Aurora B Phosphorylates Multiple Sites on Mitotic Centromere-associated Kinesin to Spatially and Temporally Regulate Its Function[□]

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Chromosome congression and segregation require the proper attachment of microtubules to the two sister kinetochores. Disruption of either Aurora B kinase or the Kinesin-13 mitotic centromere-associated kinesin (MCAK) increases chromosome misalignment and missegregation due to improper kinetochore-microtubule attachments. MCAK localization and activity are regulated by Aurora B, but how Aurora B phosphorylation of MCAK affects spindle assembly is unclear. Here, we show that the binding of MCAK to chromosome arms is also regulated by Aurora B and that Aurora B-dependent chromosome arm and centromere localization is regulated by distinct two-site phosphoregulatory mechanisms. MCAK association with chromosome arms is promoted by phosphorylation of T95 on MCAK, whereas phosphorylation of S196 on MCAK promotes dissociation from the arms. Although targeting of MCAK to centromeres requires phosphorylation of S110 on MCAK, dephosphorylation of T95 on MCAK increases the binding of MCAK to centromeres. Our study reveals a new role for Aurora B, which is to prevent excess MCAK binding to chromatin to facilitate chromatin-nucleated spindle assembly. Our study also shows that the interplay between multiple phosphorylation sites of MCAK may be critical to temporally and spatially control MCAK function.

INTRODUCTION

The bipolar mitotic spindle is composed of a dynamic array of microtubules (MTs) and MT-associated proteins, which function to segregate the chromosomes during mitosis (Gadde and Heald, 2004). Dynamic MTs are critical for the organization and function of the mitotic spindle. There are multiple proteins that regulate MT dynamics, including both MT-stabilizing and -destabilizing proteins. Mitotic centromere-associated kinesin (MCAK) is an MT-destabilizing protein that plays important roles in mitotic spindle assembly and chromosome segregation (for review, see Moore and Wordeman, 2004). Depletion or inhibition of MCAK in cells or from *Xenopus* egg extracts causes abnormal spindles with long and less dynamic MTs, as well as improperly attached chromosomes that lead to chromosome missegregation (Wordeman and Mitchison, 1995; Walczak *et al.*, 1996; Maney *et al.*, 1998; Kline-Smith and Walczak, 2002; Kline-Smith *et al.*, 2004; Stout *et al.*, 2006; Ohi *et al.*, 2007).

The role of dynamic MTs in spindle assembly is not yet fully understood. The conventional model of search and capture posits that in early mitosis, MTs undergo rapid growth and shrinkage with their plus ends distal to the spindle pole until they are connected and stabilized by kinetochores, the specialized protein complexes around the centromere region of the chromosomes (for review, see Compton, 2000; McIntosh *et al.*, 2002; Kline-Smith *et al.*, 2004). Although centrosomes are at the major MT nucleation center in most cells and they play fundamental roles in spindle assembly, they are not absolutely required for spindle assembly. Removing the centrosomes by laser ablation does not disturb bipolar spindle formation or mitotic progression of the cells (Khodjakov *et al.*, 2000; Hinchcliffe *et al.*, 2001). It is thought that in the absence of centrosomes, the chromatin can also nucleate MTs to help form the bipolar spindle. In support of this idea, in some systems that lack centrosomes, such as plant cells and female meiosis, chromatin provides the major driving force for MT nucleation and spindle assembly (for review, see Wadsworth and Khodjakov, 2004). These studies support a model in which the chromosomes can change the surrounding cytoplasm to favor MT nucleation and polymerization. Although the kinetochore itself can nucleate MTs to facilitate spindle formation (Rieder, 2005), chromatin without kinetochores is also sufficient to nucleate MTs and to form bipolar spindles in *Xenopus* egg extracts (Heald *et al.*, 1996).

The mechanism of chromatin-nucleated MT assembly is starting to be uncovered, and it includes the action of the small GTP-binding protein Ran (Carazo-Salas *et al.*, 1999; Kalab *et al.*, 1999; Ohba *et al.*, 1999; Wilde and Zheng, 1999;

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Abbreviations used: GFP, green fluorescent protein; MT, microtubule; PAGE, polyacrylamide gel electrophoresis.

Zhang *et al.*, 1999; Gruss *et al.*, 2001). It is thought that chromosome-bound RCC1, a guanine nucleotide exchange factor for the small GTPase Ran, locally generates a Ran-GTP gradient, which releases spindle assembly factors from their inhibitors, called importins (Carazo-Salas *et al.*, 1999; Gruss *et al.*, 2001; Nachury *et al.*, 2001; Wiese *et al.*, 2001; Wilde *et al.*, 2001; Ems-McClung *et al.*, 2004). Recent studies showed that Aurora B, a mitotic kinase, acts in a separate Ran-independent pathway through MCAK and Op18 to facilitate chromatin-mediated spindle formation (Sampath *et al.*, 2004; Gadea and Ruderman, 2005, 2006; Kelly *et al.*, 2007). Inhibition or depletion of Aurora B kinase leads to spindle disassembly around chromatin in *Xenopus* egg extracts (Sampath *et al.*, 2004), indicating that Aurora B controls the ability of chromatin to nucleate MTs. In contrast, depletion of MCAK restores MT formation in Aurora B complex-depleted extracts and cells (Sampath *et al.*, 2004; Tulu *et al.*, 2006), which indicates that MCAK is necessary in the Aurora B-regulated chromatin-induced spindle assembly pathway.

Aurora B is a member of the chromosome passenger complex together with Incenp, Survivin, Borealin/Dasra B, and TD-60 (Cooke *et al.*, 1987; Andreassen *et al.*, 1991; Adams *et al.*, 2000; Bolton *et al.*, 2002; Mollinari *et al.*, 2003; Sampath *et al.*, 2004; Vagnarelli and Earnshaw, 2004). This complex is named based on its localization during mitosis, in which its components localize to chromosomes/centromeres early in mitosis and then target to other destinations, such as the central spindle, in late mitosis (Earnshaw and Bernat, 1991). The initial loading on the chromosome in early mitosis is thought to provide a mechanism of conveyance for their later positioning (Earnshaw and Bernat, 1991). Aurora B regulates multiple processes in mitosis, including chromatin condensation, chromosome-MT attachments, chromosome segregation, and cytokinesis through phosphorylation of multiple substrates (for review, see Carmena and Earnshaw, 2003; Vagnarelli and Earnshaw, 2004). For example, histone H3 Ser 10 phosphorylation by Aurora B dissociates heterochromatin protein 1 from the chromosome, which may affect heterochromatin formation (Fischle *et al.*, 2005; Hirota *et al.*, 2005). In addition, Aurora B is essential for the completion of cytokinesis, and its substrates during this process include, but are not limited to, myosin II regulatory chain, vimentin, desmin, and glial fibrillary acidic protein (Carmena and Earnshaw, 2003). Of particular interest are the studies showing that Aurora B also plays a crucial role in kinetochore-MT mal-attachment correction through regulation of MCAK and Ndc80/Hec1 (Cheeseman *et al.*, 2002, 2006; Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004; Deluca *et al.*, 2006).

Aurora B phosphorylates MCAK at multiple sites in both mammalian cells and in *Xenopus* egg extracts. Aurora B phosphorylation of MCAK within its neck region at S196 inhibits its MT depolymerization activity (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004). In addition, inhibition or depletion of Aurora B abolishes the ability of MCAK to target to centromeres (Andrews *et al.*, 2004; Lan *et al.*, 2004), and phosphorylation-deficient mutant derivatives of MCAK have different turnover kinetics at centromeres, suggesting that Aurora B regulation of centromeric MCAK may be particularly critical (Andrews *et al.*, 2004). Centromeric MCAK is important for correcting mal-attachments of kinetochores to MTs, which is crucial for proper chromosome alignment and segregation (Walczak *et al.*, 2002; Kline-Smith *et al.*, 2004). These studies highlight the important roles of Aurora B phosphorylation of MCAK in chromosome alignment, but they do not address the molecular mechanism by which Aurora B regulates MCAK function.

