The Chromosomal Passenger Complex Controls Spindle Checkpoint Function Independent from Its Role in Correcting Microtubule–Kinetochore Interactions^[D] ^[V]

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Submitted April 12, 2007; Revised July 24, 2007; Accepted August 8, 2007
Monitoring Editor: Orna Cohen-Fix

The chromosomal passenger complex (CPC) is a critical regulator of chromosome segregation during mitosis by correcting nonbipolar microtubule-kinetochore interactions. By severing these interactions, the CPC is thought to create unattached kinetochores that are subsequently sensed by the spindle assembly checkpoint (SAC) to prevent premature mitotic exit. We now show that spindle checkpoint function of the CPC and its role in eliminating nonbipolar attachments can be uncoupled. Replacing the chromosomal passenger protein INCENP with a mutant allele that lacks its coiled-coil domain results in an overt defect in a SAC-mediated mitotic arrest in response to taxol treatment, indicating that this domain is critical for CPC function in spindle checkpoint control. Surprisingly, this mutant could restore alignment and cytokinesis during unperturbed cell divisions and was capable of resolving syntelic attachments. Also, Aurora-B kinase was localized and activated normally on centromeres in these cells, ruling out a role for the coiled-coil domain in general Aurora-B activation. Thus, mere microtubule destabilization of nonbipolar attachments by the CPC is insufficient to install a checkpoint-dependent mitotic arrest, and additional, microtubule destabilization-independent CPC signaling toward the spindle assembly checkpoint is required for this arrest, potentially through amplification of the unattached kinetochore-derived checkpoint signal.

INTRODUCTION

During mitosis, equal chromosome segregation is essential to maintain a stable genome (reviewed in Kops *et al.*, 2005). Microtubule capture by chromosomes occurs in a stochastic process that eventually results in bipolar attachments of all paired sister chromatids to microtubules of the mitotic spindle. Bipolarity produces tension across the two sister chromatids generated by the pulling forces of the mitotic spindle that are opposed by cohesion between sister chromatids. During the stochastic attachment process all but bipolar attachments need to be destabilized to allow for new rounds of attachments. The centromeric Aurora-B kinase destabilizes these nonbipolar microtubule-kinetochore attachments (Biggins and Murray, 2001; Tanaka *et al.*, 2002; Lampson *et al.*, 2004). Aurora-B phosphorylates several key microtubule capture factors on the kinetochore (e.g., the Dam1 and Hec1/Ndc80 complexes) and by doing so it is thought to influence microtubule-binding affinity of the kinetochore

(Cheeseman *et al.*, 2002, 2006). Inhibition of Aurora-B kinase results in an increase in syntelic (with both sister chromatids attached to microtubules from the same pole) attachments (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Besides correcting nonbipolar attachments, cells have evolved a control mechanism called the spindle assembly checkpoint (SAC) that delays anaphase onset until all chromosomes are attached in a bipolar manner. Combined, these two systems allow cells to only segregate their duplicated genome once all sister chromatids are attached in a bipolar manner (Kops *et al.*, 2005). The SAC can detect the presence of unattached kinetochores and a lack of tension between two opposing kinetochores (e.g., lack of bipolarity). For the latter, the Aurora-B kinase has been shown to play a critical role (Biggins and Murray, 2001). It is clear that a major contribution of Aurora-B to activation of the SAC is its ability to generate unattached kinetochores in response to nonbipolar attachments (Pinsky *et al.*, 2006). However, it is unclear whether Aurora-B or its interaction partners can communicate to the SAC in an alternative manner independent of their role in creating unattached kinetochores.

In mammalian cells, Aurora-B functions in a quaternary chromosomal passenger complex (CPC) consisting of Inner Centromere Protein (INCENP), Survivin, and Borealin/Dasra-B (hereafter referred to as Borealin) (reviewed in Vader *et al.*, 2006b). These proteins activate and localize Aurora-B on centromeres of sister chromatids during prometaphase. INCENP contains a putative coiled-coil domain that can interact in vitro with polymerized microtubules (Wheatley *et al.*, 2001b), and this domain mediates CPC interaction with and localization on the central spindle during anaphase in *Saccharomyces cerevisiae* (Pereira and Schiebel, 2003). This

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-04-0328>) on August 15, 2007.

^[D] ^[V] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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Abbreviations used: CPC, chromosomal passenger complex; FACS, fluorescence-activated cell sorting; INCENP, inner centromere protein; NEB, nuclear envelope breakdown; SAC, spindle assembly checkpoint; wt, wild type.

domain is phosphorylated by Cdk during (pro) metaphase, and dephosphorylation by Cdc14 during anaphase triggers microtubule interaction and spindle targeting (Pereira and Schiebel, 2003).

Here, we investigated the role of the putative coiled-coil domain in INCENP in regulating CPC function on the centromere in human cells. We found that, by creating a CPC containing a coiled-coil less INCENP, the SAC-associated role of the CPC was specifically perturbed. However, during unperturbed mitoses and after recovery from monastrol-induced monopolarity, chromosomes could achieve bipolarity in this situation, showing that the correction-mechanisms that resolve nonbipolar attachments functioned properly. These findings show that besides its well-established role in creating unattached chromosomes, the CPC needs to exert an additional effect on the SAC-machinery to allow an efficient mitotic arrest.

MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used: mouse anti-Aurora-B (Transduction Laboratories, Lexington, KY), anti-Borealin (kind gift of S. Wheatley, University of Sussex, United Kingdom), sheep anti-BubR1 (kind gift of S. Taylor, University of Manchester, United Kingdom), mouse anti-CENP-A (Upstate Biotechnology, Lake Placid, NY), rabbit anti-phospho-Serine 7-CENP-A (Upstate Biotechnology), rabbit anti-CLIP-170 (kind gift of N. Galjart, Erasmus Medical Center, The Netherlands), rabbit anti-GFP (kind gift of G. Kops, University Medical Center Utrecht, The Netherlands), rabbit anti-phospho-Serine 10 Histone H3 (Upstate Biotechnology), mouse anti-Mad1 (kind gift of A. Musacchio, European Institute of Oncology, Italy), rabbit anti-Mad2 (Bethyl Laboratories, Montgomery, TX), mouse anti-MPM-2 (Upstate Biotechnology), rabbit anti-Survivin (R&D Systems, Minneapolis, MN), mouse anti- α -tubulin (Sigma, St. Louis, MO), rabbit anti- γ -tubulin (Sigma), mouse anti-VSV (Sigma), human CREST antiserum (Cortex Biochem, San Leandro, CA), peroxidase-conjugated goat anti-rabbit and peroxidase-conjugated goat anti-mouse (Dako, Carpinteria, CA) and donkey anti-mouse/Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-rabbit/Alexa-568, goat anti-rabbit/Alexa-633, goat anti-mouse/Alexa-568, and donkey anti-sheep/Alexa-568 (Molecular Probes, Eugene, OR). Reagents were from Sigma unless stated otherwise.

Plasmids

The INCENP small interfering RNA (siRNA)-vector and siRNA-resistant human wild-type (wt)-INCENP have been described (Vader *et al.*, 2006a). siRNA-resistant INCENP- Δ 539-747 was generated by looping out the DNA sequence corresponding to amino acids 539–747 by using site-directed mutagenesis on full-length human siRNA-resistant INCENP (Vader *et al.*, 2006a). Histone H2B-green fluorescent protein (GFP) and Spectrin-GFP have been described (Gerlich *et al.*, 2003; Lens *et al.*, 2003).

Cell Culture and Functional Assays

Human U2OS cells were cultured and synchronized as described (Lens *et al.*, 2003). U2OS cells were transfected with the standard calcium-phosphate protocol. ZM447392 (in DMSO) was from AstraZeneca (Wilmington, DE). Monastrol was from Sigma. Spectrin-GFP was used as transfection marker in flow cytometric assays. H2B-GFP was used as transfection marker in immunofluorescence/live imaging assays.

To analyze chromosome alignment, thymidine-synchronized cells were treated for the indicated periods with MG132, starting 12 h after release from the thymidine block to prevent mitotic exit.

For monastrol-release experiments, thymidine-synchronized cells were treated for 2 h with monastrol (with addition of MG132 during the second hour) starting 10 h after release from the thymidine block. Cells were washed and released into fresh medium containing MG132 for 90 min. DNA was stained with DAPI (4',6-diamidino-2-phenylindole).

Flow Cytometry and Time-Lapse Microscopy

The percentage of mitotic cells (MPM-2 positivity) and cell cycle distribution (DNA was stained with propidium iodide [PI]) of Spectrin-GFP positive cells was determined by flow cytometry as described (Smits *et al.*, 2000). In each experiment, 2500 GFP-positive cells were analyzed. Cells were analyzed on a FACScan flowcytometer (Becton Dickinson, Mountain View, CA), and data were processed using CellQuest software. H2B-GFP-expressing cells were followed by time-lapse microscopy as described (Lens *et al.*, 2003).

Immunoblotting, Immunoprecipitation, and Immunofluorescence

Immunoblotting and immunoprecipitation was performed as described (Smits *et al.*, 2000). Immunofluorescence was performed as described (Lens *et al.*, 2003). Images were acquired using a Zeiss Meta confocal microscope, with a 63 $\times$ 1.3 NA objective (Thornwood, NY). For Aurora-B/CENP-A and phospho-CENP-A/CENP-A-ratio calculations, images with multiple Z-planes were taken. In the projection of the different Z-planes, CENP-A staining was used to define kinetochore/centromere regions, and CENP-A intensity was used as constant reference for the kinetochore/centromere signal. Background fluorescence, from regions of the cell without kinetochores, was subtracted. Per condition, 15 cells were quantified, and from each on average 10 kinetochores were analyzed.

