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Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis

Rüdiger Neef,[1] Christian Preisinger,[1] Josephine Sutcliffe,[1] Robert Kopajtich,[1] Erich A. Nigg,[2] Thomas U. Mayer,[2] and Francis A. Barr[1,2]

[1]Intracellular Protein Transport, Independent Junior Research Group, and [2]Department of Cell Biology, Max-Planck-Institute of Biochemistry, 82152 Martinsried, Germany

We have investigated the function of mitotic kinesin-like protein (MKlp) 2, a kinesin localized to the central spindle, and demonstrate that its depletion results in a failure of cleavage furrow ingression and cytokinesis, and disrupts localization of polo-like kinase 1 (Plk1). MKlp2 is a target for Plk1, and phosphorylated MKlp2 binds to the polo box domain of Plk1. Plk1 also binds directly to microtubules and targets to the central spindle via its polo box domain, and this interaction controls the activity of Plk1 toward MKlp2. An antibody to the neck region of MKlp2 that prevents phosphorylation of MKlp2 by Plk1 causes a cytokinesis defect when introduced into cells. We propose that phosphorylation of MKlp2 by Plk1 is necessary for the spatial restriction of Plk1 to the central spindle during anaphase and telophase, and the complex of these two proteins is required for cytokinesis.

Introduction

As the chromosomes are partitioned to the cell poles in anaphase, the central spindle, an array of interdigitated antiparallel microtubule bundles oriented along the spindle axis, is formed between them (Saxton and McIntosh, 1987). The central spindle is essential for cytokinesis, and a number of key components of its structure are known. PRC1 is a microtubule-binding protein regulated by the cdk1–cyclin B mitotic kinase that is required for the formation of the microtubule bundles making up the central spindle (Jiang et al., 1998; Mollinari et al., 2002). Another well-characterized central spindle component is a complex of mitotic kinesin-like protein (MKlp) 1 and CYK-4, a GTPase-activating protein for the Rho family of GTPases (Jantsch-Plunger et al., 2000; Mishima et al., 2002). MKlp1 is thought to organize the microtubule bundles of the central spindle (Nislow et al., 1992; Adams et al., 1998; Powers et al., 1998; Raich et al., 1998; Matuliene and Kuriyama, 2002), and CYK-4 to regulate the activation state of a Rho family GTPase at the cell cortex (Jantsch-Plunger et al., 2000). The GDP–GTP exchange factor ECT2 is another Rho regulator required for cytokinesis found at the central spindle and cleavage furrow (Prokopenko et al., 1999; Tatsumoto et al., 1999; Somma et al., 2002). The fly homologues of ECT2 and CYK-4 interact, and this interaction may be necessary to communicate the position of the spindle to the cell cortex to ensure proper temporal and spatial control of actomyosin contractile ring formation (Somers and Saint, 2003). There is also a direct link between the central spindle and the actin cytoskeleton because the CHO1 splice variant of MKlp1 contains an actin-binding domain, which is absent from MKlp1 (Kuriyama et al., 2002). Thus, by controlling the activation state of a Rho family GTPase and directly binding to actin, the complex of MKlp1/CHO1, ECT2, and CYK-4 may regulate cleavage furrow ingression. Other proteins required to control actin function at the site of cell division are the septins (Cooper and Kiehart, 1996) and anillin (Field and Alberts, 1995; Oegema et al., 2000). Septins are filament-forming GTPases needed to organize the actin filaments that form the contractile ring at the cell cortex, and act in concert with the actin-binding adaptor protein anillin (Kinoshita et al., 2002).

A second group of proteins implicated in cytokinesis are the passenger proteins, which are found at the kinetochores in metaphase and then move to the central spindle in anaphase, where they remain until cytokinesis (Adams et al.,

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Address correspondence to Francis Barr, Max-Planck-Institute of Biochemistry, Dept. of Cell Biology, Am Klopferspitz 18a, 82152 Martinsried, Germany. Tel.: 49-89-8578-3135. Fax: 49-89-8578-3102.
email: barr@biochem.mpg.de

J. Sutcliffe's present address is Adolph Butenandt Institut, Lehrstuhl für Molekularbiologie, Schillerstr. 44, 80336 Munich, Germany.

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Abbreviations used in this paper: MKlp, mitotic kinesin-like protein; Plk1, polo-like kinase 1; siRNA, small interfering RNA.

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2001). The passenger proteins include the aurora-B kinase, a large coiled-coil protein INCENP, and an inhibitor of apoptosis motif containing protein survivin (Schumacher et al., 1998; Adams et al., 2001; Wheatley et al., 2001; Bolton et al., 2002). These three proteins form a complex, and interference with their function results in defects in metaphase chromosome alignment, chromosome segregation, and cytokinesis (Kaitna et al., 2000; Severson et al., 2000; Adams et al., 2001; Giet and Glover, 2001; Wheatley et al., 2001). INCENP acts as a scaffold protein activating and targeting the aurora-B kinase (Kaitna et al., 2000; Adams et al., 2001; Wheatley et al., 2001; Bishop and Schumacher, 2002), and although the role of survivin is unclear, it does not appear to be acting as an inhibitor of apoptosis, but rather as a regulator of aurora-B function (Bolton et al., 2002).

Another protein found at both the kinetochores and central spindle that could also be termed a passenger protein is polo-like kinase 1 (Plk1; Golsteyn et al., 1995). The founder member polo was first described in fruit flies as a mitotic regulator (Sunkel and Glover, 1988; Llamazares et al., 1991), and since then, homologous kinases with functions in cell division have been described in other eukaryotes (Glover et al., 1998; Nigg, 1998). Tumor cell–associated mutations in the human Plk1 gene have also been described (Simizu and Osada, 2000), underlining its importance as a mitotic regulator. Plk1 localizes to the centrosomes, kinetochores, and central spindle during mitosis, and is required for the formation of a normal metaphase spindle (Sunkel and Glover, 1988; Llamazares et al., 1991; Lane and Nigg, 1996) and cytokinesis (Carmena et al., 1998; Nigg, 1998). Its activity is regulated at the level of protein stability and by the action of upstream kinases, so that it peaks in metaphase and drops as cells exit mitosis (Golsteyn et al., 1995; Qian et al., 1998). An additional mechanism for Plk1 regulation is suggested by an experiment showing that the COOH-terminal polo box domain binds phosphopeptides in vitro and is able to specifically recognize a subset of mitotic phosphoproteins (Elia et al., 2003). Although a number of targets for Plk1 associated with its functions in mitotic entry and regulation of the metaphase to anaphase transition have been identified (Kumagai and Dunphy, 1996; Alexandru et al., 2001; Sumara et al., 2002), little is known about how Plk1 is regulated during cytokinesis in animal cells and which proteins it targets for phosphorylation (Glover et al., 1998). Mitotic kinesins have been suggested to be targets for polo kinases because reports in flies have shown that pavarotti (the fly homologue of MKlp1) and the polo kinase can be coprecipitated, and that in *pav* embryos, polo fails to localize to the centrosomes and central spindle in mitosis (Adams et al., 1998).

Previously, we have investigated rabkinesin-6, a kinesin originally identified as a binding partner for the rab6 GTPase involved in protein transport at the Golgi apparatus (Echard et al., 1998), and found that it is needed for cytokinesis (Hill et al., 2000). Throughout the present work, we have now referred to rabkinesin-6/RAB6-KIFL as MKlp2 for the following reasons. We (and subsequently others) have demonstrated that this kinesin displays a cell cycle–regulated expression pattern being essentially absent from interphase cells and abundant in mitotic cells (Hill et al., 2000; Fontijn et al., 2001). It localizes to the central spindle in anaphase, but not to Golgi structures at any time during the cell cycle, and interference with its function by antibody microinjection blocks cytokinesis (Hill et al., 2000; Fontijn et al., 2001). These observations are similar to those made on the known mitotic kinesin MKlp1/CHO1 (Nislow et al., 1992; Kuriyama et al., 2002), and furthermore, MKlp2/rabkinesin-6 is most similar to this kinesin (Echard et al., 1998; Hill et al., 2000). Therefore, we believe that rabkinesin-6 should be reclassified as a mitotic kinesin under the name MKlp2. Here, we characterize the functions of MKlp2 and the mitotic kinase Plk1 during central spindle formation and cytokinesis in human cells.