Although the regulation of MCAK function by Aurora B has been studied intensely, there are still some key questions that remain unanswered. Aurora B phosphorylates MCAK at multiple sites, but the contribution of each of these sites to MCAK function is not clear. In addition, the mechanism by which Aurora B promotes MCAK localization to centromeres is still unknown. Here, we present an analysis of the multisite phosphoregulation of MCAK in both *Xenopus* egg extracts and in somatic cells. We find that chromosome arm-bound MCAK plays a critical role in chromatin-driven spindle assembly and that MCAK localization to centromeres and chromosome arms is controlled by distinct two-site Aurora B phosphoregulatory mechanisms.

MATERIALS AND METHODS

Cloning, Protein Expression, and Fluorescent Labeling

The MCAK phosphorylation point mutant constructs were constructed by using site-directed mutagenesis of a parental plasmid containing full-length *Xenopus* MCAK by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to directions of the manufacturer. They were then subcloned into either a 6His-green fluorescent protein (GFP) or a pFast-Bac1 (pFB) vector to make 6His-GFP-MCAK-CEN and pFB-MCAK mutant constructs. GST-MCAK-2-149 and GST-MCAK-187-263 mutant constructs were mutated directly using the QuikChange mutagenesis kit. The point mutations resulted in the following amino acid (aa) changes in the expressed protein: S37A, S38A, T95A, S110A, S125A, S161A, T162A, S165A, S177A, S196A, S230A, T246A, and S253A. All constructs were confirmed by sequencing.

6HisGFP-tagged MCAK-CEN (aa2-263 + aa630-664) and its mutant constructs were expressed in BL21 cells and purified through nickel-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA) as described previously (Kline-Smith *et al.*, 2004). The purified protein was dialyzed into buffer 1 (10 mM HEPES, pH 7.2, 100 mM KCl, 25 mM NaCl, 50 mM sucrose, 0.1 mM EDTA, and 0.1 mM EGTA), aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C . Glutathione S-transferase (GST)-tagged MCAK-2-149 and GST-tagged Neck-187-263 mutant constructs were expressed in DH5 α bacterial cells and purified through immobilized glutathione 4% beaded agarose (Pierce Chemical, Rockford, IL) as described previously (Walczak *et al.*, 1996). The purified GST-fusion proteins were dialyzed into buffer 1, aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C . Full-length MCAK (FL-MCAK) or its phosphorylation site mutants (T95A, S110A, S196A, T95E, S196E, T95ES196E, and T95ES196A) and full-length GFP-MCAK (GMCAK) (aa2-731) and its truncations (aa187-731, aa187-592, and aa263-592) were expressed in SF9 or High Five insect cells and purified using conventional chromatography as described previously (Desai *et al.*, 1999b; Ems-McClung *et al.*, 2007). His-RanL43E was purified as described previously (Ems-McClung *et al.*, 2004).

GST-tagged MCAK-2-149 mutant proteins were fluorescently labeled with Alexa Fluor protein labeling kits (succinimidyl ester) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Protein concentrations are expressed in terms of monomer concentration, and they were quantified from colloidal Coomassie blue G-250-stained SDS-polyacrylamide gels and densitometry using bovine serum albumin (BSA) as a standard. Densitometry was done using ImageJ software (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>).

In Vitro Kinase Assays

For kinase assays of MCAK-CEN, Aurora B kinase was purified as described previously (Lan *et al.*, 2004). For kinase assays of MCAK-2-149, Aurora B kinase was purified as reported previously (Resnick *et al.*, 2006). The kinase assays were performed as follows: for every 10 μl of reaction, 100 ng of kinase was incubated with 5 μM of substrate and ATP mix (0.15 mM ATP and 0.4 μCi of [γ - ^{32}P] ATP) in cytosolic factor (CSF)-XB (10 mM HEPES, pH 7.2, 2 mM MgCl_2 , 0.1 mM CaCl_2 , 100 mM KCl, 5 mM EGTA, and 50 mM sucrose) at room temperature (RT) for 30–60 min. Reactions were stopped by the addition of 2X sample buffer (0.125 M Tris, pH 6.8, 3% SDS, 20% glycerol, and 2% β -mercaptoethanol) and boiled for 5 min. Equal volumes of each reaction were separated on 10 or 15% gels by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were stained with Coomassie G250 and scanned. The band intensity was quantified with ImageJ. ^{32}P incorporation was quantified using a Typhoon PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). For each assay, the phosphate incorporation was determined for each protein by normalizing for the protein loaded from Coomassie-stained gels, and the relative phosphate incorporation for MCAK-2-149(T95A), MCAK-2-149(S110A) and MCAK-2-149(T95A-S110A) was then determined by making the wild-type (Wt) phosphate incorporation equal to

100%. The values reported represent the mean $\pm$ the SD from three separate experiments. All calculations were done in Excel (Microsoft, Redmond, WA).

Antibody Production, Affinity Purification, and Western Analysis

Phosphorylated peptide pT95 (QNHKRR-pT-ISKIPA-C) and the corresponding nonphosphorylated peptide T95 (QNHKRR-T-ISKIPA-C) were synthesized by the Protein Chemistry Core Laboratory (Baylor College of Medicine, Houston, TX) and coupled to mKLLH (Pierce Chemical) according to the manufacturer's instructions. Rabbit polyclonal antibodies were made against the conjugated peptides using the services of Covance Research Products (Denver, PA). Phosphopeptide affinity columns were made by coupling the phosphopeptides to SulfoLink-coupling gel (Pierce Chemical) according to the manufacturer's instructions. The immune serum was affinity-purified on the phosphopeptide affinity column as described previously (Lan *et al.*, 2004).

For all Western blots, samples were separated by 10 or 15% SDS-PAGE and transferred to Protran nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). For characterization of the phospho-specific antibodies, egg extracts were treated with control dimethyl sulfoxide (DMSO) or 1–5 μ M microcystin-LR, and then they were incubated at RT for 30–40 min before the samples were resuspended in 2X sample buffer. All blots were blocked in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl [TBS], 0.1% Tween 20 [TBST], and 5% nonfat dry milk) for 1 h at RT. Primary antibodies, α -NT2-XMCAK (0.5 μ g/ml) (Walczak *et al.*, 1996), α -pS196 (1.6 μ g/ml) (Lan *et al.*, 2004), α -T95 (2 μ g/ml), α -pT95 (2 μ g/ml), DM1 α anti-tubulin antibody (1/5000) (Sigma-Aldrich, St. Louis, MO), or α -XKID serum (1/1000; a kind gift from Hiro Funabiki, Rockefeller University) were diluted in Ab Dil-T (TBST, 2% BSA, and 0.1% sodium azide). α -pH3-Ser 10 (Cell Signaling Technology, Danvers, MA) was diluted 1/1000 in blocking buffer. Blots were incubated with primary antibodies for 1 h, washed in TBST, incubated for 1 h with 1/5000–1/10,000 dilution of goat anti-mouse or donkey anti-rabbit horseradish peroxidase secondary antibodies (GE Healthcare) diluted in blocking buffer, washed in TBST, washed in TBS, and then developed with SuperSignal West Pico Chemiluminescence Substrate (Pierce Chemical). Densitometry with ImageJ was used for quantification of signals on Western blots. For Figure 1D, the amount of MCAK in control or drug-treated samples was quantified by calculating the band intensity of MCAK, which was normalized to the amount of XKID, a loading control for the amount of pelleted chromatin.

