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Relocation of Aurora B from centromeres to the central spindle at the metaphase to anaphase transition requires MKlp2

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Mitotic kinases of the Polo and Aurora families are key regulators of chromosome segregation and cytokinesis. Here, we have investigated the role of MKlp1 and MKlp2, two vertebrate mitotic kinesins essential for cytokinesis, in the spatial regulation of the Aurora B kinase. Previously, we have demonstrated that MKlp2 recruits Polo-like kinase 1 (Plk1) to the central spindle in anaphase. We now find that in MKlp2 but not MKlp1-depleted cells the Aurora B–INCENP complex remains at the centromeres and fails to relocate to the central spindle. MKlp2 exerts dual control over Aurora B localization, because it is a binding partner for Aurora B, and furthermore for the phosphatase Cdc14A. Cdc14A can dephosphorylate INCENP and may contribute to its relocation to the central spindle in anaphase. We propose that MKlp2 is involved in the localization of Plk1, Aurora B, and Cdc14A to the central spindle during anaphase, and that the integration of signaling by these proteins is necessary for proper cytokinesis.

Introduction

Normal animal cell division involves the duplication and spatial segregation of the genetic material followed by cytokinesis. To ensure daughter cells receive the correct complement of chromosomes, cytokinesis typically occurs at the middle of the cell between the two separated DNA masses. Chromosomal passengers are a group of proteins implicated in the coordination of chromosome segregation with cytokinesis that localize to the kinetochores throughout metaphase and then move to the central spindle in anaphase (Earnshaw and Bernat, 1991; Adams et al., 2001a). Perturbing their function results in defects in metaphase chromosome alignment, chromosome segregation, and cytokinesis (Terada et al., 1998; Kaitna et al., 2000; Severson et al., 2000; Adams et al., 2001b; Giet and Glover, 2001; Wheatley et al., 2001). Prominent passenger proteins are the kinase Aurora B, INCENP, and survivin (Schumacher et al., 1998; Adams et al., 2001b; Wheatley et al., 2001; Bolton et al., 2002). INCENP acts as a scaffold protein targeting and activating Aurora B (Kaitna et al., 2000; Adams et al., 2001b; Wheatley et al., 2001; Bishop and Schumacher, 2002; Honda et al., 2003), and whereas the precise role of survivin is unclear, like INCENP it appears to act as a regulator of Aurora B kinase activity (Bolton et al., 2002). Cdk1-cyclin B activity is required to maintain the Aurora B–INCENP complex at centromeres (Murata-Hori et al., 2002), and in *Saccharomyces cerevisiae* the release of this complex from centromeres at the metaphase to anaphase transition is controlled by the phosphatase Cdc14 (Pereira and Schiebel, 2003). At least some aspects of this mechanism are likely to be conserved because Cdc14 family phosphatases are found throughout the eukaryotic kingdom and have been implicated in control of cytokinesis in the *Caenorhabditis elegans* early embryo (Gruneberg et al., 2002) and of cell division in human tissue culture cells (Kaiser et al., 2002; Mailand et al., 2002). Whether or not this is the only mechanism controlling the localization of the Aurora B–INCENP complex in higher eukaryotes remains to be determined. There is some evidence that mitotic kinesins contribute to Aurora B localization to the central spindle in anaphase. Depletion of Zen-4, the homologue of the human mitotic kinesin MKlp1/CHO1, results in a loss of Aurora B from the central spindle

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Abbreviations used in this paper: MKlp2C^t, MKlp2 stalk-tail domain; MKlp2N^t, MKlp2 lacking the cargo-binding tail domain; Plk1, Polo-like kinase 1.

