Phosphorylation Regulates Kinase and Microtubule Binding Activities of the Budding Yeast Chromosomal Passenger Complex in Vitro*

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The chromosomal passenger complex (CPC) is a key regulator of mitosis in eukaryotes. It comprises four essential and conserved proteins known in mammals/yeasts as Aurora B/Ipl1, INCENP/Sli15, Survivin/Bir1, and Borealin/Nbl1. These subunits act together in a highly controlled fashion. Regulation of Aurora B/Ipl1 kinase activity and localization is critical for CPC function. Although regulation of CPC localization and kinase activity in vivo has been investigated elsewhere, studies on the complete, four-subunit CPC and its basic biochemical properties are only beginning. Here we describe the biochemical characterization of purified and complete Saccharomyces cerevisiae four-subunit CPC. We determined the affinity of the CPC for microtubules and demonstrated that the binding of CPC to microtubules is primarily electrostatic in nature and depends on the acidic C-terminal tail (E-hook) of tubulin. Moreover, phosphorylation of INCENP/Sli15 on its microtubule binding region also negatively regulates CPC affinity for microtubules. Furthermore, we show that phosphorylation of INCENP/Sli15 is required for activation of the kinase Aurora B/Ipl1 and can occur in trans. Although phosphorylation of INCENP/Sli15 is required for activation, we determined that a version of the CPC lacking the INCENP/Sli15 microtubule binding region (residues Glu-91 to Ile-631) is able to form an intact complex that retains microtubule binding activity. Thus, we conclude that this INCENP/Sli15 linker domain plays a largely regulatory function and is not essential for complex formation or microtubule binding.

Background: The chromosomal passenger complex (CPC) is essential to ensure faithful segregation of chromosomes.

Results: Phosphorylation of the yeast CPC on INCENP-Sli15 activates CPC kinase and reduces CPC binding to microtubules.

Conclusion: CPC kinase activity and microtubule binding are highly regulated.

Significance: A protocol to purify milligram quantities of CPC allows for biochemical and structural studies of its functions and regulation.

The chromosomal passenger complex (CPC), also called the Aurora B complex, has been shown to play a key role in orchestrating spindle activities, checkpoint signaling, establishing chromosome bi-orientation, and in controlling cytokinesis (3–5). The CPC is composed of four proteins in mammals: Aurora B, INCENP, Survivin, and Borealin. The Aurora B kinase subunit is critical for all known functions of this complex, and inhibition of its catalytic activity by specific drugs has shown promise for cancer chemotherapy (6). During progression through mitosis, the CPC undergoes a very dynamic and precise sequence of changes in localization that involve moving from centrotermes during metaphase to the spindle midzone during anaphase (for review, see Ref. 7).

Among eukaryotes, the CPC is highly conserved. The CPC in the budding yeast Saccharomyces cerevisiae, also called the Ipl1 complex, is composed of four subunits: Ipl1, Bir1, Sli15, and Nbl1, which are the orthologs of the mammalian Aurora B, Survivin, INCENP, and Borealin, respectively (8, 9). Key properties of the CPC in yeast, including its dynamic localization and biologic functions, are similar to those described for the CPC of more complex organisms (9).

The kinase activity of Aurora B/Ipl1 is essential for CPC activity and is fully activated by INCENP/Sli15 phosphorylation (10–13). INCENP/Sli15 has a modular organization (Fig. 1A). Its domains include (i) an IN-box domain that binds Aurora B/Ipl1, this domain is also phosphorylated by Aurora B/Ipl1 and activates the kinase activity of Aurora B/Ipl1 (14); (ii) a microtubule binding domain (MTB) involved in binding of the CPC complex to microtubules (MTs) (15); and (iii) a three-helix bundle (THB) domain that binds Survivin/Bir1 and Borealin/Nbl1 (9, 16). INCENP/Sli15 serves as a linker between the Aurora B/Ipl1 kinase subunit and the Survivin/Bir1 and Borealin/Nbl1 subunits (Fig. 1A). These other CPC subunits (Survivin/Bir1 and Borealin/Nbl1) are thought to direct the CPC to its different locations during mitosis (for review, see Ref. 17).

