Plk1 and Aurora A regulate the depolymerase activity and the cellular localization of Kif2a

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Summary

The microtubule depolymerase Kif2a controls spindle assembly and dynamics and is essential for chromosome congression and segregation. Through a proteomic analysis, we identified Kif2a as a target for regulation by the Polo-like kinase Plk1. Plk1 interacts with Kif2a, but only in mitosis, in a manner dependent on its kinase activity. Plk1 phosphorylates Kif2a and enhances its depolymerase activity in vitro. Inhibition or depletion of Plk1 decreases microtubule-associated Kif2a signals and increases the spindle microtubule intensity in vivo. Interestingly, Aurora A also interacts with and phosphorylates Kif2a. Phosphorylation of Kif2a by Aurora A suppresses its depolymerase activity in vitro, and inhibition of Aurora A increases the microtubule-associated Kif2a signals and reduces the spindle microtubule intensity in vivo. Thus, Kif2a is regulated positively by Plk1 and negatively by Aurora A. We propose that this antagonistic regulation confers differential stability to microtubules in the spindle versus the pole versus in the cytosol, and that this spatial differential stability is important for spindle assembly and function.

Key words: Kif2a, Plk1, Aurora A, Microtubule depolymerase, Mitotic spindle, Mitosis

Introduction

Spindle dynamics, which generates the pulling force for chromosome congression and segregation in mitosis, is mediated through active microtubule polymerization and depolymerization. The Polo-like kinase Plk1, a key regulator of spindle dynamics (Kline-Smith and Walczak, 2004), interacts with and directly phosphorylates Kif2a during mitosis (Gadde and Heald, 2004). Plk1 and Aurora A regulate the depolymerase activity and stability of the microtubule depolymerase Kif2a.

Results

Kif2a interacts with Plk1 during mitosis

To investigate the function of Plk1, the Plk1 complexes were purified from G2 and mitotic cells expressing a tandem tagged GFP-S-Plk1, and associated proteins were analyzed by mass spectrometry (Seki et al., 2008a; Seki et al., 2008b; Zhu et al., 2008). We identified Kif2a as a Plk1-interacting protein with high confidence, as reflected in the high XCorr and DeltaCN scores (Fig. 1A). Next, we determined the cellular localization of Kif2a and Plk1 during the cell cycle. Kif2a localized to the spindle MTs and spindle poles from prophase to metaphase, which partially overlapped with the centrosomal localization of Plk1 (Fig. 1B). By contrast, Kif2a and Plk1 seem to have distinct and non-overlapping localizations from anaphase to cytokinesis.

An interaction between Kif2a and Plk1 was directly confirmed in a transient transfection experiment. Myc-Plk1 was co-transfected with GFP-Kif2a or GFP into 293T cells. Myc-Plk1 was co-precipitated with GFP-Kif2a, but not with GFP, in nocodazole-arrested mitotic cells (Fig. 1C).

The endogenous Plk1 and Kif2a also interact. HeLa S3 cells were synchronized at the G1-S boundary by a double-thymidine (TT) treatment and then released to progress from G1 to S, G2 and then to mitosis. The Plk1-Kif2a complex was first detected in early G2, then released to progress from G1 to S, G2 and then mitosis. The Plk1-Kif2a complex was first detected in early G2, then released to progress from G1 to S, G2 and then mitosis. The Plk1-Kif2a complex was first detected in early G2, then released to progress from G1 to S, G2 and then mitosis.
control the function of Kif2a. To analyze this complex during mitotic exit, HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole (TN) treatment and then released into fresh medium containing the proteasome inhibitor MG132 (20 μM) and one of the following kinase inhibitors: 1 μM BI 2536 [a Plk1 inhibitor (Lenart et al., 2007)], 25 μM Purvalanol A [a Cdk1 inhibitor (Skoufias et al., 2007)] and 5 μM VX680 [an Aurora A inhibitor (Harrington et al., 2004)]. Cells were harvested 2 hours later. Cell lysates and the anti-Plk1 immunoprecipitates were analyzed by western blotting.

We conclude that at least a subpopulation of the Plk1-Kif2a complex is likely to act as a soluble complex in the cytosol.