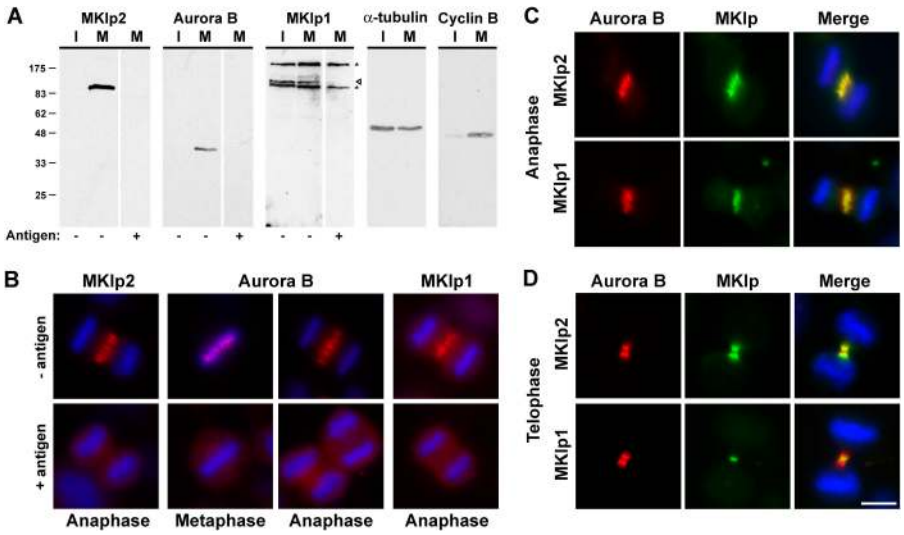

Figure 1. **Aurora B colocalizes with MKlp2 at the central spindle.** Antibodies to Aurora B, MKlp2, and MKlp1 were preincubated for 1 h in the presence or absence of a 50-fold excess of the appropriate recombinant protein antigen. They were then used to (A) probe Western blots of 20 μg extracts from interphase, I, and nocodazole arrested, M, HeLa cells, or (B) stain HeLa cells. The asterisks indicate cross-reactions of the rabbit anti-MKlp1 antibody that are not competed by the antigen, and the arrowhead marks MKlp1. (C and D) HeLa cells were fixed and stained with antibodies to Aurora B (red), and MKlp1 and MKlp2 (green). The two panels show representative staining in (C) anaphase and (D) telophase cells. DNA was stained with DAPI. Bar, 10 μm.

in *C. elegans* and these two proteins can interact in vitro (Severson et al., 2000). Furthermore, inspection of the *C. elegans* interactome reveals that Zen-4 displays two-hybrid interactions with Aurora B (Li et al., 2004). The situation is likely to be more complex in vertebrates because they possess two mitotic kinesins, MKlp1/CHO1 and MKlp2/rabkinesin-6 (Mishima et al., 2002; Neef et al., 2003). Here, we investigate the roles of MKlp1 and MKlp2 in the localization of Aurora B during anaphase in human cells.

Results and discussion

Aurora B colocalizes with MKlp2 at the central spindle in anaphase

To document their specificity, antibodies to MKlp2, Aurora B, and MKlp1 were used for Western blotting of extracts prepared from interphase and nocodazole arrested mitotic HeLa cells (Fig. 1 A); where indicated, corresponding antigens were added for competition. Equal loading and enrichment of a mitotic marker in the nocodazole arrested samples was confirmed by probing the same filters with antibodies to α-tubulin and cyclin B (Fig. 1 A). MKlp2 and Aurora B antibodies recognized single proteins of the predicted sizes, 100 and 40 kD, respectively. As expected, signals were strong in mitotic but barely detectable in interphase samples (Fig. 1 A). Immunocytochemistry revealed that MKlp2 localized to the central spindle, and Aurora B to the centromeres in metaphase and the central spindle in anaphase (Fig. 1 B), in line with previous reports (Terada et al., 1998; Hill et al., 2000). Moreover, both staining patterns were competed by the appropriate antigens (Fig. 1 B). The MKlp1 antibody detected three proteins in cell extracts, but only one of these, migrating at the expected molecular mass of 110 kD, was competed by the antigen (Fig. 1 A, arrowhead). Furthermore, the characteristic central spindle staining observed with this MKlp1 antibody was abolished by antigen addition, suggesting that MKlp1 is largely responsible for the staining pattern (Fig. 1 B). MKlp1 and MKlp2 showed similar but not identical localizations at the central spindle in anaphase, which became more distinct in telophase cells when MKlp1 was restricted to the midbody