RESULTS

Identification of a Domain in INCENP Required for SAC Function of the CPC

It has been shown that both checkpoint arrest in response to lack of tension and the execution of cytokinesis rely on CPC function, because chemical inhibition or siRNA-mediated depletion of any CPC subunit impairs both of these functions (Carvalho *et al.*, 2003; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Honda *et al.*, 2003; Lens *et al.*, 2003; Gassmann *et al.*, 2004; Sampath *et al.*, 2004; Vader *et al.*, 2006a). We systematically tested a series of truncation mutants of human INCENP in an siRNA-protein replacement assay (Lens *et al.*, 2003, 2006; Vader *et al.*, 2006a) to search for mutants that could separate distinct functions of the CPC. These mutants were designed based on previous domain mapping studies that focused on CPC localization during mitotic progression (Mackay *et al.*, 1993; Ainsztein *et al.*, 1998; Adams *et al.*, 2000; Wheatley *et al.*, 2001a). Initially, these mutants were tested on CPC functionality during prometaphase, and cytokinesis by using previously established flow cytometric assays (Lens *et al.*, 2003, 2006; Vader *et al.*, 2006a). The first assay detects the capability of cells to arrest in mitosis after exposure to the microtubule-stabilizing poison taxol, an indicator of SAC defects. This capability is a reflection of centromeric CPC function. The second assay detects the percentage of tetraploid and polyploid cells in (a)synchronous cultures, an indicator of cytokinesis errors and a reflection of CPC function at the spindle midzone/midbody. When testing these INCENP-mutants we found that cells, in which endogenous INCENP was depleted and an INCENP-mutant lacking the putative coiled-coil domain (INCENP- Δ 539-747) was expressed, were unable to sustain a taxol-induced SAC arrest (Figure 1A). However, reconstituting INCENP-depleted cells with this mutant partially alleviated the appearance of cells containing a tetra- and polyploid DNA content in synchronized cultures and in asynchronously growing cultures (Supplementary Figure 1, C–E), showing that this mutant is capable of (partially) restoring the cytokinetic function of the CPC. In chicken INCENP, the coiled-coil domain is needed for interphase microtubule bundling, but not for central-spindle localization during anaphase of INCENP (Mackay *et al.*, 1993). Surprisingly, human INCENP lacking the homologous coiled-coil domain was partially impaired in localization to the central spindle in anaphase but did show midbody-localization of INCENP during telophase/cytokinesis (Supplementary Figure 2A). Similar observations were made in fixed and live cells by using a GFP-tagged version of INCENP- Δ 539-747 (Supplementary Figure 2B and data not shown). These data thus suggest that central spindle localization of the CPC during early anaphase is not required per se for cytokinesis in human cells. Here, we will focus on the centromeric role of the CPC and therefore do not further elaborate on this observation.

Figure 1. A spindle checkpoint defect in cells expressing INCENP- Δ 539-747. (a) U2OS cells were transfected with the indicated plasmids and GFP-Spectrin. Cells were synchronized using thymidine. Eighteen hours after release into taxol, cells were harvested and mitotic percentages ($\pm$ SEM) were determined by MPM-2/PI staining and FACS analysis. (b) U2OS cells were transfected with the indicated plasmids and pBabe-puro. Cells were selected for puromycin-resistance for 36 h to enrich for transfected cells. Cells were lysed and analyzed by immunoblotting with the indicated antibodies. Alpha-tubulin was used as loading control. (c) and (d) U2OS cells were grown on glass-bottom culture dishes and cotransfected with H2B-GFP and the indicated plasmids. Cells were synchronized using thymidine and were followed by time-lapse imaging starting 10 h after release from thymidine. Time in the bottom right corner refers to the elapsed time (hours:minutes) from the time of nuclear envelope breakdown (NEB). Quantification ($\pm$ SEM) of live cell imaging data are shown in d. (e) U2OS cells were transfected with the indicated plasmids and GFP-Spectrin. Cells were synchronized using thymidine. Eighteen hours after thymidine release, cells were harvested, and mitotic percentages ($\pm$ SEM) were determined by MPM-2 staining and FACS analysis. (f) Western blotting analysis of panel e.

The SAC defect in cells expressing the INCENP- Δ 539-747-protein was confirmed by live cell imaging of cells that were

treated with taxol (Figure 1, C and D, and Supplementary Movies 1–3). Western blotting of this deletion mutant

Figure 2. INCENP-Δ539-747 localizes Aurora-B to centromeres during (pro) metaphase. (a–d) U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (depicted in blue). Cells were synchronized using thymidine. Fourteen hours after thymidine release, cells were fixed and immunostained with the indicated antibodies. Insets show magnified images of the indicated regions. (c and d) The percentage ($\pm$ SEM) of transfected cells with centromeric Aurora-B staining (c) and Aurora-B-levels on individual centromeres (d; $\pm$ SEM) was quantified. See *Materials and Methods* for more detail on how Aurora-B levels on individual centromeres were determined.

showed that it was expressed in amounts similar to that of the wt-INCENP (Figure 1B), and expression of increasing amounts of the INCENP-mutant did not have a significant effect on the ability of this mutant to restore checkpoint function (Supplementary Figure 1, A and B). This demonstrates that the observed defect is not due to a difference in protein expression. In addition, several lines of evidence argue that deletion of the coiled-coil domain did not create an inert CPC at the centromere. First, immunofluorescence analysis showed that the coiled-coil-less INCENP protein was capable of localizing to (pro)metaphase centromeres, similar to that of wt-INCENP (Figure 2A), and more importantly, it was capable of restoring centromeric Aurora-B localization to wt levels in an siRNA-replacement assay (Figure 2, B–D). Second, reconstitution of INCENP-depleted cells with this mutant brought back Aurora-B and Survivin protein levels that are severely diminished in INCENP-depleted cultures (Honda *et al.*, 2003; Vader *et al.*, 2006a; Figure 1B). Third, INCENP-Δ539-747 was capable of binding Sur-

vin, Borealin, and Aurora-B in a coimmunoprecipitation assay (Supplementary Figure 2, C and D).

