Cell, Vol. 102, 817–826, September 15, 2000, Copyright ©2000 by Cell Press

CENP-E as an Essential Component of the Mitotic Checkpoint In Vitro

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Summary

Accurate chromatid separation is monitored by a checkpoint mechanism that delays anaphase onset until all centromeres are correctly attached to the mitotic spindle. Using *Xenopus* egg extracts, the kinetochore-associated microtubule motor protein CENP-E is now found to be required for establishing and maintaining this checkpoint. When CENP-E function is disrupted by immunodepletion or antibody addition, extracts fail to arrest in response to spindle damage. Mitotic arrest can be restored by addition of high levels of soluble MAD2, demonstrating that the absence of CENP-E eliminates kinetochore-dependent signaling but not the downstream steps in checkpoint signal transduction. Because it directly binds both to spindle microtubules and to the kinetochore-associated checkpoint kinase BUBR1, CENP-E is a central component in the vertebrate checkpoint that modulates signaling activity in a microtubule-dependent manner.

Introduction

One of the most dramatic events in the cell cycle is the accurate splitting and subsequent segregation of the duplicated chromosome pairs during anaphase. Before initiating this irreversible step, cell cycle advance is restrained by a mitotic checkpoint mechanism that blocks chromosome segregation until both kinetochores on each duplicated chromosome pair have successfully attached to spindle microtubules (Rieder et al., 1995). A preponderance of evidence now firmly links unattached kinetochores as generators of an inhibitory signal that blocks the transition to anaphase by inhibiting the activity of the cellular ubiquitin ligase known as the anaphase-promoting complex (APC). Whereas anaphase initiation requires APC-mediated ubiquitination of various substrates, which targets them for destruction by the proteasome, activation of the mitotic checkpoint in response to chromosome misalignment leads to inhibition of APC activity and concomitant mitotic arrest resulting from the inability to degrade the securin proteins (Cohen-Fix et al., 1996; Zou et al., 1999), which are involved in sister chromatid cohesion. However, once both centromeres of every chromosome pair attach to the spindle, the checkpoint is satisfied, the APC is derepressed, and the metaphase–anaphase transition is triggered by ubiquitination and degradation of the securin proteins (Ciosk et al., 1998).

Genetics in yeast initially identified six components—BUB1, BUB2, and BUB3 (Hoyt et al., 1991) and MAD1, MAD2, and MAD3 (Li and Murray, 1991)—each of which was essential for establishing or maintaining the mitotic checkpoint. Vertebrate homologs of several of these have now been shown to be kinetochore-associated, including BUB1 (Taylor and McKeon, 1997; Jablonski et al., 1998), BUB3 (Taylor et al., 1998), MAD1 (Chen et al., 1998), MAD2 (Chen et al., 1996; Li and Benezra, 1996), and BUBR1, a protein kinase (Chan et al., 1999) that appears to be a hybrid of BUB1 and MAD3 (Cahill et al., 1998; Taylor et al., 1998). Active MAP kinase has also been implicated in the vertebrate context both in the checkpoint (Minshull et al., 1994; Wang et al., 1997) and as a kinetochore component (Shapiro et al., 1998; Zecevic et al., 1998). Interestingly, it has been shown that binding of MAD2 to the CDC20/fizzy/p55 protein (which activates APC for ubiquitination of PDS1/securin) can directly inhibit APC activity and prevent the metaphase–anaphase transition (Dawson et al., 1995; Visintin et al., 1997; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Wassmann and Benezra, 1998).

One current model is that targeting of MAD2 to kinetochores by MAD1 (Chen et al., 1998) forces dimer- or oligomerization of MAD2, which is then rapidly released from kinetochores. The oligomer, which is the active form of MAD2, then binds to and inhibits APC/CDC20 (Fang et al., 1998). Production of this soluble MAD2 oligomer is silenced at individual kinetochores by microtubule attachment (Waters et al., 1998; Yu et al., 1999) and/or subsequent microtubule-dependent tension developed across each kinetochore pair (Nicklas et al., 1995; Yu et al., 1999). This correlates with the release from kinetochores of BUB1 (Taylor and Mckeon, 1997), BUB3 (Martinez-Exposito et al., 1999), MAD1 (Chen et al., 1998), and MAD2 (Chen et al., 1996; Li and Benezra, 1996), as well as active MAP kinase (Shapiro et al., 1998; Zecevic et al., 1998). Addition of excessive levels of MAD2 protein is sufficient to replace kinetochore-dependent signaling (Li et al., 1997; Chen et al., 1998), producing a chronic inhibition of APC activity on mitotic substrates.

Although controversy remains about whether silencing of kinetochore signaling is mediated by microtubule attachment or the subsequent tension generated, what seems very clear is a requirement for a component(s) that can sense or directly link microtubule attachment and/or tension generation to the downstream signaling cascade. One potential component is CENP-E, a microtubule-dependent motor protein that extends at least 100 nm from the surface of kinetochores (Cooke et al., 1997; Yao et al., 1997) and remains so positioned throughout all phases of mitotic chromosome movement (Brown et al., 1996). As such, CENP-E is likely to be at least one of the components that directly tether spindle microtubules to the kinetochore. Moreover, inhibition of CENP-E function with antibodies injected into mammalian cells (Yen et al., 1991; Schaar et al., 1997), by antibody-mediated depletion from *Xenopus* extracts (Wood et al., 1997) or by antisense oligonucleotide suppression of its synthesis in mammalian cells (Yao et al.,

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2000), leads to failure to establish stable bi-oriented kinetochore–microtubule attachment, consistent with a direct role as a linker molecule and/or motor. Moreover, even for those kinetochore pairs that do achieve bi-oriented attachment with diminished levels of CENP-E, tension is reduced (Yao et al., 2000), consistent with a role for CENP-E both in kinetochore linkage to spindle microtubules and in generating tension.

To this background, absence of CENP-E in the mammalian context by antibody injection (Schaar et al., 1997) or antisense approaches (Yao et al., 2000) leads to a profound mitotic arrest with misaligned chromosomes, some of which apparently continue to generate the checkpoint stop signal, as evidenced both by the mitotic arrest and by the continued presence at kinetochores of components such as MAD2. All of these combine to support the view that CENP-E is required for stable, bi-oriented attachment of microtubules to kinetochores. Thus, at a minimum, through its role in microtubule capture, CENP-E is involved in satisfying the checkpoint, whose activation early in mammalian mitosis is a normal event that occurs immediately after nuclear envelope disassembly.