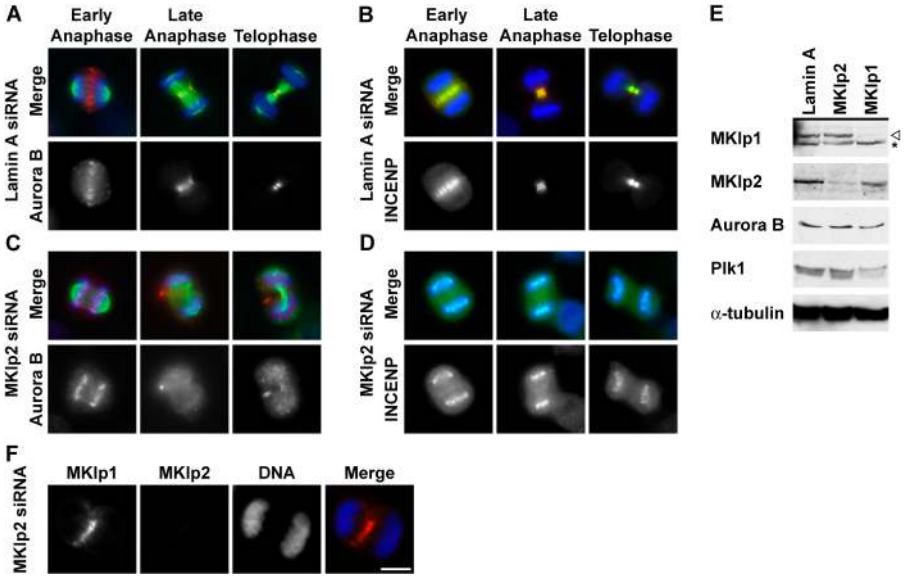

Figure 2. **Aurora B targeting to the central spindle requires MKlp2.** HeLa cells were treated with siRNA duplexes for (A and B) lamin A or (C and D) MKlp2 for 30 h and then stained with antibodies to (A and C) Aurora B (red) and tubulin (green) or (B and D) INCENP (red) and MKlp2 (green). The images show representative staining in early and late anaphase, and telophase cells. (E) Western blots of HeLa cells transfected with siRNA duplexes for MKlp1, MKlp2, and lamin A for 30 h. The open arrowhead marks MKlp1 and an asterisk indicates a further protein cross reacting with this antibody. (F) HeLa cells transfected with MKlp2 siRNA duplexes for 30 h were stained with antibodies to MKlp1 (red) and MKlp2 (green). DNA was stained with DAPI. Bar, 10 μm.

whereas MKlp2 staining was most prominent in two bands on either side of this structure (Neef et al., 2003). The localization of Aurora B overlapped with both mitotic kinesins in anaphase, although to a greater extent with MKlp2 than MKlp1 (Fig. 1 C). More revealingly, in telophase, Aurora B was found in two bands on either side of the midbody that overlapped with MKlp2 and were discrete from MKlp1 (Fig. 1 D). Therefore, MKlp2 appears to be a good candidate for a partner of Aurora B at the central spindle in anaphase and telophase, given their colocalization at this structure.

Aurora B targeting to the central spindle requires MKlp2

To investigate the requirement for MKlp2 in the localization of the Aurora B–INCENP complex to the central spindle, targeted depletion with siRNA was used. In control cells depleted for lamin A, Aurora B and INCENP were found at the central spindle throughout anaphase and telophase (Fig. 2, A and B). Aurora B–INCENP localization was strikingly different in MKlp2-depleted cells (Fig. 2, C and D), and Western blots suggested that this effect is due to the depletion of MKlp2 because the levels of other proteins found at the central spindle were unchanged (Fig. 2 E). In the absence of MKlp2, Aurora B and INCENP remained on centromeres in early anaphase and did not relocate to the central spindle. In some late anaphase and telophase cells Aurora B was not present on either centromeres or the central spindle (Fig. 2 C). Furthermore, although the central spindle microtubules were less organized in MKlp2-depleted cells (Fig. 2, A and C, compare tubulin staining in green), MKlp1 was still present in the central spindle region (Fig. 2 F) making it unlikely that this kinesin contributes to Aurora B localization.