Expression of INCENP-Δ539-747 in cultures that were not depleted of endogenous INCENP caused a dose-dependent decrease in spindle checkpoint response after taxol-treatment, showing that INCENP-Δ539-747 exerts a dominant-negative effect on SAC-associated CPC function to a similar extent as an established dominant-negative mutant lacking the centromere-targeting domain (INCENP-Δ1-47; Vader *et al.*, 2006a; Figure 1, E and F). Collectively, these results indicate that removing the coiled-coil domain from INCENP impairs a specific CPC function at the centromere, but does not affect several other aspects of INCENP function within the CPC.

INCENP-Δ539-747-expressing Cells Can Efficiently Correct Syntelic Attachments and Display No Alignment Defects

Impaired CPC function is characterized by the presence of persistent misaligned chromosomes during prometaphase, which are presumably caused by the inability of the cells to

Figure 3. Normal mitotic progression in cells expressing INCENP-Δ539-747. (a and b) U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (blue). Cells were synchronized using thymidine, and 12 h after thymidine release MG132 was added for the final 3 h. Cells were fixed and immunostained with the indicated antibodies. (a) Typical examples of cells found in different samples. (b) Quantification ($\pm$ SEM) of the observed metaphase configurations. (c) Similar to a and b, with the exception that cells were treated with MG132 for 45 (black bars ■) or 90 min (white bars □). Percentage ($\pm$ SEM) of the metaphase cells within the total mitotic population is shown. (d and e) U2OS cells were grown on glass-bottom culture dishes and cotransfected with H2B-GFP and the indicated plasmids. Cells were synchronized and were followed by time-lapse imaging starting 10 h after release from a thymidine block. Time in the bottom right corner refers to the elapsed time (hours:minutes) from the time of nuclear envelope breakdown (NEB). (e) Quantifications ($\pm$ SEM) of alignment state upon anaphase onset.

correct nonbipolar (syntelic) microtubule-kinetochore attachments, a process that critically depends on CPC function. It is generally believed that the failure to correct nonbipolar attachments is the underlying reason of the SAC defect in CPC-compromised cells after exposure to taxol (Pinsky *et al.*, 2006). Therefore, the fact that cells expressing the INCENP-Δ539-747 fail to arrest in the SAC after taxol treatment suggested that these cells would also show a

failure to correct nonbipolar attachments and to create unattached kinetochores.

To investigate this, we performed three different assays. First, we prevented mitotic exit through inhibition of proteasome function by treating the cells for 3 h with MG132 (Figure 3, A and B). This prevents degradation of Cyclin B and Securin and will therefore trap cells at the metaphase-to-anaphase transition. In the majority (77.6%) of the mock

Figure 4. Syntelic attachments can be resolved in cells expressing INCENP-Δ539-747. (a) Schematic representation of experimental setup. U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (blue). Cells were synchronized using thymidine. Ten hours after thymidine release monastrol was added for 2 h, and during the second hour MG132 was added. Cells were washed and released into fresh medium containing MG132. Cells were fixed after 90 min and immunostained with the indicated antibodies. (b and c) Typical examples (b) and quantification (c; $\pm$ SEM) from each category. (d–f) U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (blue). Cells were synchronized using thymidine, and 12 h after thymidine release taxol and MG132 were added for 3 h. Cells were fixed and immunostained with the indicated antibodies. (e) The percentage ($\pm$ SEM) of transfected cells with CLIP-170 kinetochore staining was quantified. (f) Quantification of the number ($\pm$ SEM) of CLIP-170 positive kinetochores per cell. (g and h) U2OS cells

siRNA cells that were treated with MG132 all chromosomes were aligned in a metaphase configuration. A large percentage (72.0%) of INCENP siRNA cells showed persistent misalignments, indicating that these cells indeed failed to correct nonbipolar attachments. Surprisingly, INCENP-depleted cells that were reconstituted with INCENP- Δ 539-747 were indistinguishable from mock-depleted or wt-INCENP-reconstituted cells, and the vast majority (74.5%) of these cells showed properly aligned metaphase chromosomes (Figure 3, A and B). Experiments in which cells were treated with MG132 for shorter periods (45 and 90 min) showed identical results (Figure 3C). Live cell imaging corroborated these findings, as the majority (73.0%) of the INCENP- Δ 539-747 reconstituted cells showed normal chromosome alignment before entering anaphase (Figure 3, D and E, and Supplementary Movies 4–7). However, a slight increase of cells entering anaphase with one misaligned chromosome was observed in these cells, suggesting the presence of a minor SAC defect.