Results

MKlp2 is essential for cytokinesis

Microinjection of antibodies to MKlp2 into HeLa cells results in a cytokinesis defect and the production of binucleated cells (Hill et al., 2000). To demonstrate that this is due to an essential requirement for MKlp2 in cytokinesis, and not simply a result of the formation of a dominant-negative antibody–MKlp2 complex, depletion analyses using small interfering RNAs (siRNAs) were performed. MKlp2 and the control target lamin A can be depleted by specific siRNAs (Fig. 1 A). MKlp2-depleted cells show a highly penetrant cytokinesis defect, and after 48 h, nearly 90% of cells were binucleated (Fig. 1 B and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200306009/DC1), whereas in lamin A–depleted cells, the levels and localization of MKlp2 were normal, and no cytokinesis defect was observed (Fig. 1 and Fig. S1). Live-cell imaging revealed that MKlp2-depleted cells aligned and segregated their chromosomes normally, but had a defect in both the extent and timing of cleavage furrow ingression during anaphase and failed to perform cytokinesis, whereas control cells depleted for lamin A divided normally (Fig. 1, C and D). Therefore, MKlp2 is required for the normal cell division of human cells, specifically for normal cleavage furrow ingression and cytokinesis.

Localization of Plk1 to the central spindle requires MKlp2

A survey of central spindle components revealed that MKlp2 showed extensive colocalization with Plk1 on central spindle microtubules during anaphase and telophase (Fig. 2 A), discrete from components such as PRC1 (Fig. S2 A). The effects of MKlp2 depletion on the localization of MKlp1, PRC1, and Plk1 were then examined. Control cells depleted for lamin A showed normal staining for MKlp1, MKlp2, and Plk1 (Fig. 2, B and C). Although MKlp1 and PRC1 stainings showed some abnormalities in MKlp2-depleted cells, they were still present in the central spindle region, indicating that this structure was not totally absent in these cells (Fig. 2 B and Fig. S2; summarized in Table S1). In contrast, Plk1 was absent from the central spindle in MKlp2-depleted cells during anaphase and telophase (Fig. 2 C). This was not due to the destabilization of central spindle components in general because Plk1 and

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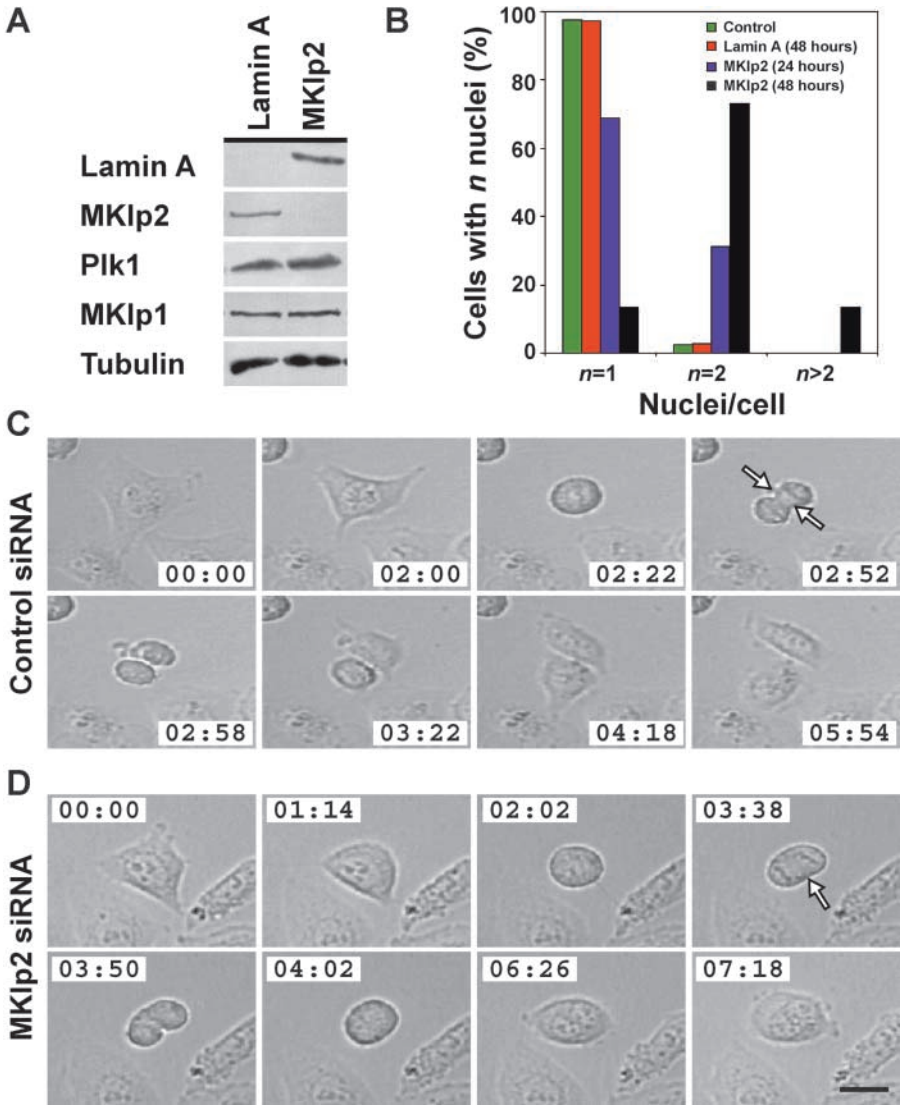

Figure 1. **MKlp2 is required for normal cell division.** (A) HeLa S3 cells were treated with the MKlp2 siRNA duplex for 36 h and the lamin A siRNA duplex for 72 h, arrested with 90 ng/ml nocodazole for 12 h to enrich for mitotic cells that express MKlp2, and then lysed in sample buffer. Western blot analysis of 25 μg protein was performed with antibodies to MKlp2, lamin A, MKlp1, Plk1, and α-tubulin. (B) The number of binucleated or multinucleated cells as a percentage of the total cell population was quantitated in HeLa S3 cells treated with lamin A or MKlp2 siRNA duplexes for the times indicated (n = 400 cells). (C) Live-cell imaging of HeLa S3 cells treated with control lamin A or (D) MKlp2 siRNA duplexes for 24 h. Representative frames reflecting the different stages of mitosis in these cells are shown, and times are indicated in h:min. Arrows indicate the position of the cleavage furrow; note the partial and asymmetric furrow ingression in MKlp2-depleted cells. Bar, 10 μM.

MKlp1 were present at the same level in control lamin A–depleted and MKlp2-depleted cells (Fig. 1 A). Additionally, in PRC1-depleted cells, MKlp2, Plk1, and survivin still localized to the central spindle, although the staining patterns were more disorganized than in control cells (Table S1). Because cleavage furrow ingression fails on MKlp2 depletion, cells were stained for anillin and septins to find out if the cleavage furrow was disrupted (Fig. 2 D). Anillin was still localized in a cortical band around the center of the cell in MKlp2-depleted cells, although late anaphase cells did not show the typical pinched furrow staining (Fig. 2 D). Similar results were obtained for septin localization (unpublished data). Therefore, MKlp2 depletion affects the localization of a subset of central spindle components, including Plk1, but does not affect the recruitment of cleavage furrow components such as anillin.

Plk1 is a microtubule-binding protein

To further characterize MKlp2 and its relationship with Plk1, microtubule-binding assays were performed with purified recombinant proteins and purified repolymerized bovine brain tubulin (Fig. 3). In this assay, both Plk1 and MKlp2 sedimented in the presence (but not in the absence) of microtubules, whereas survivin did not sediment under either condition (Fig. 3 A). Previous findings on MKlp2 showing that the COOH-terminal neck and stalk region contain a microtubule-binding domain could also be confirmed (Echard et al., 1998; unpublished data). The microtubule binding of Plk1 was mapped to the COOH-terminal polo box domain (Fig. 3 B), supporting the idea that this interaction reflects specific microtubule binding. Exogenously expressed full-length Plk1 and the COOH-terminal polo box domain both targeted to the central spindle in telophase, whereas the kinase domain displayed diffuse cytoplasmic fluorescence (Fig. 3 C) consistent with previous observations (Seong et al., 2002). These findings indicate that the polo box domain has the ability to target to a subset of microtubule structures.