Mitosis is an essential process required to faithfully segregate replicated chromosomes during eukaryotic cell division. Errors in segregation give rise to chromosome aneuploidy, a condition that can promote the development of various cancers. The fidelity of mitosis is ensured by multiple checkpoints, highlighting the critical physiologic importance of accurately segregating chromosomes (1, 2).

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2 The abbreviations used are: CPC, chromosomal passenger complex; CIP, calf intestine phosphatase; KD, kinase-dead; MT, microtubule; MTB, microtubule binding; S-MT, subtilisin-treated microtubule; THB, three-helix bundle.
Despite all that is known about its subunits, little is known about the biological and biochemical activities of the complete four-subunit CPC itself: the interactions of the individual subunits, geometry of the complex, and autoregulation. Here we describe the purification of the intact yeast CPC expressed in bacteria and the biochemical characterization of the complete four-subunit complex. We show that kinase activation requires phosphorylation of INCENP/Sli15 at Aurora B/Ipl1 sites, that this phosphorylation regulates CPC kinase activity and CPC affinity for MTs, and that this phosphorylation can occur in trans. We also determined the affinity of the CPC for MTs and demonstrated that the binding of CPC to MTs is primarily electrostatic, depending critically on the acidic C-terminal (E-hook) tail of tubulin. Finally, we found that the MTB domain of INCENP/Sli15 is not only dispensable for the stability of the CPC, but also for the ability of CPC to bind MTs.

**EXPERIMENTAL PROCEDURES**

**CPC Expression/Purification**—A polycistronic vector was constructed using the pET3aTr/pST39 plasmid system (18). **BIR1**, **SLI15**, **IPL1**, and **NBL1** genes were amplified from *S. cerevisiae* genomic DNA by PCR and inserted individually into the pET3aTr plasmid using the NdeI/BamHI restriction sites. Six histidines were added C-terminally to **NBL1** together with a three-glycine linker, and silent mutations were introduced to eliminate restriction sites that would interfere with the cloning process. The coding sequences for the four individual genes along with their translation cassette (translation enhancer/Shine-Delgarno sequence/TATA box for expression in *Escherichia coli*) were cloned from pET3aTr and inserted into pST39C backbone in the following order: **SLI15**, **IPL1**, **BIR1**, and **NBL1**-His6 using the Xbal/BamHI, EcoRI/MfeI/HindIII, ScaI/KpnI, and BspEI/MluI/BasHII restriction enzymes, respectively, to make the pDD2396 plasmid.

The CPC mutant with all 17 Aurora B/Ipl1 consensus sites in INCENP/Sli15 mutated to alanines (17A-Sli15)-CPC (pDD2397) was constructed following the same procedure. 17A-Sli15 was amplified by PCR from plasmid pDD2397 described in Ref. 19.

The Ipl1 kinase-dead mutant complex (KD-CPC), pDD2399, was constructed by mutagenesis of the Ipl1 gene in the WT-CPC plasmid (pDD2396) to create the K133R mutation in Ipl1. The CPC mutant containing Ipl1 kinase-dead and Sli15-GFP (KD-GFP-CPC), pDD2400, was constructed by ligation of a GFP tag using the unique restriction site AvrII in pDD2399.

