Phosphorylation- and Polo-Box–dependent Binding of Plk1 to Bub1 Is Required for the Kinetochore Localization of Plk1

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INTRODUCTION

Polo-like kinases (Plks) are evolutionarily conserved protein serine/threonine kinases that play crucial roles during multiple stages of the cell cycle, especially in mitosis (Barr et al., 2004; Glover, 2005; Lee et al., 2005; Liu and Maller, 2005; Lowery et al., 2005; van Vugt and Medema, 2005; Xie et al., 2005). There are four Plk family members in mammals, Plk1-4 (Barr et al., 2004). Plk1 is the orthologue of Drosophila Polo and yeast Cdc5 and is the best studied Plk family member in mammals (Barr et al., 2004). Plk1 contains two signature domains: an N-terminal kinase domain and a C-terminal polo-box domain (PBD) that consists of two tandem polo-boxes (Lowery et al., 2005). The PBD of Plk1 functions as an independently folded domain that specifically recognizes phospho-serine/threonine–containing peptides (Elia et al., 2003a, b; Lowery et al., 2005). Binding of PBD to phosphopeptides has been shown to mediate the phosphorylation-dependent targeting of Plk1 to substrates, to activate the kinase activity of Plk1 allosterically, and to regulate the subcellular localization of Plk1 (Elia et al., 2003a, b; Lowery et al., 2005; van Vugt and Medema, 2005).

Plk1 exerts its multiple functions in mitosis, including centrosome maturation, spindle assembly, cohesin removal, and cytokinesis, by phosphorylating a multitude of substrates (Barr et al., 2004; Lowery et al., 2005; van Vugt and Medema, 2005). For example, phosphorylation of cyclin B1, Cdc25, and Wee1 by Plk1 contributes to the activation of cyclin-dependent kinase 1 (Cdk1), which in turn promotes the entry into mitosis (Kumagai and Dunphy, 1996; Toyoshima-Morimoto et al., 2001; Watanabe et al., 2004). Plk1 regulates centrosome maturation by phosphorylating the centrosomal protein, ninein-like protein (Nlp), and blocks its targeting to centrosomes (Casenghi et al., 2003). In prophase, Plk1 is required for the removal of cohesin from chromosomal arms by phosphorylating the SA2 subunit of cohesin (Sumara et al., 2002; Hauf et al., 2005). Plk1 also phosphorylates Emi1, an inhibitor of the anaphase-promoting complex or cyclosome (APC/C), and mediates the degradation of Emi1 in early mitosis (Hansen et al., 2004; Moshe et al., 2004). During late mitosis, Plk1 interacts with and phosphorylates the central spindle proteins MKLP1/2, Nir2, and NudC, which is required for the completion of cytokinesis (Neef et al., 2003; Zhou et al., 2003; Litvak et al., 2004; Liu et al., 2004).

More recently, several studies have revealed functions of Plk1 at the kinetochores and in the spindle checkpoint (Ahonen et al., 2005; Wong and Fang, 2005), a cell cycle surveillance mechanism that ensures the fidelity of chromosome segregation (Cleveland et al., 2003; Bharadwaj and Yu, 2004). The spindle checkpoint prevents premature sister-chromatid separation by inhibiting the ubiquitin ligase activity of APC/C until all sister kinetochores have achieved bipolar attachment to the mitotic spindle and are therefore under mechanical tension (Yu, 2002). Lack of tension across sister kinetochores creates a yet unidentified phosphopeptide at these kinetochores that is recognized by the 3F3/2 monoclonal antibody (mAb) (Cyr et al., 1988; Gorbsky and Ricketts, 1993; Nicklas et al., 1995). Plk1 has recently been shown to be responsible for generating the tension-sensing 3F3/2 phosphopeptide at the kinetochores (Ahonen et al., 2005; Wong and Fang, 2005). Furthermore, Plk1 facilitates the localization of Mad2 and other spindle checkpoint pro-
teinization to the kinetochores (Ahonen et al., 2005; Wong and Fang, 2005).

In keeping with its multiple mitotic functions, Plk1 localizes to key mitotic structures during various stages of mitosis (Barr et al., 2004; van Vugt and Medema, 2005). During early mitosis, Plk1 is localized to the centrosomes (Golsteyn et al., 1995). During late anaphase and telophase, Plk1 is recruited to the central spindle through its interactions with MKLP1/2 (Golsteyn et al., 1995; Neef et al., 2003; Liu et al., 2004). Finally, consistent with its kinetochore functions, Plk1 localizes to the kinetochores during mitosis (Arnaud et al., 1998; Ahonen et al., 2005; Wong and Fang, 2005). It is thus important to establish the mechanism by which Plk1 is recruited to the kinetochores.

Budding uninhibited by benomazole 1 (Bub1) is a protein serine/threonine kinase and has two well established roles in the spindle checkpoint (Hoyt et al., 1991; Taylor and McKeon, 1997; Yu and Tang, 2005). First, Bub1 localizes to the kinetochores in mitosis and is required for the kinetochore localization of other spindle checkpoint proteins, including Bub1-related protein (BubR1) and Mad2 (Taylor and McKeon, 1997; Sharp-Baker and Chen, 2001; Johnson et al., 2004). Surprisingly, the kinase activity of Bub1 is dispensable for its function in targeting BubR1 and Mad2 to kinetochores (Sharp-Baker and Chen, 2001). Second, human Bub1 directly phosphorylates Cdc20 and inhibits APC/C (Tang et al., 2004a). Bub1 itself is hyperphosphorylated, and its kinase activity is enhanced in mitosis (Chen, 2004; Tang et al., 2004a). In addition to these two functions in the spindle checkpoint, Bub1 is required for the kinetochore localization of the Shugoshin/MEI-5352 protein and protects centromeric cohesion (Tang et al., 2004b; Kitajima et al., 2005). Bub1 also promotes stable bipolar kinetochore-microtubule attachment in mammalian cells (Meraldi and Sorger, 2005).

In an effort to study the regulation of Bub1 during mitosis, we immunoprecipitated Bub1 from nocodazole-arrested mitotic HeLa cells and identified Plk1 as a Bub1-binding protein by mass spectrometry. Binding of Plk1 to Bub1 requires the PBD of Plk1 and phosphorylation of Bub1 at T609. Bub1 is phosphorylated at T609 in mitosis in vivo. Phosphorylation of Bub1 by Cdk1 at T609 enhances Plk1 binding and Plk1-mediated phosphorylation of Bub1 in vitro. Depletion of Bub1 by RNA interference (RNAi) diminishes the kinetochore localization of Plk1. Ectopic expression of the wild-type Bub1, but not the Bub1-T609A mutant, rescues the kinetochore localization of Plk1 in Bub1-RNAi cells. Therefore, our results suggest that Plk1 directly interacts with and phosphorylates Bub1 in mitosis and that the box-and-phosphorylation-dependent interaction between Bub1 and Plk1 helps to recruit Plk1 to kinetochores.