Second, to further investigate the ability of the CPC formed by INCENP- Δ 539-747 to correct nonbipolar attachments, we performed monastrol-washout experiments (Figure 4A). Monastrol is a well-characterized reversible inhibitor of Eg5, a mitotic kinesin that is required for bipolar spindle assembly (Kapoor *et al.*, 2000). Inhibition of Eg5 will result in cells with monopolar spindles and consequently, will greatly increase the amount of monotelic and syntelic microtubule-kinetochore interactions. After washing away monastrol, cells will quickly form a bipolar spindle, and the present syntelic attachments are resolved in an Aurora-B-dependent manner. Therefore, this is a robust method to test the microtubule-correction function of the CPC (Lampson *et al.*, 2004). To prevent premature mitotic exit during the monastrol treatment, MG132 was added to block proteasome function and to arrest cells at the metaphase-anaphase transition. The majority (75.0%) of INCENP-depleted cells failed to correct the syntelic attachments and as a consequence nearly all chromosomes failed to align on a metaphase plate (Figure 4, B and C). Expression of INCENP- Δ 539-747 restored the correction of syntelic attachments to a large extent, as judged by the percentage of metaphase-aligned cells (60.0%). However, we did observe a small increase in the percentage of cells that had a few persistent misaligned chromosomes after monastrol release (14.0%), compared with wt-INCENP-reconstituted INCENP-depleted cells (10.0%). Despite this small increase in cells with a few persistent misalignments, the majority of the cells expressing INCENP- Δ 539-747 showed the same efficacy in correcting syntelic attachments as the wt-INCENP (68.5%), indicating that microtubule correction is functioning largely normal in these cells (Figure 4, B and C).

Third, we investigated whether cells expressing INCENP- Δ 539-747 had retained the capability to create unattached kinetochores in taxol-treated cultures. Hereto, kinetochore localization of the microtubule plus-end tracking protein CLIP-170 was monitored. During mitosis, CLIP-170 localizes to unattached kinetochores and is displaced from kinetochores upon microtubule capture (Tanenbaum *et al.*, 2006). It

is therefore a good marker for unattached kinetochores. Control-depleted cells that were treated with taxol contained a subset of kinetochores with strong CLIP-170 staining, whereas in INCENP-depleted cells a profound decrease in cells with CLIP-170 positive kinetochores could be found, confirming that CPC function is required for the generation of unattached kinetochores after taxol treatment (Figure 4, D and E). Cells in which INCENP was replaced by wt-INCENP or INCENP- Δ 539-747 did contain CLIP-170 positive kinetochores, indicating that INCENP- Δ 539-747-reconstituted cells had retained the capability to create unattached kinetochores after taxol treatment in a manner comparable to wt-INCENP-reconstituted cells (Figure 4, D and E). Similar results were obtained with the spindle checkpoint protein Mad2 [which is also specifically recruited to unattached kinetochores (reviewed by Kops *et al.*, 2005; Supplementary Figure 3A)]. Moreover, CLIP-170-positive kinetochores stained positive for the checkpoint protein Mad1 (Supplementary Figure 3B). Importantly, the number of kinetochores per mitotic cell staining positive for CLIP-170 upon reconstitution of INCENP-depleted cells by either wt-INCENP or INCENP- Δ 539-747 were indistinguishable (Figure 4F). Taken together, these observations strongly suggest that the correction of nonbipolar attachments during chromosome alignment and the generation of unattached kinetochores (that recruit spindle checkpoint proteins) are restored in INCENP-depleted cells expressing INCENP- Δ 539-747.

The disability of cells expressing INCENP- Δ 539-747 to arrest in mitosis upon taxol treatment, despite the presence of unattached kinetochores, suggested that the coiled-coil domain in INCENP was required for checkpoint signaling in the presence of unattached kinetochores. To further test this, we investigated the checkpoint response upon complete microtubule depolymerization by treating cells with nocodazole. As described previously (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003), inhibition of Aurora-B function leads to a partial decrease in the efficacy of the mitotic arrest caused by complete microtubule depolymerization (after nocodazole treatment). A similar partial decrease in checkpoint response was observed in INCENP-depleted cells expressing INCENP- Δ 539-747 (Figure 4, G and H), showing that this response indeed requires INCENP's coiled-coil domain. It is important to note that the defect in checkpoint response after nocodazole treatment is much less severe compared with the defect in checkpoint function in response to taxol treatment (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Figure 4G), suggesting that cells containing a only few unattached kinetochores (e.g., after taxol treatment) more critically rely on CPC function for the mitotic arrest than cells containing a large number of unattached kinetochores (e.g., after nocodazole treatment).

The Defect in SAC Signaling Cannot Be Explained by Partial Inactivation of Aurora-B

A possible explanation for the observed specific defect in checkpoint function could be that the coiled-coil domain in INCENP was needed for complete activation of Aurora-B in vivo and that only the SAC function of the CPC might be sensitive to minor alterations in Aurora-B kinase activity. If this were the case, partial inhibition of Aurora-B should cause the same differential effect on chromosome alignment versus SAC function. To test this possibility, cells were treated with decreasing concentrations of the Aurora-B inhibitor ZM447392 (Ditchfield *et al.*, 2003). Treatment of cells with 2 μ M ZM447392 (a concentration commonly used (Ditchfield *et al.*, 2003) caused dramatic chromosome mis-

Figure 4 (cont). were transfected with the indicated plasmids and GFP-Spectrin. Cells were synchronized using thymidine. Eighteen hours after release into taxol or nocodazole, cells were harvested, and mitotic percentages were determined by MPM-2/PI staining and FACS analysis. (h) A fraction of the cells from panel f was lysed and analyzed by immunoblotting with the indicated antibodies. Asterisk denotes aspecific background signal that was used as loading control.