To examine more closely how CENP-E participates in establishing, maintaining, and silencing the mitotic checkpoint, we used *Xenopus* egg extracts that reproduce in vitro many aspects of the mitotic cycle, including kinetochore-dependent activation of the mitotic checkpoint (Minshull et al., 1994). Surprisingly, we found that depletion of CENP-E resulted in failure to establish a checkpoint arrest when challenged with microtubule-depolymerizing agents. This is mediated through suppression of the sensing apparatus rather than an effect on the downstream components of the checkpoint, because addition of high levels of MAD2 (which causes a kinetochore-independent checkpoint arrest in wild-type extracts [Chen et al., 1998]) restores the checkpoint to CENP-E-depleted extracts. Moreover, loss of the checkpoint is a specific result of CENP-E depletion because addition of purified recombinant CENP-E restores checkpoint-dependent cell-cycle arrest. Taken together, these findings implicate CENP-E as a kinetochore attachment sensor that simultaneously binds to microtubules and kinetochore-bound checkpoint components, thereby activating and/or silencing the mitotic checkpoint.

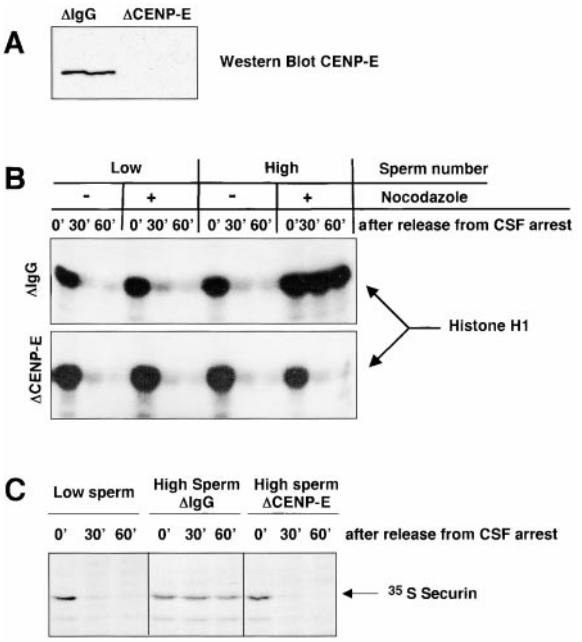

Figure 1. CENP-E Depletion Prevents the Checkpoint from Being Activated in *Xenopus* Egg Extracts

(A) Immunodepletion of CENP-E from CSF-arrested egg extracts. Extracts were immunodepleted using purified nonspecific (IgG) or anti-CENP-E rabbit polyclonal antibodies. The depleted extracts were subjected to SDS-PAGE and immunoblotted using an anti-CENP-E tail antibody.

(B) Depletion of CENP-E prevents activation of the mitotic checkpoint as determined by loss of CDK activity. CENP-E-depleted or mock-depleted extracts were induced to undergo mitosis (by addition of 0.4 mM $CaCl_2$) in the presence of high levels (9000/μl) or low levels (500/μl) of purified sperm head nuclei, and in the presence or absence of nocodazole. At the indicated times after release of the CSF arrest, aliquots were removed and assayed for CDK activity by incubation with purified bovine Histone H1 in the presence of [γ-^{32}P]ATP. Reaction products were then analyzed by SDS-PAGE and autoradiography.

(C) Depletion of CENP-E prevents activation of the mitotic checkpoint as determined by ability to degrade recombinant securin protein. In vitro–translated ^{35}S-radiolabeled securin was incubated in CENP-E-depleted and mock-depleted extracts for 20 min before release to the CSF arrest. At the indicated times, aliquots were removed and subjected to SDS-PAGE and autoradiography.

Results

CENP-E Is Required for Establishment of the Mitotic Checkpoint in *Xenopus* Egg Extracts

Although the first 12 *Xenopus* embryonic cell divisions proceed without an effective mitotic checkpoint, egg extracts previously have been demonstrated to activate the checkpoint in vitro when sufficient sperm head nuclei are added (≈9000 nuclei/μl extract) to approximate the chromosome/cytoplasm ratio present at the mid-blastula transition (the time of appearance of the checkpoint during normal development) (Minshull et al., 1994). Because the assembly of spindles from the added nuclei is not completely synchronous, the presence of an activated checkpoint can be most conveniently assayed first by using an endogenous activity known as cytostatic factor (CSF) to synchronize all spindles at metaphase, then assaying the presence of an activated checkpoint by the continued mitotic arrest after inactivation of CSF by addition of calcium (Murray, 1991) and disassembly of spindle microtubules with a microtubule inhibitor such as nocodazole.

Because earlier efforts depleting CENP-E have demonstrated that absence of CENP-E does not affect CSF-mediated mitotic arrest (Wood et al., 1997), we initially depleted CENP-E by immunoadsorption from CSF-arrested extracts (Figure 1A) to determine how absence of CENP-E affects the establishment and maintenance of the checkpoint in response to spindle damage. High numbers of sperm were added, followed by nocodazole (for 30 min) to disrupt spindle microtubule assembly, and the presence of an activated checkpoint was assessed by chromosome decondensation and CDC2/cyclin B (cyclin-dependent kinase [CDK]) histone kinase activity (Figure 1B) 0, 30, or 60 min after addition of calcium to release the CSF arrest. Whereas control extracts mock-depleted with equivalent amounts of nonimmune IgG activated the checkpoint and remained

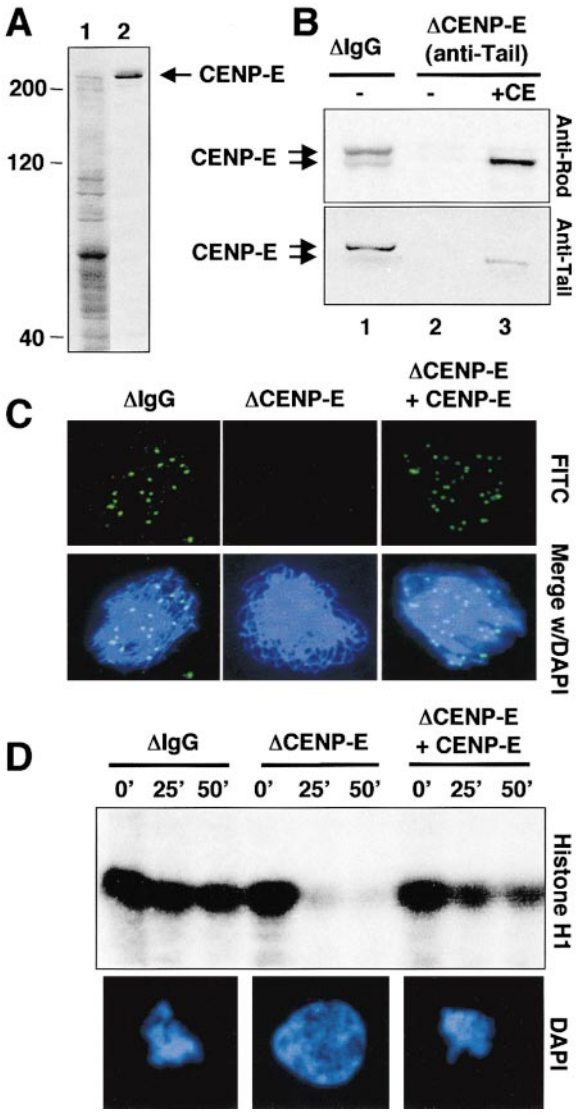

Figure 2. Recombinant CENP-E Restores the Mitotic Checkpoint in CENP-E-Depleted Extracts