Very recently, Goto et al. (2006) reported that inner centromere protein (INCENP) is phosphorylated by Cdk1 in mitosis and interacts with Plk1 in a box-and-phosphorylation-dependent manner. The INCENP–Plk1 interaction was postulated to recruit Plk1 to kinetochores (Goto et al., 2006). However, as suggested by its name, INCENP localizes to inner centromeres during mitosis (Cooke et al., 1987). In contrast, it has been reported that Plk1 localizes to mid- to outer kinetochores (Ahonen et al., 2005). In this study, we have confirmed that INCENP localizes to inner kinetochores and that there is little overlap between the INCENP staining and Plk1 staining at the kinetochores. Thus, the physical interaction between Plk1 and INCENP is unlikely to be directly responsible for the kinetochore localization of Plk1. Furthermore, INCENP is a component of the so-called “chromosome passenger complex” that also contains Aurora B, survivin, and Borealin (Pinsky and Biggins, 2005). The kinetochore localization of INCENP and Aurora B is interdependent, and Aurora B is required for the kinetochore localization of Bub1 and BubR1 (Ditchfield et al., 2003; Honda et al., 2003; Vigneron et al., 2004). We confirm that RNAi-mediated depletion of INCENP using the same small interfering RNA (siRNA) as Goto et al. (2006) diminishes the kinetochore localization of Bub1. Our findings along with a wealth of published data are consistent with the notion that INCENP controls the kinetochore localization of Bub1, which in turn facilitates the recruitment of Plk1 to kinetochores.

MATERIALS AND METHODS

Antibodies, Immunoblotting, and Immunoprecipitation

The production of rabbit α-Bub1, α-BubR1, α-Sgo1, and α-APC2 antibodies was described previously (Fang et al., 1998; Tang et al., 2001, 2004b). The following antibodies were purchased from the indicated sources: monoclonal α-Bub1 (Immunovision, Springdale, AZ), α-BubR1 (HA) and α-BubR1 (Phospho-S768) (Roche Diagnostics), and rabbit α-BubA and α-BubC (Bethyl Laboratories, Montgomery, TX). For immunoblotting, the antibodies were used at 1:1000 dilution for crude sera or 1 μg/ml for purified IgG. Monoclonal α-Bub1 antibody is only used in the experiments of costaining of Bub1 with BubR1 or INCENP.

For immunoprecipitation, affinity-purified rabbit α-Bub1 or α-Myc were coupled to Affi-Prep protein A beads (Bio-Rad, Hercules, CA) at a concentration of 1 mg/ml. HeLa cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol [DTT], 10% glycerol, 0.5 M okadaic acid, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin). After clearing by centrifugation for 30 min at 4°C at 13,000 rpm, the lysate was incubated with the antibody beads for 2 h at 4°C. The beads were washed with the lysis buffer five times. The proteins bound to the beads were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with the desired antibodies. For the large-scale purification of Bub1-containing protein complexes, the bound proteins were eluted with 100 mM glycine, pH 2.5, separated by SDS-PAGE, and visualized by silver staining.

Protein bands were excised from the gel and subjected to analysis by mass spectrometry.

Mammalian Cell Culture, RNAi, and Transfection

HeLa Tet-On (Clontech, Mountain View, CA) cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 10 mM l-glutamine. To arrest cells at G1/S, cells were incubated in the growth medium containing 2 mM thymidine (Sigma-Aldrich, St. Louis, MO) for 18 h. To arrest cells in mitosis, cells were treated with 100 ng/ml nocodazole (Sigma-Aldrich) for 16–18 h. For the roscovitine treatment, cells were first treated with nocodazole for 18 h to arrest them in mitosis, and 50 μM roscovitine was added in the medium for the indicated durations.

Plasmid transfection was performed when the cell reached a confluence of ~50% using the Effectene reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For RNAi experiments, the siRNA oligonucleotides were chemically synthesized at Dharmacon RNA Technologies (Lafayette, CO). HeLa cells were transfected as described previously (Tang et al., 2004a) and analyzed 48 h after transfection. The sequences of the siRNAs used in this study are Bub1 (CCAGGGCAUGGAGACCCUGTT and GAGU-GAUCAGCUACUUAUTT), Plk1 (GGCCGGCCUUGGCGAGUGCTT), BubR1 (GUGGAGAAGAGAGAUAAT), INCENP (GAAGACAGATTCTTCA), and Aurora B (GCGGGCAUCUCAAAUUGCTT). To establish cell lines stably expressing Myc-Bub1 or Myc-Bub1-T609A, HeLa Tet-On cells were transduced with pTRE2-Myc-Bub1 or pTRE2-Myc-Bub1-T609A constructs and then selected with 300 μg/ml hygromycin (Clontech). The surviving clones were screened for induced expression of Myc-Bub1 or Myc-Bub1-T609A in the absence or presence of 1 μg/ml doxycycline (Clontech).

Identification of Phosphorylation Sites by Tandem Mass Spectrometry (MS/MS)

Cdk1 phosphorylation sites on Bub1 were identified by a combination of precursor ion scanning and nanoelectrospray MS/MS. Briefly, Bub1 was phosphorylated by Cdk1 in vitro and separated on SDS-PAGE. The protein band was excised and digested with trypsin. The dried protein digests were dissolved in 5% formic acid and loaded onto a pulled capillary filled with POROS R2 resin. After washing, the peptides were eluted into a nanoelectrospray needle for either precursor ion scanning in negative ion mode or MS/MS in positive ion mode. All MS analyses were performed on a QSTAR Pulsar-I quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MDS Scien, Toronto, Ontario, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning, the parent ion was measured over a mass range of 1000–3000 Da and the precursor ions with charge states z ≤ 2 were selected for fragmentation. For precursor ion scanning, the parent ion was measured over a mass range of 1000–3000 Da and the precursor ions with charge states z ≤ 2 were selected for fragmentation.
scanning experiments, the instrument was set in negative ion mode, with the quadrupole Q2 pulsing function turned on, to detect the PO$_4^-$ fragment ion at m/z ~ 79. The optimum collision energies were determined for each experiment by gradually increasing the voltage of Q0 in steps corresponding to one-twentieth of the m/z value of the precursor ion. After data acquisition by precursor-ion scanning, the instrument was switched to positive ion mode, and the phosphopeptide sequence and sites of phosphorylation were identified by nanoelectrospray MS/MS. In the MS/MS scan mode, precursor ions were selected in quadrupole Q1 and fragmented in the collision cell (Q2), using argon as the collision gas.