Microtubules regulate Plk1 activity toward MKlp2

These observations raise a number of questions relating to Plk1 and MKlp2. Is Plk1 a direct and specific regulator of MKlp2 and its microtubule-binding properties, and do MKlp2 and microtubules control Plk1 activity? To answer these questions,

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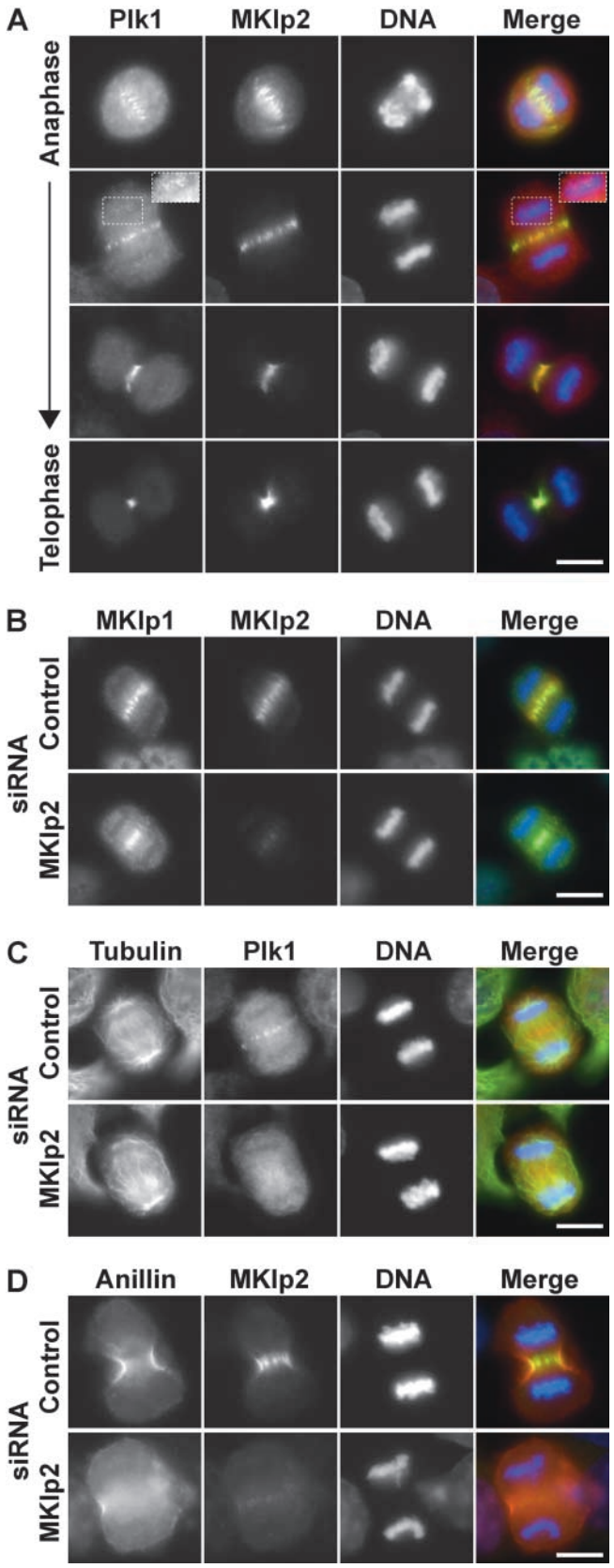

Figure 2. **Loss of Plk1 from the central spindle in MKlp2-depleted cells.** (A) HeLa cells fixed with PFA were stained with antibodies to Plk1 (red) and MKlp2 (green), and DNA was stained with DAPI (blue). Colocalization of Plk1 and MKlp2 was observed from early anaphase until cytokinesis, with the difference that MKlp2 was absent from kinetochores, unlike Plk1 (see the contrast-enhanced boxed area). (B–D) HeLa S3 cells treated with control lamin A and MKlp2 siRNA duplexes were fixed with methanol and then stained with antibodies to MKlp2, MKlp1, α-tubulin, Plk1, and anillin. Bar, 10 μM.

the abilities of Plk1 and the cdk1–cyclin B mitotic kinase to phosphorylate MKlp2 and the known mitotic spindle phosphoproteins PRC1 and MKlp1 were tested (Fig. 3 D). Equimolar amounts of substrate were used in all kinase assays to allow a direct comparison of the different substrates. The cdk1–cyclin B mitotic kinase phosphorylated histone H1, PRC1, MKlp1, and to some extent MKlp2 (Fig. 3 D). In contrast, Plk1 strongly phosphorylated MKlp2, and weakly phosphorylated MKlp1, PRC1, and the model substrate casein (Fig. 3 D). The aurora-B kinase complex was unable to phosphorylate MKlp2 or MKlp1 (unpublished data). Therefore, Plk1 is a candidate kinase for MKlp2, which is specifically phosphorylated under the conditions used. The effect of microtubules on the ability of Plk1 to phosphorylate MKlp2 and the model substrate casein was then tested (Fig. 3 E). MKlp2 was phosphorylated by Plk1 in the absence of microtubules, and this was increased 10-fold by the addition microtubules (Fig. 3 E). No significant phosphorylation of MKlp2 was observed with microtubules alone, so this was most likely due to Plk1 and not a contaminating kinase. Under the same conditions, phosphorylation of casein was decreased, suggesting that this effect was not due to activation of Plk1 by microtubules toward all substrates. Therefore, microtubules regulate the phosphorylation of MKlp2 by Plk1, supporting the idea that Plk1 regulates MKlp2 at the central spindle. If Plk1 does regulate MKlp2 at the central spindle, then it should phosphorylate MKlp2 at the same sites in vitro and during mitosis in living cells. Mass spectrometric analysis of MKlp2 phosphorylated in vitro by Plk1 and precipitated from mitotic cells revealed that a peptide corresponding to amino acids 526–537 was phosphorylated at serine 528 in both cases (Fig. 4 A). Secondary phosphorylation sites at serines 62, 662, and 668 were also identified using this approach (unpublished data). Mutation of serine 528 to alanine resulted in a fivefold reduction in the amount of MKlp2 phosphorylation by Plk1 in vitro (Fig. 4 B). Therefore, MKlp2 is phosphorylated at serine 528 during mitosis, in vitro by Plk1, and this is a major site of Plk1 phosphorylation. This residue is in the neck-linker sequence of MKlp2, and by analogy with other kinesins, this region is likely to be critical for the control of microtubule binding and motor activity (Grummt et al., 1998; Rice et al., 1999; Mishima et al., 2002; Schafer et al., 2003). Thus, Plk1 is likely to be a physiological regulator of MKlp2, and the simplest explanation is that Plk1 regulates the binding of MKlp2 to microtubules. However, this was tested, and both MKlp2^{S528A} and MKlp2 phosphorylated by Plk1 all showed similar microtubule-binding activity to MKlp2 control incubations (Fig. 4 C), and for this reason, other aspects of MKlp2 function were examined.

Plk1 regulates the microtubule-bundling activity of MKlp2

To test the hypothesis that MKlp2 and Plk1 cooperate to control microtubule organization, the effects of purified

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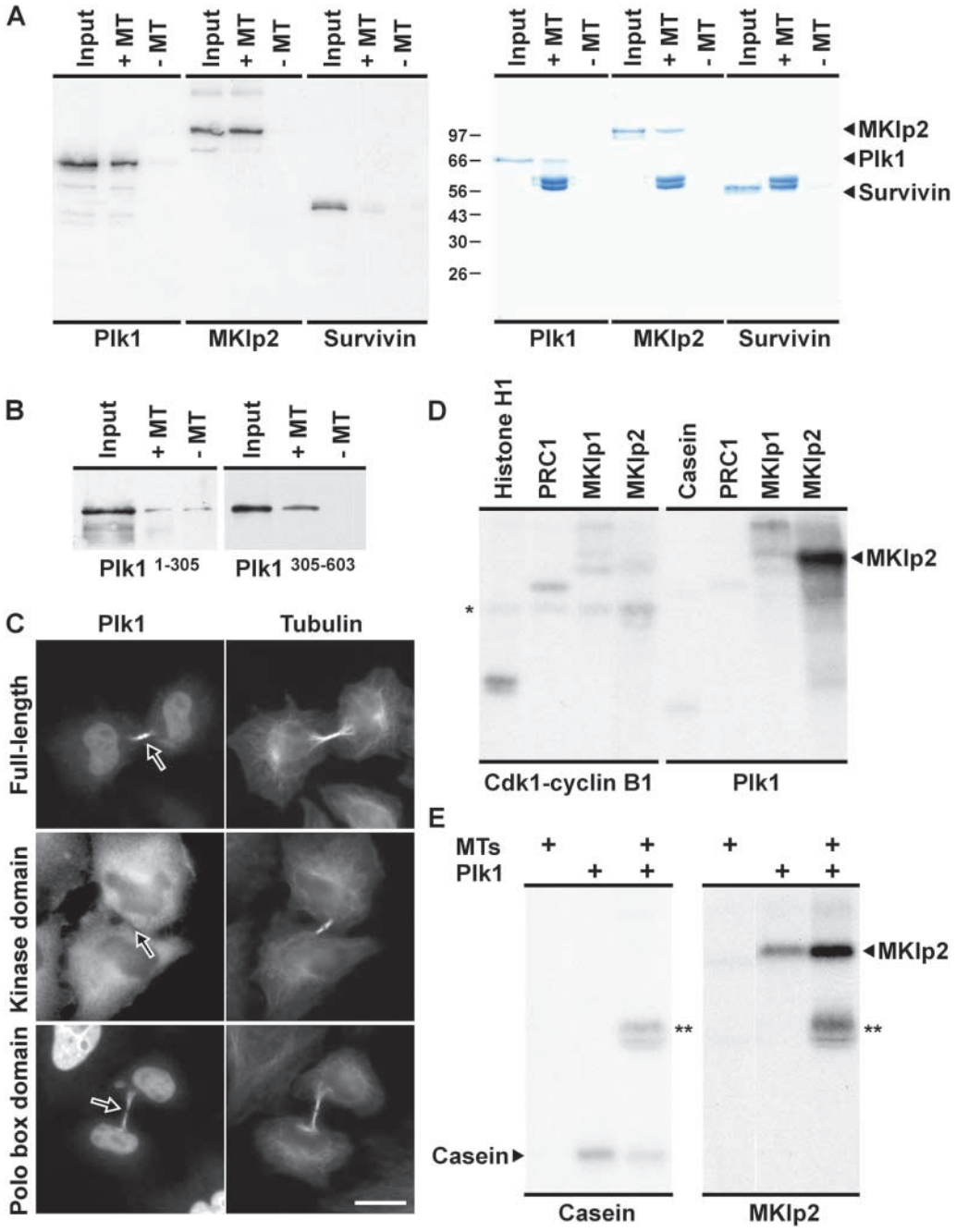