(A) Purification of recombinant XeCENP-E. Lane 1 shows an initial whole-cell extract from insect cells infected with a baculovirus encoding XeCENP-E; lane 2, purified XeCENP-E after a two-step ion exchange chromatography and visualization by SDS-PAGE and Coomassie blue staining.

(B) Immunoblotting of anti-CENP-E detects either one band (anti-Tail, bottom panel, lane 1) or two bands (anti-Rod, top panel, lane 1) in CSF-arrested extracts. Extracts were mock-depleted (IgG) or depleted of CENP-E with an anti-tail antibody (lanes 2 and 3). The resulting extracts were immunoblotted with anti-CENP-E rod or tail antibodies. Note that both bands are removed after anti-tail CENP-E depletion (lane 2, both panels). The recombinant CENP-E added to the depleted extract (lane 3, both panels) migrates as the lower band and is recognized by both antibodies.

(C) Recombinant XeCENP-E associates with kinetochores of sperm head chromatin. Extracts were either mock-depleted (ΔIgG), depleted of CENP-E (ΔCENP-E), or depleted of endogenous CENP-E and supplemented with recombinant CENP-E (ΔCENP-E + CENP-E). After addition of sperm head nuclei and nocodazole, extracts were observed by immunofluorescence with anti-CENP-E antibodies, and chromatin was visualized with DAPI (merged with CENP-E, lower panels).

(D) CSF extracts either mock-depleted (ΔIgG), CENP-E-depleted (ΔCENP-E), or CENP-E-depleted and supplemented with recombinant CENP-E (ΔCENP-E + CENP-E) were treated with high sperm and then nocodazole for 30 min to initiate the checkpoint; aliquots were assayed at the indicated times for histone H1 kinase activity (top) and chromatin decondensation (bottom) (by visualization with DAPI). Loss of checkpoint control (as indicated by loss of H1 kinase activity and decondensation of chromatin) in ΔCENP-E extracts (center) is overcome by supplementation with an equivalent amount of recombinant CENP-E (right).

arrested in mitosis as demonstrated by continued CDK activity (assayed with histone H1 as a substrate; Figure 1B) or chromosome condensation (not shown), quantitative depletion of CENP-E from these extracts before sperm addition led to rapid loss of H1 kinase activity, chromosome decondensation, and nuclear envelope reassembly.

Because it had been previously shown that APC-mediated destruction of securin is required for sister chromatid separation to occur at anaphase, we examined whether securin degradation, as well as loss of CDK activity, was CENP-E dependent after microtubule disruption. High sperm numbers were added to CENP-E-depleted or mock-depleted CSF-arrested extracts. After incubation in the presence of nocodazole for 30 min, CSF was released by addition of calcium in the presence of ^{35}S-labeled recombinant securin. At 0, 30, and 60 min after release from CSF arrest, aliquots of extract were removed and analyzed by SDS-PAGE and autoradiography. As shown in Figure 1C, securin was stable after checkpoint activation by nocodazole in mock-depleted extracts. However, consistent with failure to activate the checkpoint after depletion of CENP-E, securin was rapidly and quantitatively degraded from similarly treated CENP-E-free extracts, confirming that CENP-E is required for checkpoint-mediated inhibition of sister chromatid separation in response to nocodazole-induced spindle damage.

Recombinant CENP-E Restores the Checkpoint to CENP-E-Depleted Extracts

Because it is known that CENP-E associates in vitro with known components of the mitotic checkpoint (e.g., MAPK [Zecevic et al., 1998] or BUBR1 [Chan et al., 1998]), one mechanism through which immunodepletion of CENP-E might suppress the mitotic checkpoint was co-depletion of a CENP-E-associated protein present in limiting amounts. To determine whether the checkpoint suppression was a direct consequence of the loss of CENP-E function, we purified recombinant CENP-E protein from insect cells infected with a baculovirus encoding full-length *Xenopus* CENP-E. Recombinant CENP-E protein was purified to near homogeneity by using a combination of cation and anion exchange chromatography (Figure 2A). Two CENP-E isoforms are detected in *Xenopus* extracts (Figure 2B; Wood et al., 1997) by using antibodies either prepared against a portion of the helical rod domain (anti-rod) or against the C-terminal rod and tail domain (anti-tail). The recombinant CENP-E co-migrates with the smaller isoform (most easily seen by the increase in the lower species after re-addition to depleted extracts; Figure 2B, lane 3), which is also the predominant species present in *Xenopus* XL177 epithelial cells (results not shown). Whether the different forms arise from distinct genes or alternatively

spliced products of a single gene has not been established; however, both are efficiently depleted by the anti-tail antibody (Figure 2B, cf. lanes 1 and 2).

As an initial test of the functional properties of the full-length CENP-E, we examined whether the recombinant protein could efficiently bind to kinetochores when added to depleted extracts. As expected, no CENP-E could be detected by immunofluorescence when sperm head nuclei were added to CENP-E-depleted extracts (Figure 2C, middle). However, when recombinant CENP-E was added to depleted extracts at a level comparable to the normal concentration of CENP-E in these extracts (Figure 2B, cf. lanes 1 and 3), the recombinant CENP-E was found to bind readily in a series of punctate spots associated with chromosomes (Figure 2C, right), a pattern indistinguishable from endogenous CENP-E in undepleted extracts (Figure 2C, left). This indicates that recombinant CENP-E is able to associate with *Xenopus* kinetochores. Moreover, initial assays for microtubule motor activity revealed that the full-length protein mediates ATP-sensitive microtubule binding and powers ATP-dependent microtubule gliding with motor movement in a plus end direction, as initially demonstrated for a motor-bearing *Xenopus* CENP-E fragment (Wood et al., 1997).