**Immunofluorescence Microscopy**

HeLa Tet-On cells or various RNAi cells were cultured in chambered cover slides (Nalge Nunc International, Rochester, NY) and transfected with siRNA duplexes at ~50% efficiency. After 24 h, thymidine (at final concentration of 2 mM) was added to the medium. After another 18 h, cells were washed and released into fresh medium for 7 h and treated with 100 ng/ml nocodazole and 50 μM MCI32 for 4 h to arrest cell in mitosis. Cells were washed once with physiologic-buffered saline (PBS) and fixed with freshly made 4% paraformaldehyde for 15 min at room temperature. After washing with PBS three times, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed with the same buffer, and blocked with 5% nonfat milk in permeabilizing solution for 30 min. Cells were then incubated with the appropriate primary antibodies (diluted to 1 μg/ml in blocking solution) for 1 h, washed three times with 0.2% Triton X-100 in PBS, and incubated with cross-absorbed fluorescent secondary antibodies (Invitrogen) at 1:500 dilution. After washing and staining with 4,6-diamidino-2-phenylindole (DAPI), slides were mounted, and examined using a 63× objective on a Zeiss Axiovert 200M microscope. Images were acquired and processed with the Slidebook software (Intelligent Imaging Innovations, Denver, CO) and pseudocolored in Photoshop (Adobe Systems, Mountain View, CA). A series of z-stack images were captured at 0.2-μm intervals and deconvolved using the nearest neighbor algorithm. The maximum z-projection was then created for the deconvoluted images. For quantification of kinetochore staining, a mask was generated to mark all kinetochores based on CREST staining in the projected image. After background subtraction, the mean intensity for each channel and for each object in the mask was measured. These values were then exported and plotted with the Prism software (GraphPad Software, San Diego, CA). For each condition, kinetochore staining of at least 10 cells was measured with the average and SD plotted.

**In Vitro Kinase and Protein-binding Assays**

The expression and purification of human Bub1-ΔKD, Bub1-ΔKD-S99A, Bub1-ΔKD-T609A, PIK1-T210D, and the α90-cyclin B1/Cdk1 complex from Sf9 cells were performed exactly as described previously (Tang and Yu, 2004). The kinase assay was carried out in the kinase buffer (50 mM Tris-HCl, pH 7.7, 100 mM KCl, 10 mM MgCl$_2$, and 1 mM DTT) containing 200 μM nonradioactive ATP, 0.1 μM Ci/ml [γ-$^32$P]ATP, 1 μM Bub1-ΔKD, and 100 nM Plk1 with or with out 100 nM Cdk1. For the two-step kinase assay, the purified Bub1-ΔKD and mutant proteins were first immobilized on α-Bub1 beads. After washing, the protein-bound to beads were used as substrates in the first step reaction in the presence of 200 μM nonradioactive ATP. The Bub1-ΔKD-containing beads were washed three times with the high-salt buffer (lysis buffer plus 300 mM KCl) and twice with the kinase buffer to completely remove Cdk1. The protein-bound to beads were subjected to the second kinase reaction with PIK1-T210D and [γ-$^32$P]ATP. The reactions were then incubated at room temperature for 30 min, stopped by SDS sample buffer, separated by SDS-PAGE, and analyzed using a phosphorimager (Fuji, Tokyo, Japan).

Glutathione S-transferase (GST), GST-PBD, and GST-PBD-H538A/K540M proteins were expressed in bacteria and purified using glutathione-agarose beads. In protein-binding assays, the proteins were immobilized on the glutathione-agarose beads, blocked with Tris-buffered saline (TBS) plus 5% nonfat milk, and incubated with the lysate of HeLa cells transfected with Myc-Bub1 for 2 h at 4°C. The bound proteins were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with α-Myc. For the binding assays that involved Cdk1-phosphorylated Bub1-ΔKD, Bub1-ΔKD was incubated first with 100 nM Cdk1 in the kinase buffer with or without 200 μM nonradioactive ATP for 1 h. The reaction mixtures were then applied to beads containing various GST proteins that had been blocked with TBS plus 5% nonfat milk.

**RESULTS**

**Plk1 Interacts with Bub1 in Mitosis**

To identify proteins that interacted with Bub1 in mitosis, we immunoprecipitated Bub1-containing protein complexes from nocodazole-arrested mitotic HeLa cells. As shown in Figure 1A, in addition to Bub1, Bub3, and several degradation products of Bub1, a protein band that migrated around 65 kDa was present in the α-Bub1 immunoprecipitates (IP), but not in the IP of control IgG. Mass spectrometry analysis revealed that this band belonged to human Plk1. To confirm the interaction between Plk1 and Bub1, we performed IP-Western type of experiments. Lysates of HeLa cells that were arrested at G$_1$/S or mitosis by thymidine or nocodazole, respectively, were immunoprecipitated with α-Bub1. The α-Bub1 IP was blotted with α-Plk1. Plk1 was clearly detected in the α-Bub1 IP, but not in the IPs of control IgG or α-Mps1 (a spindle checkpoint kinase) (Fisk et al., 2004), from mitotic HeLa cells (Figure 1B and Supplemental Figure 1). The interaction between Bub1 and Plk1 was not observed in cells arrested at the G$_1$/S boundary by thymidine (Figure 1B). Similar levels of Bub1 were present in the lysates from thymidine- or nocodazole-treated cells and in the α-Bub1 IPs from these cells. Plk1 was also present in the lysate of thymidine-arrested cells, albeit at a lower level compared with nocodazole-treated cells. However, the difference in the amounts of Plk1 present in the α-Bub1 IPs from thymidine-
and nocodazole-arrested cells was much greater than the difference between Plk1 levels in the lysates of the two types of cells. Thus, our results suggest that Bub1 specifically interacts with Plk1 in mitosis.

To further confirm the interaction between Plk1 and Bub1, HeLa cells were transfected with plasmids encoding Myc-Bub1 together with HA-Plk1-WT, HA-Plk1-K82R (kinase-inactive mutant), or HA-Plk1-H538A/K540M and arrested in mitosis with nocodazole. The cell lysates and α-Myc IP were blotted with α-Myc and α-HA. (C) HeLa cells were transfected with plasmids encoding Myc-Bub1 and arrested in mitosis with nocodazole. The cell lysates were incubated with glutathione-agarose beads that contained GST, GST-PBD-WT, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Myc (top) or stained with Coomassie blue (bottom).