The interaction between Plk1 and Kif2a is phospho-dependent, as incubation of the Plk1 immuno-complex purified from G2 (TT9) or M (TN0) cells with λ-phosphatase removed the associated Kif2a (Fig. 1F). To determine which kinase is responsible for this phospho-dependent interaction, the Plk1-Kif2a complex was immunoprecipitated with an anti-Plk1 antibody from HeLa S3 cells harvested after a thymidine-nocodazole treatment (TN0) or collected at 9 hours after the release from a double-thymidine arrest (TT9). The immunoprecipitates were incubated with or without λ-phosphatase (λ-PPase), washed to remove dissociated Kif2a, and then analyzed by western blotting.
Plk1 directly phosphorylates Kif2a and activates its depolymerase activity. (A) Recombinant His-Kif2a was incubated with wild-type (WT) or kinase-dead (KD) Plx1 in the presence of radioactive ATP. Casein, a common substrate of Plx1, was included as a control. The amounts of His-Plx1 and His-Kif2a used in the reaction were visualized by silver staining (for Plx1) and by Coomassie blue staining (for Kif2a). (B) Recombinant Histagged Kif2a (10 nM) was phosphorylated with wild-type or kinase-dead Plx1 and the reaction mix was incubated with Taxol-stabilized MTs for 3 minutes. MTs and Kif2a were then analyzed in a co-pelleting assay, followed by detection of MTs with silver staining and detection of Kif2a and Plx1 with western blotting. sup., supernatant. (C) His-Kif2a (10 nM) was phosphorylated by Plx1 and the reaction mix was then incubated with Taxol-stabilized MTs, fixed, and sedimented onto coverslips. Shown are representative immunofluorescence images of β-tubulin. Scale bar: 20 μm.

phospho-dependence, prometaphase cells arrested by the TN treatment was incubated with MG132, an inhibitor of proteasomes, together with BI 2536 (a Plk1 inhibitor) (Lenart et al., 2007), Purvalanol A (a Cdk1 inhibitor) (Skoufias et al., 2007) or VX680 (an Aurora A inhibitor) (Harrington et al., 2004) (Fig. 1G). The presence of MG132 prevents mitotic exit upon inhibition of various mitotic kinases (Seki et al., 2008b; Skoufias et al., 2007). Although the abundance of the Plk1-Kif2a complex was slightly increased in cells treated with either Aurora A or Cdk1 inhibitor (Fig. 1G, lanes 2-4), this complex dissociated in the presence of the Plk1 inhibitor, even though the levels of the Plk1 and Kif2a proteins in cell lysates were not altered by inhibition of Plk1 (Fig. 1G, lanes 5 and 6). We conclude that the kinase activity of Plk1 is required for the complex formation.

Plk1 promotes the depolymerase activity of Kif2a

As Kif2a is a Plk1-interacting protein (Fig. 1), we investigated whether Plk1 directly phosphorylates Kif2a in vitro. Recombinant Kif2a and Plx1, the Plk1 homolog from Xenopus, were expressed in and purified from SF9 cells. As shown in Fig. 2A, Kif2a was phosphorylated by the wild-type (WT) Plx1, but not by the inactive Plx1-N172A (kinase-dead; KD) (Qian et al., 1998), indicating that Kif2a is a substrate of Plx1 (and Plk1).

Next, we examined whether the depolymerase activity of Kif2a is regulated by Plx1. Recombinant Kif2a and Plx1 or Plx1-KD were first incubated with ATP and then with pre-polymerized MTs. The MTs remaining at the end of the incubation were pelleted through a glycerol cushion and assayed by silver-staining and western blotting (Fig. 2B) or by immunofluorescence (Fig. 2C). Incubation of MTs with Kif2a reduced the amounts of MTs in the pellet, indicating the presence of depolymerase activity (Fig. 2B, lanes 3 and 4 vs 1 and 2), whereas neither Plx1 nor Plx1-KD altered the amounts of MTs in the pellets (Fig. 2B, lanes 7, 8, 11 and 12 vs 1 and 2; Fig. 2C). Interestingly, incubation of Kif2a with Plx1 greatly enhanced the depolymerase activity of Kif2a (Fig. 2B, lanes 5 and 6 vs 3, 4, 7 and 8; Fig. 2C). This enhancement specifically resulted from the phosphorylation of Kif2a by Plx1, as Plx1-KD failed to stimulate Kif2a (Fig. 2B, lanes 9 and 10 vs 3, 4, 11 and 12). We conclude that Plk1 directly phosphorylates Kif2a and enhances its ability to depolymerize MTs.