Figure 3. **Microtubules regulate the phosphorylation of MKlp2 by Plk1.** (A) Microtubule-binding assays were performed with 5 pmol Plk1, MKlp2, and survivin. Samples were then analyzed by Western blotting and detection with antibodies to the hexahistidine tag, and on Coomassie brilliant blue–stained SDS-PAGE 10% minigels (right). (B) Microtubule-binding assays were performed with 5 pmol GST-tagged Plk1 amino acids 1–305 and 305–603. Samples were then analyzed by Western blotting and detection with antibodies to GST. (C) Constructs corresponding to the full-length myc-tagged human Plk1, amino acids 1–305, and 305–603 were expressed in HeLa S3 cells for 24 h using transient transfection. Cells were fixed with methanol and stained with antibodies to the myc-epitope and α-tubulin. The localization of the exogenously expressed Plk1 in telophase cells is shown; arrows mark the position of the microtubule bridge, central spindle structure. Bar, 10 μM. (D) Phosphorylation assays were performed with 1 pmol purified histone H1, PRC1, MKlp1, and MKlp2 using 50 ng recombinant cdk1–cyclin B kinase, and with 1 pmol purified casein, PRC1, MKlp1, and MKlp2 using 50 ng recombinant Plk1. Autoradiographs of samples separated by SDS-PAGE on 12% minigels are shown. The asterisk marks phosphorylated cyclin B. (E) Phosphorylation assays were performed with 1 pmol purified casein and MKlp2 using 10 ng recombinant Plk1 in the presence and absence of microtubules. Autoradiographs of samples separated by SDS-PAGE on 12% minigels are shown. The double asterisk marks a nonstoichiometric phosphorylation of tubulin by Plk1.

MKlp2 on microtubules in vitro were examined (Fig. 5) because full-length MKlp2 (but not the motor or neck and stalk domains alone) can bundle microtubules when expressed in cells (Fig. S3). Fluorescently labeled microtubules were incubated alone, or with MKlp2 and Plk1, in the presence and absence of ATP. MKlp2-bundled microtubules in the absence and addition of ATP resulted in the formation of loops from these bundles and fraying at the bundle ends,

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whereas Plk1, although able to bind microtubules, caused no microtubule bundling. MKlp2 treated with Plk1 did not form the regular microtubule bundles seen with MKlp2 only; many single microtubules were seen instead, and the bundles that were formed were loose parallel arrays rather than the dense bundles seen with untreated MKlp2. Therefore, Plk1 negatively regulates the microtubule-bundling properties of MKlp2. MKlp2^{S528A} bundled microtubules even after Plk1 treatment, indicating that serine 528 is a regulatory site. Microtubule bundling by PRC1, which is not a substrate of Plk1, was unaffected by Plk1 treatment. The effects of Plk1 treatment on MKlp2-mediated microtubule bundling are therefore unlikely to be due to direct interference of Plk1 with the assay, and these results therefore support the proposal that MKlp2 function on central spindle microtubules involves regulation by Plk1.

Phosphorylation of MKlp2 regulates binding of Plk1

In MKlp2-depleted cells, Plk1 fails to localize to the central spindle region, despite the fact that other components such as PRC1 and MKlp1 are still able to do so. The simplest explanation for this observation is that Plk1 binds to MKlp2. To test this, Plk1 and MKlp2 were precipitated from cells arrested in mitosis with nocodazole, and were then released for 2 h to allow the cells to enter anaphase. Plk1 immune precipitates contain MKlp2, and the converse was also true, whereas a control antibody did not precipitate either protein (Fig. 6 A). MKlp1 was not precipitated by any of the antibodies used (Fig. 6 A). Therefore, Plk1 and MKlp2 are in a complex in mitotic cells, as they coprecipitate under conditions where another central spindle protein does not. Because the Plk1 polo box domain is a phospho-specific adaptor (Elia et al., 2003), in addition to the microtubule-binding properties described in Fig. 3 A, it was possible that it might bind directly to phosphorylated MKlp2. The Plk1 polo box domain strongly bound to MKlp2 phosphorylated by Plk1 on ligand blots, whereas untreated MKlp2 gave a weak signal and the control protein PRC1 did not bind either before or after cdk1–cyclin B1 phosphorylation (Fig. 6 B). This recognition was dependent on phosphorylation of serine 528 because MKlp2^{S528A} showed a fivefold reduction in binding to the polo box domain (Fig. 6 C). Plk1 purified from insect cells shows extensive autophosphorylation (unpublished data) and cyclin B1 is phosphorylated by Plk1; however, these phosphorylations were not recognized by the polo box domain. The Plk1 kinase domain did not bind to any of the proteins under these conditions (Fig. S4). Therefore, Plk1