**Figure 2.** The PBD of Plk1 mediates the binding between Plk1 and Bub1. (A) Schematic drawing of the domain structure of Plk1 and the locations of two critical phosphate-binding residues. (B) HeLa cells were transfected with plasmids encoding Myc-Bub1 together with HA-Plk1-WT, HA-Plk1-K82R (kinase-inactive mutant), or HA-Plk1-H538A/K540M and arrested in mitosis with nocodazole. The cell lysates and α-Myc IP were blotted with α-Myc and α-HA. (C) HeLa cells were transfected with plasmids encoding Myc-Bub1 and arrested in mitosis with nocodazole. The cell lysates were incubated with glutathione-agarose beads that contained GST, GST-PBD-WT, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Myc (top) or stained with Coomassie blue (bottom).

**The PBD of Plk1 Mediates Its Interaction with Bub1**

Plk1 contains an N-terminal kinase domain and two polo-boxes at its C-terminal region (Figure 2A). The two poloboxes of Plk1 have been shown to fold into one intact domain (PBD) that binds to phosphorylated serine/threonine motifs and targets Plk1 to its substrates and proper subcellular locations (Elia et al., 2003a, b). We tested whether the PBD of Plk1 mediated its interaction with Bub1. HA-Plk1-PBD bound to Bub1 as efficiently as did the full-length HA-Plk1, indicating that the PBD of Plk1 was sufficient for Bub1 binding (Figure 1C). Two residues in the second polobox of Plk1, H538, and K540, form direct contact with the phosphate group and are required for the selective binding between the PBD and phosphopeptides (Elia et al., 2003b). To determine whether the Bub1–Plk1 interaction required the intact PBD of Plk1, we introduced two mutations into the PBD, H538A and K540M, which were known to disrupt the phosphopeptide-binding activity of the PBD (Figure 2A) (Elia et al., 2003b). As shown in Figure 2B, HA-Plk1-H538A/K540M bound to Bub1 much more weakly than did the wild-type HA-Plk1. The kinase-inactive mutant of Plk1, Plk1-K82R, bound to Bub1 as efficiently as Plk1-WT (Figure 2B), suggesting that the secondary phosphorylation on Bub1 by Plk1 (Figure 4C) might not be required for the Bub1–Plk1 interaction.

We also performed an in vitro protein-binding assay. Purified recombinant GST-PBD and GST-PBD-H538A/K540M fusion proteins were bound to glutathione-agarose and incubated with lysate of nocodazole-arrested HeLa cells that had been transfected with Myc-Bub1. Myc-Bub1 selectively bound to beads containing the wild-type GST-PBD, but it did not bind to beads containing GST or GST-PBD-H538A/K540M (Figure 2C). These results indicated that binding of Plk1 to Bub1 required an intact PBD. Moreover, it has been shown previously that Bub1 is hyperphosphorylated in mitosis and the hyperphosphorylated species of Bub1 migrated slower on SDS-PAGE (Chen, 2004; Tang et al., 2004a). Myc-Bub1 that was bound to the wild-type GST-PBD migrated slower on SDS-PAGE, compared with Myc-Bub1 in the cell lysate (Figure 2C). This suggests that the PBD of Plk1 might preferentially bind to phosphorylated Bub1.

**Bub1 Is Phosphorylated on T609 In Vivo and Phosphorylation of T609 Is Required for the Plk1–Bub1 Interaction**

Yaffe and coworkers have shown that the PBD of Plk1 prefers to bind to phosphorylated S-pS/pT-P motifs (Elia et al., 2003a). Inspection of the amino acid sequence of Bub1 revealed that human Bub1 contains two such S-S/T-P motifs, one of which, T609, is conserved among vertebrate Bub1 proteins (Figure 3A). To determine whether any of the two S-S/T-P motifs of Bub1 were phosphorylated in vivo, we tested whether either of the two sites was recognized by the MPM-2 phosphospecific mAb that can detect certain pS/pT-P motifs (Yaffe et al., 1997). The wild-type Myc-Bub1 from mitotic HeLa cells contained a phosphoepitope that was detected by the MPM-2 antibody (Figure 3B). The S99A mutation did not alter the MPM-2 antigen within Bub1, whereas the T609A mutation significantly attenuated the MPM-2 reactivity of Myc-Bub1 (Figure 3B). This suggests that Bub1 is phosphorylated at T609 in mitosis, and this phosphoepitope on Bub1 can be detected by the MPM-2 antibody. However, the MPM-2 antibody still recognized Bub1-T609A to some extent, suggesting that Bub1 contained other MPM-2 epitopes in addition to T609.

Our results in Figure 3B cannot rule out the possibility that the MPM-2 antibody does not directly detect phosphorylation at T609 but detects phosphorylation at other S/T-P sites whose phosphorylation might be in turn dependent on phosphorylation at T609. Human Bub1 contains 13 S/T-P sites, seven of which are conserved in vertebrates, including T441, T452, S459, S593, T609, S655, and S661. To determine whether T609 of Bub1 was phosphorylated in mitosis and whether phospo-T609 was detected by MPM-2, we constructed a Bub1-7A mutant in which the serines/threonines in all seven conserved S/T-P motifs are mutated to alanines and a Bub1-6A-T609 mutant in which T609 was not mutated but the other six sites were mutated. Myc-Bub1-7A immunoprecipitated from nocodazole-arrested HeLa cells was not recognized by MPM-2 (Figure 3C). Bub1-6A-T609 was detected by MPM-2 (Figure 3C), suggesting that Bub1 is most likely phosphorylated at T609, and this phosphorylation event can be recognized by MPM-2. The MPM-2 reactivity of Bub1-6A-T609 was much weaker compared with Bub1-WT (Figure 3C), consistent with the notion that Bub1 contains
Lysates of HeLa cells treated with thymidine or nocodazole were blotted with the MPM-2 antibody (top) and  
Bub1 protein was immunoprecipitated from either thymidine-treated G1/S or nocodazole-treated mitotic HeLa cells. The Bub1 protein was phosphorylated at T609 in mitosis. Moreover, although Myc-Bub1-WT and Myc-Bub1-S99A interacted strongly with HA-Plk1, Myc-Bub1-T609A failed to interact with HA-Plk1 in mitotic HeLa cells (Figure 3E). This suggests that phosphorylation of Bub1 at T609 creates a docking site for the PBD of Plk1 and is required for efficient Plk1 binding.

Interestingly, the T609-containing S-S/T-P motif is conserved in BubR1 proteins (Figure 3A). Co-IP experiments confirmed that the endogenous BubR1 interacted with Plk1 in mitosis (Supplemental Figure 1). BubR1 is also hyperphosphorylated in mitosis (Taylor et al., 2001). Although we do not know whether the corresponding S-S/T-P motif of BubR1 is phosphorylated, it is very likely that Plk1 interacts with BubR1 in a manner similar to its binding to Bub1. The functional consequence of the association between Plk1 and BubR1 is not further explored in this study.