**Microtubule Co-sedimentation Assay**—The MT co-sedimentation assay was done under the same conditions to get a more pure CPC. The fractions containing the CPC were pooled and concentrated to 500 μl using centrifugal concentrators (Ultracel 30K; Amicon). The concentrated sample was injected onto a Superose 6 10/300 GL size-exclusion column (GE Healthcare) equilibrated with 10% glycerol, 50 mM Tris- HCl, pH 7.4, 300 mM KCl, 5 mM MgCl2, 5 mM ATP, 1 mM DTT, 1 mM PMSF, 20 mM imidazole, 1% Triton X-100, and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science) for 50 ml of lysis buffer, sonicated for 3 min at 1-min intervals, and centrifuged at 66,000 × g for 20 min at 4 °C. The supernatant was collected and injected onto a HisTrap HP column (GE Healthcare), and the following buffer was used to wash the column: 10% glycerol; 50 mM Tris-HCl, pH 7.4, 300 mM KCl, 5 mM MgCl2, 5 mM ATP, 1 mM DTT, 1 mM PMSF, 20 mM imidazole. The tagged protein was eluted using the same buffer containing 500 mM imidazole. The fractions containing CPC were pooled and incubated with 1 ml Chitin Beads (NEB) for 30 min at 4 °C to remove chaperone proteins (20). The supernatant was concentrated to 500 μl using centrifugal concentrators (Ultracel 30K; Amicon). The concentrated sample was injected onto a Superose 6 10/300 GL size-exclusion column (GE Healthcare) equilibrated with 10% glycerol, 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 50 mM arginine, 50 mM glutamate. Fractions containing the CPC were pooled and concentrated to 500 μl using centrifugal concentrators (Ultracel 30K). Another round of size-exclusion chromatography was done under the same conditions to get a more pure CPC. The fractions containing the CPC were pooled and concentrated to the appropriate volume using centrifugal concentrators (Ultracel 30K) and then flash frozen in liquid nitrogen and stored at −80 °C. The yield was ~250 μg of CPC/liter of *E. coli* culture.

**Microtubule Co-sedimentation Assay**—The MT co-sedimentation assays have been conducted as described previously (19). To summarize, bovine tubulin was assembled into MTs with GTP at 37 °C and then stabilized with Taxol. Those Taxol-stabilized MTs were used at different concentrations and mixed with a fixed amount of CPC in a buffer containing 20% glycerol,
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FIGURE 2. Phosphorylation of INCENP/Sli15 by Aurora B/Ipl1 regulates the CPC kinase activity. A, WT-CPC was phosphorylated on INCENP/Sli15 when expressed in bacteria. Purified WT-CPC (first lane), mock-treated (second lane), or treated with alkaline phosphatase (third lane) were run on SDS-PAGE and immunoblotted with anti-Sli15 antibody. B, purified WT-CPC retained its kinase activity and specificity. Aurora B/Ipl1 kinase activity was assessed by quantifying the amount of \( ^{32}P \) transferred by Aurora B/Ipl1 kinase from \( [\gamma-^{32}P]ATP \) to its substrate (here the Dam1 complex, left lane, or histone H1, right lane) in an autoradiogram (bottom panel). The top panel shows the same SDS-polyacrylamide gel stained with Coomassie Blue. C, Aurora B/Ipl1 subunit of CPC phosphorylated INCENP/Sli15. WT-CPC or KD-CPC was incubated with radiolabeled ATP, and phosphorylation events on INCENP/Sli15 were revealed by autoradiography. D, INCENP/Sli15 was phosphorylated by Aurora B/Ipl1 in trans. WT-CPC or a mutant containing all Aurora B/Ipl1 consensus sites on INCENP/Sli15 mutated to alanines (17A-Sli15)-CPC was incubated alone or mixed with CPC containing a kinase-dead mutant of Aurora B/Ipl1 and a GFP-tagged INCENP/Sli15 (KD-GFP-CPC) in the presence of radiolabeled ATP. Phosphorylation of the target protein was revealed by autoradiography. E, phosphorylation on INCENP/Sli15 was necessary for Aurora B/Ipl1 kinase activation. WT-CPC, phosphatase-treated CPC (CIP-CPC) or (17A-Sli15)-CPC were incubated alone or with the substrate Dam1. Phosphorylation of Dam1 was assessed by autoradiography.

RESULTS

Purification of the Complete Yeast Aurora B/Ipl1 Complex from E. coli—To obtain intact CPC for biochemical characterization, we cloned the four full-length protein subunits of the complex (Survivin/Bir1, INCENP/Sli15, Aurora B/Ipl1, and Borealin/Nbl1; Fig. 1A) into a polycistronic vector for expression in E. coli (18). The smallest subunit, Borealin/Nbl1, was tagged at its C-terminal end with a His\(_6\) tag separated by a 3-glycine amino acid linker, and the complex was purified using a nickel affinity column (Fig. 1B) followed by size-exclusion chromatography (Fig. 1C). A second round of size-exclusion chromatography further purified the complex (Fig. 1D). We

50 mM HEPES, pH 7.4, 50 mM NaCl, 50 mM arginine, 50 mM glutamate, 1 mM PMSF, and 10 μM Taxol and incubated for 20 min at room temperature. Samples were spun down, and supernatant and pellet fractions were collected, run on SDS-PAGE, transferred onto nitrocellulose membrane, and blotted using anti-Sli15 or anti-Bir1 antibodies.

