Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor–mediated metaphase arrest

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In cells containing disrupted spindles, the spindle assembly checkpoint arrests the cell cycle in metaphase. The budding uninhibited by benzimidazole (Bub) 1, mitotic arrest-deficient (Mad) 1, and Mad2 proteins promote this checkpoint through sustained inhibition of the anaphase-promoting complex/cyclosome. Vertebrate oocytes undergoing meiotic maturation arrest in metaphase of meiosis II due to a cytoplasmic activity termed cytostatic factor (CSF), which appears not to be regulated by spindle dynamics. Here, we show that microinjection of Mad1 or Mad2 protein into early Xenopus laevis embryos causes metaphase arrest like that caused by Mos. Microinjection of antibodies to either Mad1 or Mad2 into maturing oocytes blocks the establishment of CSF arrest in meiosis II, and immunodepletion of either protein blocked the establishment of CSF arrest by Mos in egg extracts. A Mad2 mutant unable to oligomerize (Mad2 R133A) did not cause cell cycle arrest in blastomeres or in egg extracts. Once CSF arrest has been established, maintenance of metaphase arrest requires Mad1, but not Mad2 or Bub1. These results suggest a model in which CSF arrest by Mos is mediated by the Mad1 and Mad2 proteins in a manner distinct from the spindle checkpoint.

Introduction

Vertebrate eggs undergo an arrest of the cell cycle with an intact spindle at metaphase of meiosis II while awaiting fertilization. This arrest is a result of inhibition of the anaphase-promoting complex/cyclosome (APC/C), and is mediated by an activity generated during oocyte maturation, termed cytostatic factor (CSF; Masui and Markert, 1971). In amphibians, CSF activity disappears 30–40 min after fertilization in response to an increase in the level of free calcium. Many of the molecular components that constitute CSF activity have been identified since its initial description over 30 years ago (for review see Tunquist and Maller, 2003). Three independent pathways are thought to contribute to CSF arrest. One pathway arises from the synthesis of Cdk2 and cyclin E proteins during oocyte maturation. Cdk2 and cyclin E are present at very low levels in resting oocytes, and their levels increase dramatically during entry into meiosis II (Rempel et al., 1995). Injection of maturing oocytes with antisense oligonucleotides specific for Cdk2 attenuates CSF arrest in vivo (Gabrielli et al., 1993), and addition of a constitutively active form of cyclin E/Cdk2 to Xenopus laevis egg extracts is sufficient for APC/C inhibition and metaphase arrest (D’Angiolella et al., 2001; Tunquist et al., 2002). The mechanism by which cyclin E/Cdk2 inhibits the APC/C is not clear, but is likely related to the general mechanism by which G1 Cdks turn off the degradation of G2 cyclins by the APC/C in G1 (Amon et al., 1994; Zachariae et al., 1998).

A second pathway involved in APC/C inhibition and CSF arrest in the egg involves the recently identified vertebrate homologue of the regulator of cyclin A1, early mitotic inhibitor 1 (Emi1; Reimann et al., 2001a; Reimann and Jackson, 2002). Emi1 binds directly to the only known APC/C activator in the egg, termed Cdc20, to prevent premature activation of the APC/C. Overexpression of Emi1 in CSF-arrested egg extracts prevents cyclin B and Mos proteolysis upon addition of either calcium or a constitutively active form of calcium/calmodulin-dependent protein kinase II (Reimann and Jackson, 2002), and overexpression of Emi1 in blastomeres causes cleavage arrest (Reimann et al., 2001a). Immunodepletion of Emi1 from CSF extracts
has been reported to cause release from the arrest in the absence of calcium addition (Reimann and Jackson, 2002).