**Phosphorylation of Bub1 by Cdk1 Promotes the Plk1–Bub1 Interaction and Facilitates Plk1-mediated Phosphorylation of Bub1 In Vitro**

Previous studies have revealed that Cdk1 is the “priming” kinase that initially phosphorylates S-S/T-P motifs within several Plk1 substrates, such as Nir2, GRASP65, and Cdc25C and generates the docking sites for the PBD of Plk1 (Elia et al., 2003a; Litvak et al., 2004; Preisinger et al., 2005). We thus tested whether Cdk1 phosphorylated Bub1 in vitro. Because Bub1 is itself a kinase and undergoes autophosphorylation, we expressed and purified from Sf9 cells a truncation mutant of human Bub1 (residues 1-726) that lacked the kinase domain, referred to as Bub1-ΔKD, and used it as the substrate in the kinase assays. Purified recombinant cyclin B1/Cdk1 complex (referred to as Cdk1 for simplicity) phosphorylated Bub1-ΔKD (Figure 4A). This phosphorylation was blocked by roscovitine, a chemical inhibitor of Cdk1 (Figure 4A). Using mass spectrometry, we mapped the phosphorylation sites of Bub1 that had been phosphorylated by Cdk1 in vitro. Two major Cdk1 phosphorylation sites on Bub1 were identified, including S593 and T609 (Figure 4B). This result demonstrates that Cdk1 can phosphorylate Bub1 on T609 in vitro. We next examined the effect of Cdk1-mediated phosphorylation of Bub1 on the Plk1–Bub1 interaction. Bub1-ΔKD was first incubated with Cdk1 in the presence or absence of ATP. The reaction mixtures were then incubated with beads containing GST, GST-PBD, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Bub1 (Figure 4C). A preincubation of Bub1-ΔKD with Cdk1 in the presence of ATP enhanced its binding to GST-PBD (Figure 4C, compare lanes 2 and 5). The Cdk1-enhanced binding between Bub1-ΔKD and PBD required the intact phosphopeptide-binding pocket of the PBD, because less Bub1-ΔKD was bound to GST-PBD-H538A/K540M (Figure 4C, compare lanes 2 and 3). These results suggest that phosphorylation of Bub1 by Cdk1 facilitates the PBD-dependent binding of Plk1 to Bub1.

We next tested whether Plk1 phosphorylated Bub1-ΔKD and whether the Cdk1-enhanced interaction between Plk1 and Bub1 also facilitated the phosphorylation of Bub1 by Plk1. To do so, we developed a two-step sequential kinase assay. In this assay, Bub1-ΔKD, Bub1-ΔKD-S99A, or Bub1-ΔKD-T609A proteins were first incubated with or without Cdk1 in the presence of nonradioactive ATP. The Bub1 proteins were immunoprecipitated with α-Bub1 beads. After extensive washing to remove Cdk1, the Bub1 proteins bound to beads were further incubated with or without Plk1-T210D (a constitutively active mutant of Plk1) in the presence of [γ-32P]ATP (Qian et al., 1999). The removal of Cdk1 by the washing procedure was complete, because Bub1 was not phosphorylated in the absence of Plk1 (Figure 4D, lanes 3, 7, and 11). Plk1 phosphorylated Bub1 in the absence of a pretreatment by Cdk1 (Figure 4D, lane 2). A preincubation with Cdk1 enhanced the Plk1-mediated phosphorylation of Bub1-ΔKD and Bub1-ΔKD-S99A, but not Bub1-ΔKD-T609A, as judged by the retarded gel mobility.
Bub1 is phosphorylated by Cdk1 on T609, which creates a binding site for the PBD of Plk1, thus enhancing the Plk1–Bub1 interaction and phosphorylation of Bub1 by Plk1.

We also noticed that Plk1 phosphorylated Bub1–ΔKD and Bub1–ΔKD-S99A more efficiently than Bub1–ΔKD-T609A even in the absence of a pre-incubation with Cdk1 (Figure 4C, compare lanes 2 and 6 with lane 10). Consistently, recombinant Bub1–ΔKD expressed and purified from Sf9 cells was detected by the MPM-2 antibody (Figure 4E). The MPM-2 reactivity of Bub1–ΔKD was abolished by λ-phosphatase treatment and greatly diminished by the T609A mutation (Figure 4E). These results suggested that a fraction of Bub1–ΔKD had already been phosphorylated at T609 by kinase(s) in Sf9 cells.

Next, we sought to obtain evidence to suggest that Cdk1 phosphorylated Bub1 in vivo and that this phosphorylation was required for the Bub1–Plk1 interaction. Nocodazole-arrested mitotic HeLa cells were treated briefly with the Cdk1 inhibitor roscovitine (Mishima et al., 2004). The MPM-2 reactivity of Bub1 was lost in cells treated with roscovitine for only 15 min (Figure 4F, top). Consistently, Bub1 from roscovitine-treated cells migrated faster on SDS-PAGE (Figure 4F, middle) and failed to interact with Plk1 (Figure 4F, bottom). Thus, our data are consistent with the notion that Bub1 is phosphorylated by Cdk1 on T609, and this phosphorylation is required for its interaction with Plk1.

Bub1 is Required for the Kinetochore Localization of Plk1

To explore the functions of the binding between Plk1 and Bub1, we examined whether the Plk1–Bub1 interaction is required for the kinetochore localization of Bub1 or Plk1 in mitosis. It has been shown that improper kinetochore–microtubule attachment in certain situations can cause a microtubule-dependent depletion of kinetochore proteins. For example, the kinetochore concentrations of Mad1 and Mad2 are much lower in human cells that are depleted for components of the Ndc80 complex by RNAi (Martin-Luemsma et al., 2002; DeLuca et al., 2003; Bharadwaj et al., 2004). However, the kinetochore localization of Mad1 and Mad2 is restored when these cells are treated with nocodazole to depolymerize their microtubules (DeLuca et al., 2003; Bharadwaj et al., 2004). Because both Bub1 and Plk1 have been implicated in proper kinetochore–microtubule attachment (A honen et al., 2005; Meraldi and Sorger, 2005; Wong and Fang, 2005) and because Bub1 and Plk1 do not undergo mitotic arrest efficiently in the presence of nocodazole (Tang et al., 2004a; Meraldi and Sorger, 2005), we adopted the following experimental scheme (Supplemental Figure 2A). Twenty-four hours after HeLa cells were transfected with siRNA against Bub1, they were arrested at the G1/S boundary by the addition of thymidine for 18 h. The cells were then released into fresh medium to allow cell cycle progression. Nocodazole and the proteasome inhibitor MG132 were added 7 h later to arrest cells in mitosis with depolymerized microtubules and were fixed for immunostaining 4 h later.