The defect in Aurora B localization in MKlp2-depleted cells was rescued by transfection of myc-MKlp2 (Fig. 3). In the absence of MKlp2 no Aurora B staining was observed on the central spindle, whereas all cells expressing myc-MKlp2 showed central spindle staining in both anaphase and telophase (Fig. 3, A and B). In contrast, a deletion mutant of MKlp2 lacking the cargo-binding tail domain (MKlp2N^t) was unable to rescue Aurora B localization to either the central spindle (Fig. 3 A) or the midbody (not depicted). Cells expressing a construct comprising only the MKlp2 stalk-tail domain (MKlp2C^t) did not show rescue in anaphase (Fig. 3 A), and only showed faint Aurora B staining on midbody-like structures in telophase (Fig. 3 B). However, unlike telophase cells rescued by the full-length MKlp2, those rescued by the MKlp2C^t construct still showed a significant pool of Aurora B at the centromeres (Fig. 3 B). Moreover, targeting of the MKlp2C^t mutant to the midbody required much higher levels of expression than the near-complete rescue of localization by full-length MKlp2 (Fig. 3 C). Thus, these results indicate that efficient localization of MKlp2, and hence Aurora B, to the central spindle requires not only the motor domain of MKlp2, but also its COOH terminus.

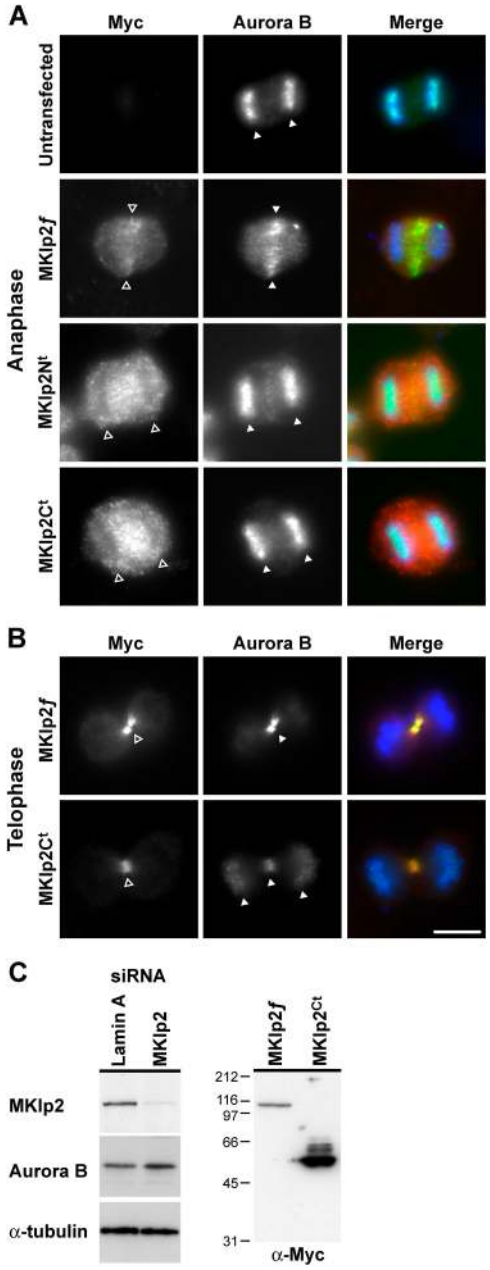

Figure 3. **Rescue of the MKlp2 siRNA phenotype.** HeLa cells treated with siRNA duplexes targeting MKlp2 for 6 h, were then transfected with plasmids encoding full-length MKlp2 (myc-MKlp2*f*), the MKlp2 motor domain (myc-MKlp2N^t) or the MKlp2 stalk and tail region (myc-MKlp2C^t) for a further 24 h. Cells were stained with antibodies to Aurora B (green) and the myc-epitope to detect the transfected MKlp2 (red). Aurora B and myc-MKlp2 localization, filled and open arrowheads mark, respectively, is shown in (A) anaphase and (B) telophase cells. DNA was stained with DAPI. Bar, 10 μm. (C) Western blots of HeLa cells transfected with siRNA duplexes for lamin A and MKlp2 for 30 h, and MKlp2 siRNA cells rescued with myc-MKlp2*f* and myc-MKlp2C^t for 24 h.

Aurora B is a binding partner for MKlp2 but not MKlp1

To confirm that Aurora B targeting in anaphase is independent of MKlp1, cells were depleted of MKlp1 with siRNA (Fig. 4). In control cells depleted for lamin A, MKlp1 and Aurora B showed a similar but only partially overlapping staining pattern at the central spindle in anaphase cells (Fig. 4 A). In contrast to MKlp2-depleted cells, Aurora B localization to the kinetochores and central spindle was unaltered by the depletion of MKlp1 (Fig. 4 B). Importantly, Mklp2 showed no dependence on MKlp1 for its localization to the central spindle (Fig. 4, A and B, bottom). Aurora B targeting