The third and most well-characterized pathway involved in CSF arrest is initiated by Mos, a germ cell–specific MAPK kinase kinase (MAPKKK), synthesized during oocyte maturation in response to progesterone administration (for review see Tunquist and Maller, 2003). Mos phosphorylates and activates the MAPK kinase, MAPK/Erk kinase 1 (MEK1), which in turn phosphorylates and activates MAPK. Finally, MAPK phosphorylates and activates the 90-kD ribosomal protein S6 kinase (p90\textsuperscript{Rsk}) (for review see Tunquist and Maller, 2003). Mos phosphor-

Results

Mad1 and Mad2 arrest cleaving Xenopus embryos in metaphase

Mad1 and Mad2 proteins are present in the early embryo even though nocodazole does not affect cell cycle progression until the gastrula stage (Minshull et al., 1994; Clute and Masui, 1995). However, if CSF components are introduced into cells of the embryo, the cell cycle arrests in metaphase of the next mitosis (Masui and Markert, 1971; for review see Tunquist and Maller, 2003). To determine whether Mad1 and Mad2 possess CSF activity, we injected recombinant forms of each protein into blastomeres of two-cell stage Xenopus embryos (Fig. 1), which divide every 26 min. The amount injected (50 ng) represents an ~10–20-fold increase over the endogenous level of each protein in one blastomere (unpublished data). Control blastomeres were injected with 50 ng of either BSA or Mos. Injection of one blastomere of a two-cell embryo with recombinant Mos resulted in the immediate arrest of cell division in metaphase as shown by DNA and spindle staining (Fig. 1 E), as reported previously (Sagata et al., 1989; Gross et al., 1999). In contrast, blastomeres injected with BSA (control) continued cell division (Fig. 1 E). Blastomeres injected with Mad1 arrested cell division with kinetics similar to that seen after Mos injection, and Mad1-arrested blastomeres also arrested in metaphase, as witnessed by DNA and spindle morphology (Fig. 1 E). For comparison, we injected blastomeres with Mad2, which has previously been reported to cause cleavage arrest (Fang et al., 1998). Injection of recombinant Mad2 protein resulted in a cell cycle arrest identical to that of Mad1- or Mos-injected blastomeres (Fig. 1 E). Therefore, even though the two-cell embryo has not yet developed a nocodazole-induced spindle checkpoint mechanism (Clute and Masui, 1995), modest overexpression of either the Mad1 or Mad2 spindle checkpoint protein is sufficient to cause metaphase arrest in the early embryo.

The ability of recombinant Mad2 to cause cell cycle arrest in blastomeres was originally reported to occur with tetramer (but not monomeric) forms of human Mad2 (Fang et al., 1998). However, subsequent structural and biochemical analyses suggested that both monomeric and oligomerized forms of Mad2 bind to Cdc20 and to Mad1 (Sironi et al., 2001, 2002). Moreover, both wild-type Mad2 and a point mutant unable to oligomerize (Mad2 R133A; Sironi et al., 2001) are able to activate the spindle checkpoint when co-
overexpressed with Mad1 (Sironi et al., 2001). To investigate whether oligomerization is required for CSF arrest in vivo, blastomeres were injected with equal amounts (50 ng) of wild-type \textit{Xenopus} Mad2 or \textit{Xenopus} Mad2 R133A. As shown in Fig. 1, the mutant impaired for oligomerization was unable to cause CSF arrest. This phenotype was not due to a rapid degradation of Mad2 R133A protein because extracts obtained from injected embryos contained equal levels of recombinant Mad2 proteins, as visualized by Western blotting (unpublished data). This is consistent with the report of Fang et al. (1998) that a biochemically purified monomeric form of Mad2 is unable to cause cleavage arrest in blastomeres.

\textbf{Mad1 and Mad2 are required for the establishment of CSF arrest by Mos in \textit{Xenopus} egg extracts}

In addition to the classical blastomere injection assay, CSF arrest can also be induced by addition of Mos to extracts prepared from eggs crushed 45 min after activation by the calcium ionophore A23187 (Bhatt and Ferrell, 1999; Tunquist et al., 2002). At this time, all endogenous Mos protein has been degraded (Watanabe et al., 1991), and all maternal \textit{mos} mRNA has been deadenylated (Ueno and Sagata, 2002). Such extracts can then undergo up to three rounds of DNA replication and mitosis in vitro with cycles of activation and inactivation of cyclin B/Cdc2 histone H1 kinase.
activity, due to the synthesis and degradation of cyclin B (Murray and Kirschner, 1989). However, if Mos or other elements involved in CSF arrest (such as Emi1 or cyclin E/Cdk2) are introduced, metaphase arrest occurs at the next mitosis (Bhatt and Ferrell, 1999; Reimann et al., 2001b; Tunquist et al., 2002). An advantage of the egg extract system is that it is amenable to depletion/reconstitution experiments—essentially a biochemical knockout approach that can define elements necessary or sufficient for CSF arrest.