Transfection of HeLa cells with siRNA against Bub1 or Plk1 efficiently knocked down the protein levels of Bub1 or Plk1 in mitosis (Supplemental Figure 2B). Depletion of Bub1 did not affect the protein levels of Plk1, and vice versa (Supplemental Figure 2B). Interestingly, Bub1 from Plk1–RNAi cells migrated faster on SDS-PAGE, consistent with the notion that Plk1 was involved in the phosphorylation of Bub1 in nocodazole-arrested mitotic HeLa cells (Supplemental Figure 2B). Mitotic Plk1- and Bub1–RNAi cells were stained with α-Bub1 (middle) and α-Plk1 (bottom).

and the increased amount of 32P incorporation (Figure 4D). Although phosphorylation of Bub1–ΔKD by Plk1 retarded the gel mobility of Bub1–ΔKD in the autoradiograph, Bub1–ΔKD was not up-shifted in the corresponding Western blot (Figure 4D), indicating that only a small fraction of Bub1–ΔKD was phosphorylated in these reactions. We also tested whether Bub1 phosphorylated Plk1–K82R and failed to detect any such phosphorylation (our unpublished data). Our results are consistent with the notion that phosphorylation of Bub1 at T609 by Cdk1 creates a binding site for the PBD of Plk1, thus enhancing the Plk1–Bub1 interaction and phosphorylation of Bub1 by Plk1.

Figure 4. Cdk1 phosphorylates Bub1 and promotes Plk1-binding and Plk1-mediated phosphorylation of Bub1. (A) Recombinant purified Bub1–ΔKD (a Bub1 truncation mutant with its kinase domain deleted) was incubated with [γ-32P]ATP and cyclin B1/Cdk1 in the absence or presence of 10 μM roscovitine for 30 min at room temperature. The reaction was quenched and analyzed by SDSPAGE followed with autoradiography. Bottom, Coomassie-stained gel of the same reactions to indicate that similar amounts of Bub1–ΔKD were present in the reactions. (B) Two major in vitro Cdk1 phosphorylation sites on recombinant Bub1 mapped by mass spectrometry. (C) The Bub1–ΔKD protein was incubated with Cdk1 in the presence or absence of ATP. The reaction mixtures were incubated with glutathione-agarose beads that contained GST, GST-PBD-WT, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Bub1. Bottom, Coomassie-stained gel of the same reactions to indicate that similar amounts of GST, GST-PBD-WT, and GST-PBD-H538A/K540M were present in the reactions. (D) Bub1–ΔKD and mutant proteins were incubated with Cdk1 in the kinase buffer containing nonradioactive ATP for 1 h at room temperature. The Bub1 proteins were then immunoprecipitated and subjected to a second kinase reaction with purified Plk1–T210D and [γ-32P]ATP. After 1 h, the reactions were quenched and analyzed by SDSPAGE followed by autoradiography (top). The same samples were blotted with α-Bub1 to show that similar amounts of Bub1 proteins were present (bottom). (E) Recombinant Bub1–ΔKD or Bub1–ΔKD-T609A from Sf9 cells were either untreated or treated with λ-phosphatase and blotted with MPM-2. (F) Nocodazole-arrested HeLa cells were incubated with 50 μM roscovitine for the indicated times and then lysed and subjected to α-Bub1 IP. The IPs were blotted with the MPM-2 antibody (top), α-Bub1 (middle), and α-Plk1 (bottom).
affect the kinetochore localization of Bub1 in mitosis (Figure 5, A and C). In contrast, the kinetochore localization of Plk1 was significantly reduced in mitotic Bub1-RNAi cells (Figure 5A). Similar results were obtained in prometaphase and metaphase cells from asynchronized Bub1-RNAi cells (our unpublished data). Costaining of Bub1-RNAi cells with Plk1 and /H9253-tubulin showed that the centrosomal localization of Plk1 was unaffected by Bub1-RNAi (Figure 5B). These results indicate that Bub1 is required for the kinetochore localization, but not the centrosomal localization, of Plk1 in mitosis.

**The Kinetochore Localization of Plk1 Does Not Require BubR1**

Bub1 is required for the kinetochore localization of other checkpoint proteins, such as BubR1 and Mad2 (Sharbaker and Chen, 2001; Johnson et al., 2004). We have also detected an interaction between BubR1 and Plk1 (Supplemental Figure 1). To test whether the kinetochore localization of Plk1 was also dependent on BubR1, we examined the kinetochore localization of Plk1 in HeLa cells transfected with siRNA against BubR1. RNAi-mediated depletion of BubR1 was efficient (Supplemental Figure 2B). Both Bub1 and Plk1 localized normally to mitotic kinetochores in BubR1-RNAi cells (Figure 5, C and D, and Supplemental Figure 2, C and D), indicating that BubR1 is not required for the kinetochore localization of Plk1. Consistent with previous reports (Sharbaker and Chen, 2001; Johnson et al., 2004; Ahonen et al., 2005; Wong and Fang, 2005), the kinetochore localization of BubR1 was significantly reduced in Bub1-RNAi and Plk1-RNAi cells (Supplemental Figure 3), suggesting that BubR1 lies downstream of Bub1 and Plk1 in the hierarchy of kinetochore association.

**The Kinetochore Localization of Plk1 Requires the Intact Polo-Box-binding Motif on Bub1**

The kinetochore localization of Plk1 is dependent on Bub1 in HeLa cells. We next sought to test whether the PBD- and phosphorylation-dependent binding between Plk1 and Bub1 was required for the kinetochore localization of Plk1. To do so,