Figure 5. Checkpoint function and chromosome alignment are equally sensitive to Aurora-B inhibition. (a and b) U2OS cells were synchronized using thymidine. Directly after release cells were treated with the indicated concentrations of ZM447392 or DMSO with or without taxol. Eighteen hours later, cells were harvested and mitotic percentages (right panel) and percentages ($\pm$ SEM) of cells with 4n DNA content (left panel) were determined by MPM-2 and PI staining and analyzed by FACS. (b) Cells were treated with DMSO or the indicated concentrations of ZM447392 in combination with nocodazole for 15 h. Mitotic cells were obtained by mechanical detachment and analyzed by immunoblotting with the indicated antibodies. (c and d) U2OS cells grown on coverslips were synchronized by thymidine treatment. Directly after release cells were treated with the indicated concentrations of ZM447392 or DMSO. Cells were treated with MG132 for 3 h starting 12 h after release from thymidine. (d) After fixation, mitotic figures were quantified ($\pm$ SEM).

alignments, a failure to arrest in mitosis in response to taxol treatment, a significant increase in polyploidy and disappearance of the phosphorylated form of serine-10 Histone H3 during mitosis (data not shown). Decreasing the ZM447392 concentration to 1 μ M caused equally severe phenotypes, and therefore we chose this concentration as the highest concentration in our titration experiments. Treating cells with decreasing concentrations of the Aurora-B inhibitor caused a gradual decrease in the severity of the defects in checkpoint response, chromosome alignment and execution of cytokinesis, but importantly, all phenotypes were equally sensitive to the concentration of ZM447392 that was used (Figure 5, A–D).

To test whether Aurora-B could be properly activated by INCENP- Δ 539-747 in vivo, the phosphorylation status of serine-7 CENP-A, an established centromeric Aurora-B substrate was investigated (Zeitlin *et al.*, 2001). Although INCENP depletion caused a dramatic decrease in the amount of phosphorylated CENP-A, reconstitution with INCENP- Δ 539-747 caused a restoration of the amount of phosphorylated CENP-A that was indistinguishable from wt-INCENP reconstitution in unperturbed mitoses (Figure 6, A–C). Because of the reported microtubule-binding capac-

ity of the coiled-coil domain, it was investigated whether the presence of microtubules influenced Aurora-B activity in vivo. Hereto, we performed the same analyses in the presence of the microtubule depolymerizing drug nocodazole or of taxol (combined with MG132 to prevent mitotic exit). As shown in Figure 6, D and E, treatment with taxol or nocodazole did not significantly alter the amount of phosphorylated CENP-A in either wt-INCENP or INCENP- Δ 539-747-reconstituted cells. Taken together, these data suggest that the loss-of-checkpoint function in cells expressing INCENP- Δ 539-747 is not due to partial loss of Aurora-B activity. Additionally, this suggests that the specific loss of SAC function after removal of the coiled-coil domain is most likely a qualitative effect, also because partial chemical inhibition of Aurora-B causes equal defects in both chromosome alignment and SAC function.

BubR1 Retention on Kinetochores Is Not Altered in INCENP- Δ 539-747-expressing Cells

The SAC protein BubR1 is recruited to unattached kinetochores during prometaphase. During a mitotic arrest triggered by microtubule-stabilizing (taxol) or -destabilizing

Figure 6. In vivo activation of Aurora-B is normal in cells expressing INCENP-Δ539-747. (a–d) U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (blue). Cells were synchronized using thymidine. Fourteen hours after thymidine release, cells were fixed and immunostained with the indicated antibodies. (b–d) Quantification of the percentage ($\pm$ SEM) of transfected cells with positive phospho-CENP-A staining (b) and phospho-CENP-A levels on individual centromeres (c and d; $\pm$ SEM). See *Materials and Methods* for more detail on how phospho-CENP-A levels on individual centromeres were determined. (d) Experiments similar to panel a, with the exception that 3 h before fixation, taxol (in combination with MG132) was added to the cells. (e) Experiments similar to panel a, with the exception that upon release from thymidine block, nocodazole was added to the cells.

drugs (nocodazole) BubR1 is retained at high levels on kinetochores. Interference with CPC function has been shown to cause a premature loss of BubR1 from kinetochores in nocodazole- or taxol-treated mitotic cells, suggesting a tight relation between CPC function and BubR1 kinetochore recruitment (Carvalho *et al.*, 2003; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003; Lens *et al.*, 2003). To investigate whether BubR1 retention was altered after reconstitution of INCENP-depleted cells with INCENP-Δ539-747, transfected cells were treated with nocodazole, and BubR1 kinetochore localization was monitored. As shown in Figure 7, A and B, BubR1 kinetochore recruitment was not impaired upon microtubule destabilization in INCENP-Δ539-747-reconstituted cells compared with wt-INCENP-reconstituted cells, indicating that the CPC influences SAC function independently from BubR1 recruitment to kinetochores.