Cell Culture and Immunofluorescence

Xenopus S3 cells or XL177 cells were cultured in L-15 medium (60% Leibovitz-15, 10% fetal bovine serum [FBS], and 1% Pen-Strep) (Invitrogen). For hesperadin treatment, 250 or 500 nM hesperadin was used to treat cells for 4–5 h, and then the cells were processed for immunofluorescence. For immunofluorescence, cells were plated on poly-L-lysine (Sigma-Aldrich)-coated coverslips for 2–3 d, washed in PBS (12 mM phosphate, pH 7.4, 137 mM NaCl, and 3 mM KCl), fixed in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO₄, pH 7.0) containing 0.5% Triton X-100, 2% formaldehyde for 20 min, and then washed two times in TBS-TX (TBS and 0.1% Triton X-100) for at least 10 min each wash. The cells were blocked in Ab Dil-Tx (TBS-TX, 2% BSA, and 0.1% Azide) for 1 h at RT or 4°C overnight; incubated in primary antibodies α -NT2-XMCAK (0.5 μ g/ml), α -T95 (2 μ g/ml), α -pT95 (2 μ g/ml) or α -GST (0.5 μ g/ml) for 30 min; and washed with TBS-TX and incubated in 1/50 dilution of goat anti-rabbit fluorescein isothiocyanate secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. All primary and secondary antibodies were diluted in Ab Dil-Tx. Cells were washed three times in TBS-TX. DNA was stained with 2 μ g/ml Hoechst diluted in TBS-TX after secondary antibody application, washed, and mounted in mounting media (90% glycerol, 20 mM Tris-HCl, pH 8.8, and 0.5% *p*-phenylenediamine). For peptide competition experiments, we added a fivefold molar excess of the corresponding phospho- or dephospho-peptide in the primary antibody solution, preincubated them at 4°C for 1 h, and then processed the samples as described above.

For staining cells with two rabbit antibodies, we fluorescently labeled each of them with Alexa Fluor protein labeling kits (succinimidyl ester) according to the manufacturer's instructions (Invitrogen). Specifically, α -NT2-XMCAK (α -MCAK) was labeled with Alexa 488 (α -MCAK-Alexa 488) or Alexa 594 (α -MCAK-Alexa 594), α -pT95 was labeled with Alexa 594 (α -pT95-Alexa 594), and α -pS196 was labeled with Alexa 488 (α -pS196-Alexa 488). For each labeling reaction, 500 μ l of 1 mg/ml antibody was used. Fixed cells were costained with two differently labeled primary antibodies (1/400 dilution, a final concentration of 1 μ g/ml) for 1 h at RT, washed in TBS-TX, and mounted with mounting media. DNA was stained with Hoechst.

Immunodepletion and Spindle/Kinetochore Assembly in *Xenopus* Egg Extracts

CSF-arrested *Xenopus* egg extracts were prepared as described previously (Murray, 1991). Immunodepletion was done with 10 μ g of antibody/25 μ l of protein G-Dynabeads (Invitrogen)/100 μ l extract. α -MCAK, nonimmune immunoglobulin (IgG), or α -Aurora B antibodies were incubated with the beads at 4°C for 2 h in PBS and then washed two times in PBS or TBS and two times

in CSF-XB. The beads were isolated on a magnet, resuspended in extract, and incubated on ice for 1.5 h without further perturbation. After incubation, the extracts were separated from the beads using a magnet for 30 min in aliquots of no >150 μ l.

X-Rhodamine-labeled tubulin was included in all extracts at 50 μ g/ml to visualize MTs. For drug addition, the stock hesperadin concentration was 2 mM, and the stock ZM447439 concentration was 10 mM in DMSO. Hesperadin and ZM447439 stocks were diluted in extracts to make a 100 and 400 μ M dilution stock, respectively. As a control, DMSO was added at the same dilution as hesperadin and ZM447439 to the extracts. The 100 μ M hesperadin or 400 μ M ZM447439 dilution stock was further diluted 1/20 into extracts to make a 5 μ M hesperadin or 20 μ M ZM447439 reaction concentration. The highest final concentration of a DMSO control was 0.25%. The 5 μ M hesperadin or 0.25% DMSO extract was further diluted 1/5 in 20- μ l reactions to make a final reaction concentration of 1 μ M hesperadin or 0.05% DMSO. Before spindle assembly, IgG-depleted extracts or MCAK-depleted extracts were supplemented with control CSF-XB buffer, MCAK, or MCAK mutant proteins so that the final concentrations of all proteins were identical and did not exceed 1/10 the volume of the extract. For assays of kinetochore targeting and chromatin binding, sperm nuclei were added directly to CSF extracts without cycling. For cycled spindle assembly, sperm nuclei were added to CSF extracts that were depleted with control IgG or with anti-MCAK antibodies. MCAK proteins were added to MCAK-depleted extracts at 2 times the endogenous MCAK concentration and then cycled into interphase by the addition of CaCl₂ and incubated for 60–90 min at RT. After 20 min on ice, fresh control or MCAK-depleted CSF extracts were added, and spindles were assembled for 90 min as described previously (Desai *et al.*, 1999a). Quantification of the MT structures was as reported previously (Ems-McClung *et al.*, 2007).

For kinetochore assembly on chromatin, recombinant proteins were diluted to equal concentrations in CSF-XB and added to the kinetochore assembly reactions to a dilution of 1/20. Chromatin was assembled for 45 min in noncycled extracts in which 10 μ g/ml nocodazole was added to inhibit MT polymerization (Walczak *et al.*, 2002). After spindle or kinetochore assembly, the structures were fixed in 2% formaldehyde, 30% glycerol, BRB80 (80 mM 1,4-piperazinediethanesulfonic acid [PIPES], pH 6.8, 1 mM MgCl₂, and 1 mM EGTA), 0.5% Triton X-100, and 2.5 mM MgCl₂ for 10 min and then spun onto coverslips through a 40% glycerol cushion in BRB80 at 16°C using a Beckman JS7.5 rotor (Beckman Coulter, Fullerton, CA) for 20 min. Coverslips were postfixed in –20°C methanol for 5 min and rehydrated by washing two times in TBS-TX for 10 min (Desai *et al.*, 1999a). All other processing for immunofluorescence is as described above for immunofluorescence.

DNA-coated chromatin beads were prepared essentially as described previously (Heald *et al.*, 1996). Before adding to extracts, the beads were washed three times in CSF-XB. Beads were added to extracts at 10 μ l of beads/100 μ l of extract, and the extracts were cycled through interphase and then back into mitosis with a second CSF extract addition. The beads were isolated on a magnet stand for 30 min and washed three times with CSF-XB and three times with TBS-TX. The beads were resuspended in 2X sample buffer at a concentration of 5 to 30 μ l of original extract and then boiled before running on 10% SDS-PAGE gels.

Ran or DMSO Aster Assembly in Hesperadin-treated *Xenopus* Egg Extracts

To assemble Ran asters, His-RanL43E (Wilde and Zheng, 1999) was added to a final concentration of 25 μ M on ice to CSF extracts that contained X-rhodamine tubulin and hesperadin or control DMSO. Hesperadin or control DMSO was added to the extracts as described above. DMSO asters were assembled by adding DMSO to a final concentration of 5%. Reactions were incubated at RT for 30 min, fixed, and sedimented onto coverslips as described for spindle assembly reactions except that the centrifugation step was for 30 min and then processed for immunofluorescence as described above.

Imaging and Quantitative Immunofluorescence Analysis

Images were acquired with a 40 $\times$ 1.0 Plan Apo, 60 $\times$ 1.4 Plan Apo VC, or a 100 $\times$ 1.4 Plan Apo VC objective mounted on a Nikon 90i microscope with a Photometrics CoolSNAP HQ cooled charge-coupled device camera. The microscope, camera, and filter wheels were controlled by MetaMorph (Molecular Devices). For control and experimental reactions in each experiment, images were taken with the same exposure time and scaled identically. For Figure 2A, the exposure times of individual proteins are different to make the chromatin staining visible, but they are the same between the control and hesperadin-treated reactions. Quantification of immunofluorescence staining was determined using MetaMorph and Excel. All images were processed in Adobe Photoshop (Adobe Systems, Mountain View, CA) and assembled in Adobe Illustrator.