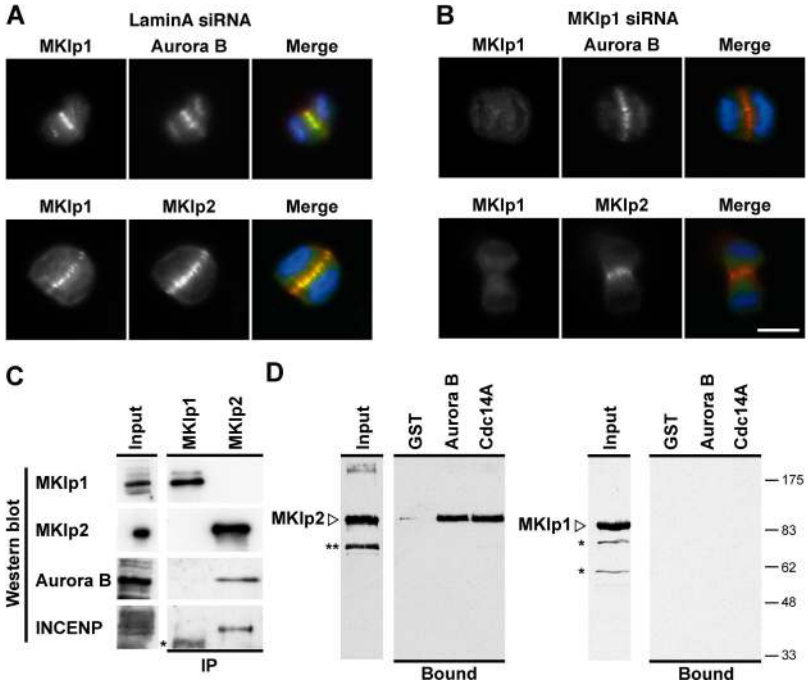

Figure 4. **Targeting of the Aurora B–MKlp2 complex is independent of MKlp1.** HeLa cells were treated with siRNA duplexes for (A) lamin A or (B) MKlp1 for 48 h and then stained with antibodies to Aurora B (red), and MKlp1 and MKlp2 (green). The images in the top panels show representative staining for Aurora B and MKlp1 in anaphase cells. The bottom panels show the effects of depleting MKlp1 on MKlp2 localization. DNA was stained with DAPI. Bar, 10 μm. (C) MKlp1 and MKlp2 were immune precipitated from anaphase HeLa cell extracts, and then Western blotted. The asterisk on the rabbit anti-INCENP blot marks a cross-reaction of the donkey anti–rabbit secondary antibody with the rabbit antibody used to immune precipitate MKlp1. (D) Purified His-tagged MKlp1 and MKlp2, 5 μg, were incubated with GST, GST-tagged Aurora B, or Cdc14A for 1 h at 4°C, and then recovered on glutathione agarose. Recovered complexes were analyzed by Western blotting, half of the input fraction is loaded, and all of the bound fractions. The double asterisk marks a degradation product of MKlp2 lacking the COOH terminus that does not bind to Aurora B or Cdc14A. The single asterisks mark degradation products of MKlp1.

to the central spindle in anaphase therefore requires the activity of MKlp2 but not MKlp1.

How does MKlp2 control Aurora B localization? The simplest explanation is that MKlp2 interacts with components of the Aurora B–INCENP complex. To test this possibility, MKlp1 and MKlp2 complexes were isolated from anaphase cells extracts, and then blotted for Aurora B and INCENP. This approach revealed that Aurora B and INCENP coprecipitated with MKlp2 but not MKlp1 (Fig. 4 C). It is noteworthy, that the precipitated INCENP band had a higher mobility and was sharper than the total pool of INCENP in the cell extract, perhaps indicative of differences in phosphorylation state (Fig. 4 C). In binding assays with purified proteins, MKlp2 but not MKlp1 was able to interact with Aurora B (Fig. 4 D). Therefore, MKlp2 recruitment of the Aurora B–INCENP complex to the central spindle may involve direct contact with Aurora B.