To test whether the kinetochore-associated, recombinant CENP-E was capable of restoring the checkpoint to reconstituted extracts, we monitored CDK activity and chromosome condensation after release of CSF arrest in depleted extracts to which the purified CENP-E had been added back to the normal level of CENP-E. Both histone kinase activity and chromosome decondensation revealed that depleted extracts again exited mitosis in the presence of nocodazole (Figure 2D, middle), whereas reconstituted extracts remained mitotically arrested (Figure 2D, right). This, along with a similar elimination of checkpoint signaling from the addition of CENP-E antibodies (see below), demonstrated that CENP-E, rather than some associated *Xenopus* factor, is the essential component of checkpoint signaling.

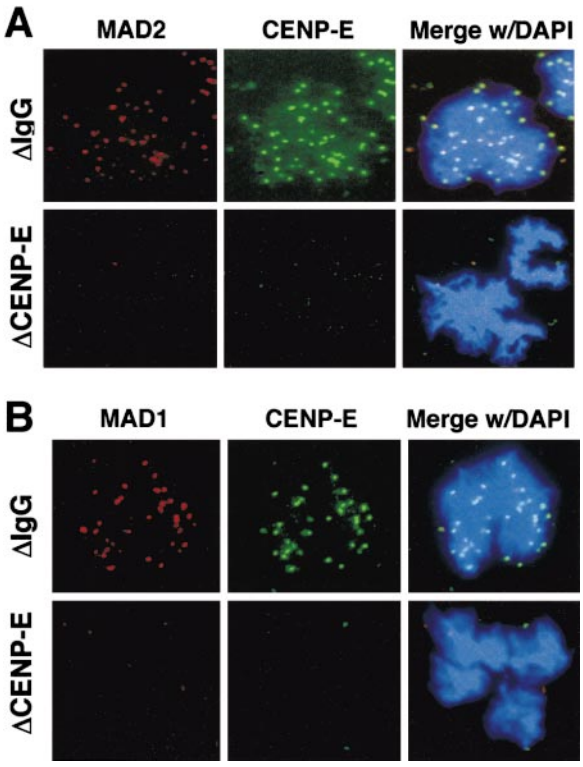

Figure 3. CENP-E Is Required for Association of MAD2 and MAD1 with Kinetochores

CSF-arrested extracts were mock-depleted (ΔIgG) or CENP-E-depleted (ΔCENP-E), and ≈9000 sperm/μl were added. Finally, 30 min after addition of nocodazole, the association of CENP-E (center column) and MAD2 (A) or MAD1 (B) (left column) with kinetochores was visualized. MAD1 and MAD2 positions were detected by appropriate primary antibodies followed by Texas red–labeled anti-rabbit IgG; chromosome-bound CENP-E was subsequently detected using anti-CENP-E rabbit antibody directly coupled to biotin and visualized with FITC-avidin. Chromatin was visualized with DAPI, and the three signals were merged (right column).

CENP-E Is Necessary for Kinetochore Signaling in the Checkpoint

A final step in the checkpoint signaling cascade is thought to be the inhibition of APC-dependent ubiquitination of substrates, of which an essential one is securin. Evidence from several groups has demonstrated that activation of APC is prevented by MAD2 in association with an APC specificity factor known as CDC20 (Visintin et al., 1997) (also known as fizzy [Dawson et al., 1995] or p55CDC [Kallio et al., 1998]). Repetitive binding of MAD2 to unattached kinetochores followed by its release in an APC inhibitory complex is generally thought to arise as the last step in kinetochore-dependent checkpoint signaling (Fang et al., 1998). Consequently, MAD2 is found associated only with kinetochores that are sending the inhibitory signal (Chen et al., 1996; Li and Benezra, 1996). To test if absence of CENP-E affected kinetochore-dependent signaling, we examined chromosomes in mock-depleted or CENP-E-depleted extracts for centromere-associated MAD2. This revealed that MAD2 binding to kinetochores was eliminated by immunodepletion of CENP-E, although it could be easily identified at centromeres in extracts with an active checkpoint (Figure 3A, top panels). Furthermore, because it is known that MAD1 is required for MAD2 to be recruited to the kinetochores (Chen et al., 1998), most likely in a MAD1/MAD2 complex, we also examined MAD1 localization in the absence of CENP-E. As shown in Figure 3B, MAD1, like MAD2, fails to localize to kinetochores in the absence of CENP-E, revealing a requirement for CENP-E in MAD1 recruitment to kinetochores.

The necessity for kinetochore-dependent signaling in the checkpoint has been previously shown to be overcome by addition of excess recombinant MAD2, which yields spontaneous formation of the inhibitory complex in the absence of high levels of kinetochores (i.e., sperm nuclei) or even spindle-disrupting agents (Chen et al., 1998). To determine whether removal of CENP-E affected signaling steps downstream of the kinetochore, we added excess recombinant *Xenopus* MAD2 at ≈15-fold the normal level of MAD2 in the extracts. Regardless of whether or not CENP-E function had previously been disrupted by CENP-E antibody depletion, excess MAD2 was still able effectively to block mitotic progression, as observed by the maintenance of high CDK activity (Figure 4) or chromosome condensation (not shown). Thus, the CENP-E function in establishing the checkpoint occurs at a kinetochore-dependent step in the

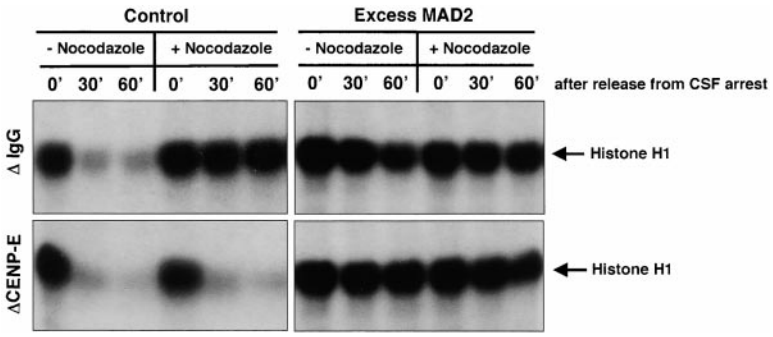

Figure 4. Addition of High Levels of MAD2 Demonstrates That Depletion of CENP-E Does Not Disrupt Checkpoint Steps Downstream of Kinetochore Signaling

CSF-arrested extracts depleted of CENP-E (ΔCENP-E) or mock-depleted (ΔIgG) were prepared as in Figure 3, except that excess MAD2 protein was added before addition of nocodazole. After incubation for 30 min, CSF arrest was released, and aliquots of the extracts were removed at the indicated times and assayed for histone H1 kinase activity as described above. Progression through mitosis is prevented by addition of excess recombinant MAD2 protein, regardless of whether CENP-E is present.

signaling cascade, upstream of MAD2-mediated inhibition of APC/CDC20.