To quantify the centromere/chromosome arm ratio in cells, three to five cell images were taken from each of two coverslips for each treatment in three independent experiments for a total of 6–10 cells/experiment and ~25 cells total. For each cell, approximately five centromeres/chromosome arm regions were quantified so that ~120 centromeres/chromosome arm regions were quantified in total. To quantify the chromosome arm fluorescence intensity,

the fluorescence intensity was measured in a box of 20×20 pixels drawn on the chromosome arm minus a background 20×20 pixel box drawn outside of the cell. To calculate the centromere fluorescence intensity, a box of 20×20 pixels was drawn on an area including a single centromere, and then a background measurement was taken on the chromosome arm so that the fluorescence intensity would represent that only of the centromere and not the surrounding chromosome arm. We then used the values of chromosome arm and centromere fluorescence intensity to calculate the ratio of the centromere to chromosome arm staining. To prevent oversampling of data from a single cell, we took an average of all of the centromere and chromosome arm staining intensities from a single cell and then calculated the ratio of these averages for a given cell. To do a statistical analysis, we averaged the ratios of centromere-to-chromosome arm intensities in all 6–10 images from a single experiment, which was used as the mean and then calculated the SEM from the three independent experiments. To carry out this analysis on chromatin assembled in egg extracts, a similar protocol was followed except that the chromosome arm and centromere intensities were obtained from approximately five centromere or chromosome regions on an individual chromatin mass. Each chromatin mass was treated equivalently to a cell, and the data were obtained from three independent extracts.

RESULTS

Inhibition of Aurora B Causes Excessive MCAK Binding to Chromosome Arms

The Kinesin-13 MCAK was originally identified as a protein that localizes to centromeres and was later shown to function there to correct improper kinetochore-MT attachments (Wordeman and Mitchison, 1995; Walczak *et al.*, 1996; Kline-Smith *et al.*, 2004). Immunofluorescence staining of MCAK shows that in interphase cells, MCAK is found within the nucleus, in the cytoplasm, and at the plus ends of MTs (Wordeman and Mitchison, 1995; Kline-Smith *et al.*, 2004; Moore *et al.*, 2005). In G2, MCAK localizes to the centromeres, but there is also visible nuclear/chromatin staining that is likely to be associated with the chromosome arms (Figure 1A). In late prophase/early prometaphase, MCAK staining becomes more concentrated on centromeres

and is less prominent on chromosome arms (Figure 1A). To assess the difference between MCAK localization in G2 and prophase, we determined the ratio of α -MCAK fluorescence intensity on centromeres to chromosome arms in each stage. We found that the centromere-to-chromosome arm fluorescence intensity ratio was increased approximately twofold from G2 to prophase. This increased ratio could be due to either an increased binding of MCAK to centromeres and/or to a decreased association of MCAK with chromatin. We found that the centromere-bound MCAK increased $53 \pm 11\%$ and the chromosome arm bound MCAK decreased $34 \pm 9\%$, suggesting that both the centromere- and chromosome arm-bound MCAK are affected.

Because Aurora B regulates the association of MCAK with centromeres (Andrews *et al.*, 2004; Lan *et al.*, 2004), we hypothesized that Aurora B also controlled the association of MCAK with chromosome arms. It is interesting that although inhibition of Aurora B by the small-molecule inhibitor hesperadin (Hauf *et al.*, 2003) causes a significant decrease of MCAK at the centromeres in mitotic cells during prometaphase to early anaphase (Andrews *et al.*, 2004; Lan *et al.*, 2004), it only moderately reduces the centromere-bound MCAK in prophase. However, treatment of cells with hesperadin increased the amount of MCAK in the chromatin region in prophase (Figure 1B). Quantification of the fluorescence of anti-MCAK staining in control and hesperadin-treated cells showed that hesperadin increased MCAK in the chromatin region by $81 \pm 17\%$, whereas the centromere staining decreased by $48 \pm 11\%$.

To study the regulation of the chromosome arm bound MCAK, we used *Xenopus* egg extracts in which chromatin can be assembled in vitro from sperm nuclei. We found that in the absence of MTs, MCAK bound to centromeres and chromosome arms of chromatin assembled in noncycled

Figure 1. MCAK localizes to chromosome arms in an Aurora B-dependent manner. (A–C) Immunostaining of DNA with Hoechst and MCAK with α -MCAK antibody. Bar, 5 μ m. (A) XL177 cells in G2 and prophase. (B) XL177 cells treated with control DMSO (Control) or 250 nM hesperadin (Hesp). (C) Chromatin assembled in nocodazole-treated *Xenopus* egg extracts in the presence of control DMSO (Control) or 5 μ M hesperadin (Hesp). (D) Western analysis of egg extracts treated with control DMSO (Control), 1 or 5 μ M hesperadin. Blots were probed with α -MCAK, α -pH3, α -pS196, and α -pT95. (E) Western analysis of chromatin assembled on DNA-coated beads in the presence of Aurora B inhibitors. Chromatin-coated beads were assembled in the presence of control DMSO (Control), 5 μ M Hesp, or 20 μ M ZM447439 (ZM); the beads were isolated; and chromatin proteins were analyzed with Western blot with α -MCAK or α -Xkid as chromatin loading control.

CSF extracts (Figure 1C). Addition of hesperadin abolished MCAK localization to centromeres when added at 1 μ M to chromatin assembly reactions, consistent with previous results in extracts and in cells (data not shown) (Andrews *et al.*, 2004; Lan *et al.*, 2004). However, the chromosome arm staining was not reduced. In fact, with 5 μ M hesperadin, MCAK staining on chromatin was actually increased two- to five-fold (Figure 1C). The same results were obtained in Aurora B-depleted extracts, and in extracts treated with another Aurora B inhibitor ZM447439 (data not shown), which indicates that the increased localization of MCAK on chromatin is a specific effect of Aurora B inhibition. The increased MCAK binding to chromatin was not due to increased MCAK binding at centromeres, because inhibition of Aurora B also increased MCAK on chromatin assembled on DNA beads (Figure 1E). Furthermore, the increased binding of MCAK to chromatin was specific to MCAK, because 5 μ M hesperadin abolished the localization of Ndc80, CENP E, and P150 dynactin to kinetochores (Ditchfield *et al.*, 2003) without causing a significant increase in chromosome arm binding of these proteins (Supplemental Figure S1).

It is perhaps disconcerting that 5 μ M hesperadin was necessary to cause an increase in chromatin-associated MCAK, because this concentration is much higher than what is typically used to inhibit Aurora B in cells, which is usually 100–500 nM (Hauf *et al.*, 2003; Hirota *et al.*, 2005). Perhaps hesperadin is less effective in egg extracts because of the high protein concentration in extracts. Consistent with this idea, others have used a 10-fold higher amount of ZM447439 in extracts than what was used in somatic cells to inhibit Aurora B (Gadea and Ruderman, 2005). Alternatively, it is possible that Aurora B has different phosphorylation efficiencies for each of its substrates such that inhibition of Aurora B differentially affects the phosphorylation of different substrates. In support of this idea, we found that 1 μ M hesperadin was sufficient to inhibit Aurora B phosphorylation of serine 10 of histone-H3 (pH3), but 5 μ M hesperadin was required to effectively inhibit Aurora B phosphorylation of S196 and T95 of MCAK (Figure 1D), two major Aurora B phosphorylation sites (discussed below) (Lan *et al.*, 2004; Ohi *et al.*, 2004). These results are also consistent with our *in vitro* kinase assays with purified kinase and substrates, in which 10 nM hesperadin inhibited Aurora B phosphorylation of histone H3 protein, but 100 nM was needed to inhibit phosphorylation of MCAK (Lan *et al.*, 2004). These results suggest that high concentrations of hesperadin are required to fully inhibit Aurora B in extracts and *in vitro*.