DISCUSSION

Elegant experiments in budding yeast have demonstrated that a major route through which Aurora-B contributes to spindle checkpoint function in response to a lack of stable bipolar attachments (lack of tension) is via the creation of unattached kinetochores by destabilizing microtubule-kinetochore interactions (Pinsky *et al.*, 2006). These unattached kinetochores subsequently recruit checkpoint proteins and thereby produce an inhibitory checkpoint signal that halts cell cycle progression. If this would be the only way by which Aurora-B communicates to the spindle checkpoint, impairing Aurora-B's checkpoint function should always correlate with a failure to correct nonbipolar attachments.

Here, we provide evidence that microtubule destabilization and SAC function are not necessarily coupled. We show

Figure 7. BubR1 is recruited normally to unattached kinetochores in cells expressing INCENP- Δ 539-747. (a and b) U2OS cells grown on coverslips were transfected with indicated plasmids and H2B-GFP (blue). Cells were synchronized using thymidine, and nocoda-

zole was added immediately after release from thymidine. Fourteen hours later cells were fixed and immunostained with the indicated antibodies. Typical examples (a) and quantification (b; $\pm$ SEM) from each category are shown. (c) Model depicting the bifurcated effect of the CPC on spindle checkpoint function. On the presence of nonbipolar attachments, lack of tension is detected by the CPC (in a currently unknown way). The concurrent response to the lack of tension across the sister chromatids is twofold. First, the CPC promotes destabilization of the microtubule-kinetochore interactions that do not create tension and thereby creates unattached kinetochores that subsequently produce a spindle assembly checkpoint signal. Second, the CPC (with a specific requirement for the coiled-coil domain) is required to strengthen the signal emanating from the unattached kinetochores to produce efficient checkpoint signals that are required to prevent premature anaphase onset. This effect could either be dependent or independent of Aurora-B (possibly via a Survivin/INCENP subcomplex).

that removal of the putative coiled-coil domain in INCENP critically impairs spindle checkpoint function, but essentially leaves the microtubule-correction function of Aurora-B intact. This implies that the CPC has an additional, more direct (i.e., not via microtubule destabilization) effect on SAC signaling and that mere microtubule destabilization is not sufficient for an efficient checkpoint arrest in response to lack of tension. These findings also suggest that the coiled-coil domain in INCENP is essential for this specific function the CPC in SAC signaling.

The effect that we observe cannot be explained by a failure in global activation or localization of Aurora-B. The centromeric localization of Aurora-B was normal in INCENP-depleted cells that were reconstituted with INCENP- Δ 539-747. It was shown previously that the IN-box in the carboxy-terminus of INCENP is sufficient to bind and activate Aurora-B in vitro (Sessa *et al.*, 2005), and the IN-box is present in INCENP- Δ 539-747. By using a phospho-specific antibody for serine-7 in CENP-A we could indeed show that the coiled-coil domain is dispensable for normal activation of Aurora-B in vivo, either in the presence or absence of microtubules. Additionally, titration experiments with an Aurora-B inhibitor did not reveal a specific sensitivity for checkpoint function versus chromosome alignment.

We envision that INCENP, via its coiled-coil domain, interacts with a certain, at present unknown, factor either by direct recruitment, or via its reported interaction with microtubules. One possibility is that this interaction allows Aurora-B to phosphorylate this factor and that this is a crucial event for normal SAC function. In this scenario, INCENP would facilitate Aurora-B function within the SAC. Alternatively, because important Aurora-B functions (like destabilization of defective microtubule-kinetochore interactions) are intact in cells expressing INCENP- Δ 539-747, and because a functional Survivin/INCENP subcomplex has been described (Sandall *et al.*, 2006), INCENP might play a role in checkpoint control independent of Aurora-B kinase activity. At present, we cannot rule out either possibility. However, we favor a model in which INCENP facilitates the availability of specific substrates of Aurora-B, because earlier studies have shown that Aurora-B kinase activity (independently of disruption or mislocalization of the CPC) is necessary to maintain an efficient checkpoint dependent arrest in response to microtubule depolymerization (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003).

Recently, a study in budding yeast identified Mad3 (the budding yeast homologue of BubR1) as a critical substrate of Ipl1 (the budding yeast homologue of Aurora-B) in spindle checkpoint function in response to a lack of tension (King *et al.*, 2007). Phosphorylation of Mad3 by Ipl1 was required in

zole was added immediately after release from thymidine. Fourteen hours later cells were fixed and immunostained with the indicated antibodies. Typical examples (a) and quantification (b; $\pm$ SEM) from each category are shown. (c) Model depicting the bifurcated effect of the CPC on spindle checkpoint function. On the presence of nonbipolar attachments, lack of tension is detected by the CPC (in a currently unknown way). The concurrent response to the lack of tension across the sister chromatids is twofold. First, the CPC promotes destabilization of the microtubule-kinetochore interactions that do not create tension and thereby creates unattached kinetochores that subsequently produce a spindle assembly checkpoint signal. Second, the CPC (with a specific requirement for the coiled-coil domain) is required to strengthen the signal emanating from the unattached kinetochores to produce efficient checkpoint signals that are required to prevent premature anaphase onset. This effect could either be dependent or independent of Aurora-B (possibly via a Survivin/INCENP subcomplex).