CENP-E Is Required at Kinetochores for Maintenance of an Activated Checkpoint

Although CENP-E is a transient centromere component that binds to the outermost region of the kinetochore (Cooke et al., 1997; Yao et al., 1997) only after all other known kinetochore components have associated (Chan et al., 1998), the disruption in kinetochore-dependent checkpoint signaling after depletion of CENP-E could in principle arise from disruption in basic centromere assembly rather than a more direct effect on the checkpoint. To test if this were true, CSF-arrested extracts to which high numbers of sperm nuclei had been added were released from CSF with calcium, cycled for 80 min through S phase to duplicate the chromatids, and arrested again in the subsequent mitosis by addition of CSF (i.e., by re-addition of an aliquot of the initial CSF extract). CENP-E antibodies or nonimmune IgG were then added. This yielded bipolar spindles with aligned chromosomes in nonimmune antibody-containing extracts (Figure 5A, left) that were capable of anaphase chromosome segregation within 15 min after calcium-dependent release of CSF (Figure 5A, middle). Addition of nocodazole 30 min before CSF release not only disassembled all spindle microtubules but activated the checkpoint, as seen by continued DNA condensation and elevated H1 kinase activity (Figure 5B). On the other hand, addition of CENP-E antibodies resulted in chromosome misalignment (e.g., the chromosomes marked with arrows in Figure 5C, left), as expected from the known requirement for CENP-E in chromosome congression (Wood et al., 1997). Nevertheless, anaphase chromosome movement appeared largely normal after release of CSF arrest (Figure 5C, middle), with many chromosomes clustered near the poles in a fashion fully consistent with movement mediated by microtubule forces acting at kinetochores. Thus, the added CENP-E antibodies disrupted neither the continued functional attachment of chromosomes to spindle microtubules nor apparent centromere function in anaphase. Despite this, after addition of nocodazole to disrupt spindle microtubules 30 min before CSF release, no checkpoint could be detected in extracts containing CENP-E antibodies. Instead, decondensation of chromatin and loss

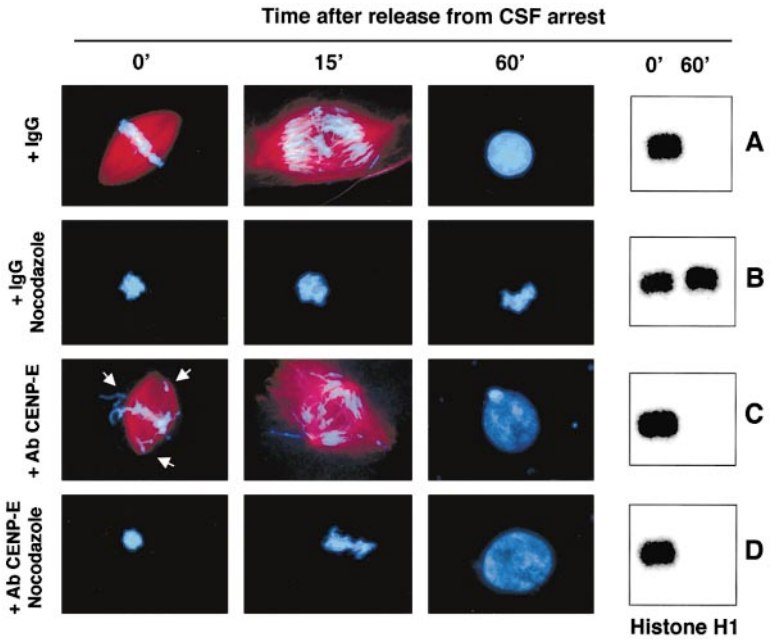

Figure 5. Addition of CENP-E Antibody Does Not Disrupt Centromere Function but Allows Mitosis Exit, Despite the Presence of High Sperm Number and Nocodazole

CSF extracts containing high levels of sperm were allowed to progress (after addition of calcium) through the cell cycle and were then arrested in the subsequent metaphase by re-addition of CSF (as described in Experimental Procedures). Either affinity-purified anti-CENP-E antibodies or a control IgG was then incubated for 30 min. At the times indicated after release from the second CSF arrest, chromatin and microtubules were visualized using DAPI and tetramethylrhodamine tubulin. Aliquots taken from the extracts were assayed for histone kinase activity as described in the legend to Figure 1B.

(A and B) Extracts plus nonimmune IgG in the absence (A) or presence (B) of nocodazole to dissemble microtubules.

(C and D) Extracts plus anti-CENP-E IgG in the absence (C) or presence (D) of nocodazole. Whereas IgG-treated extracts arrest in mitosis in response to nocodazole (B), those treated with an anti-CENP-E-antibody (D) exit mitosis (as judged by decondensation of chromatin and loss of histone kinase activity).

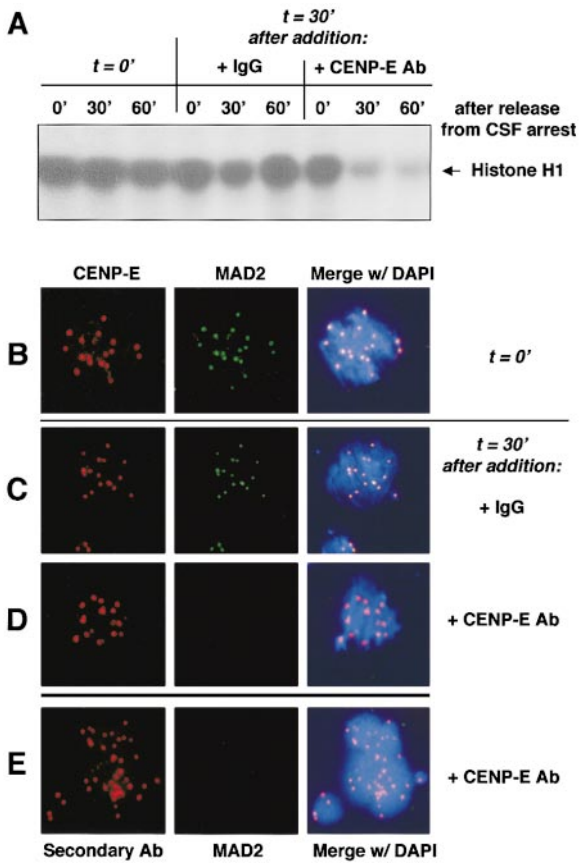

Figure 6. Kinetochore-Associated CENP-E Is Required for Maintenance of a Previously Established Mitotic Checkpoint

(A) CSF extracts in which the checkpoint had been activated by addition of ≈9000 sperm/μl and incubation in nocodazole for 30 min were then treated with anti-CENP-E or nonspecific (IgG) antibodies for 30 min before release from the CSF arrest. At the indicated times, aliquots were removed and assayed for histone kinase activity as described in the legend to Figure 1. Although the checkpoint was already activated by the presence of nocodazole for 30 min (t = 0′), incubating the extract with the anti-CENP-E antibody for 30 min (t = 30′) prevented maintenance of the checkpoint.