CPC in Vitro Kinase Assay—The CPC in vitro kinase assays were conducted as described previously (8). In Fig. 2B, CPC was used at a concentration of 10 nM, and Dam1 and histone H1 were used at a concentration of 10 μM. In Fig. 2C, CPC was used at a concentration of 1 μM. In Fig. 2D, WT-CPC or (17A-Sli15)-CPC was used at a concentration of 50 nM and (KD-GFP)-CPC was used at a concentration of 1 μM. In Fig. 2E, CPC was used at a concentration of 1 nM, and Dam1 was used at a concentration of 1 μM.

Dam1 Complex Expression and Purification—The Dam1 complex for the in vitro kinase assays was purified as described previously (21).

Determination of the CPC Stokes Radius—A mixture of proteins of known molecular mass and Stokes radii (gel filtration standard; Bio-Rad) was loaded on a gel filtration column (Superose 6). The \( K_{av} \) (partition coefficient) was determined for each protein as \( K_{av} = (V_c - V_0)/(V_t - V_0) \) where \( V_c \) is the elution volume of the particular protein, \( V_0 \) is the void volume of the column, and \( V_t \) is the total volume of the column. \( V_0 \) and \( V_t \) were determined using acetone and dextran blue. The \( K_{av} \) of CPC was determined, and the calibration curve found in Fig. 3 was then used to convert its \( K_{av} \) to its apparent molecular mass and Stokes radius.
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also assessed the quality of the purified complex final product by Coomassie Blue staining of a SDS-polyacrylamide gel, as well as by immunoblot analysis using antibodies against Survivin/Bir1, INCENP/Sli15, and Aurora B/Ipl1 (Fig. 1E). The full complex, with a calculated molecular mass of 243 kDa, eluted from the size-exclusion column at the same size as a 1.5-MDa globular protein (Stokes radius of 9.7 nm). This value is comparable with the 10.3-nm Stokes radius determined for native yeast CPC\(^3\) and indicates that yeast CPC expressed in and purified from bacteria has organization and oligomerization properties similar to those of the native CPC from yeast. The CPC elutes from size-exclusion chromatography as a larger complex than expected based on molecular mass, which is consistent with an elongated shape and/or a multimer of complexes (at most a hexamer).

Phosphorylation of CPC by Aurora B/Ipl1 on INCENP/Sli15 Regulates Its Kinase Activity—Following CPC purification, we observed that INCENP/Sli15, with a molecular mass of 79 kDa, exhibited anomalously slow migration in SDS-polyacrylamide gels, at slightly more than 100 kDa. INCENP/Sli15 has previously been shown to be phosphorylated by Aurora B/Ipl1 kinase (19, 22). To test whether the slow migration of INCENP/Sli15 was due to phosphorylation, we subjected the CPC to dephosphorylation using nonspecific calf intestine phosphatase (CIP). Treatment with CIP increased the migration rate of the INCENP/Sli15 protein band, whereas mock treatment resulted in INCENP/Sli15 migrating at the same speed as the original untreated sample (Fig. 2A). This result suggests that INCENP/Sli15 is phosphorylated when expressed in bacteria.

To determine whether the Aurora B/Ipl1 subunit of CPC could be responsible for phosphorylation of INCENP/Sli15, we tested whether Aurora B/Ipl1 retains its kinase activity after being purified from bacteria. We first evaluated the activity and specificity of Aurora B/Ipl1 kinase on a known substrate, the Dam1 complex (8), and on a histone protein known not to be a CPC substrate (histone H1 (23)). Upon addition of radiolabeled ATP, the band corresponding to Dam1 strongly incorporated radiolabeled ATP whereas no signal was detectable for histone H1 (Fig. 2B), showing that CPC retained its kinase activity and specificity toward Dam1 and not histone H1.