a situation that was characterized by a lack of tension in the complete absence of unattached kinetochores. We have tested a potential direct link between Aurora-B and BubR1 in checkpoint function in human cells, also because BubR1 contains four putative Ipl1-consensus sites (Cheeseman *et al.*, 2002). However, BubR1 was not phosphorylated by Aurora-B in vitro (C.W.A. Cruijsen and S.M.A. Lens, unpublished observations), and alanine mutations of the four putative Ipl1 sites within BubR1 did not affect the function of BubR1 within the SAC (S.M.A. Lens, unpublished observations). Most importantly, mass spectrometric analysis of BubR1 isolated from checkpoint-arrested mitotic cells failed to identify phosphorylated serine or threonine residues that map within Ipl1-consensus motifs (G. Kops, personal communication). Taken together, these findings suggest that in human cells, BubR1 is most likely not a direct substrate of Aurora-B during checkpoint signaling. Furthermore, although King *et al.* (2007) suggested the existence of a tension-specific (Aurora-B-dependent) checkpoint branch, the fact that our experimental set-up (in which we evidently detect a number of unattached kinetochores in the presence of taxol; Figure 4, C–E) is clearly different from their system precludes us from drawing any conclusions regarding the presence or absence of such a separate checkpoint branch in human cells.

Why is the coiled-coil domain essential for this specific function? Recent data by Sandall *et al.* (2006) implied the coiled-coil domain in Sli15 (the budding yeast orthologue of INCENP) in regulation of Aurora-B at centromeres. They showed that this domain interacts with microtubules, whereas the CPC is localized at the centromere. Deletion of this domain from Sli15 caused a defect in chromosome segregation, and this domain was therefore suggested to function as a tension sensor at the kinetochore/centromere that could somehow relay the absence of tension toward Aurora-B. At present we do not know whether microtubule binding by the coiled-coil domain is the underlying cause of the phenotype we observed. Regardless, the effect we find due to removal of the coiled-coil domain is fundamentally different from the effect described by Sandall *et al.* (2006). We find a specific effect in spindle checkpoint function, but we do not detect significant changes in efficacy of chromosome alignment and correction of nonbipolar microtubule-kinetochore interactions. This suggests that if the coiled-coil domain functions as a tension sensor in our system and if this is the specific function we disturb, it only has an effect on Aurora-B function toward the spindle checkpoint and not toward microtubule-kinetochore interactions. A possible explanation for the difference in effect caused by removal of the coiled-coil domain found here and by Sandall *et al.* (2006) could lie in the fundamental difference in organization of the microtubule-kinetochore interface between budding yeast and human kinetochores. Yeast kinetochores bind a single microtubule, whereas human kinetochores interact with up to 20 individual microtubule fibers and need to coordinately regulate all these interactions (Maiato *et al.*, 2004). Therefore, microtubule binding by the CPC might have evolved to play a differential role in chromosome dynamics in budding yeast versus other eukaryotes.

Finally, an important implication of this work is that the CPC influences SAC function additionally to its ability to create unattached kinetochores. We propose that in the case of nonbipolar attachments the CPC responds to this lack of tension in a bifurcated manner: 1) through the creation of unattached kinetochores via destabilization of these incorrect microtubule-kinetochore interactions, and 2) through the generation of a signal that is required to amplify the

“wait anaphase” signal produced at the unattached kinetochores (Figure 7C). This amplification step is essential to halt mitotic progression when a low number of kinetochores is unattached and could explain why this CPC function only becomes essential in cases when only a few kinetochores are unattached (upon taxol treatment) or in a spindle checkpoint arrest after prolonged nocodazole treatment. These findings are not unprecedented because inhibition of Aurora-B was shown to influence the efficacy of the mitotic arrest imposed by microtubule depolymerization, implicating the presence of microtubule-destabilization-independent mechanisms by which Aurora-B communicates to, and strengthens the mitotic checkpoint (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Furthermore, in budding yeast, overexpression of the spindle checkpoint kinase Mps1 resulted in an Aurora-B-dependent checkpoint arrest. This arrest does not require functional kinetochores, again arguing that Aurora-B is also required for checkpoint function independently of kinetochore-microtubule interactions (Biggins and Murray, 2001). Evidently, the CPC influences spindle checkpoint signaling through multiple pathways to control faithful chromosome segregation, and these findings open the way for the identification of new CPC interactors and/or Aurora-B substrates that are critical in these events.

ACKNOWLEDGMENTS

We thank A. Musacchio, S. Wheatley, S. Taylor, and N. Galjart for sharing reagents. We thank G. Kops for sharing reagents and unpublished data. We acknowledge AstraZeneca for supplying ZM447392. This work is supported by Dutch Cancer Society (NKI 2002-2764) and the Netherlands Organization for Scientific Research (Vidi 917.66.332).

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