(B–E) Antibody-mediated perturbation of CENP-E prevented continued MAD2 localization to kinetochores, despite continuing presence of kinetochore-bound CENP-E. Immunofluorescence detection of CENP-E and MAD2 visualized before (B) and 30 min after (C–E) adding antibodies, as in (A). Samples were fixed, and CENP-E location was determined using a Texas red anti-rabbit antibody in the presence (A–D) or absence (E) of anti-CENP-E added during the immunofluorescence detection. Subsequently, MAD2 was detected using a biotinylated anti-MAD2 followed by avidin-FITC.

of CDK activity occurred within 60 min (Figure 5D). Taken together with the immunodepletion of CENP-E (Figures 1 and 2), these results indicate that CENP-E is required for establishment of the mitotic checkpoint in *Xenopus* extracts but not for basic centromere assembly/function.

To test whether CENP-E is also required for maintaining an already established mitotic arrest, the checkpoint was activated by addition of nocodazole to a CSF extract with high numbers of sperm as before, and CENP-E antibodies were then added. As shown in Figure 6A, even though the checkpoint had already been activated, antibody-induced inhibition of CENP-E function caused exit from mitosis, as indicated by the loss of histone kinase activity. Therefore, CENP-E appears to be required for maintaining checkpoint signaling even after full activation.

CENP-E Acts at Kinetochores to Activate or Silence Checkpoint Signaling

Suppression of kinetochore signaling from addition of CENP-E antibodies could arise from antibody-mediated release of CENP-E or by inhibition at the kinetochore of an essential CENP-E activity in checkpoint signaling. To distinguish between these two mechanisms, the checkpoint was activated with high sperm and nocodazole followed by addition of antibodies to the CENP-E tail (including part of the rod; Wood et al., 1997). Whether CENP-E remained kinetochore associated was determined both by standard indirect immunofluorescence localization (Figure 6D) and by simple addition of a secondary antibody coupled to Texas red to detect the position of the added anti-CENP-E IgG (Figure 6E). This revealed that the suppression of checkpoint signaling by added CENP-E antibody arose despite retention of normal levels of CENP-E at kinetochores (cf. Figures 6B and 6C with 6D and 6E). Thus, CENP-E antibody-induced loss of maintenance of checkpoint signaling (or its activation; not shown) occurs despite retention of normal levels of kinetochore-associated CENP-E. Further, addition of CENP-E antibodies, but not nonimmune IgG, to extracts with an already activated checkpoint provoked a time-dependent release of MAD2 (cf. Figures 6D and 6E with 6B and 6C), consistent with checkpoint silencing.

Discussion

Use of immunodepletion and antibody addition has demonstrated that CENP-E is absolutely required for establishing and maintaining the mitotic checkpoint in *Xenopus* egg extracts. It is CENP-E that is required for the checkpoint, because addition of purified recombinant CENP-E protein to depleted extracts restores the ability to arrest in mitosis in response to spindle damage, and simple antibody inhibition of CENP-E is sufficient to silence signaling. Further, CENP-E action is necessary at the centromeres in checkpoint signaling because antibody addition eliminates CENP-E function in the checkpoint without displacing it from kinetochores.

How does CENP-E contribute to the checkpoint in *Xenopus* extracts? The vertebrate checkpoint is normally activated just after nuclear envelope disassembly, as unattached kinetochores acquire the signaling machinery. Three lines of evidence converge to support the view that CENP-E directly captures spindle microtubules and/or generates tension through its microtubule binding motor domain distal to the kinetochore, while its proximal portion modulates the activity(ies) of other components of the kinetochore signaling cascade. First, CENP-E is localized at the kinetochore outer surface, with the CENP-E microtubule motor domain positioned for microtubule capture (Cooke et al., 1997; Yao et al., 1997). Second, mammalian CENP-E has been shown to bind directly to BUBR1 (Chan et al., 1998; Yao et al., 2000), a checkpoint kinase (Chan et al., 1999). Thus, physical spindle attachment and/or tension development could easily be sensed and transmitted to BUBR1 through potential alterations in CENP-E conformation. Third, antibody binding to kinetochore-associated CENP-E is itself sufficient to silence successful check-

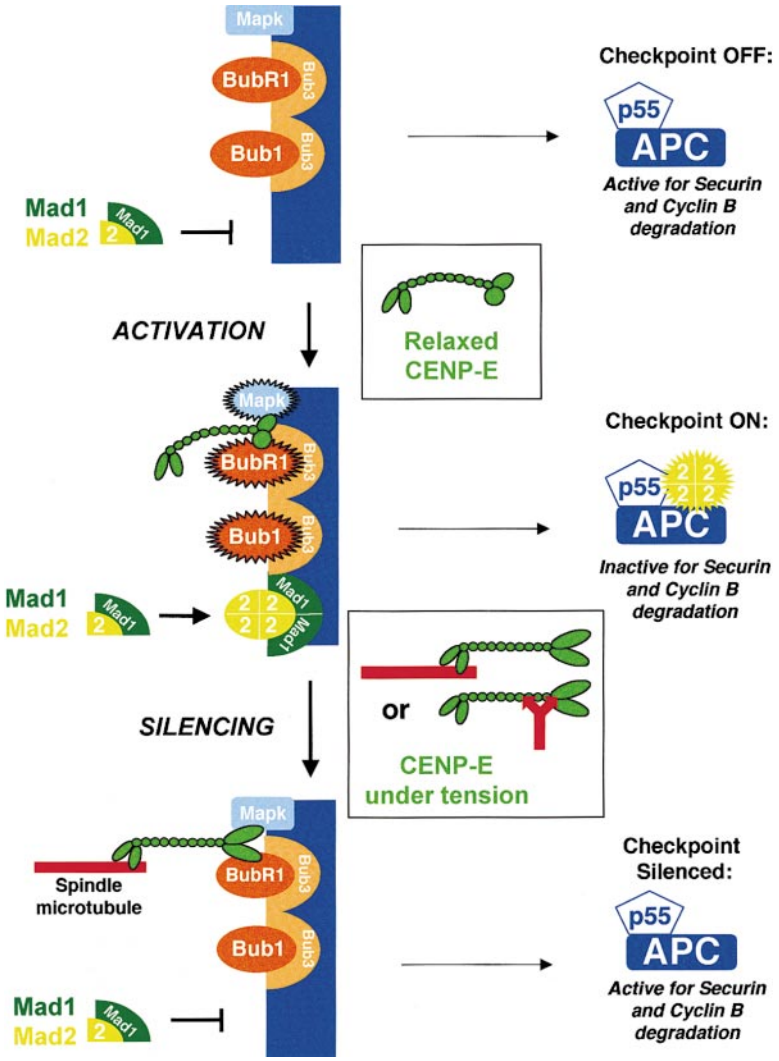

Figure 7. Model for the Role of CENP-E in the Mitotic Checkpoint in *Xenopus* Extracts