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**Figure 5.** Bub1-RNAi diminishes the kinetochore localization of Plk1. (A) HeLa cells that were either mock transfected or transfected with siRNAs against Bub1 or Plk1 were fixed and stained with α-Plk1, α-Bub1, CREST, and DAPI. In the merge, DAPI is pseudocolored blue, CREST in red, and Plk1 (top two panels) or Bub1 (bottom) in green. Bar, 5 μm. (B) HeLa cells that were either mock transfected or transfected with siRNA against Bub1 were fixed and stained with α-Plk1, CREST, γ-tubulin, and DAPI. In the merge, DAPI is pseudocolored blue, γ-tubulin red, and Plk1 green. The centrosomes are marked by arrows. Bar, 5 μm. (C) The kinetochore signal of Bub1 was quantified in mock, Plk1- and BubR1-RNAi cells. Kinetochores from more than 10 mitotic cells were analyzed and normalized using the CREST staining. The mean and SD are shown. (D) The kinetochore staining of Plk1 was quantified in mock, Bub1-, and BubR1-RNAi cells. Kinetochores from more than 10 mitotic cells were analyzed and normalized using the CREST staining. The mean and SD are shown.
so, we attempted to rescue the defective kinetochore localization of Plk1 in Bub1-RNAi cells by stable transfection of plasmids that encoded Myc-Bub1-WT and Myc-Bub1-T609A (the Bub1 mutant that lacked the priming phosphorylation site required for Plk1 binding). Because these Bub1-expressing plasmids also contained silent mutations in the region that was targeted by Bub1-RNAi, the expression of the Bub1 transgenes was not knocked down by Bub1-RNAi. Both Myc-Bub1-WT and Myc-Bub1-T609A were expressed at levels comparable with that of the endogenous Bub1 (Figure 6A) and localized normally to kinetochores in mitosis (Figure 6B). The finding that Myc-Bub1-T609A exhibits normal kinetochore localization is consistent with the notion that Plk1 is not required for the kinetochore localization of Bub1 (Figure 5C) (Ahonen et al., 2005). Ectopic expression of Myc-Bub1-WT or Myc-Bub1-T609A largely restored the kinetochore localization of Plk1 (Figure 6, B and D). In contrast, expression of Myc-Bub1-T609A failed to restore the kinetochore localization of Plk1 in Bub1-RNAi cells (Figure 6, B and D). Because Myc-Bub1-T609A localizes to kinetochores normally, this result strongly suggests that the Bub1–Plk1 interaction is required for the kinetochore localization of Plk1.

In addition to its function in the spindle checkpoint, Bub1 also protects centromeric cohesion by targeting the Sgo1–PP2A complex to kinetochores (Tang et al., 2004b, 2006; Yu and Tang, 2005). We thus examined the regulation of Plk1 by INCENP. We first examined the localization of INCENP and Plk1 by immunofluorescence. INCENP interacts with Plk1 in a phosphorylation- and PBD-dependent manner and is required for the kinetochore localization of Plk1. We therefore investigated the regulation of Plk1 by INCENP. We first examined the localization of INCENP and Plk1 by immunofluorescence. INCENP localized to a single dot in the inner kinetochore between the two Plk1 dots, and there was little overlap between the INCENP and Plk1 staining (Figure 7A). Therefore, while Bub1 and Plk1 colocalize at the outer kinetochores (Figure 1D),
INCENP does not colocalize with Plk1, which is inconsistent with the notion that the INCENP–Plk1 interaction directly recruits Plk1 to kinetochores. We next used the same siRNA that had been used by Goto et al. (2006) to deplete INCENP from HeLa cells (Figure 7E). Consistent with Goto et al. (2006), INCENP-RNAi reduced the kinetochore localization of Plk1 (Figure 7, A and C), albeit to a lesser extent than Bub1-RNAi. Importantly, the kinetochore localization of Bub1 was greatly reduced in INCENP-RNAi cells (Figure 7, B and D). These results suggest that INCENP might regulate the localization of Plk1 through Bub1.

**DISCUSSION**

**Polo-Box- and Phosphorylation-dependent Binding of Plk1 to Bub1 in Mitosis**

Ever since Yaffe and coworkers discovered the phosphopeptide-binding activity of the PBD of Plk1 (Elia et al., 2003a), numerous studies have established a role of the Plk1 PBD in targeting Plk1 to its binding partners and substrates (Litvak et al., 2004; Yoo et al., 2004; Fabbro et al., 2005; Preisinger et al., 2005). The prevailing view that emerged from these studies is that the Plk1-binding protein is first phosphorylated by a “priming” kinase, which creates a docking site for the PBD of Plk1. Binding of the phosphopeptide to PBD recruits Plk1 to its binding partner and allosterically activates the kinase activity of Plk1, thus facilitating the phosphorylation of Plk1 substrates. In addition to being Plk1 substrates, certain Plk1-binding proteins also target Plk1 to various mitotic structures. Our results presented herein suggest that binding of Plk1 to Bub1 seems to exploit a similar mechanism and targets Plk1 to kinetochores.

Because the optimal PBD-binding motif is S-pS/pT-P, the priming kinase for Plk1-binding proteins is likely a proline-directed protein kinase. Not surprisingly, the master mitotic kinase, Cdk1, has been shown to be the priming kinase for the majority of Plk1-binding proteins, including Cdc25C, GRASP65, Cep55, and Nir2, although the MAP kinase Erk2 has also been implicated in the priming phosphorylation of Cep55 (Elia et al., 2003a; Litvak et al., 2004; Fabbro et al., 2005; Preisinger et al., 2005). In the case of Bub1, we have shown that Cdk1 is sufficient to phosphorylate T609 and facilitates the binding of Plk1 to Bub1 and the subsequent phosphorylation of Bub1 by Plk1. We have also shown that Bub1 is phosphorylated at T609 in mitotic HeLa cells. Inhibition of Cdk1 by roscovitine in cells causes the dephosphorylation of Bub1 and disrupts its interaction with Plk1. The fission yeast Bub1 is also phosphorylated by Cdk1 (Yamaguchi et al., 2003). These results strongly suggest that Cdk1 is the prim-
Requirement for Bub1 in the Kinetochore Localization of Plk1

It has become increasingly clear that Plk1 has important functions at the kinetochores during mitosis (Ahonen et al., 2005; Wong and Fang, 2005). It is thus critical to understand how Plk1 itself is recruited to kinetochores. Numerous elegant studies in Xenopus egg extracts and mammalian cells have established the interdependency and hierarchy of a large collection of mitotic regulatory proteins with respect to their localization at the kinetochores (Sharp-Baker and Chen, 2001; Johnson et al., 2004; Vigneron et al., 2004). Our results presented herein have established a requirement for Bub1 in the kinetochore localization of Plk1. Our findings are consistent with the notion that the kinetochore localization of Plk1 is facilitated by its polo-box- and phosphorylation-dependent binding to Bub1.