Plk1 enhances the localization of Kif2a to spindle MTs and spindle poles

It has been reported that Plk1 controls spindle assembly and function in mitosis (Lenart et al., 2007; Sumara et al., 2004). We demonstrate here that Kif2a is a spindle-associated target for Plk1 regulation. Inhibition of Plk1 by a specific inhibitor, BI 2536, leads to a monopolar spindle (Lenart et al., 2007). However, a sub-population of cells incubated with BI 2536 reached metaphase with unaligned chromosomes (Fig. 3A), presumably because of a partial inhibition of Plk1. We quantified the effect of Plk1 inhibition on the localization of Kif2a and other spindle-pole-associated proteins, such as γ-tubulin and NuMA (nuclear mitotic apparatus protein), to the bipolar spindle in metaphase cells. Inhibition of Plk1 reduced the amounts of Plk1 associated with centrosomes (Fig. 3C), consistent with a previous report (Lenart et al., 2007). Interestingly, inhibition of Plk1 also reduced Kif2a signals on spindle MTs and poles, even though the amounts of total cellular Kif2a did not alter (Fig. 3B,C). Consistent with this, the amounts of spindle MTs were increased by 30% upon inhibition of Plk1 (Fig. 3C), despite the fact that the spindle-pole-associated polymerization activity, as indicated by γ-tubulin signals, was also substantially reduced (Fig. 3D). This reduction in Kif2a signals was even more significant when normalized to the spindle MT intensity (Fig. 3C). As a control, spindle pole signals for an unrelated protein, NuMA, were not altered by BI 2536 when normalized to the MT intensity (Fig. 3E). We conclude that efficient targeting of Kif2a to spindle MTs and poles requires the kinase activity of Plk1.
Recruitment of Plk1 to centrosomes appears independent of Kif2a

We analyzed the effect of Kif2a on the localization of Plk1 (Fig. 4A). Knockdown of Kif2a to the indicated level neither altered the steady-state levels of the Plk1 protein in mitosis, nor changed the centrosomal Plk1 signals (Fig. 4A-C). Similarly, γ-tubulin and NuMA signal intensities around spindle poles were not substantially affected by Kif2a (Fig. 4D,E). As expected from its depolymerase activity, depletion of Kif2a increased the spindle MT intensity (Fig. 4C), consistent with our previous report (Jang et al., 2008).

Aurora A interacts with Kif2a and inhibits its depolymerase activity

Spindle- and spindle pole-associated Kif2a only partially overlaps with the localization of Plk1, but colocalizes with the kinase Aurora A in mitosis (Fig. 5A) (Barr and Gergely, 2007), suggesting that Aurora A may be another regulator of Kif2a. Indeed, in a transient transfection experiment, Myc-Aurora A co-precipitated with GFP-Kif2a, but not with GFP (Fig. 5B), indicating a direct interaction.