In *Xenopus* extracts, CENP-E is required for establishing the checkpoint. Before spindle microtubule capture, kinetochore-bound CENP-E is necessary for activating BUBR1, MAPK, and/or BUB1-dependent kinetochore signaling, culminating in production of active MAD2, which inhibits APC/p55. Depletion of CENP-E yields failure to activate BUBR1, MAPK, and/or BUB1 signaling. Checkpoint signaling is normally satisfied by spindle microtubule capture or subsequent tension, which alters CENP-E conformation and its interaction with other kinetochore components, especially BUBR1. CENP-E antibody binding to the C-terminal rod and tail of kinetochore-bound CENP-E mimics spindle microtubule capture by CENP-E, thereby altering CENP-E interactions that affect the activity of other kinetochore signaling components.

point signaling (Figure 6). This last effect may be most easily explained if the antibody (which binds to the CENP-E tail and rod regions) mimics the effect(s) of microtubule binding and/or tension, for example, thereby affecting CENP-E interaction with BUBR1 and altering BUBR1 activity (as illustrated in Figure 7).

A Model for CENP-E and Checkpoint Signaling

The evidence here strongly supports the following model for checkpoint signaling in the *Xenopus* extracts (Figure 7). On chromosomes that are not attached to the mitotic spindle, CENP-E initiates checkpoint signaling by binding to and activating one or more signaling factors (e.g., the BUBR1 protein kinase with which it is known to associate in vivo). This would lead to a kinetochore-associated signaling cascade with generation of an active form of MAD2. Once activated, MAD2 would detach from kinetochores, preventing anaphase initiation by binding to APC/p55 and thus preventing the degradation of securin. When kinetochore-associated CENP-E binds to spindle microtubules (or mimicked by CENP-E antibody binding to kinetochore-associated CENP-E; Figure 7), CENP-E undergoes some structural change that renders it incapable of contributing to the activation of signaling factors such as BUBR1. Thus, once both centromeres on each chromosome pair attach to spindle microtubules, CENP-E transduces this information to silence kinetochore-dependent checkpoint signaling and the cell cycle transitions to anaphase. Interaction of kinetochore-bound CENP-E with one or more kinetochore components, especially BUBR1, is a central feature of signaling in this model; hence, at least in this context the checkpoint cannot be activated without CENP-E (Figure 7). Thus, each chromatid (indeed, each kinetochore-bound molecule of CENP-E/BUBR1) would be potentially capable of arresting the cell cycle because each kinetochore can generate the inhibitory signal, a property that accounts for the observation that even a single unattached kinetochore can inhibit anaphase (Rieder et al., 1995).

How could CENP-E sense kinetochore attachment status? There is an ongoing controversy about whether the checkpoint senses microtubule attachment (Waters et al., 1998) or tension at the kinetochore (Nicklas et al., 1995; Yu et al., 1999). A microtubule binding protein would be strictly required for sensing either of the two phenomena. It is tempting to speculate that CENP-E could be a tension-sensing molecule because kinesin-like proteins undergo microtubule-dependent conformational changes that lead to the production of force.

Conversely, it is plausible that the application of tensile forces on a motor protein could cause it to undergo conformational changes that allosterically affect its ability to interact with other proteins that are involved in checkpoint signaling. In either event, the results described herein identify the requirement of one particular microtubule binding protein in establishing vertebrate mitotic checkpoint signaling.

The Checkpoint Paradox

Inhibition of CENP-E function in mammalian systems has given results that seemingly contradict those reported here in *Xenopus*: addition of neutralizing antibodies that remove CENP-E from kinetochores (Chan et al., 1999) or antisense oligonucleotide-mediated suppression of CENP-E accumulation (Yao et al., 2000) causes a G2/M arrest apparently arising from a chronically activated mitotic checkpoint characterized by monopolar chromosomal attachment, chromosome misalignment, and reduced tension at bi-oriented chromosome pairs. Although all of the evidence clearly points to CENP-E as a critical link between spindle microtubules and the checkpoint machinery, the key underlying difference(s) in checkpoint signaling between the *Xenopus* and mammalian systems could simply be what residual activity remains in a partial kinetochore complex. Indeed, in considering a minimal three-component signaling/silencing complex (spindle microtubule, CENP-E, and the kinetochore-associated signaling kinase BUBR1), there can be no clear de novo prediction of whether removal of the linker (CENP-E) would lead to a constitutively activated or silenced activity of a component such as BUBR1. In the *Xenopus* egg system, removal of CENP-E prevents MAD2 from associating with kinetochores, thereby blocking the checkpoint by preventing MAD2 from becoming activated at kinetochores (to inhibit APC/CDC20). In contrast, depletion of CENP-E in human HeLa cells leaves active BUBR1 and MAD2 associated with unattached kinetochores (Yao et al., 2000). The differences between the frog and mammalian systems may reflect species-specific distinctions, the difference between the meiotic state of egg extracts versus the mitotic state of cultured cells, or greater redundancy in microtubule linkers in the mammalian somatic cells. A resolution of the seeming contradiction will require identification and purification of the *Xenopus* BUBR1 homolog and comparison with the corresponding properties of the mammalian counterpart.

The differential apparent activity of the signaling kinases in the presence or absence of CENP-E in the mammalian and *Xenopus* systems highlights a paradox. The checkpoint pathway is itself widely conserved, yet individual components are not. In budding yeast, where genetic evidence has unequivocally identified seven components required for the checkpoint (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996), search of the complete genome reveals that there is no CENP-E-like protein. Moreover, BUBR1, the mammalian checkpoint component with which CENP-E directly interacts, is itself an apparent fusion of yeast MAD3 with the kinase domain of BUB1 (Taylor et al., 1998). In any event, although the component(s) that links spindle microtubules to centromeres in yeast remains unidentified, current evidence makes it clear that CENP-E is a key vertebrate component for linking spindle microtubule binding to centromere-dependent checkpoint signaling and/or silencing.