Because INCENP/Sli15 is a known target of Aurora B/Ipl1, we next incubated the CPC by itself with radiolabeled ATP. After incubation, we observed a strong signal at the level of the INCENP/Sli15 band that was not observed when using a kinase-dead mutant of the complex (KD-CPC, Fig. 2C). Aurora B/Ipl1 is thus capable of phosphorylating the INCENP/Sli15 subunit.

Another question we asked is whether phosphorylation of INCENP/Sli15 might occur in vivo, with the Aurora B/Ipl1 kinase subunit of one CPC phosphorylating the INCENP/Sli15 subunit of another CPC. If true, we would expect that incubating WT-CPC with a mutant containing Ipl1 kinase-dead and Sli15-GFP (KD-GFP-CPC) would lead to phosphorylation of Sli15-GFP by Ipl1 from the WT complex. This was indeed the result with KD-GFP-CPC not phosphorylating its own INCENP/Sli15, but with this subunit being phosphorylated by the WT–CPC in the reaction mix (Fig. 2D). We also noticed that a CPC mutant version with the 17 Aurora B/Ipl1 consensus sites in INCENP/Sli15 mutated to alanines (described in Ref. 19) named (17A-Sli15)-CPC was not able to phosphorylate its own Sli15 or the Sli15-GFP of the kinase-dead CPC complex. The fact that (17A-Sli15)-CPC lacks kinase activity suggests that INCENP/Sli15 phosphorylation by AuroraB/Ipl1 kinase might be associated with an active state of the Aurora B/Ipl1 kinase CPC complex (Fig. 2D).

To investigate further the requirement of CPC phosphorylation for kinase activity, we asked whether the key residues for CPC kinase activation are contained within the Sli15 subunit of the complex. To test this, we used the Dam1 complex as a substrate and compared the kinase activity of WT–CPC, CPC that had been dephosphorylated by treatment with CIP (CIP-CPC), and (17A-Sli15)-CPC. As expected, WT–CPC is active whereas CIP-treated CPC has a dramatically reduced activity (Fig. 2E, fifth and sixth lanes). Moreover, (17A-Sli15)-CPC does not have activity (Fig. 2E, seventh lane). These results suggest that the specific phosphorylation sites responsible for CPC activation are indeed phosphorylation sites for Aurora B/Ipl1 on INCENP/Sli15.

These results are consistent with the conclusion that the kinase activity is dependent upon the CPC phosphorylation state. They also indicate that INCENP/Sli15 phosphorylation by Aurora B/Ipl1 might play a major regulatory role in activating CPC kinase activity in vivo.

Phosphorylation of CPC by Aurora B/Ipl1 on INCENP/Sli15 Inhibits CPC Binding to Microtubules—To explore the MT binding activity of the CPC, we incubated a fixed amount of CPC with varying amounts of Taxol-stabilized MTs and pelleted the mixture by ultracentrifugation. We then measured how the CPC distributes between supernatant (unbound) and pellet (bound). In this experiment, we found that the bacterially expressed CPC binds to MTs (Fig. 4A). Moreover, we noticed that a fraction of the CPC could not be pelleted even at high MT concentrations. Interestingly, this nonbinding fraction of the CPC migrates at a higher apparent molecular mass than the

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3 Y. Nakajima, unpublished data.
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FIGURE 4. Phosphorylation of INCENP/Sli15 by Aurora B/Ipl1 regulates CPC binding to MTs. A, WT-CPC containing slow migrating INCENP/Sli15 (hyperphosphorylated) does not bind to MTs whereas WT-CPC containing fast migrating INCENP/Sli15 (hypophosphorylated) does bind to MTs. Increasing concentrations of MTs were incubated with 0.1 µM WT-CPC, pellet, and the supernatants (S) and pellets (P) were run on SDS-PAGE, stained with Coomassie Blue (control for tubulin pelletting, data not shown), or immunoblotted with anti-Sli15 antibody. B, CPC-CPC, as well as (17A-Sli15)-CPC, show enhanced binding to MTs. WT-CPC, CIP-CPC, or (17A-Sli15)-CPC was incubated with or without 5 µM MTs and processed as in A. C, graph represents a variation of (17A-Sli15)-CPC binding to MTs using various amounts of MTs. This graph is used to fit a binding curve and determine the $K_d$ of (17A-Sli15)-CPC for MTs, yielding a value of 0.1 µM.

