006), except that the GST-ROCK-Rho-binding-domain (aa934-1015) was used instead of GST-Rhotekin to pull down active RhoA.

Preparation of mitotic microtubules
Preparation of mitotic microtubules was performed essentially as described by Silljé and Nigg (Silljé and Nigg, 2006).

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References


A

KIF3b-wt (aa 1-747)

KIF3b-NT (aa 1-345)

KIF3b-CT (aa 346-747)

B

RFP  p0071  enlargement

RFP  KIF3b-NT

RFP  KIF3b-CT
p0071-wt-DsRed

Δ
dp0071rep1-DsRed

KIF3b siRNA

control siRNA

DAPI α-tub

p0071

p0071

DAPI α-tub

p0071

p0071