To further investigate the relationship between Aurora A and Kif2a, we inhibited the kinase activity of Aurora A with a specific inhibitor, VX-680 (Harrington et al., 2004). Inactivation of Aurora A in metaphase cells did not alter the steady-state levels of the Kif2a protein, but increased the amounts of Kif2a associated with spindle MTs and spindle poles and decreased the amounts of spindle MTs (Fig. 5C-E). However, depletion of Kif2a did not alter the amounts of Aurora A associated with spindle MTs and
Fig. 5. Aurora A colocalizes and interacts with Kif2a and inhibits its recruitment and its depolymerase activity. (A) Maximum projections from deconvolved z-stacks of representative HeLa cells stained for Kif2a (green), Aurora A (red) and DNA (blue). (B) Myc-Aurora A was co-transfected with GFP-Kif2a or GFP into 293T cells. Twelve hours after transfection, cells were treated with 100 ng/ml of nocodazole for 18 hours. Cell lysates and anti-GFP immunoprecipitates (IP) were analyzed by western blotting. (C-E) HeLa cells were treated with 500 nM VX680 for 15 minutes, fixed and stained for Kif2a (green), β-tubulin (red) and DNA (blue). (C) Maximum projections from deconvolved z-stacks of representative control or VX680-treated HeLa cells. (D) Immunofluorescence intensities for β-tubulin and Kif2a on metaphase spindle were quantified and plotted (n=10 cells for each quantification). (E) Levels of endogenous proteins in VX680-treated cells were analyzed by western blotting with p38MAPK as a loading control. In D, *P<6.6×10^{-4}; **P<8.5×10^{-5}; ***P<1.1×10^{-4} (two-tailed t-test). A.U., arbitrary unit. (F) Recombinant His-Kif2a was incubated with recombinant His-Aurora A with or without VX680 in the presence of radioactive ATP and phospho-Kif2a was analyzed by SDS-PAGE. The amount of His-Kif2a used in the reaction was visualized by Coomassie blue staining. (G) Recombinant His-Kif2a (25 nM) was phosphorylated by His-Aurora A with or without VX680 and the reaction mix was incubated with Taxol-stabilized MTs for 10 minutes either in the presence or absence of VX680. MTs were then analyzed in a co-pelleting assay, followed by detection of MTs with silver staining and detection of Kif2a and Aurora A with western blotting. As controls, incubation of MTs with Aurora A alone or with VX680 alone did not alter the amounts of MTs pelleted (data not shown). sup., supernatant; –/–, no VX680 added; –/+ , VX680 added during the incubation with MTs, but not during the phosphorylation reaction with Aurora A; +/+, VX680 present both during the phosphorylation reaction and during the incubation with MTs. (H) His-Kif2a (25 nM) was phosphorylated by Aurora A and the reaction mix was then incubated with Taxol-stabilized MTs for 10 minutes, fixed, and sedimented onto coverslips. Shown are representative immunofluorescence images of β-tubulin. For optimal analysis of the inhibition of Kif2a by Aurora A, a higher concentration of Kif2a and a longer incubation time were used for samples in G and H compared to those in Fig. 2B,C. Scale bars: 5 μm (A,C); 20 μm (H).
with poles (Fig. 4F). Thus, Aurora A controls Kif2a localization and the MT density in the mitotic spindle, a conclusion independently confirmed in cells depleted of Aurora A in a siRNA experiment (data not shown).

Biochemically, recombinant Aurora A directly phosphorylated recombinant Kif2a and this phosphorylation was inhibited by VX680 (Fig. 5F). Furthermore, phosphorylation of Kif2a by Aurora A decreased its depolymerase activity in a manner dependent on the kinase activity of Aurora A (Fig. 5G, H). We conclude that Aurora A negatively regulates Kif2a by promoting the dissociation of Kif2a from the spindle MTs and poles and through inhibiting its depolymerase activity.

Discussion
Antagonistic regulation of Kif2a depolymerase by Plk1 and Aurora A
Kif2a is a MT depolymerase that plays a critical role in spindle assembly and dynamics in mitosis (Cameron et al., 2006; Desai et al., 1999; Ferenz and Wadsworth, 2007; Ganem and Compton, 2004; Ganem et al., 2005; Jang et al., 2008; Manning et al., 2007; Ohi et al., 2007). We report here the regulation of Kif2a in mitosis. We show that the biochemical and cellular activity of the Kif2a depolymerase is under the control of two mitotic kinases, Plk1 and Aurora A. Plk1 positively regulates Kif2a by enhancing its depolymerase activity and by promoting its recruitment to the spindle MTs and poles, whereas Aurora A inhibits Kif2a by decreasing its depolymerase activity and by interfering with its cellular recruitment.

Mechanistically, Plk1 and Aurora A directly interact with and phosphorylate Kif2a. The interaction between endogenous Plk1 and Kif2a is phospho-dependent and mitosis-specific. Substrates of Plk1 tend to interact with the Polo-box domain in Plk1 after being prime-phosphorylated by another kinase. Although Cdk1 has been shown to be a common priming kinase for Plk1 substrates (Barr et al., 2004), inhibition of Cdk1 enhances the Kif2a-Plk1 interaction, indicating that Cdk1 is not the priming kinase. Similarly, Aurora A is not the priming kinase. Instead, Plk1 itself appears to be the priming kinase that is required for the Kif2a-Plk1 interaction, as inhibition of the kinase activity of Plk1 abolished this interaction.