Experimental Procedures

Xenopus Egg Extracts

CSF-arrested egg extracts were prepared as previously described (Murray, 1991), except that the checkpoint was activated in the presence of ≈9000 sperm head nuclei/μl and 10 μg/ml nocodazole (Minshull et al., 1994). Typically, the checkpoint is activated in the presence of high sperm nuclei and nocodazole for 30 min before assay by release from the CSF arrest. Microtubules were visualized by addition of 50 μg/ml tetramethylrhodamine-labeled bovine tubulin. To follow sister chromatid separation (Figure 5), we added sperm to CSF extracts, which were then induced to enter interphase and replicate DNA when we added 0.4 mM calcium chloride. These extracts were then re-arrested in mitosis by the addition of half volume of fresh CSF extract, as previously described (Chen and Murray, 1997). Final concentration of added sperm was 9000/μl. After fixation of the sample in formaldehyde containing DAPI (1 μg/ml), microtubules and chromatin were visualized using a Zeiss Axiophot microscope at 60× with epifluorescence illumination. Images were recorded using a Princeton Instruments ST138/KAF1400 cooled CCD camera that was controlled by Metamorph software (Universal Imaging, West Chester, PA).

CENP-E was depleted from 100 μg of CSF extracts by using 100 μl of Protein A beads (Dynal) coupled to 100 μg of anti-tail CENP-E-purified antibodies. To prevent release of the antibodies in the extract during the depletion, CENP-E IgG were covalently coupled to the Protein A beads by using DMP coupling (Harlow and Lane, 1999). For antibody addition experiments, affinity purified CENP-E IgG were added at 100 μg/ml.

Histone H1 Phosphorylation Assay

At indicated time points after calcium addition, 1 μl of extract was frozen in liquid nitrogen. Extract samples were thawed by the addition of 9 μl of histone H1 mix that included [γ-^{32}P]ATP (Chen and Murray, 1997) and incubated for 10 min at room temperature. The reactions were terminated by the addition of 10 μl of 2× protein sample loading buffer and run on 12% SDS–polyacrylamide gels. The gels were dried and subjected to autoradiography to visualize phosphorylated histone H1.

Securin Degradation

The securin-encoding plasmid PCS2FA-WT (a gift of H. Zou) was in vitro transcribed and translated in reticulocyte lysate by using the TNT SP6 polymerase kit (Promega) in the presence of [^{35}S]methionine. One microliter of the in vitro translation reaction was incubated with 20 μl of extract, and 2 μl were taken at the indicated time points after calcium addition. Samples were diluted with 2× protein sample loading buffer, electrophoresed on SDS–polyacrylamide gels, and visualized by autoradiography.

CENP-E Production and Purification

Baculoviruses expressing *Xenopus* CENP-E were prepared using the Bac-to-Bac expression system (GIBCO/Life Technologies, Bethesda, MD). A full-length cDNA encoding *Xenopus* CENP-E (Wood et al., 1997) was cloned into the baculovirus expression vector pFastBac1 (GIBCO–BRL, Bethesda, MD), and recombinant bacmids were produced as directed by the manufacturer. The protein was expressed by infection of ≈4 × 10^7 Hi5 cells at a multiplicity of infection of 5–10 for 48 hr. Infected cells were isolated by centrifugation, frozen in liquid nitrogen, and stored at −70° C until use. Whole-cell extracts were prepared by thawing the cell pellet in 10 ml of PK100 buffer (80 mM PIPES [pH 6.8], 100 mM KCl, 0.5 mM EGTA, 4 mM $MgCl_2$, 1 mM ATP, 10 μg/ml leupeptin/pepstatin/chymostatin, and 1 mM PMSF) and briefly sonicated. Extracts were centrifuged for 20 min at 12,000 rpm in a Sorval SA600 rotor. The supernatant was loaded onto a 1-ml HiTrap SP Sepharose ion-exchange column on an FPLC chromotography system (Pharmacia, Piscataway, NJ). The protein was eluted using a linear gradient from 100 mM to 1 M KCl in IEX buffer (10 mM PIPES [pH 6.8], 0.5 mM EGTA, 2 mM $MgCl_2$, and 50 μM ATP). The peak fraction (at ≈200 mM KCl) was diluted 1:1 with IEX buffer lacking KCl and loaded onto a 1-ml Source 15Q column. The protein was eluted using a linear gradient from 100 mM to 1 M KCl in IEX buffer. The peak

fraction eluted at ≈200–250 mM KCl. Powdered sucrose was added to a final concentration of 10%, and the peak fraction was frozen in liquid nitrogen and stored at −70° C.

Immunofluorescence

Immunofluorescence with *Xenopus* extracts was performed as previously described (Wood et al., 1997). MAD2 antibodies were raised into rabbits after prokaryote expression of pQE-Xmad2 (a gift of R. H. Chen) and purified against the same protein. MAD2 protein was detected using Texas red anti-rabbit antibody (Figure 3). The anti-MAD1 was a gift from R.H. Chen, and MAD1 was detected using Texas red anti-rabbit antibody (Figure 3). CENP-E was detected using anti-tail CENP-E polyclonal affinity-purified antibodies (Wood et al., 1997) followed by Texas red anti-rabbit antibody (Figures 6B–6D) or using the Texas red anti-rabbit antibody only (Figure 6E). For double-labeling experiments, the anti-CENP-E and anti-MAD2 antibodies were previously labeled with Biotin (long arm) NHS (Vector Laboratories) and detected using avidin-FITC (Figure 3 for CENP-E and Figures 6B–6E for MAD2). In those co-staining experiments, the nonbiotinylated primary antibody was used first, followed by Texas red–conjugated anti-rabbit antibody; then the biotinylated primary antibody was used, followed by avidin-FITC.

Acknowledgments

We are grateful to H. Zou and M. Kirschner for providing the PDS1 constructs and to R. H. Chen for providing the pQE-Xmad2 plasmid and the anti-MAD1 antibody, as well as helpful hints on the checkpoint technique in *Xenopus* egg extracts. This work has been supported by a grant from the NIH to D. W. C. A. A. has been supported by ARC (Association pour la Recherche contre le Cancer) and HFSP (Human Frontier Science Program). J. A. K. was supported by postdoctoral fellowship DRG-1567 of the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation and by a fellowship from the Bank of America/Giannini Foundation. D. W. C. is a Member of the Ludwig Institute for Cancer Research, which provides salary support for him.

Received May 3, 2000; revised July 24, 2000.

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