Previous studies showed that depletion of the Aurora B complex or addition of 20 μ M ZM447439 to extracts abolishes spindle formation around chromatin and that this effect is MCAK dependent, suggesting that the cytoplasmic pool of MCAK mediates this phenotype (Sampath *et al.*, 2004; Gadea and Ruderman, 2005). To test the effects of Aurora B inhibition on cytoplasmic MTs, we added 5 μ M hesperadin to extracts, and then we induced MT aster formation by the addition of Ran or DMSO. We found that addition of 5 μ M hesperadin did not affect the ability of the extract to form asters (Supplemental Figure S2A), suggesting that regulation of cytoplasmic MCAK by Aurora B cannot explain why spindles fail to form around chromatin in hesperadin-treated extracts. In sperm-induced spindle assembly reactions, and consistent with Incenp depletion (Sampath *et al.*, 2004), the addition of 5 μ M hesperadin results in MT nucleation around the centrosomes of sperm nuclei at early time points without MT nucleation close to chromatin that results in the loss of spindle formation at later time points (Supplemental Figure S2B). This result indicates that chromatin is an

inhibitory factor for spindle assembly in Aurora B-inhibited extracts. In addition, chromatin-bound MCAK in Aurora B-inhibited extracts is active, because it is hypophosphorylated at S196 (data not shown), which is the site that Aurora B phosphorylates, resulting in the inhibition of MCAK depolymerization activity. Our findings support the model that inhibition of Aurora B causes an increased amount of active MCAK binding to chromosome arms, which depolymerizes MTs that are nucleated by chromatin. Thus, through Aurora B phosphorylation activity, MCAK contributes to chromatin-mediated spindle assembly by regulating MT formation on chromatin.

Aurora B Regulates MCAK Binding to Chromosome Arms through a Two-Site Regulatory Mechanism

The Neck Region of MCAK Regulates its Chromosome Arm Binding, and Binding Is Inhibited by Phosphorylation of S196. To understand how Aurora B regulates MCAK binding to chromosome arms, we first mapped the region and the site that is responsible for the increased arm binding in the presence of hesperadin. We assayed the binding of different GFP-MCAK truncation mutants to chromatin assembled in extracts in the presence or absence of hesperadin. Full-length GFP-MCAK (2–731) increased on chromosome arms in 5 μ M hesperadin (Figure 2A). GFP-MCAK (GMCAK) derivatives lacking the N terminus (187–731) or both the N and C termini (187–592) also had increased arm binding, suggesting that neither the N- nor the C-terminal domains of MCAK were needed for the increased chromatin arm binding of MCAK in the absence of Aurora B activity. However, the catalytic domain of MCAK alone (263–592) bound chromosome arms poorly in the presence of 5 μ M hesperadin (Figure 2A), suggesting that the neck region (187–263) is the crucial Aurora B-regulated domain involved in chromosome arm binding. To confirm this finding, a GST-tagged version of MCAK containing only the neck (GST-Neck-187-263) was added to chromatin assembly reactions in *Xenopus* egg extracts in the presence and absence of 5 μ M hesperadin. GST-Neck-187-263 chromatin binding increased in the presence of hesperadin, indicating that binding is negatively regulated by Aurora B activity (Figure 2B).

To identify which site(s) within the neck region of MCAK regulates chromatin arm binding, point mutations of the predicted Aurora B phosphorylation sites in the neck domain of MCAK (S196, S230, T246A, and S253A) were made in GST-Neck-187-263, and the expressed proteins were tested in Aurora B kinase assays *in vitro* and for their ability to bind to chromosome arms in the presence or absence of 5 μ M hesperadin in chromatin assembly assays. In our *in vitro* Aurora B kinase assay, we found that S196 is the major Aurora B phosphorylation site in the neck domain of MCAK (Figure 2C). To determine whether phosphorylation of S196 regulates the binding of MCAK to chromosome arms, the mutant neck proteins were tested for their ability to bind chromatin in the presence or absence of hesperadin. GST-Neck-187-263(S196A) bound strongly to chromosome arms in the absence of hesperadin (control), and its binding did not increase after 5 μ M hesperadin treatment, suggesting that phosphorylation of S196 inhibits MCAK chromosome arm binding (Figure 2B). In contrast, the other neck mutants behaved like the wild-type neck construct in that hesperadin treatment increased chromosome arm binding (Figure 2B), suggesting that S196 in these proteins is mostly phosphorylated under control conditions but becomes hypophosphorylated upon hesperadin treatment. These results indicate

Figure 2. Aurora B regulates MCAK binding to chromosome arms through S196. (A and B) Chromatin was assembled in the presence of control DMSO (Control) or 5 μ M Hesp in nocodazole-treated CSF extracts, pelleted onto coverslips, stained with Hoechst, and immunostained with α -GFP. Bar, 5 μ m. (A) The neck region of MCAK (aa187–263) regulates chromosome arm binding. Full-length GFP-MCAK(2-731), GFP-MCAK(187-731), GFP-MCAK(187-592), or GFP-MCAK(263-592) was added to chromatin assembly reactions. GFP staining is shown. (B) Mutation of S196 to alanine in GST-Neck-187-263 promotes chromosome arm binding independent of Aurora B activity. Fluorescently labeled wild-type GST-Neck-187-263 (Wt), GST-Neck-187-263(S196A), GST-Neck-187-263(S230A), or GST-Neck-187-263(T246A-S253A) proteins were added to chromatin assembly reactions in the presence of control DMSO (Control) or 5 μ M Hesp. The fluorescent signal of the labeled MCAK protein (top) and the DNA staining (bottom) are shown. (C) Aurora B does not phosphorylate GST-Neck-187-263(S196A). The phospho-mutants in (B) were assayed for their ability to be phosphorylated by Aurora B in vitro by using an Aurora B kinase assays. Autoradiogram (top) and Coomassie-stained gel (bottom).

that Aurora B limits MCAK binding to the chromatin through phosphorylation of S196 in the neck domain of MCAK.

Phosphorylation of T95 Regulates the Binding of MCAK to Chromatin. Two observations suggested that Aurora B has a second mechanism to regulate MCAK binding to chromatin. First, MCAK(S196A) does not bind to the chromosome arms tightly in the absence of hesperadin (data not shown), suggesting that domains other than the neck region also con-

Figure 3. Aurora B regulates MCAK binding to chromosome arms through T95. (A) Aurora B poorly phosphorylates MCAK-CEN with T95A and S196A mutations. In vitro Aurora B kinase assays were performed on wild-type MCAK-CEN (aa2–263 + 630–664) (Wt) or alanine phospho-mutants of MCAK-CEN. The phospho-mutants are indicated with their respective mutated amino acid(s) above the lanes. Autoradiogram (top) and Coomassie-stained gel (bottom). (B) MCAK-2-149(T95A) has decreased arm binding and increased centromere binding. GST (not shown), MCAK-2-149 (Wt) or MCAK-2-149(T95A) proteins were added to chromatin assembly reactions, chromatin was pelleted onto coverslips, and then processed for immunofluorescence with α -GST antibodies. (C) MCAK-2-149 (T95A) binds chromatin less well than wild-type MCAK-2-149. Fusion proteins as in B were added to extracts (input) in which chromatin was assembled on DNA-coated beads. The beads were isolated, and equal volumes were electrophoresed on SDS-PAGE and analyzed by Western blot. The blot was probed with α -GST to identify the fusion protein or with α -XKl as a loading control. (D) α -T95 and α -pT95 are MCAK dephosphorylation- and phosphorylation-specific antibodies, respectively. Equal amounts of untreated, microcystin-LR-treated, or MCAK-depleted microcystin-LR-treated CSF extracts were run on SDS-PAGE and processed by Western blots with α -MCAK, α -tubulin (DM1 α), α -T95, or α -pT95. Lanes 1, 4, and 7, untreated egg extracts. Lanes 2, 5, and 8, egg extracts treated with 1 μ M microcystin-LR. Lanes 3, 6, and 9, MCAK-depleted egg extracts treated with 1 μ M microcystin-LR. (E) α -T95 and α -pT95 stained centromeres and chromatin similarly in somatic *Xenopus* cells and egg extracts. *Xenopus* XL177 cells (top) or *Xenopus* egg extract chromatin assembly reactions were immunostained with α -T95 or α -pT95 antibodies. Bar, 5 μ m.

tribute to chromosome arm binding. Second, in vitro Aurora B kinase assays show that although S196 is the major site within MCAK-187-731 (Lan *et al.*, 2004), mutation of this site in MCAK-CEN (MCAK-2-263 + 630–664) did not significantly reduce phosphorylation of this substrate by Aurora B (Figure 3A, compare lanes Wt and S196A). These results indicate that there must be at least one additional Aurora B phosphorylation site outside of the neck domain, but within MCAK-CEN. To identify additional Aurora B phosphorylation site(s) in MCAK-CEN without contributing phosphorylation by S196, we made point mutations of both identified and predicted Aurora B phosphorylation sites in MCAK-CEN(S196A), and we tested them in Aurora B kinase assays

in vitro. Mutation of T95 in MCAK-CEN(T95A-S196A) resulted in a dramatic reduction in Aurora B phosphorylation (Figure 3A), suggesting that T95 is a major Aurora B phosphorylation site in the N terminus of MCAK.