CPC binding to microtubules is electrostatic and depends on the C-terminal (E-hook) tail of tubulin—To investigate the nature of the interaction of the CPC with MTs and to evaluate the contribution of electrostatic and/or hydrophobic components to binding, we performed an assay in which (17A-Sli15)-CPC (containing the mutant Sli15 subunit that cannot be phosphorylated and thus forming a CPC complex that binds strongly to MTs) was incubated with MTs and increasing concentrations of NaCl. Because increasing concentrations of NaCl disrupt electrostatic interactions but stabilize hydrophobic interactions, this approach can be used as a method for evaluating the nature of the interaction. At a high NaCl concentration (200 mM), the interaction between CPC and MTs was completely abolished (Fig. 5A), suggesting that electrostatic interactions provide the main component of the binding energy between CPC and MTs.

An electrostatic interaction could result from binding to a specific site on the MT lattice, or it could occur without a specific footprint on the MT but through interactions with the charged $\alpha$- and $\beta$-tubulin C termini. These two interaction possibilities are not mutually exclusive. In an attempt to distinguish between these possibilities, we performed a MT binding assay using subtilisin-treated MTs (S-MTs). Subtilisin is a protease that cleaves the unstructured C-terminal tail of tubulin (E-hook) (24). Under our experimental conditions, the C-terminal tails of both $\alpha$- and $\beta$-tubulin were proteolyzed (Fig. 6). Both WT-CPC and (17A-Sli15)-CPC exhibited a decreased affinity for S-MTs compared with untreated MTs (Fig. 5B). We calculated the affinity of (17A-Sli15)-CPC for S-MTs to be 0.6 µM (data not shown) compared with 0.1 µM for MTs. This suggests that a major component of WT-CPC and (17A-Sli15)-CPC binding to MTs is through general electrostatic interactions with the C-terminal tail of tubulin in the absence of a specific CPC footprint on the MT lattice. However, because subtilisin treatment did not completely abrogate binding to MTs, binding also depends in part on other, nonelectrostatic interactions.

The MITD Domain of INCENP/Sli15 Is Not Required for the CPC Microtubule Binding Activity—To investigate the contribution of the MITD domain of INCENP/Sli15 to the overall integrity and microtubule binding ability of the CPC, we designed a mutant CPC complex in which the INCENP/Sli15
MTB domain is deleted (amino acids 91–631), and the remaining N-terminal (from amino acids 1 to 90) and C-terminal portions of INCENP/Sli15 (from amino acids 632 to 698) flanking this deleted MTB domain are themselves synthesized as two separate and distinct polypeptides. This mutant complex is called (ΔM-Sli15)-CPC. To express the CPC with these two INCENP/Sli15 distinct segments, we created a polycistronic plasmid that expresses Survivin/Bir1, Aurora B/Ipl1, Borealin/Nbl1-His6, the N-terminal sequences of INCENP/Sli15 (from amino acids 1 to 90), and the C-terminal sequences of INCENP/Sli15 distinct segments, we created a polycistronic plasmid that expresses Survivin/Bir1, Aurora B/Ipl1, Borealin/Nbl1-His6, the N-terminal sequences of INCENP/Sli15 (from amino acids 1 to 90), and the C-terminal sequences of INCENP/Sli15 (from amino acids 632 to 698) for a total of five open reading frames. We then followed the same purification protocol as with the full complex (described above). We found that even though the N- and C-terminal domains of INCENP/Sli15 were expressed as distinct proteins, an intact “hybrid” CPC was generated that contains all five polypeptides: the three proteins expected of a CPC complex (Survivin/Bir1, Aurora B/Ipl1, and Borealin/Nbl1) plus the two N- and C-terminal polypeptides of INCENP/Sli15 (Fig. 7B). Although we determined that the full-length INCENP/Sli15 is not present in this hybrid complex (Fig. 7B), we were not able to detect any of the two INCENP/Sli15 polypeptides generated due to their small size (the N-terminal and the C-terminal fragments are 11 and 8 kDa, respectively) and possibly due to the absence of the epitope(s) necessary for detection with anti-Sli15 antibody. However, when we tried to express the CPC without INCENP/Sli15 (expressing only Aurora B/Ipl1, Survivin/Bir1, and Borealin/Nbl1), we could not detect any complex assembly (data not shown), indicating that the CPC is not stable when it is missing either the N-terminal or the C-terminal part of INCENP/Sli15. This result is also supported by the fact that Sli15 is destabilized when complex formation through the THB is disrupted (25). This might be due to the necessity of the presence of the THB domain of INCENP/Sli15 to bind Survivin/Bir1 to Borealin/Nbl1 (16).