To investigate the role of Mad2 in CSF arrest, extracts were immunodepleted of Mad2 and analyzed for arrest in response to Mos. Fig. 2 A shows the histone H1 kinase activity of a control extract that cycles after the addition of sperm nuclei and warming to 22°C. These extracts enter M phase at 15–30 min after warming, as evidenced by an increase in histone H1 kinase activity at least two- to threefold above the interphase level (compare Fig. 2 A with Fig. 3 A) and by condensed chromosomes (compare Fig. 2 F with Fig. 3 E). By 30–45 min, histone H1 kinase activity has returned to the basal level and nuclei are in interphase (Fig. 2, A and F). However, addition of Mos protein to these extracts while in interphase causes them to arrest in the next M phase with cyclin B/Cdc2 histone H1 kinase activity greater than threefold above the interphase level, and with condensed chromosomes arranged on metaphase spindles (compare Fig. 2, B and F with Fig. 3, B and E). Cell cycle arrest by Mos can be maintained in these extracts for 1 h. We generated rabbit pAbs against Mad2 that were capable of removing all endogenous Mad2 protein (Fig. 2 G). Immunodepletion of Mad2 prevented arrest at M phase by Mos, as evidenced by a decrease in

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**Figure 2.** **Mad2 is required for establishment of CSF arrest by Mos.** (A–E) Histone H1 kinase activity. Extracts in interphase at 4°C were treated with protein A Dynabeads with or without bound anti-Mad2 antibodies to immunodeplete endogenous Mad2 protein (G), and then were supplemented with 500 sperm nuclei/μl. (A) Mock-depleted extract supplemented with Mos injection buffer. (B) Mock-depleted extract supplemented with Mos protein (60 ng/μl). (C) Mad2-depleted extract supplemented with Mos protein. (D) Mad2-depleted extract supplemented with Mos and recombinant Mad2 protein (30 ng/μl). (E) Mad2-depleted extracts supplemented with Mos and recombinant Mad2 R133A protein (30 ng/μl). Cycling was initiated by warming to 22°C, and aliquots of the extracts were monitored for histone H1 kinase activity. The “M” bar in each panel refers to the length of M phase as determined by DAPI staining (F). (F) Effect of Mad2 depletion on nuclear morphology in cycling extracts. The samples shown in A–E were monitored for progression of the cell cycle by DAPI staining and fluorescence microscopy at the indicated times. Nuclei in all samples entered mitosis by 15 min. Addition of Mos at 0 min caused a prolonged M phase in the presence, but not the absence, of Mad2; arrest by Mos could be restored by the addition of wild-type, but not R133A Mad2 protein. (G) Immunodepletion of Mad2. Affinity-purified rabbit pAb to Mad2 was bound to protein A Dynabeads and incubated with a cycling extract in interphase for 45 min at 4°C (lanes 3–5). Mock-depleted extract (lane 1 and lane 2) was generated by incubation of extract with beads bound with control IgG. The supernatants were collected and immunoblotted for Mad2.

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Histone H1 kinase activity by 45 min to less than twofold above the basal level, and by the formation of interphase nuclei after M phase (Fig. 2, C and F). This suggests that Mad2 is required downstream of Mos in the establishment of CSF arrest. Mad1 and Mad2 have previously been reported to form a complex in *Xenopus* egg extracts that can be coimmunoprecipitated (Chung and Chen, 2002). To rule out the possibility that the attenuation of the Mos-mediated arrest by Mad2 immunodepletion is due to depletion of additional proteins, such as Mad1, we added back a physiological concentration of recombinant Mad2 protein to the Mad2-depleted extract and found that arrest by Mos could be restored (Fig. 2, D and F). This final reconstituted concentration was approximately fivefold above the endogenous concentration, and had no effect on cell cycle progression by itself (Fig. 4).