To test whether T95 could also regulate the association of MCAK with chromatin, we tested the ability of Wt and a T95A-mutated version of MCAK-2-149 (T95A) to bind to chromatin assembled in egg extracts. We used the MCAK-2-149 construct because we showed previously that this is the minimum region that is required for MCAK to target to the centromeres (Walczak *et al.*, 2002). In addition, this construct does not have the neck region so the complexity of additional regulation through phosphorylation at S196 can be reduced. The chromatin assembly assays showed that MCAK-2-149 bound to chromosome arms and centromeres (Figure 3B, left), which was consistent with what we observed with anti-MCAK staining of endogenous MCAK. Surprisingly, MCAK-2-149(T95A) had significantly reduced chromosome arm binding, and an apparent increase in centromere binding (Figure 3B, right). To demonstrate the decreased arm binding of MCAK-2-149(T95A) in the absence of centromeres, we assembled chromatin on DNA-coated beads in the presence of GST, MCAK-2-149 (Wt) or MCAK-2-149(T95A) (T95A). We found that although MCAK-2-149 bound to chromatin beads, MCAK-2-149(T95A) had significantly reduced binding (Figure 3C, compare lane Wt and T95A). These results suggest that T95 phosphorylation directly promotes MCAK binding to chromosome arms.

To explore the phosphorylation pattern of T95 in vivo, we made phospho- and dephospho-specific antibodies for the region surrounding T95 of MCAK, and we tested their specificity by immunoblotting and immunofluorescence. MCAK is hyperphosphorylated in mitotic egg extracts as shown by the slower mobility band recognized by α -MCAK antibodies in mitotic extracts in the presence of the phosphatase inhibitor microcystin (Figure 3D, lane 2). In Western blots, the α -T95 antibodies recognized only dephosphorylated MCAK in egg extracts that were not treated with microcystin (Figure 3D, lane 4), whereas the α -pT95 specifically recognized phosphorylated MCAK in mitotic egg extracts that were treated with microcystin (Figure 3D, lane 8). Neither antibody stained any bands in extracts that were immunodepleted of MCAK (Figure 3D, lanes 6 and 9). Using these antibodies, we immunostained *Xenopus* somatic cells and chromatin assembled in *Xenopus* egg extracts (Figure 3E). The α -T95 antibodies specifically stained centromeres, but the α -pT95 preferentially stained chromosome arms with low centromere staining. This staining could be competed by inclusion of the corresponding peptide (data not shown), further demonstrating the specificity of the antibodies. Quantification of the fluorescence of α -T95 and α -pT95 staining in *Xenopus* egg extracts showed that the centromere/chromosome arm ratio of α -T95 was at least twofold higher than α -pT95. These results indicate that T95-phosphorylated MCAK is preferentially associated with chromosome arms, which is consistent with our analysis of T95A mutants on chromatin beads. Our cellular data also show that MCAK association with chromosome arms is not restricted to meiotic chromatin in extracts, suggesting that MCAK association with chromosome arms is likely physiologically important in somatic cells as well.

Aurora B Regulates MCAK Targeting to Centromeres through a Second Two-Site Regulatory Mechanism

Because the α -T95 antibody mainly stains centromeres and the α -pT95 antibody mainly stains chromosome arms, this suggests that dephosphorylation at this site may increase

Figure 4. MCAK-2-149(T95A) has higher binding to centromeres than wild-type MCAK-2-149. (A) Chromatin was assembled in CSF egg extracts in the presence of 1.35 μ M, 1 μ M or 100 nM concentrations of MCAK-2-149 (top) or MCAK-2-149(T95A) (bottom), pelleted onto coverslips, immunostained with α -GST, α -P150 (to show centromere localization) and Hoechst (to show DNA). (B) Chromatin was assembled in MCAK-depleted extracts in the presence of different concentrations of full length MCAK or MCAK(T95A), pelleted onto coverslips, and immunostained with α -MCAK. Scale bars, 5 μ m.

centromere binding of MCAK. In previous work, we established that the centromere targeting domain of MCAK (MCAK-2-149) needed to be added to extracts at a 10- to 20-fold molar excess over endogenous MCAK ($\sim$ 100 nM) to displace endogenous MCAK. We found that 100 nM MCAK-2-149(T95A) was able to target to centromeres as least as well as 1.35 μ M MCAK-2-149 (Figure 4A). This indicates that MCAK-2-149(T95A) bound more readily to centromeres than did MCAK-2-149. To address whether this increased binding of T95A was also found in FL-MCAK, we made a FL-MCAK(T95A) mutant. We added different concentrations of MCAK or MCAK(T95A) to MCAK-depleted extracts, and found that 10 nM MCAK(T95A) can bind to centromeres as well as 80 nM Wt MCAK (Figure 4B), confirming that dephosphorylation of MCAK at T95 promotes strong binding to centromeres. The finding that dephosphor-

Figure 5. Aurora B regulates MCAK targeting to centromeres through T95 and S110. (A) Sequences around the three Aurora B phosphorylation sites of MCAK. The phosphorylated residues are in bold. (B) T95A and S110A inhibit Aurora B phosphorylation of MCAK-2-149. Aurora B in vitro kinase assays were performed on MCAK-2-149 and its phospho-mutant derivatives: no GST fusion protein (–), GST control (GST), wild-type MCAK-2-149 (Wt), MCAK-2-149(T95A) (T95A), MCAK-2-149(S110A) (S110A), and MCAK-2-149(T95A-S110A) (AA). Top, autoradiogram. Bottom, Coomassie-stained gel. (C) Quantification of the in vitro kinase assays from three independent experiments in which the wild-type phosphate incorporation is normalized to 100%. The mean percentages of phosphate incorporation are graphed $\pm$ SD. (D) The S110A mutation abolished MCAK-2-149 centromere targeting, but the T95A centromere targeting phenotype is dominant to S110A. Fluorescently labeled wild-type MCAK-2-149 (Wt), MCAK-2-149(T95A), MCAK-2-149(S110A) or MCAK-2-149(T95A-S110A) (AA) proteins were added to *Xenopus* CSF extract chromatin assembly reactions and pelleted onto coverslips. The fluorescence signals of the labeled proteins are shown. Bar, 5 μ m.

ylation of T95 promotes centromere binding is confusing, because we and others found that Aurora B phosphorylation is required to target MCAK to centromeres (Andrews *et al.*, 2004; Lan *et al.*, 2004; Ohi *et al.*, 2004). This inconsistency implies that there is another Aurora B site that positively regulates centromere association. In support of this idea, we found that in the absence of Aurora B activity, MCAK-2-149(T95A) still cannot target to the centromeres (Supplemental Figure S3). An additional candidate site is S110 in the centromere-targeting domain of MCAK. The residues around S110 have a weak Aurora B phosphorylation consensus sequence relative to the other Aurora B sites (Figure 5A), and S110 was identified as an Aurora B substrate by mass spectrometry (Ohi *et al.*, 2004).