Cdc14 targeting to the central spindle requires MKlp2

The phosphatase Cdc14 was recently shown to regulate release of the Aurora B–INCENP complex from centromeres in yeast by dephosphorylation of INCENP (Pereira and Schiebel, 2003). This raised the possibility that the Aurora B–INCENP complex is under dual control by Cdc14 and MKlp2 in human cells. To test this idea antibodies were raised against human Cdc14A that react with a protein of the expected size in HeLa cell extracts, but do not cross react with Cdc14B (Fig. 5 A). Consistent with its proposed functions in spindle formation and cytokinesis (Kaiser et al., 2002; Mailand et al., 2002), a portion of Cdc14A was present on the spindle poles, and at the central spindle (Fig. 5 B). Furthermore, Cdc14A depended on MKlp2 for its localization to the central spindle but not the spindle poles (Fig. 5, C and D). Given the similar localizations of MKlp2 and Cdc14A in anaphase and the dependence of Cdc14A on MKlp2 for targeting to the central spindle, it seemed likely that these proteins could interact. A two-hybrid analysis showed that Cdc14A and MKlp2 do indeed interact and mapped the Cdc14 binding site to the stalk and tail of MKlp2 (Fig. 5 E), discrete from the Polo-like kinase 1 (Plk1) binding neck region (Neef et al., 2003). Binding assays with purified proteins confirmed that MKlp2 but not MKlp1 was able to bind to immobilized Cdc14A (Fig. 4 D). Furthermore, human Cdc14A was able to dephosphorylate INCENP (Fig. 5 F). MKlp2 could therefore exert dual control over the localization of the Aurora B–INCENP complex by direct interaction with Aurora B, and through Cdc14A regulation of INCENP.

This work, together with previous studies on MKlp2 (Hill et al., 2000; Fontijn et al., 2001; Neef et al., 2003), uncovers a difference between the mammalian cell division apparatus and that of invertebrates such as *Drosophila melanogaster* and *C. elegans*. In *C. elegans* the MKlp1 homologue Zen-4 is an important component of the central spindle required for cytokinesis (Powers et al., 1998; Raich et al., 1998). One of its major binding partners is the GAP Cyk-4 needed to control the activity of Rho GTPases in cytokinesis, and this complex is conserved in human cells where it has a similar function (Jantsch-Plunger et al., 2000; Mishima et al., 2002). In *D. melanogaster*, the MKlp1 homologue Pav is needed for the localization of the Polo kinase to the central spindle, and defects in either of these components cause a failure in cytokinesis (Adams et al., 1998). However, mammalian cells possess two mitotic kinesins with clearly distinct roles. MKlp1 is needed to bring the Rho GAP hsCyk-4 to the central spindle where it regulates late stages of cytokinesis (Kuriyama et al., 2002; Mishima et al., 2002), whereas MKlp2 is responsible for the localization of the Polo family kinase Plk1 (Neef et al., 2003). We now show that MKlp2 but not MKlp1 is needed for the recruitment of the Aurora B–INCENP complex to the central spindle from centromeres. This is consistent with studies demonstrating that the release of the