To investigate whether oligomerization of Mad2 is required to reconstitute Mos-dependent CSF arrest, we compared the ability of wild-type Mad2 and Mad2 R133A to support CSF arrest after depletion of endogenous Mad2. Only wild-type Mad2 reconstituted CSF arrest downstream of Mos upon addition to depleted extracts (compare Fig. 2, D and F with Fig. 2, E and F), suggesting that Mad2 oligomerization is required for CSF arrest in vivo and in vitro. Combined with the failure of Mad2 R133A to cause CSF ar-

Figure 3. **Mad1 is required for establishment of CSF arrest by Mos.** (A–D) Histone H1 kinase activity. Extracts in interphase at 4°C were treated with Dynabeads coupled to protein A with or without bound anti-Mad1 antibodies to immunodeplete endogenous Mad1 (F), then supplemented with 500 sperm nuclei/µl extract. (A) Mock-depleted extract supplemented with Mos injection buffer. (B) Mock-depleted extract supplemented with 60 ng/µl Mos protein. (C) Mad1-depleted extract supplemented with Mos protein. (D) Mad1-depleted extract supplemented with 60 ng/µl Mos and 10 ng/µl Mad1 protein. Cycling was initiated by warming to 22°C, and aliquots of the extracts were monitored for histone H1 kinase activity. The “M” bar in each panel refers to the length of M phase as determined by DAPI staining (E). (E) Effect of Mad1 depletion on nuclear morphology in cycling egg extracts. The samples shown in A–D were monitored for progression of the cell cycle by DAPI staining and fluorescence microscopy at the indicated times. Nuclei in all samples entered mitosis by 30 min, and extracts treated with control beads exited from M phase by 45 min. Addition of Mos at 0 time (45 min after CSF release) caused a prolonged M phase in the presence, but not the absence, of Mad1; arrest by Mos could be restored by the addition of recombinant Mad1 protein. (F) Immunodepletion of Mad1. Rabbit antibodies to Mad1 were bound to protein A Dynabeads and incubated with a cycling extract in interphase for 45 min at 4°C (lane 3 and lane 4). Mock-depleted extracts (lanes 1 and 2) were generated by incubation of extract with beads bound with control IgG. The supernatants were collected and immunoblotted for Mad1.
The finding that both Mad1 and Mad2 are required for the establishment of M phase arrest downstream of the MAPK pathway in cycling egg extracts (Fig. 2 and Fig. 3) strongly suggests that these proteins contribute to CSF activity arising in meiosis II in oocytes. To evaluate this possibility, antibodies were microinjected into immature oocytes, and maturation was initiated by the addition of progesterone. Controls were injected with antibodies raised against either protein were microinjected into oocytes. To evaluate this possibility, antibodies were microinjected into immature oocytes, and maturation was initiated by the addition of progesterone. Controls were injected with antibodies from the same rabbits lacking Mad1 or Mad2 immunoreactivity as described in the Materials and methods section.

Upon activation of the spindle assembly checkpoint, Mad1 and Mad2 have been suggested to form a tetrameric complex of equal stoichiometry that is critical for Mad2-mediated inhibition of the APC/C (Chung and Chen, 2002; Sironi et al., 2002). Thus, it was important to determine whether Mad1 is also required for the establishment of CSF arrest downstream of the Mos/MAPK pathway. Cycling extracts were either mock depleted or immunodepleted for Mad1 and assayed for the ability of Mos to cause arrest in M phase (Fig. 3). Similar to the Mad2 depletion experiment (Fig. 2), it was found that Mad1 immunodepletion abrogated Mos-mediated arrest (Fig. 3, C and E). Importantly, reconstitution of the extracts through the readdition of recombinant Mad1 to a physiological level restored the ability of Mos to arrest the extract in M phase (Fig. 3, D and E), indicating the block to CSF arrest was due to the absence of Mad1 rather than an associated protein. Mad1 addition by itself at this final concentration (approximately twofold above endogenous wild-type levels) did not cause arrest in M phase (Fig. 4). Previous experiments in cycling egg extracts have shown that overexpression of Mad2 by 20–100-fold causes a cell cycle arrest in M phase (Li et al., 1997; Chen et al., 1998; Reimann et al., 2001b). However, as shown in Fig. 4, overexpression of Mad1 or Mad2 approximately two- to fivefold, respectively, above the endogenous level had no effect on cell cycle progression (Fig. 4).