To determine whether S110 is an Aurora B site that is important for centromere targeting, we compared the Aurora B phosphorylation levels of the T95A, S110A, or T95A-S110A double mutants by using in vitro kinase assays, and we assayed the mutant proteins for centromere targeting in chromatin assembly assays in *Xenopus* egg extracts. Although T95 is a major Aurora B phosphorylation site within the N terminus of MCAK, we found that S110 was also an Aurora B phosphorylation site (Figure 5B). Quantification of the phosphorylation levels showed that the S110A mutation resulted in an approximate twofold reduction in the ability of Aurora B to phosphorylate wild-type MCAK-2-149, whereas the T95A mutation caused an approximate fivefold reduction in phosphorylation (Figure 5C). In centromere targeting assays, MCAK-2-149(T95A) targeted robustly to centromeres, whereas the S110A mutation abolished the ability of MCAK-2-149 to target to centromeres (Figure 5D). This indicates that phosphorylation of S110 positively regulates MCAK targeting to centromeres. Surprisingly, we found that the double mutant MCAK-2-149(T95A-S110A) behaved identically to MCAK-2-149(T95A), suggesting that dephosphorylation of T95 overrides or is dominant to that of S110 (Figure 5D). We conclude that there are two Aurora B sites in the N terminus of MCAK that have opposite effects on centromere binding; phosphorylation on S110 promotes

centromere binding, whereas phosphorylation on T95 limits binding.

Phospho-Mutants of MCAK Have Altered Spindle Assembly Activities in *Xenopus* Egg Extracts

To address whether alteration of the three phosphorylation sites had any effect on spindle assembly, *Xenopus* CSF extracts were depleted of endogenous MCAK and either Wt or the different phospho-mutant derivatives of FL-MCAK were added back to the extract to near endogenous levels (Figure 6A). As described previously, MCAK depletion caused the assembly of large asters, which could be rescued by the addition of Wt MCAK (Figure 6, B and C) (Walczak *et al.*, 1996). Addition of MCAK(T95A) or MCAK(S110A) to MCAK-depleted extracts rescued spindle assembly, but there was a small but significant increase in the percentage of spindles with misaligned chromosomes (Figure 6, B–E). This is not surprising given that both mutations alter the ability of MCAK to target properly to centromeres. MCAK(S196A) was also able to rescue spindle assembly, but surprisingly it caused a large and significant increase in the percentage of spindles with misaligned chromosomes (Figure 6, B–E). Because preventing MCAK phosphorylation at S196 increases chromosome arm binding, it may be that this indirectly affects centromere targeting or the turnover kinetics of MCAK at centromeres. Alternatively, it is possible that the increased depolymerization activity of MCAK on the chromosome arms makes it more difficult to properly attach kinetochores to the spindle. Unlike MCAK(T95A), MCAK(T95E) had a significant reduction in its ability to rescue spindle assembly, and the spindles often had excess MT polymer (Figure 6, B and C). This lack of rescue is not likely due to reduced MT depolymerization activity, because MCAK(T95E) was as active as Wt MCAK when tested with in vitro MT depolymerization assays (data not shown). Thus, this lack of rescue must be due to differential binding of this mutant to chromosomes and kinetochores. MCAK(S196E) was un-

Figure 6. The phospho-mutants of MCAK have altered ability to rescue spindle assembly. CSF extracts were depleted of endogenous MCAK or mock depleted with IgG and cycled through interphase and back into mitosis. (A) Extracts were depleted of endogenous MCAK and then MCAK or its phospho-mutant derivatives were added back to near endogenous levels. The blots were probed with anti-MCAK antibodies (top) or anti-tubulin antibodies (bottom). (B) Images of representative structures from each of the add-back experiments. Chromatin is shown in green, and MTs are shown in magenta. Bar, 10 μ m. (C) Quantification of the MT structures that are associated with chromatin. The data are represented as the mean $\pm$ SD from three independent extracts in which 100 structures were counted per reaction. An asterisk represents significant differences in which $p < 0.05$. (D) Images of representative localizations of MCAK and two MCAK phospho-mutant derivatives on chromatin. Bar, 10 μ m. (E) Quantification of the percentage of bipolar spindles with aligned or misaligned chromosomes. The data are represented as the mean $\pm$ SD. An asterisk represents significant differences in which $p < 0.05$.

able to rescue spindle assembly, which is consistent with our previous hypothesis that S196 phosphorylation inhibits MCAK activity (Figure 6, B and C) (Lan *et al.*, 2004).

Because chromatin binding of MCAK is controlled by both T95 and S196 phosphorylation, we made two double mutants that contained mutations at both sites. Like the MCAK(T95E) and MCAK(S196E) single mutants, the MCAK(T95E-S196E) double mutant was unable to rescue spindle assembly (Figure 6, B and C). In contrast, the MCAK(T95E-S196A) double mutant was able to rescue spindle assembly (Figure 6C). However, we noticed that the spindles were often much smaller in size (Figure 6B). In addition, we found the percentage of chromatin that had no spindles surrounding it ($28 \pm 11\%$) was much higher than with Wt MCAK addition ($3 \pm 2\%$). This is not surprising given that MCAK(T95E-S196A) bound strongly to chromatin (Figure 6D). Together, these results show that not only does alteration of MCAK phosphorylation status affect MT depolymerization activity, but also has varying effects on spindle assembly that must be attributed to defects in chromatin and centromere targeting. Furthermore, our data shows that a mutated form of MCAK that targets strongly to chromatin disrupts chromatin-driven spindle assembly, which supports our model that chromosome-bound MCAK plays a physiologically important role in this process.

Temporal and Spatial Regulation of T95, S110, and S196

Our results revealed that Aurora B phosphorylates three distinct sites on MCAK, but their temporal order of phosphorylation *in vivo* is not known. To test when and where these phosphorylations occur relative to each other, we took advantage of our phospho-specific antibodies to stain somatic cells in interphase and in mitosis. Although it would have been ideal to compare α -pS110 staining together with α -pT95 and α -pS196, we were unable to generate a phospho-specific antibody to S110. Immunostaining of *Xenopus* somatic cells showed that MCAK localized to the nucleus, chromatin, and to the centromeres in late G2 before mitosis (Figure 7, A and B). At this stage, α -pT95 stained chromatin

strongly and centromeres lightly, indicating that T95 is phosphorylated by this point in the cell cycle (Figure 7A). These results are consistent with the function of T95 phosphorylation to target MCAK to chromatin. In contrast, α -pS196 did not stain chromatin or centromeres in G2, indicating that S196 is not phosphorylated at this point in the cell cycle (Figure 7A).

During prophase when chromosomes become notably condensed but before nuclear envelope breakdown, both α -pT95 and α -pS196 staining were visible on centromeres, indicating the presence of MCAK phosphorylated at T95 and S196 (Figure 7). In addition, it seemed as though phosphorylated T95 MCAK was enriched on chromosome arms relative to total MCAK or to phosphorylated S196 MCAK, consistent with phosphorylation of T95 promoting chromatin binding and phosphorylation of S196 inhibiting chromatin binding. These data imply that the MCAK population is phosphorylated on T95 before it is phosphorylated on S196 in cells. In addition, *in vitro* kinase assays showed that MCAK was phosphorylated by Aurora B more rapidly at T95 than at S196 (data not shown). Together, these results suggest that Aurora B not only spatially restricts phosphorylation of MCAK (in the cytoplasm, on the chromatin, or at the centromere) but also temporally controls the phosphorylation during late interphase and early mitosis.

DISCUSSION

Our study combined a functional domain analysis with single- and multisite mutagenesis to reveal distinct roles of three Aurora B phosphorylation sites of MCAK. We demonstrated that chromosome arm binding and centromere targeting are regulated by distinct two-site phosphoregulatory mechanisms involving residues T95, S110, and S196. This is the first study that clearly dissects the detailed regulatory mechanism of MCAK centromere targeting and chromosome arm binding by Aurora B phosphorylation.