Intriguingly, although Plk1 also binds to BubR1 in mitosis, the kinetochore localization of Plk1 is not significantly affected by BubR1 depletion. There are several possible explanations for this observation. First, depletion of Bub1 might have caused the loss of other yet unidentified Plk1-binding proteins at the kinetochores. For the first possibility to be correct, the kinetochore localization of this putative Plk1-binding protein would also have to require T609 of Bub1, because expression of Bub1-T609A fails to restore the kinetochore localization of Plk1. Second, the concentration of BubR1 at the kinetochores might be lower than that of Bub1. Thus, loss of the BubR1-bound pool of Plk1 in BubR1-RNAi cells does not significantly alter the concentration of Plk1 at the kinetochores. Third, our results showed that the kinetochore localization of BubR1 requires Plk1 (Supplemental Figure 3). Studies in yeast have also revealed that the mitotic phosphorylation of Mad3, the yeast orthologue of BubR1, requires Plk1 (Rancati et al., 2005). It is possible that Plk1 also mediates the mitotic phosphorylation of BubR1 in mammalian cells. Binding between Plk1 and BubR1 might simply be a result of a kinase–substrate relationship and only occur following the recruitment of Plk1 to the kinetochores by Bub1. Consistent with this notion, the central spindle protein Nir2 binds to Plk1 and is a Plk1 substrate, but Nir2 is not required for the localization of Plk1 at the central spindle (Litvak et al., 2004). Regardless of which possibility is correct, our results clearly indicate that Bub1, but not BubR1, is upstream of Plk1 with respect to kinetochore localization.

Relationship between Bub1 and INCENP in the Kinetochore Targeting of Plk1

In a recent report, Goto et al. (2006) show that Plk1 binds directly to INCENP and that the Plk1–INCENP interaction depends on the PBD of Plk1 and Cdk1-mediated phosphorylation of INCENP at T388. Depletion of INCENP by RNAi resulted in inefficient kinetochore targeting of Plk1, which can be rescued by ectopic expression of the wild-type INCENP, but not the T388A mutant of INCENP. Contrary to a published report (Honda et al., 2003), Goto et al. (2006) further show that depletion of Aurora B by RNAi does not affect the kinetochore localization of INCENP and thus does not affect the kinetochore localization of Plk1. These results led Goto et al. (2006) to propose that INCENP directly recruits Plk1 to the kinetochores in an Aurora B-independent manner.

However, the model by Goto et al. (2006) is inconsistent with the following observations. First and foremost, as mentioned previously, INCENP localizes to the inner kinetochores, whereas Plk1 localizes to outer kinetochores (Figure 7A), suggesting that the bulk of kinetochore-bound pools of these two proteins does not associate with each other. Bub1 and Plk1 colocalize to outer kinetochores, consistent with their direct physical interaction. Second, the mechanisms of kinetochore targeting of many kinetochore components are conserved between mammalian cells and Xenopus egg extracts. T388 of INCENP is not conserved in Xenopus INCENP. Third, the kinetochore localization of INCENP and Aurora B is interdependent (Honda et al., 2003). We have confirmed that the kinetochore localization of INCENP is indeed diminished in Aurora B-RNAi cells (Supplemental Figure 4). Rather expectedly, we also show that the kinetochore localization of Plk1 is diminished in Aurora B-RNAi cells (Supplemental Figure 4). These observations contradict the findings of Goto et al. (2006) that INCENP and Plk1 localize normally to kinetochores in Aurora B-RNAi cells and challenge their conclusion that the kinetochore localization of Plk1 is independent of Aurora B. One possible explanation is that the siRNAs against Aurora B used by Goto et al. (2006) did not deplete the levels of Aurora B as efficiently. Finally, numerous studies have shown that the Aurora B–INCENP complex is one of the most upstream components in the signaling cascades that control the kinetochore targeting of many kinetochore proteins. In particular, the Aurora B–INCENP complex is required for the localization of other spindle checkpoint proteins, such as Bub1, BubR1, and Mad2 (Johnson et al., 2004; Vigneron et al., 2004). Given that the kinetochore localization of Plk1 also requires Bub1 (this study), we favor the notion that the Aurora B–INCENP complex targets Bub1 to kinetochores, which in turn helps to recruit Plk1 to kinetochores. However, it remains possible that the Aurora B–INCENP complex is required to recruit Plk1 from the cytoplasm to the kinetochores initially, which enables Plk1 to associate with Bub1 and other PBD-docking proteins at kinetochores. The kinetochore localization of Plk1 is then maintained by Bub1. This latter model explains the defective kinetochore localization of Plk1 in INCENP-T388A–expressing cells.

Functions of Plk1 at Kinetochores and in the Spindle Checkpoint

Recently, two independent studies in Xenopus egg extracts and mammalian cells have demonstrated that Plk1/Plx1 creates the tension-sensing 3F3/2 phosphoepitope at the kinetochores in response to the lack of mechanical tension across sister kinetochores (Ahonen et al., 2005; Wong and Fang, 2005). Loss of proper Plk1/Plx1 function not only re-
duces the 3F3/2 signal at the kinetochores but also decreases the concentrations of other proteins at the kinetochores, including Hec1/Ndc80, Spc24, Mad2, Cdc20, CENP-E, and possibly BubR1 (Ahonen et al., 2005; Wong and Fang, 2005). These findings strongly suggest that Plk1 is crucial for spindle checkpoint signaling, at least in response to the lack of tension at kinetochores. Paradoxically, RNAi-mediated depletion of Plk1 in mammalian cells causes abnormal mitotic spindles and a spindle checkpoint-dependent mitotic arrest (Sumara et al., 2004; van Vugt et al., 2004). It is possible that the residual levels of Plk1 in these Plk1-RNAi cells are sufficient for its function in the spindle checkpoint, but they are insufficient for its earlier mitotic function in spindle assembly. Our study establishes a role of Bub1 upstream of Plk1 in the hierarchy of kinetochore localization, and, quite possibly, in the tension-sensing pathway. That Bub1, a well-established spindle checkpoint protein, controls the kinetochore localization of Plk1 further supports a role of Plk1 in the spindle checkpoint. Unfortunately, we have so far failed to detect spindle checkpoint defects in Bub1-T609A-expressing cells that are treated with Bub1 RNAi. For example, expression of Bub1-T609A restores nocodazole/taxol-dependent mitotic arrest of Bub1-RNAi cells (our unpublished data). There are two possible explanations for this finding. Loss of Bub1-dependent kinetochore targeting of Plk1 may not completely deplete Plk1 at kinetochores. Alternatively, the kinetochore localization of Plk1 might not be absolutely required for the spindle checkpoint. Soluble, cytoplasmic pools of Plk1 might be sufficient to generate the 3F3/2 phosphopeptide.

In conclusion, we have shown that the Bub1–Plk1 interaction is required for efficient kinetochore targeting of Plk1 in mitosis. It has been shown previously that Bub1 and Plk1 are required for the kinetochore localization of Mad2 and other downstream checkpoint proteins in a manner that does not require the kinase activity of Bub1 (Sharp-Baker and Chen, 2001; Johnson et al., 2004; Ahonen et al., 2005; Wong and Fang, 2005). Together, these findings are consistent with the following model: phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 recruits Plk1 to kinetochores. The kinetochore-bound Plk1 then facilitates the kinetochore localization of BubR1, Mad2, and other checkpoint components.

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