**Mad1 and Mad2 are required for the establishment of CSF arrest during Xenopus oocyte maturation**

The results presented thus far suggest that the activities of Mad1 and Mad2 are required for the establishment of CSF arrest downstream of the Mos/MAPK pathway. However, an important question concerns whether the Mad proteins are required for the maintenance of this arrest in the egg once it has been established. This laboratory and others have previously found that the activities of Cdk2/cyclin E, MEK1, MAPK, p90, and Bub1 are not required for the maintenance of CSF arrest (Bhatt and Ferrell, 1999; Chung and Chen, 2002; Sironi et al., 2002). As shown in Fig. 5 (A and C), the level of total histone H1 kinase activity from control and antibody-injected oocytes decreased transiently during the meiosis I to II transition, and this correlated with a transient reduction in cyclin B2 protein as judged by immunoblotting (Fig. 5, B and D). Cyclin B reaccumulation and elevated histone H1 kinase activity returned by 2 h after germinal vesicle breakdown and were maintained for at least 1 h during CSF arrest in the control oocytes. However, oocytes injected with antibody against Mad2 (Fig. 5, A and B) or Mad1 (Fig. 5, C and D) entered meiosis II normally, but failed to undergo CSF arrest, as witnessed by an immediate decline in histone H1 kinase activity and in cyclin B2 protein. The inability of these oocytes to establish CSF arrest provides compelling evidence that these proteins are required for the establishment of CSF arrest in vivo. Interestingly, the ability of these antibodies to inhibit the function of Mad1 and Mad2 in vivo suggests that they disrupt the interaction between these proteins and the APC/C or its regulators because cyclin B2 was not degraded after reaching the metaphase level.

**Mad1 (but not Mad2) is essential for the maintenance of CSF arrest once it has been established**

Rest in blastomeres (Fig. 1 F), this result indicates that Mad2 oligomerization is required for arrest downstream of Mos as well as for arrest due to Mad2 overexpression.

Figure 4. **Effect of Mad1 and Mad2 addition to cycling egg extracts.** Mad1 or Mad2 was added to a cycling extract at a final concentration of 10 ng/μl extract (Mad1) or 30 ng/μl extract (Mad2), and cell cycle progression was monitored by DAPI staining. Cell cycle progression was not altered by Mad1 or Mad2 relative to the control, whereas addition of 60 ng/μl Mos did cause M phase arrest. The total concentration of Mad1 and Mad2 in the extract was identical to that in the reconstitution experiments in Fig. 2 and Fig. 3.
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Sharp-Baker and Chen, 2001; Tunquist et al., 2002), although these proteins are important for its establishment (Sagata et al., 1989; Haccard et al., 1993; Kosako et al., 1994; Bhatt and Ferrell, 1999; Gross et al., 1999; Tunquist et al., 2002). On the other hand, Emi1, a protein capable of inhibiting the APC/C through the direct binding of Cdc20 in a non-checkpoint-dependent manner, is necessary for CSF maintenance (Reimann and Jackson, 2002). Given the identification of Mad1 and Mad2 as required elements for CSF activity, and the fact that these proteins function during the spindle assembly checkpoint through inhibition of the APC/C, it was important to investigate whether these proteins are required for CSF maintenance.

Both gain-of-function and loss-of-function approaches were undertaken to determine whether Mad1 and/or Mad2 are necessary for maintenance of CSF arrest once it has been established. These experiments use extracts prepared from CSF-arrested eggs in metaphase that enter anaphase after calcium addition due to activation of calcium/calmodulin-dependent protein kinase II (Lorca et al., 1991b, 1993). CSF release is monitored by a decrease in histone H1 kinase activity, as a consequence of cyclin B degradation (Murray, 1991; Bhatt and Ferrell, 1999; Tunquist et al., 2002), by loss of cyclin B immunostaining, and by an increase in electrophoretic mobility of Cdc20 indicative of dephosphorylation (Taieb et al., 2001). CSF-arrested control extracts supplemented with BSA exhibit a decrease in histone H1 kinase activity shortly after the addition of calcium ions (Fig. 6). It has previously been reported that an additional way to stimulate CSF release in these extracts is through addition of the phosphatase inhibitor microcystin (Lorca et al., 1991a). Control extracts that received 1 μM microcystin also exhibited a decline in histone H1 kinase activity similar to that seen with calcium addition. Interestingly, overexpression of either Mad1 or Mad2 proteins in these extracts to a level 20-fold greater than endogenous maintained CSF arrest in the presence of either calcium or microcystin (Fig. 6). Inhibition of CSF release by an even higher level of Mad2 has been reported previously (Chen et al., 1998; Reimann et al., 2001b), and overexpression of Mad2 protein by ~150-fold in MII oocytes was previously reported to prevent cyclin...
degradation and the metaphase II–anaphase II transition upon electrical activation (Peter et al., 2001). Thus, it appears that either Mad protein is sufficient to mediate inhibition of the APC/C and to maintain CSF arrest even in the presence of activating stimuli.