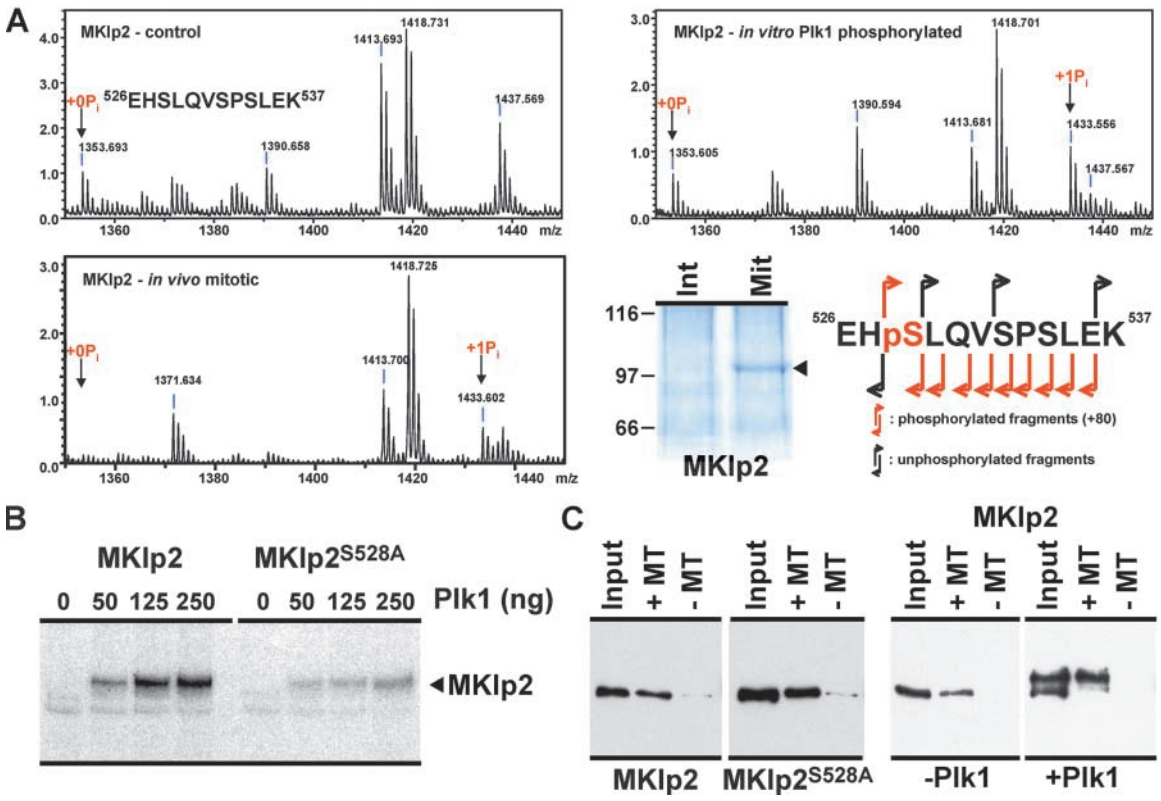

Figure 4. **Analysis of the sites on human MKlp2 phosphorylated in vivo and in vitro by Plk1.** (A) MKlp2, MKlp2 treated with Plk1 in vitro, and MKlp2 immune precipitated from 2.5 mg extract prepared from asynchronous cells (Int) and cells arrested with nocodazole and released for 2 h (Mit) were digested with trypsin and then analyzed by mass spectrometry. Note that as expected, MKlp2 is highly enriched in the mitotic cells. A region of the spectra showing phosphorylation of a peptide from 526 to 537 in Plk1-treated and in vivo MKlp2 is shown. Fragmentation of this peptide showed that the serine at 528 is phosphorylated by Plk1 and in vivo. (B) MKlp2 and MKlp2^{S528A} (1 μg) were treated with the indicated amounts of Plk1 for 1 h at 30°C, and were then analyzed by gel electrophoresis and autoradiography. (C) Microtubule-binding assays were performed with 5 pmol MKlp2 and MKlp2^{S528A} treated with buffer or Plk1. Samples were then analyzed by Western blotting and detection with antibodies to the hexahistidine tag.

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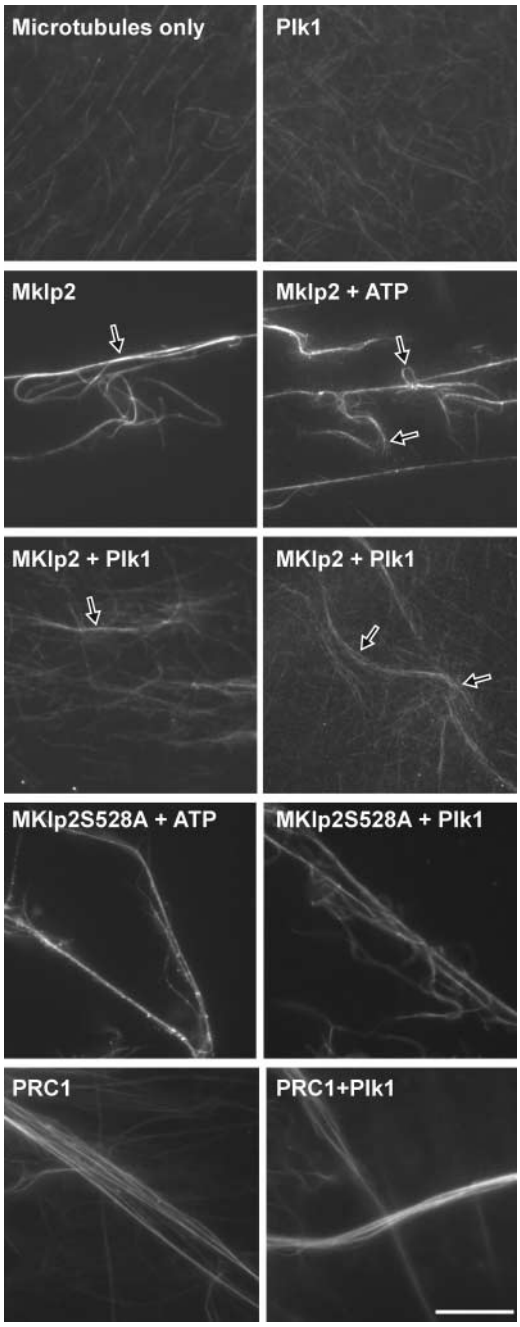

Figure 5. **Plk1 regulates the microtubule-bundling activity of MKlp2.** Microtubules labeled with fluorescein were incubated with buffer alone, MKlp2, or MKlp2^{S528A} in the presence and absence of 1 mM ATP, Plk1, MKlp2 or MKlp2^{S528A} treated with Plk1 in the presence of 1 mM ATP, and PRC1 treated with 1 mM ATP or Plk1 and 1 mM ATP. Bundling was observed in the presence of MKlp2 and PRC1, but not Plk1 or with microtubules alone. Exposure time was 500 msec for all images, and representative images from 14 independent experiments are shown. Bar, 10 μM.

exists in a complex with MKlp2 in mitotic cells, and binds directly to phosphorylated MKlp2 by its polo box domain.

Plk1 targeting to the central spindle requires MKlp2 phosphorylation

To show that the interaction of Plk1 with phosphorylated MKlp2 is relevant for the targeting of Plk1 to the central spindle, MKlp2 and a phosphorylation site mutant of MKlp2 were expressed in cells depleted of MKlp2 using siRNA (Fig. 7). Wild-type MKlp2 expressed in MKlp2-depleted cells localized to the central spindle, and in addition, rescued Plk1 targeting to the central spindle (Fig. 7). In contrast, the phosphorylation site mutant of MKlp2, although localized to the central spindle, did not rescue Plk1 targeting to this structure (Fig. 7). Therefore, MKlp2 targeting to the central spindle does not require phosphorylation by Plk1, but phosphorylation is needed for the recruitment of Plk1 to the central spindle.

Phosphorylation of MKlp2 by Plk1 is required for cytokinesis

Antibodies to the neck and stalk region of MKlp2 can block cytokinesis when microinjected into HeLa cells (Hill

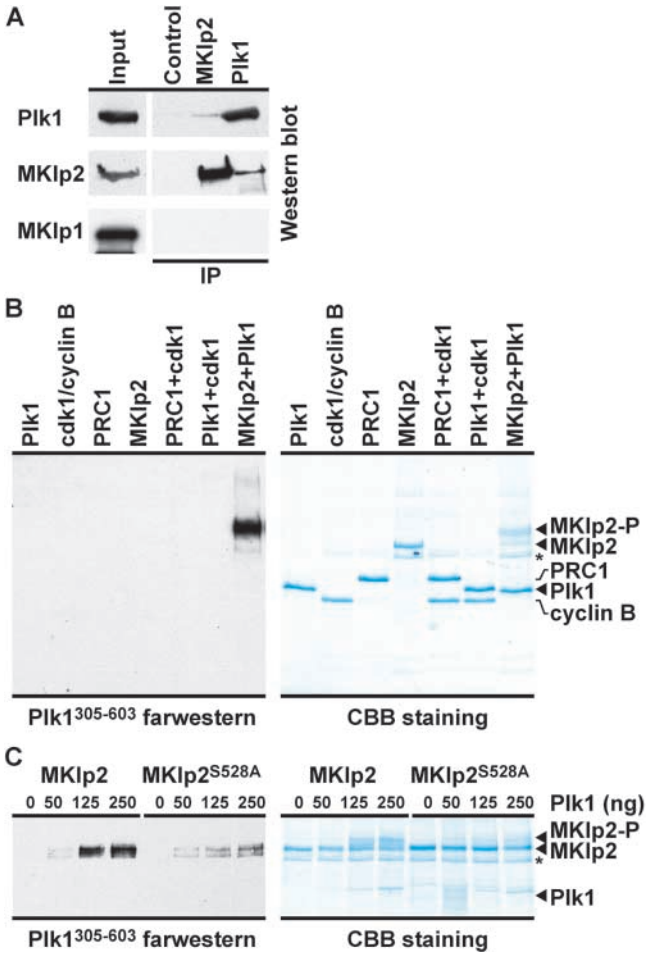

Figure 6. **The Plk1 polo box domain binds to phosphorylated MKlp2.** (A) HeLa S3 cells grown in suspension were arrested with 90 ng/ml nocodazole for 20 h, and then released for 2 h. Immune precipitations with affinity-purified antibodies to MKlp2, Plk1, and the nonspecific control GFP were performed from 100-μg soluble extracts prepared from these cells. Western blot analysis was performed on 20 μg cell extract, and one fifth of the immune precipitated material. (B) Ligand blots were performed with Plk1 amino acids 305–603 as the probe, and Plk1, cdk1/cyclin B, PRC1, MKlp2, treated with Plk1 or cdk1/cyclin B as targets. (C) Ligand blots were performed with Plk1 amino acids 305–603 as the probe, and MKlp2 or MKlp2^{S528A} treated with Plk1 as targets. The asterisks mark a truncation product in the MKlp2 preparations.