The hybrid (ΔM-Sli15)-CPC eluted from the size-exclusion column at the same volume as does the wild-type CPC (data not shown), indicating that a normal sized CPC is able to assemble and that the integrity of this (ΔM-Sli15)-CPC complex is preserved relative to the wild-type CPC without affecting its oligomerization properties.

Because both the MTB domains of INCENP/Sli15 and of Aurora B/Ipl1 independently bind directly to MTs (22), we wanted to know whether the MTB domain of INCENP/Sli15, in the context of this fully reconstituted hybrid complex, is necessary for CPC binding to MTs. To ask this question, we incubated (ΔM-Sli15)-CPC with MTs and found that this CPC complex does still retain the ability to bind MTs (Fig. 7C), but with an affinity reduced to 4.5 μM (Fig. 7D).

DISCUSSION

In this article, we report for the first time a protocol for the expression and purification of the intact, complete yeast CPC expressed in bacteria, which yields enough material for detailed studies of the biochemical properties of the complex. Using this...
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reagent, we obtained new insights into CPC kinase activity, stability, and MT interaction.

The function of the CPC is directly linked to its localization and enzymatic activity. Our bacterially expressed yeast CPC retains the same subunit composition and oligomerization properties as the CPC purified from yeast, but we can achieve a significantly higher yield of 50 mg of protein/g of bacteria. As shown for higher eukaryote CPC subcomplexes (10, 26) and the yeast CPC subcomplex containing Aurora B/Ipl1-INCENP/Sli15 (22), our recombinant four-subunit CPC retains its kinase activity and substrate specificity. Moreover, our data shed light on the role of CPC phosphorylation of the INCENP/Sli15 subunit, an event responsible for the activation of the full kinase activity of the CPC possibly similar to an activation loop. We also showed that the Aurora B/Ipl1 kinase subunit of one CPC complex is able to phosphorylate the INCENP/Sli15 subunit of another CPC complex in *trans in vitro*. This finding suggests that oligomerization might play a role in CPC activation *in vivo* by bringing several complexes in close proximity in such a manner that this activating phosphorylation can occur. This may be particularly relevant during late anaphase in yeast, when accumulation of CPC at the midzone coincides with a high activity of CPC that phosphorylates and activates several spindle-destabilizing factors (27).

The binding of CPC to MTs is essential for many of its functions *in vivo*, and its regulation by phosphorylation has a critical role in the timely localization of CPC along the mitotic spindle (19, 28). Our CPC expressed in bacteria binds to MTs and phosphorylation of the INCENP/Sli15 subunit leads to a loss of affinity of the CPC for those MTs. We also found that dephosphorylated CPC and a mutant form of the CPC that mimics the dephosphorylated state, (17A-Sli15)-CPC, have similar affinities for MTs, the *Kₐ* of (17A-Sli15)-CPC and phosphatase-treated CPC for MTs both being ~0.1 μM, whereas the highly phosphorylated version of recombinant CPC exhibited virtually no MT binding activity. These results are consistent with CPC *in vivo* activity. Indeed, in a *S. cerevisiae* strain expressing (17A-Sli15)-CPC, the CPC is found prematurely associated with the spindle and anomalously enriched at spindle pole bodies during metaphase, as well as prematurely enriched at the midzone during late anaphase (19). Because the phosphorylation of INCENP/Sli15 regulates the affinity of the CPC for MTs, CPC phosphorylation may have an important role in regulating the spatio-temporal association of CPC with the spindle.