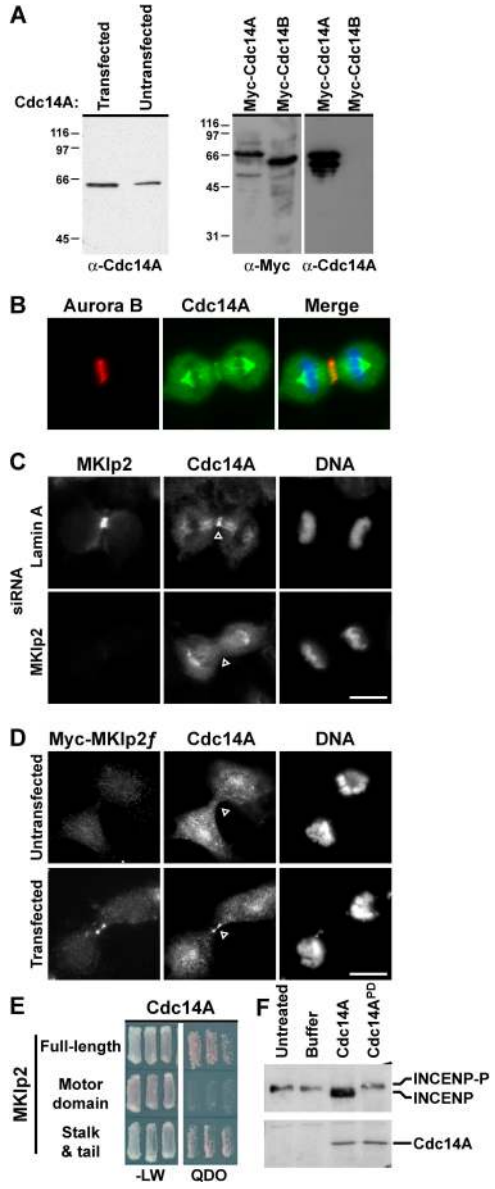

Figure 5. **Cdc14A targeting to the central spindle requires MKlp2.** (A) Extracts, 5 μg, from HeLa cells left untransfected or transfected with plasmids encoding untagged Cdc14A, myc-Cdc14A, and Cdc14B were Western blotted with affinity purified rabbit anti-Cdc14A antibodies or a mAb to the myc-epitope. (B) U2OS cells were fixed and stained with antibodies to Aurora B (red) and Cdc14A (green). A representative staining in anaphase cells is shown. (C) U2OS cells were treated with siRNA duplexes for lamin A or MKlp2 for 36 h and then costained with antibodies to MKlp2 and Cdc14A. (D) U2OS cells treated with siRNA duplexes targeting MKlp2 for 6 h, were then transfected with myc-MKlp2*f* for a further 30 h. Cells were stained with antibodies to Cdc14A and the myc-epitope to detect the transfected MKlp2. Arrowheads indicated central spindle staining. DNA was stained with DAPI. Bars: (C and D) 10 μm. (E) Bait constructs comprising the full-length MKlp2, the MKlp2 motor domain, or the COOH-terminal stalk and tail region were tested against full-length human Cdc14A for the ability to grow on selective medium (QDO), compared with nonselective medium (−LW) in the yeast two-hybrid system. (F) Aurora B–INCENP complexes were immune precipitated from mitotic HeLa cell extracts with antibodies to Aurora B. Samples before and after dephosphorylation with buffer, 50 ng human Cdc14A, or phosphatase dead Cdc14A ($Cdc14A^{PD}$) were then blotted for INCENP and Cdc14A. Note that dephosphorylated form of INCENP is detected more efficiently than the phosphorylated form with the antibody used for this experiment.

Aurora B–INCENP complex from centromeres at the metaphase to anaphase transition occurs via a microtubule-dependent mechanism (Canman et al., 2002; Murata-Hori and Wang, 2002), and the suggestion that chromosomes supply microtubules with factors promoting cytokinesis (Canman et al., 2003). Obvious candidates for these factors are the chromosomal passenger proteins (Earnshaw and Bernat, 1991), including the Aurora B–INCENP complex and Plk1. In the absence of MKlp2 both the Aurora B–INCENP complex and Plk1 fail to localize correctly during anaphase and telophase, and cleavage furrow ingression and cytokinesis fail (Neef et al., 2003). Therefore, we propose that MKlp2 promotes the microtubule-dependent localization of Plk1, Aurora B, and Cdc14A to the central spindle during anaphase.

Materials and methods

Antibody reagents

Antibodies that were used are as follows: DM1a 1 mg/ml mouse monoclonal to α-tubulin (Sigma-Aldrich); goat polyclonal to cyclin B SC-5235 0.2 mg/ml (Santa Cruz Biotechnology, Inc.); rabbit polyclonal to human MKlp1 SC-867 0.2 mg/ml (Santa Cruz Biotechnology, Inc.); affinity purified 1 mg/ml sheep polyclonal to human MKlp2 (Hill et al., 2000); AIM-1 0.25 mg/ml mouse monoclonal to Aurora B (Becton Dickinson); affinity purified 0.1 mg/ml rabbit antibodies to Cdc14A; and rabbit polyclonal to INCENP (Honda et al., 2003). Secondary antibodies conjugated to HRP, CY2, and CY3 were obtained from Jackson ImmunoResearch Laboratories.