MCAK Localization to Chromatin and Centromeres Is Regulated through Aurora B Phosphorylation at Multiple Sites

Our results are consistent with previous studies showing that Aurora B is necessary to target MCAK to centromeres, but because multiple Aurora B sites were mutated simultaneously in these studies, the site that controls MCAK centromere targeting was not uncovered (Andrews *et al.*, 2004; Lan *et al.*, 2004). Our study extends earlier findings by identifying the sites that control MCAK centromere targeting, and it demonstrates that MCAK centromere targeting is achieved by a balance between phosphorylation at S110 and dephosphorylation at T95 (Figure 8). Andrews *et al.* (2004) mutated five Aurora B phosphorylation sites on CgMCAK (equivalent to *Xenopus* MCAK T95, E123, S125, T129, and S196) and found that the MCAK-AAAAA (MCAK-5A) mutant localizes to centromeres with a shift to the inner centromere. Their results are consistent with our findings that the single T95A mutant targets prominently to centromeres. In addition, their fluorescent recovery after photobleaching analysis showed MCAK-5A having a slower turnover rate at centromeres, further substantiating that MCAK-5A has a high centromere affinity (Andrews *et al.*, 2004). In a separate study, Ohi *et al.* (2004) mutated four sites on *Xenopus* MCAK (T95, S110, S177, and S196) and found that MCAK-4A forms large aggregates on centromeres, which is similar to our T95A single mutant phenotype. We presume that if Ohi and colleagues lowered the MCAK-4A mutant concentration in their extract experiments to levels below endogenous

Figure 7. T95 is phosphorylated earlier in the cell cycle than S196. *Xenopus* XL177 cells in G2 or prophase/early prometaphase were stained for DNA with Hoechst and costained with α -MCAK-Alexa 488 and α -pT95-Alexa 594 (A) or α -MCAK-Alexa 594 and α -pS196-Alexa 488 (B) antibodies. In A and B, the top panels are cells in G2, and the bottom panels are cells in mitosis. Bar, 10 μ m.

Figure 8. Aurora B regulates chromosome arm binding and centromere targeting through distinct two-site phospho-regulatory mechanisms. Schematic diagram summarizing the MCAK Aurora B phosphorylation sites that regulate MCAK chromatin binding, centromere targeting, and MT depolymerization activity. Green indicates a positive effect of phosphorylation, and red represents an inhibitory effect.

MCAK concentrations, MCAK-4A would target to centromeres at levels similar to wild-type MCAK, as we found with MCAK(T95A). Our finding that the T95A mutant phenotype is dominant to the S110A phenotype explains why previous work was unable to uncover the positive centromere-targeting site of MCAK, because combining single site mutation analysis with individual functional domains of MCAK proved to be necessary to unravel this complicated two-site phosphoregulatory mechanism.

Also in this study, we show that T95 and S196 regulate MCAK binding to the chromosome arms by another Aurora B two-site regulatory system. Our group previously showed that Aurora B inhibits MCAK MT depolymerase activity by phosphorylating S196, which is important for chromosome alignment and error correction (Lan *et al.*, 2004). Phosphorylation of T95 and S196 occur at different cell cycle stages and in different locations. T95 is phosphorylated in late G₂, and it is present mainly on chromosome arms, but S196 is phosphorylated only after cells enter mitosis. During mitosis, S196 phosphorylation may not only inhibit the centromeric MCAK activity but also keep MCAK off the condensed chromosome arms so that the chromatin can effectively nucleate MTs (Figure 8).

We propose a model that explains MCAK localization and regulation by Aurora B during mitosis using these two-site regulatory mechanisms. We hypothesize that before cells enter mitosis, T95 phosphorylation may enhance MCAK binding to chromatin so that MCAK is poised to bind centromeres. In this way, chromosome arms provide a “ride” for MCAK to load on centromeres, similar to the chromosome passenger proteins. When cells enter mitosis, particularly during prophase, chromosome arm-bound MCAK is driven off by phosphorylation at S196 to reduce the amount of MCAK on chromosomes to make chromatin favorable for MT nucleation during prometaphase. From late G₂ through telophase, Aurora B phosphorylates MCAK at S110 directly at the centromeres or in the cytoplasm to ensure proper centromeric MCAK levels. This centromeric MCAK is highly dynamic (Andrews *et al.*, 2004; Kline-Smith *et al.*, 2004), and its activity, localization, and amount are under the control of Aurora B depending on the MT attachment status (Andrews *et al.*, 2004; Kline-Smith *et al.*, 2004; Knowlton *et al.*, 2006). When improper attachments occur, Aurora B locally increases MCAK concentration to help correct attachments (Knowlton *et al.*, 2006), probably through S110 phosphorylation. However, enriched centromeric MCAK at mal-attached kinetochores is hypophosphorylated at S196 (Knowlton *et al.*, 2006), making it

an active MT depolymerase. Overall, this mechanism tightly regulates the activity and localization of MCAK, which can be altered rapidly depending on the MT attachment status.

Aurora B Prevents Excessive MCAK Binding to Chromosome Arms to Enable Chromatin-induced MT Nucleation

Our results also reveal an important regulatory mechanism for Aurora B in chromatin-induced spindle assembly. During mitosis, MTs are nucleated in the vicinity of chromatin and at centrosomes. Previous work found that depletion or inhibition of the Aurora B complex caused MCAK dependent spindle disassembly (Sampath *et al.*, 2004; Gadea and Ruderman, 2005). Our work clearly uncovers the molecular mechanism by showing that excess MCAK accumulation on chromatin is the major reason why Aurora B inhibition blocks spindles assembly. This is supported not only by the observation that full inhibition of Aurora B increases the amount of MCAK on chromosomes and decreases spindle assembly but also because the MCAK(T95E/S196A) mutant binds strongly to chromatin and decreases spindle assembly around chromatin (Figure 6D). The story is not that simple though because we know that S196 phosphorylation also controls MCAK depolymerase activity so that the cytoplasmic MCAK activity may also be increased in this mutant. Our finding that an MCAK(S196A) mutant can rescue spindle assembly with normal-sized spindles strongly suggests that it is the increased amount of chromosome bound MCAK(T95E/S196A) that is responsible for the smaller spindle size. Overall, our findings support a model in which Aurora B activity is needed to control both where MCAK will be localized and how active MCAK will be at that location. This is probably advantageous to be able to rapidly turn on and turn off MCAK activity to respond to the dynamic changes in MT attachments, and both global and local MT dynamics.

Aurora B Phosphorylation of MCAK Occurs Temporally and Spatially

Aurora B localizes to different places and has multiple substrates in cells. Many MCAK subcellular locations are identical to Aurora B locations, so it is perhaps surprising that phosphorylation at the three major Aurora B sites on MCAK is spatially distributed in cells and extracts. This raises a question of how Aurora B can selectively phosphorylate one site but not the others at a given time. It is possible that MCAK folds into alternate conformations at different times in mitosis so that the accessibility of each site to Aurora B is different. For example, before prophase, MCAK may fold in a compact form so that only T95 and S110 are accessible to Aurora B. After MCAK is phosphorylated at T95 and S110, its neck region may be exposed, allowing Aurora B to phosphorylate S196. In support of this idea, we recently showed that there is a complex interplay between the N- and C-terminal domains of MCAK that is critical for MCAK function (Ems-McClung *et al.*, 2007). The ability of Aurora B to spatially distribute and precisely time its phosphorylation events may not be unique to MCAK. Multiple other substrates of Aurora B are distributed diversely in cells and have distinct functions during mitosis (Carmena and Earnshaw, 2003). Even at the centromere, Aurora B phosphorylates multiple substrates involved in mediating kinetochore-MT attachments. In yeast, the Dam1 complex is an Aurora B substrate essential for mediating proper attachments (Jones *et al.*, 2001; Shang *et al.*, 2003). In addition, Aurora B phosphorylates Ndc80/Hec1 to ensure proper attachments (Cheeseman *et al.*, 2006; Deluca *et al.*, 2006). To-

gether, these studies probably represent just a fraction of the complex interactions that occur at this dynamic interface. These studies also highlight the intricate phosphoregulatory mechanism that occurs at the centromere to establish and maintain proper kinetochore–MT interactions, which help ensure accurate chromosome segregation.

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