A large number of MT-associated proteins (MAPs) use the C-terminal negatively charged tail of tubulin (the E-hook) either to bind to and diffuse along the surface of MTs (29, 30) or to serve as a flexible, low affinity tether that augments a higher specificity binding site spaced at regular intervals along the MT surface (31). However, for some MAPs, binding does not depend on tubulin C-terminal tail (e.g. myosin 5 (32)). Here we showed that the CPC binds to MTs through electrostatic interactions and that the C-terminal tail of tubulin contributes strongly to this interaction. Thus, the CPC may bind to the MT lattice with a combination of an electrostatic interaction perhaps combined with a precisely defined footprint. These interaction modes of a diffusive mobility pattern, together with cooperative CPC oligomerization, might account for the observations of CPC punctae at the surface of MTs both *in vitro* and *in vivo* (19).

Interactions between CPC subunits and their individual contributions to CPC integrity and function are not completely understood. Other than some clear interactions highlighted by the crystal structures of globular Aurora B/Ipl1-INCENP/Sli15 (12) and Survivin/Bir1-INCENP/Sli15-Borealin/Nbl1 (16) subcomplexes, how the two globular regions of the complex associate has not been determined. Similar to higher eukaryotes, the yeast Aurora B/Ipl1 interacts with the N terminus of INCENP/Sli15, and all three proteins Survivin/Bir1-INCENP/Sli15-Borealin/Nbl1 come together through the formation of a three-helix bundle (9). Current models imply that the extended coiled-coil domain of INCENP/Sli15, containing its MTB domain, acts as a flexible linker to join the two lobes of the CPC complex. However, which subunit-subunit interactions are critical for CPC integrity are not known. Furthermore, a direct interaction between Aurora B/Ipl1 and Survivin/Bir1 has been reported, but the interaction is thought to be either extremely strong (15) or very weak (33). To test the role of the INCENP/Sli15 MTB domain in linking Aurora B/Ipl1 and Borealin/Nbl1, we purified a version of the CPC that does not contain the INCENP/Sli15 MTB (540 amino acids), but which retains the N- and C-terminal domains of INCENP/Sli15 as two individual polypeptides without covalent linkage. This five-subunit CPC remains stable through dilution by size-exclusion chromatography, supporting the idea that the Aurora B/Ipl1 lobe of the complex is able to bind directly to the Survivin/Bir1-Borealin/Nbl1 lobe with substantial affinity in the absence of the INCENP/Sli15 MTB domain to link them together.

Previously it was established that both Aurora B/Ipl1 and the MTB region of INCENP/Sli15 interact directly with MTs *in vitro* (22). Using the mutant CPC five-subunit complex described above, we showed that the MTB region of INCENP/Sli15 is not required for MT binding of the CPC, but does contribute significantly to the overall affinity of the complete CPC for MTs. It is likely that Aurora B/Ipl1 provides basal affinity of the CPC for MTs and that the MTB region of INCENP/Sli15, which is phosphorylated by Cdk1 and Aurora B/Ipl1, confers additional affinity that can potentially be finely regulated to facilitate the CPC characteristic localization changes throughout the cell cycle. Understanding how Aurora B/Ipl1 kinase activity is regulated in the context of the complete CPC is of major importance to understand transitions between the successive localizations of the CPC and how these transitions contribute to the control of major mitotic events and cell cycle phases.

Our data shed new light on the CPC core biochemical characteristics. We determined its affinity for MTs and showed that its binding to MTs depends significantly on electrostatic interactions with the C-terminal tail of tubulin. This binding is also negatively regulated by phosphorylation of INCENP/Sli15, which leads to the activation of Aurora B/Ipl1 kinase activity. Our data also provide a better understanding of the CPC architecture, showing that the proposed linker activity of INCENP/Sli15 is neither necessary for the structural integrity of the CPC nor for its MT binding ability. In the future, the ability to purify
large amounts of the yeast CPC from bacteria using our protocol will allow further elucidation of the CPC structure, function, and regulatory mechanisms.

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