Even though the interaction between endogenous Kif2a and Aurora A has escaped our detection so far (data not shown), presumably because of the transient nature of a weak interaction, Kif2a and Aurora A colocalize, and ectopically expressed proteins form a complex. It is interesting to note that MCAK, a member of the kinesin-13 family of MT depolymerases, is also phosphorylated by Aurora A and Aurora B, and phosphorylation controls the localization of MCAK and inhibits its depolymerase activity (Andrews et al., 2004; De Luca et al., 2008; Knowlton et al., 2006; Lan et al., 2004; Ohi et al., 2004; Sampath et al., 2004; Zhang et al., 2008; Zhang et al., 2007).

In theory, phosphorylation of Kif2a by either Plk1 or Aurora A may change its affinity to MTs, the rate of MT depolymerization or the processivity of the depolymerase. In addition, phosphorylation may also alter the mode of interaction between Kif2a and MTs (binding to the MT lattice versus binding to MT ends). We have tried extensively, but failed because of technical difficulties, to map the sites of phosphorylation in endogenous Kif2a purified from mitotic cells as well as in recombinant Kif2a phosphorylated in vitro by either Aurora A or Plk1. Although the sites of phosphorylation of MCAK by Aurora A and Aurora B have been analyzed previously (Andrews et al., 2004; Lan et al., 2004; Zhang et al., 2008; Zhang et al., 2007), these sites are not highly conserved in Kif2a. Thus, the exact biochemical mechanisms by which phospho-Kif2a alters its depolymerase activity remain a subject for future investigation.

Physiological function of antagonistic regulation on Kif2a
We propose that the antagonistic nature of Kif2a regulation by Plk1 and Aurora A provides a mechanism for spatial control of the spindle assembly and MT dynamics. During mitosis, MTs polymerized from chromatin are sorted into a spindle structure by molecular motors and by MT-associated proteins (Gadde and Heald, 2004). Aurora A phosphorylates and inhibits Kif2a on spindle MTs, thereby stabilizing the minus-ends of MTs before they are anchored onto centrosomes. Through its regulation of Kif2a, Aurora A promotes MT growth and spindle assembly. However, once the minus-ends of MTs are incorporated into spindle poles, centrosomal Plk1 then binds to Kif2a and activates its depolymerase activity, which promotes MT dynamics and generates the pulling force to establish tension across sister kinetochores, consistent with the fact that Plk1 is required for the interkinetochore tension (Sumara et al., 2004). Thus, the antagonistic regulation of Kif2a by Plk1 and Aurora A may result in differential dynamics of MTs on the spindle and at the poles, and this differential dynamics may be essential for efficient assembly and proper function of the mitotic spindle.

Regulation of Kif2a by Plk1 is probably not restricted to spindle poles alone, as the Plk1-Kif2a complex is abundant in nocodazole-arrested prometaphase cells, in which Kif2a does not colocalize with Plk1 on centrosomes (data not shown). We propose that the Plk1-Kif2a complex has a general role in cytosol to depolymerize MTs generated outside the mitotic spindle. In this model, cytosolic Kif2a is predominantly associated with Plk1, not with Aurora A, consistent with the differential stability between Plk1-Kif2a and Aurora A-Kif2a complexes. Plk1 stimulates the depolymerase activity of the cytosolic Kif2a and reduces the amounts of MTs polymerized in cytosol in general. However, chromatin, through the Ran-GTP pathway, recruits Aurora A (Ducat and Zheng, 2004; Goodman and Zheng, 2006), which inhibits Kif2a and promotes MT polymerization. Thus, Aurora A and Plk1 confer a differential stability to MTs existing inside compared with those outside of the mitotic spindle, thereby ensuring that MTs are predominantly polymerized and assembled around the chromatin into the mitotic spindle. We speculate that the antagonistic regulation of Kif2a by Plk1 and Aurora A provides spatial cues for spindle assembly.