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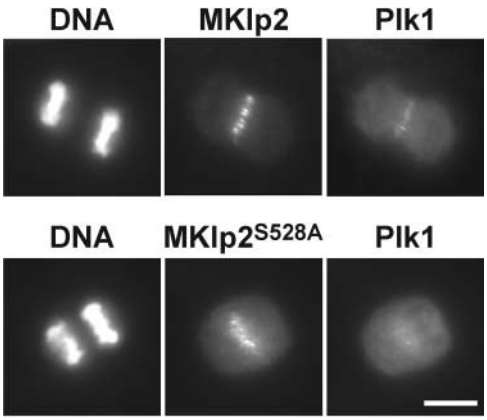

Figure 7. **A phosphorylation site mutant of MKlp2 fails to rescue targeting of Plk1 to the central spindle in MKlp2-depleted cells.** HeLa S3 cells were treated with the MKlp2 siRNA duplex for 18 h and were then transfected with siRNA-resistant GFP-tagged MKlp2 or a phosphorylation site mutant for 12 h. Cells were fixed with methanol and then stained with antibodies to Plk1, GFP was directly visualized, and DNA was stained with DAPI. Bar, 10 μM.

et al., 2000), and we decided to reinvestigate these findings in the light of the data presented in the current paper on Plk1 regulation of MKlp2. HeLa cells microinjected with affinity-purified antibodies to the neck region of MKlp2 failed to complete cytokinesis, whereas cells injected with a control antibody divided normally (Fig. 8 A). When MKlp2 was preincubated with these antibodies before use as a substrate in Plk1 kinase assays, a 10-fold reduction in the phosphorylation of MKlp2 was observed relative to the control conditions (Fig. 8 B). Incubation of the Plk1 used in these assays with antibodies to the polo box domain also prevented phosphorylation of MKlp2 (Fig. 8 B), indicating the importance of this domain for Plk1 function. Pre-incubation of MKlp2 with specific antibodies not only reduced its phosphorylation by Plk1, but this MKlp2 showed a reduced ability to bind Plk1 on ligand blots (Fig. 8 C). However, when the MKlp2 antibodies were added to the blocking reaction of the ligand blot, they were unable to prevent binding of Plk1 to phosphorylated MKlp2 (Fig. 8 C). The effect of these antibodies on the behavior of MKlp2 in microtubule binding was then tested, but no obvious differences were seen between buffer, MKlp2, and control antibody incubations (Fig. 8 D). In bundling assays, the MKlp2 antibodies caused a slight reduction in the length of the microtubule bundles compared with control incubations with MKlp2 alone or with GFP antibodies without affecting the number or morphology of the bundles formed (Fig. 8 E). The failure of cytokinesis in HeLa cells injected with antibodies to the MKlp2 neck and stalk region could therefore be due to a failure of Plk1 to phosphorylate MKlp2 because antibody binding has little effect on the other properties of the kinesin investigated here. These observations support the idea that phosphorylation of MKlp2 by Plk1 is necessary for MKlp2 function in Plk1 localization to the central spindle, and for the control of cytokinesis in human cells.

Discussion

We have investigated the functions of the kinesin MKlp2 and the mitotic kinase Plk1 during central spindle formation and cytokinesis, and uncovered a regulatory mechanism linking these two proteins. Depletion of MKlp2 using siRNAs shows that it is essential for cytokinesis, and that in these cells, Plk1 fails to localize to the central spindle during anaphase and telophase. The dependence of Plk1 on MKlp2 for localization to the central spindle can be explained by the fact that these proteins interact directly, and phosphorylation of MKlp2 by Plk1 controls this interaction. This interpretation is supported by the demonstration that a phosphorylation site mutant of MKlp2 targets to the central spindle, but fails to rescue Plk1 localization when expressed in MKlp2-depleted cells. This extends previous observations in flies showing that the mitotic kinesin pavarotti and the polo kinase can be coprecipitated, and in *pav* embryos, polo fails to localize to the central spindle (Adams et al., 1998). We were unable to confirm a previous report that mouse MKlp1/CHO1 is a target for Plk1 (Lee et al., 1995). A possible explanation is that the kinesin seen in this previous report was in fact MKlp2 because it was not known at the time that mammalian cells have two related mitotic kinesins, whereas worms and flies have only one. In addition, cleavage furrow ingression fails in MKlp2-depleted cells, although contractile ring components localize normally, suggesting that the MKlp2–Plk1 complex is required for signaling from the central spindle to the cell cortex. This is different from published reports where MKlp1/CHO1 function was blocked and furrow ingression occurred normally, but cytokinesis failed at a late stage (Kuriyama et al., 2002). Therefore, the two mitotic kinesins MKlp1/CHO1 and MKlp2 appear to have discrete functions at the central spindle essential for the cytokinesis of mammalian cells.

Substrates for polo kinases in cytokinesis

Other than the mitotic kinesins found in animal cells described above, which proteins are targets for polo kinases in cytokinesis? In the fission yeast *Schizosaccharomyces pombe*, the polo kinase Plo1p is required for septum formation (Ohkura et al., 1995), and mutations in the plo1 gene cause defects in the localization of the division plane (Bahler et al., 1998). One potential Plo1p target that is conserved in other eukaryotes is the anillin homologue Mid1p, needed for correct positioning of the actin contractile ring (Chang et al., 1996; Sohrmann et al., 1996; Paoletti and Chang, 2000). A link between Plk1 function and actin function at the cell cortex is also suggested by our observation that in MKlp2-depleted cells, where Plk1 fails to localize correctly, cleavage furrow ingression either fails entirely or is asymmetric. Anillin localization to the contractile ring does not appear to require MKlp2 function, implying that if it were a target for the MKlp2–Plk1 complex, then this phosphorylation would be required for a process downstream of recruitment to the cleavage furrow, but upstream of the initiation of furrow ingression. Together, these observations indicate a conserved function for polo kinases in animal cell cytokinesis and its yeast-equivalent

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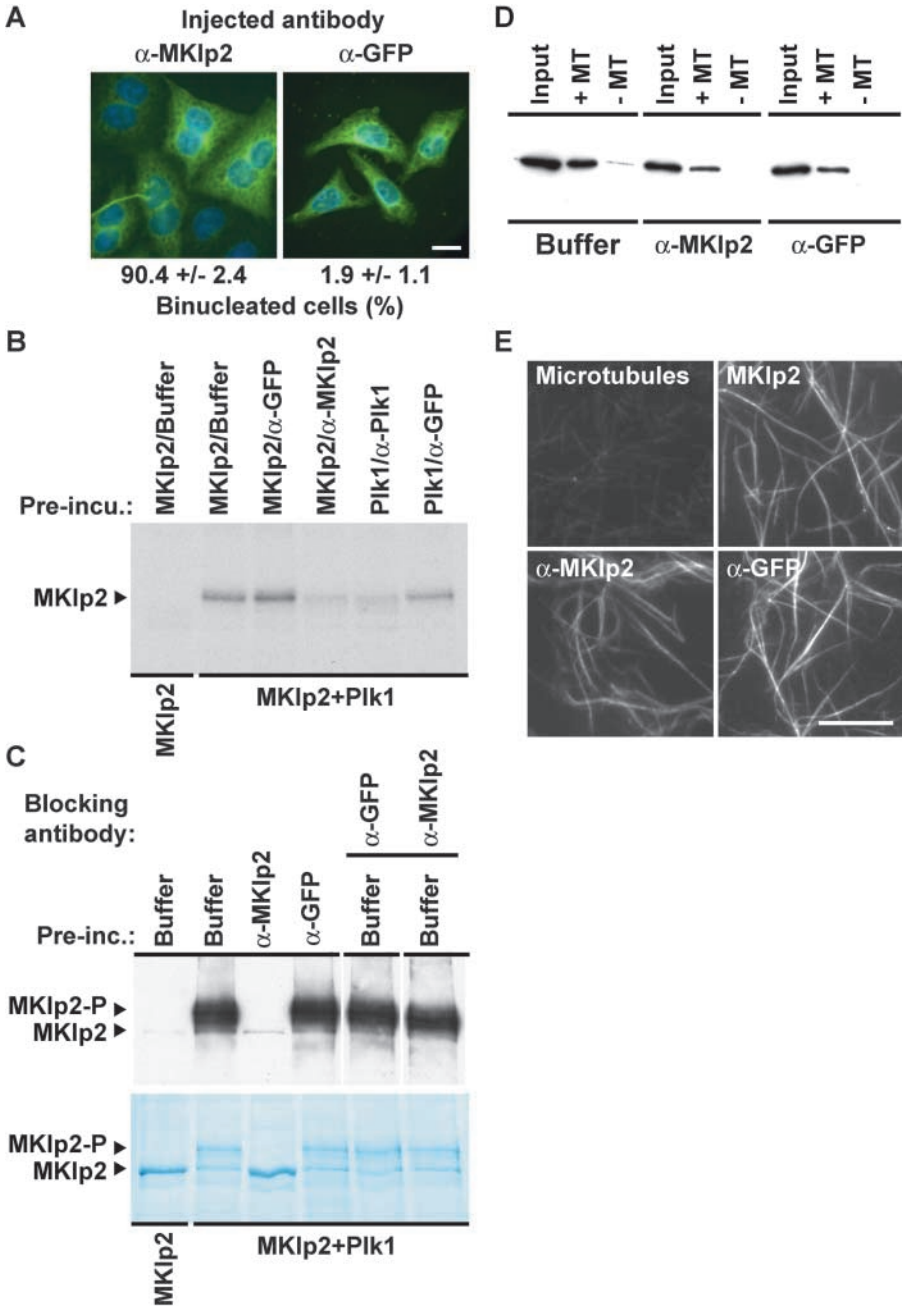