Molecular biology and protein expression

Human MKlp2, Aurora B, and Cdc14A were amplified from human testis cDNA (Becton Dickinson) using the pfu polymerase (Stratagene) and cloned in pCRII-TOPO (Invitrogen). All constructs were confirmed by DNA sequencing (Medigenomix). Mammalian expression constructs were made in pcDNA3.1+ (Invitrogen). Yeast two-hybrid assays were performed according to the yeast protocol handbook (CLONTECH Laboratories, Inc.). For baculovirus expression the pAcSG2 vector (Becton Dickinson) modified to include the hexahistidine-tag from pQE32 (QIAGEN) was used. Baculoviruses were produced and proteins expressed in Sf9 cells according the manufacturer's protocols (Becton Dickinson). Bacterial expression was performed using the T7 polymerase hexahistidine-GST expression vector pGAT2 and the GST expression vector pGEX5X1. An insert encoding Cdc14A was inserted into pGEX5X1, or for Aurora B into pGAT2 and the proteins expressed in BL21(DE3) cells. Fusion proteins were purified over glutathione Sepharose (Amersham Biosciences) or nickel-NTA agarose (QIAGEN). GST–Aurora B and GST were dialyzed against 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM DTT, and GST-Cdc14A was desalted into 10 mM Hepes-NaOH, pH 7.3, 150 mM NaCl, 5% (vol/vol) glycerol, 1 mM DTT with HiTrap 5 ml desalting columns (Amersham Biosciences) then aliquots frozen in liquid nitrogen for storage at –80°C.

Cell culture and RNA interference

HeLa S3 cells and U2OS were cultured at 37°C and 5% CO_2 in DME containing 10% FCS. Plasmid transfection and RNA interference were performed as described previously (Neef et al., 2003). MKlp2 was targeted with 5′-aaccacctatgtaatctcatg-3′, MKlp1 with 5′-aagcagtcttccaggtcatct-3′, and the lamin-A control was described previously (Neef et al., 2003).

Image acquisition

Cells to be imaged were fixed for 20 min in 3% (wt/vol) PFA, quenched for 10 min with 50 mM ammonium chloride, then permeabilized with 0.1% (vol/vol) Triton X-100 for 5 min. All solutions were made in PBS, and antibody staining was performed for 60 min using a 1,000-fold dilution of antiserum or purified antibody at a final concentration of 1 μg/ml. Coverslips were mounted in 10% (wt/vol) Moviol 4-88, 1 μg/ml DAPI, 25% (wt/vol) glycerol in PBS. Images were collected using an Axioskop-2 with a 63× Plan Apochromat oil immersion objective of NA 1.4, standard filter sets (Carl Zeiss MicroImaging, Inc.), a 1,300 by 1,030 pixel cooled CCD camera (model CCD-1300-Y; Princeton Instruments) and Metavue software (Visitron Systems). Images were cropped in Adobe Photoshop 7.0 then sized and placed using Adobe Illustrator 10.0 (Adobe Systems).

Immune precipitations

HeLa S3 cells were grown and arrested with 1.6 μg/ml aphidicolin for 19 h, released for 6 h in fresh growth medium, and then arrested for 14 h with 100 ng/ml nocodazole. Mitotic cells obtained by shake off were plated in fresh growth medium and released for 70 min. Cell pellets were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 40 mM β-glycerophosphate, 10 mM NaF, 1% [vol/vol] IGEPAL, 0.1% deoxycholate, 100 μM ATP, 100 μM $MgCl_2$, 2 mM Pefabloc and complete protease inhibitor cocktail; Roche Diagnostics). For immune precipitations, 2 μg of affinity purified antibody, 20 μl protein G or A Sepharose, and 8 mg of extract in a total volume of 500 μl were incubated for 2 h at 4°C. Beads were washed twice with 1 ml of lysis buffer, then twice with 1 ml of wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 40 mM β-glycerophosphate, 10 mM NaF, 0.1% [vol/vol] IGEPAL, and 1 mM Pefabloc). Bound proteins were eluted in 50 μl of sample buffer and then analyzed by Western blotting.

MKlp binding assays

His-tagged MKlp1 or MKlp2, 5 μg, expressed in baculovirus-infected Sf9 cells was incubated with 10 μg either GST-tagged Aurora B, Cdc14A, or GST alone for 1 h at 4°C in HNTM buffer (50 mM Hepes-NaOH, pH 7.2, 150 mM NaCl, 0.1% [vol/vol] Triton X-100, 1 mM $MgCl_2$, 1 mM DTT, complete protease inhibitor cocktail; Roche Diagnostics) in the presence of 20 μl glutathione Sepharose and 100 μM ATP in a total volume of 400 μl. Beads were washed three times with 1 ml HNTM, the bound proteins eluted in 30 μl of reducing sample buffer and then analyzed by Western blotting.

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