Materials and Methods
Plasmids and antibodies
GFP-Kif2a, Myc-Plk1, and Myc-Aurora A were subcloned into pCS2+ containing a N-terminal GFP or myc tag. Recombinant His-Kif2a (a generous gift from Claire Walczak, Indiana University, Bloomington, IN) was expressed in Sf9 cells for 56 hours and purified using a NTA-agarose column (Quagen, Inc.) in 1× BRB80 buffer (80 mM Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) containing 125 mM KCl, 2 mM EGTA, 1 mM DTT, 10 μg/ml each of leupeptin, pepstatin and chymostatin. Recombinant His-Plk1 and His-Plk1-N172A (kinase-dead, KD) (Qian et al., 1998) were expressed in Sf9 cells for 44 hours and then treated with 250 nM okadaic acid for 4 hours to activate the kinase activity of wild-type Plk1 (Kumagai and Dumphry, 1996). Plk1 was purified with Talon beads (BD Bioscience).

The anti-GFP sera were raised against the full-length recombinant GFP and affinity purified. The anti-Kif2a antibody was a generous gift from Claire Walczak (Indiana University, Bloomington, IN). The anti-NumA antibody was kindly provided by Duane Compton (Dartmouth Medical School, Hanover, NH). Anti-β-tubulin E7 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank.

The following antibodies were from commercial sources: anti-γ-tubulin (Sigma); anti-Kif2a (Novus); anti-Plk1, anti-Aurora A, anti-Myc, and anti-p38MAPK antibodies (Santa Cruz Biotechnology).
Cell Culture, siRNAs and transfection

HeLa and HeLa S3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) and antibiotics. Cells were synchronized at the G1-S boundary by a double-thymidine treatment or at prometaphase by a thymidine-nocodazole treatment, as previously described (Fang et al., 1998a; Fang et al., 1998b). siRNA duplexes were synthesized by Dharmacon, Inc. The sequence targeting Plx1 was 5’-AGATTGGTCCTAGGTTCT-3’ and Kif2a was 5’-GGAGTCGTCCTCCCTGAACTTT-3’. The control siRNA (siGLO2) was 5’-CGTACGGGAAATCTGATT-3’. siRNAs were transfected into HeLa cells using DharmaFECT 1 (Dharmacon, Inc.).

DNA transfection was performed using Effectene (Qiagen) or Lipofectamine 2000 (Invitrogen) as instructed by the manufacturers. Cells co-transfected with GFP-Kif2a and Myc-Plk1 or Myc-Aurora A were analyzed at 36 hours post-transfection.

Immunoprecipitation

Antibodies against Plk1, Aurora A or GFP were coupled to Affi-Prep Protein A beads (Bio-Rad, Richmond, CA) at a concentration of 0.3 mg/ml. HeLa or HeLa S3 cells were lysed in NP-40 lysis buffer (50 mM HEPES, pH 7.4, 200 mM KCl, 0.3% NP-40, 10% glycerol, 1 mM EGTA, 1 mM MgCl2, 0.5 mM DTT, 0.5 μM microcystin, 10 μg/ml each of leupeptin, pepstatin and chymostatin). Lysates were centrifuged, incubated at 4°C for 1 hour with Protein A beads coupled to pre-immune rabbit IgG, and then incubated at 4°C overnight with Protein A beads coupled to specific antibodies. Antibody beads were recovered by centrifugation, washed five times with the lysis buffer and then twice with the lysis buffer in the presence of 500 mM KCl, analyzed by SDS-PAGE, and immunoblotted with appropriate antibodies.

Immunofluorescence

HeLa cells on coverslips were fixed with –20°C methanol for 30 minutes. Alternatively, cells were extracted briefly with the BRB80-T buffer (80 mM Pipes, pH 6.8, 1 mM MgCl2, 5 mM EGTA, analyzed by SDS-PAGE, and immunoblotted with appropriate antibodies. 500 mM KCl, 15 mM MgCl2, 1 mM DTT, 1 mM ATP and 15 μM Taxol at room temperature for 3 minutes (Fig. 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Cell Biol. 117, 304-315.


References


Plk1 and Aurora A control Kif2a activity