Figure 8. **Antibodies to the neck and tail region of MKlp2 prevent phosphorylation by Plk1 and cytokinesis.** (A) HeLa cells were injected with affinity-purified antibodies to the MKlp2 neck region and control affinity-purified antibodies to GFP. Cells were fixed with methanol 24 h after injection and then stained with a CY2-conjugated secondary antibody to detect the injected antibody (green) and with DAPI for the DNA (blue). The number of binucleated cells was counted and is expressed as a percentage of total injected cells detected ($n = 3$). Bar, 10 μM. (B) 1 μg MKlp2 preincubated with buffer, affinity-purified antibodies to MKlp2 or GFP was treated for 1 h at 30°C with 125 ng Plk1, itself preincubated with affinity-purified antibodies to Plk1 or GFP, then analyzed by gel electrophoresis and autoradiography. (C) 1 μg MKlp2 preincubated with buffer, affinity-purified antibodies to MKlp2 or GFP was treated with 250 ng Plk1, and then was used as a target in a ligand blot with Plk1 amino acids 305–603 as the probe. Two strips containing MKlp2 phosphorylated by Plk1 were incubated with 2 μg affinity-purified antibodies to MKlp2 or GFP before probing with the Plk1 polo box domain. (D) Microtubule-binding assays were performed with 5 pmol MKlp2 and MKlp2 preincubated on ice for 60 min with 1 μg affinity-purified antibodies to MKlp2 or GFP. Samples were then analyzed by Western blotting and detection with antibodies to the hexahistidine tag. (E) Microtubules labeled with fluorescein were incubated with buffer alone, MKlp2, or MKlp2 preincubated for 60 min on ice with 1 μg affinity-purified antibodies to MKlp2 or GFP. Exposure time was 1,000 msec for all images, and representative images from five independent experiments are shown. Samples were renumbered and scored for bundling activity by two different people. Bar, 10 μM.

septum formation, and suggest the existence of conserved substrates such as Mid1p/anillin needed for the control of these events. Another potential target in animal cells linking the central spindle to events at the cleavage furrow is the Rho-GTPase exchange factor ECT2, which is known to be regulated by phosphorylation, although the kinase has not yet been identified (Tatsumoto et al., 1999). Further work will be needed to investigate whether any of these proteins are indeed targets for the MKlp2–Plk1 complex, and the consequences of phosphorylation for cleavage furrow ingression and cytokinesis.

Microtubules and Plk1 function

Both MKlp2 and Plk1 bind to microtubules in vitro, and MKlp2 phosphorylation is stimulated by the addition of microtubules. These findings are consistent with the fact that Plk1 is usually found on microtubule-associated structures at all points of the cell cycle (Golsteyn et al., 1995), and by the presence of the active polo kinase in microtubule fractions of *Drosophila* embryos (Tavares et al., 1996) and mammalian cells (Feng et al., 1999). The conserved COOH-terminal polo box domain of Plk1 and related kinases is reported to regulate both their kinase activity and localization (Mundt et al., 1997), and in our hands is responsible for the binding of Plk1 to phosphorylated MKlp2. The Plk1 polo box domain has recently been reported to bind a phosphorylated peptide motif related to the cdk1–cyclin B consensus (Elia et al., 2003). Therefore, it is interesting that Plk1, which recognizes a consensus sequence for phosphorylation distinct from cdk1–cyclin B (Kelm et al., 2002), is capable of pro-

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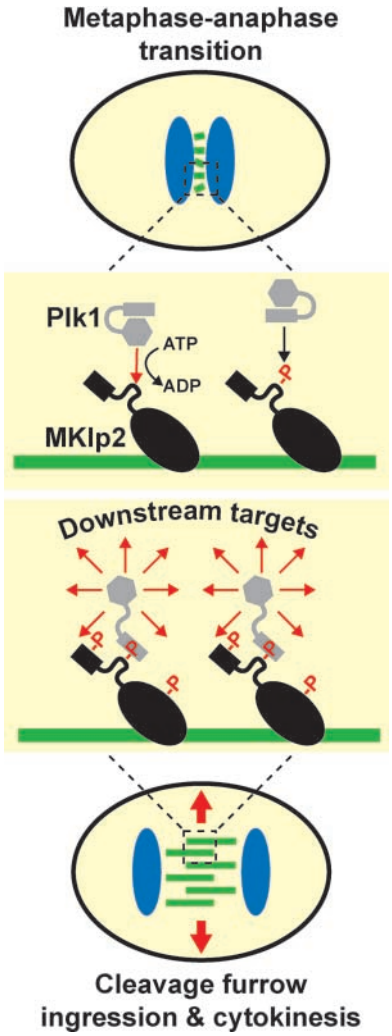

Figure 9. **Plk1 and MKlp2 form a microtubule-sensing device at the central spindle.** Early in anaphase as the chromosomes (blue) are partitioned, MKlp2 is recruited to the forming microtubule bundles that will give rise to the central spindle. Plk1, although able to weakly associate with microtubules (green), is not present on all microtubule structures, and therefore must have additional specificity factors in order to localize properly; MKlp2 appears to be one such factor. Plk1 associates with microtubules and phosphorylates MKlp2, to which it can then bind and thus maintain a stable association with the central spindle. We propose that this MKlp2-associated and thus spatially restricted pool of Plk1 can then phosphorylate other targets at the central spindle and cleavage furrow to control cleavage furrow ingression and cytokinesis. Red arrows indicate phosphorylation events.

ducing a binding site for itself on MKlp2. A related issue is the mechanism of Plk1 binding to microtubules, which does not appear to depend on phosphorylation because alkaline phosphatase treatment of microtubules does not abolish this interaction (unpublished data). Recognition of specific targets by the Plk1 polo box domain in vivo may therefore involve both phosphorylation-dependent and -independent mechanisms, possibly explaining the presence of the two independent polo box motifs in this domain. Characterization of further binding partners for Plk1, and their modes of interaction with the polo box domain, will be needed to clarify these issues.

Spatial regulation of Plk1 activity

Spatial control of mitotic kinases is becoming an increasingly important area for the understanding of how cells regulate many events during cell division (Pines, 1999). Our data suggest a mechanism for the spatial restriction of Plk1 signaling to the central spindle during anaphase and telophase (Fig. 9). This is not the only example where spatially restricted Plk1 signaling may play a key role in regulation of cell division. Plk1 is important for the localized activation of the cdk1–cyclin B1 complex at the centrosomes and for the triggering of mitosis (Jackman et al., 2003). Although it is unknown how Plk1 is regulated in this case, its association with the key cellular microtubule-nucleating structure, the centrosome, indicates that microtubules are also likely to be important for controlling Plk1 activity in this context. Similarly, the identity of the microtubule-sensing component at the kinetochores monitoring chromosome capture is still unknown, and our results suggest that Plk1 could be a component of such a microtubule sensor. These proposals highlight the general importance of the finding that Plk1 binds to and is regulated by microtubules. The identification and characterization of further Plk1 substrates and interacting partners at the central spindle, kinetochores, and centrosomes will help address the generality of this proposal.

In summary, we have identified a regulatory mechanism for Plk1 in cytokinesis, involving association with microtubules and a microtubule-associated protein, MKlp2. This mechanism, in conjunction with other microtubule-associated proteins, could also be relevant for spatial control of Plk1 function at the centrosomes and kinetochores during mitotic spindle formation and chromosome segregation (Glover et al., 1998; Nigg, 2001).

Materials and methods

Materials

Chemicals were obtained from Sigma-Aldrich or Merck unless otherwise specified. All cell culture media and sera were obtained from Invitrogen. DNA primers were synthesized by Thermo-Hybaid, and synthetic RNA duplexes by Dharmacon Research, Inc. Restriction enzymes were purchased from New England Biolabs, Inc., and reagents for the purification of DNA purchased from QIAGEN. Antibodies were obtained as follows: DM1a mouse monoclonal to α-tubulin (Sigma-Aldrich); 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, affinity-purified 1 mg/ml goat polyclonals to human Plk1 and MKlp2, rabbit polyclonal to MKlp2, and rabbit polyclonal to Plk1 (Upstate Biotechnology); rabbit polyclonal to PRC1 (a gift from W. Jiang, New York University Medical Center, New York, NY); rabbit polyclonal to human survivin NB 500–201 K3 (Novus Biologicals); mouse monoclonal to lamin A L34B4, rabbit polyclonals to human anillin, and septin (a gift from Dr. C. Field; Harvard, University, Cambridge, MA); and rabbit polyclonal to GST (a gift from Dr U. Grüneberg, Max-Planck-Institute of Biochemistry, Martinsried, Germany). Secondary antibodies to sheep/goat, rabbit, and mouse conjugated to HRP, CY2, and CY3 prepared at 0.5 mg/ml were obtained from Jackson ImmunoResearch Laboratories. Human aurora-B/INCENP/survivin complex purified from insect cells was a gift from Dr. R. Honda (Max-Planck-Institute of Biochemistry, Martinsried, Germany).

Molecular biology and protein expression

Full-length human MKlp1, MKlp2, PRC1, survivin, aurora-B, cyclin B1, and Plk1 were amplified from human testis cDNA (CLONTECH Laboratories, Inc.) using the pfu polymerase (Stratagene) and cloned in pCRII-TOPO (Invitrogen). Point mutants were constructed using the QuikChange® mutagenesis protocol (Stratagene). All constructs were con-

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firmed by DNA sequencing (Medigenomix). Mammalian expression constructs were made in pcDNA3.1+ (Invitrogen) and pEGFP-C2 (CLONTECH Laboratories, Inc.). MKlp2 rescue constructs resistant to siRNA were made by introducing silent mutations into the MKlp2 siRNA target site. For baculovirus expression, the pAcSG2 vector (Becton Dickinson) modified to include the hexahistidine tag from pQE32 (QIAGEN) was used. Baculoviruses were produced and proteins were expressed in Sf9 cells according the manufacturer's protocols (Becton Dickinson). Bacterial expression was performed using the T7 polymerase hexahistidine-GST expression vector pGAT2. Inserts encoding survivin and Plk1 amino acids 1–305 and 305–603 were inserted into pGAT2, and proteins were expressed in BL21(DE3) cells. Proteins were purified over nickel-NTA agarose (QIAGEN). Proteins were desalted into 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 1 mM DTT with HiTrap™ 5-ml desalting columns (Amersham Biosciences), and then aliquots were snap frozen in liquid nitrogen for storage at −80°C.

Cell culture and microscopy

HeLa-S3 cells were cultured, transfected, and processed for immunofluorescence microscopy as described previously (Short et al., 2001). RNA interference and live-cell imaging were performed as described previously (Elbashir et al., 2001; Short et al., 2001). MKlp2, lamin A, and PRC1 were targeted with siRNA duplexes designed from the sequences 5′-aagatcagggttgtgtccgtatt-3′, 5′-aactggacttccagaagaacatc-3′, and 5′-aaggcttctaggcgtgaggagtt-3′, respectively. For microinjection experiments, antibodies were dialyzed into PBS, diluted to 1 mg/ml, then centrifuged at 20,000 *g* for 15 min at 4°C before use. Cells were arrested for 16 h in 2.5 μg/ml aphidicolin, and were then released into fresh medium before injection with antibodies. Microinjection conditions were 150 hPa injection pressure and 0.2 s injection time at RT, and between 300 and 500 cells were injected per coverslip. For immunofluorescence, primary antibodies were diluted to 1 μg/ml or 1:1,000 for sera, and secondary antibodies to 0.5 μg/ml; staining was performed for 1 h at RT, with five washes in PBS before and after the incubations. Coverslips were mounted in Moviol 4–88 containing 1 μg/ml DAPI to stain the DNA.

Microtubule binding and bundling assays

Microtubules were polymerized from 25 μl bovine brain tubulin (10 mg/ml) by incubation for 30 min at 37°C in a total of 70 μl BRB80 (80 mM Pipes, pH 6.8, 1 mM $MgCl_2$, and 1 mM EGTA) containing 4 mM $MgCl_2$, 1 mM GTP, and 4.8% DMSO. The reaction was then centrifuged for 15 min at 35°C through a 50-μl a cushion of 40% glycerol in BRB80 at 55,000 rpm in a rotor (model TLA100; Beckman Coulter). The pellet was washed once with 50 μl BRB80 containing 50 μM paclitaxel, centrifuged again, and then resuspended in 50 μl BRB80 containing 50 μM paclitaxel. The final concentration of microtubules was 50 μM (5 mg/ml). Microtubule binding and bundling assays were performed as follows: 5 pmol of the recombinant protein to be tested was incubated with 2-μl microtubules for 20 min at RT in a total volume of 20 μl BRB80 containing 10 μM paclitaxel. Microtubule bundling was performed with or without 1 mM ATP using rhodamine-labeled microtubules, and was analyzed directly after incubation under a microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) using a 63× oil immersion lens. For pulldown experiments, the reaction was centrifuged for 20 min at 25°C through a 50-μl cushion of 40% glycerol in BRB80 at 55,000 rpm in a rotor (model TLA100; Beckman Coulter). The pellet was washed once with 50 μl BRB80 containing 50 μM paclitaxel, centrifuged again, and then resuspended in 30 μl sample buffer.

Protein phosphorylation and Far Western ligand blots

Protein phosphorylation was performed using 1 pmol of each substrate, 1 mM ATP, 0.1 μl γ[^{32}P]ATP, 3,000 Ci/mmol, and 10 mCi/ml, in a total volume of 20 μl BRB80 for 30 min at 30°C. For assays with microtubules, 2 μl polymerized tubulin was added. Cold phosphorylations were performed using 5 pmol substrate and only nonlabeled ATP. Far Western ligand blots were performed in TBST (50 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, and 4% wt/vol skim milk powder). Assays performed with 500 ng PRC1, Plk1, and MKlp2 treated with 500 ng Plk1 or cdk1/cyclin B for 60 min at 30°C were separated on 7.5% minigels, Western blotted, then probed with 1 μg/ml GST-tagged fragments of Plk1 for 6 h at 4°C. For antibody-blocking experiments, the MKlp2 was pretreated with 1 μg antibody for 60 min on ice before phosphorylation with Plk1. Bound protein was detected using affinity-purified rabbit antibodies to GST.

Immune precipitations and mass spectrometry

HeLa S3 cells were grown and arrested with nocodazole, and extracts were prepared from them as described previously (Barr et al., 1997). For immune precipitations, 2 μg affinity purified antibody, 20 μl packed protein G–Sepharose, and 100 μg extract in a total volume of 500 μl KHMT (25 mM Hepes-KOH, pH 7.3, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, and 0.1% Triton X-100) were incubated for 1 h at 4°C rotating continuously. Beads were washed four times with KHMT, 50 μl of reducing sample buffer was added, and then beads were heated at 95°C for 3 min followed by analysis on 7.5% minigels. Proteins were digested in-gel with sequencing grade porcine trypsin (Promega) and extracted using a published method (Shevchenko et al., 1996). Extracted peptides were analyzed using a MALDI-TOF instrument (Reflex III; Bruker) and probability database searching (Perkins et al., 1999), or separated by nano-HPLC and analyzed by tandem mass spectrometry (Wilm et al., 1996).

Online supplemental material

The localization of a variety of central spindle components in cells depleted for lamin A, MKlp2, or PRC1 using siRNA is summarized in Table S1. Additional images of the MKlp2 siRNA phenotype are shown in Fig. S1 and Fig. S2. The analysis of MKlp2 targeting is shown in Fig. S3; note that only full-length MKlp2 can bundle microtubules when overexpressed in HeLa cells. Control experiments showing that the polo box domain (and not the kinase domain) of Plk1 mediates the interaction of Plk1 with phosphorylated MKlp2 are presented in Fig. S4. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200306009/DC1.

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