In the second type of experiment, we used immunodepletion techniques with CSF-arrested extracts. If either Mad1 or Mad2 protein is required to maintain CSF arrest, then the loss of either of these proteins should be sufficient for CSF release in the absence of calcium addition. Fig. 6 B shows a mock-immunodepleted CSF-arrested extract that maintains the M phase level of histone H1 kinase activity for 45 min. However, calcium addition 45 min after immunodepletion caused these extracts to subsequently exit CSF arrest with declining histone H1 kinase activity and loss of cyclin B2 immunostaining. This confirms that these extracts are viable and capable of CSF release upon exposure to calcium. Upon warming to 22°C, the extract immunodepleted of endogenous Mad1 protein (Fig. 6 C) underwent CSF release in the absence of calcium addition as judged by a loss of histone H1 kinase activity, degradation of cyclin B (Fig. 6 B), and an electrophoretic downshift of Cdc20 (Fig. 6 C) known to occur at the metaphase–anaphase transition (Taieb et al., 2001). This suggests that CSF arrest cannot be maintained in these extracts without Mad1 even in the presence of near endogenous levels of Mad2, although a small fraction of total Mad2 was coimmunoprecipitated with Mad1 (Fig. 6 C). To verify that CSF release and cyclin B degradation observed in Mad1-depleted extracts reflected only the loss of Mad1, GST-Mad1 protein was preincubated with Mad1-depleted extracts before warming; it rescued cyclin B2 stability and CSF arrest as judged by the level of histone H1 kinase activity in the extract (Fig. 6 D). In contrast, immunodepletion of Mad2 had no effect on CSF maintenance (Fig. 6 B), even though a small fraction of Mad1 was coimmunoprecipitated with Mad2 (Fig. 6 C). This result was reproducible, but unexpected because it suggests that Mad2, a direct binding partner for Cdc20, is not required to maintain APC/C inhibition, and raises the possibility that maintenance of CSF arrest by Mad1 may operate, at least in part, through a mechanism independent of Mad2. Lastly, CSF extracts immunodepleted for both Mad1 and Mad2 were unable to maintain CSF arrest, most probably due to the loss of Mad1 (Fig. 6 B).
Chung and Chen (2002) previously reported an association of Mad2 with Cdc20 in CSF-arrested extracts, and ablation of Cdc20 is known to block CSF release by calcium (Lorca et al., 1998; Taieb et al., 2001). To rule out the possibility that failure of the Mad2 immunodepletion to cause release from CSF arrest was due to the indirect depletion of bound Cdc20, we immunoblotted both the immunoprecipitates and supernatants for Cdc20 protein. Fig. 6 C shows an equal level of Cdc20 protein in all extracts, and no Cdc20 was detectable in the immunoprecipitates of either Mad1, Mad2, or Mad1 plus Mad2. This suggests that the antibodies raised against Mad1 and Mad2 disrupt any interaction between Mad1 and Cdc20, as well as between Mad2 and Cdc20. A similar disruption of Cdc20 binding appears to occur with Emi1 during immunoprecipitation (Reimann and Jackson, 2002). The failure of Mad2 immunodepletion to cause CSF release suggests that other Cdc20-interacting proteins present in the egg, such as Emi1 (Reimann et al., 2001), are (Reimann et al., 2001) or is not (Reimann et al., 2002). It is perhaps noteworthy that depending on the antibody, Emi1 (Reimann and Jackson, 2002), may be sufficient to bind Cdc20 and prevent release during depletion of Mad2 from the extract.

Discussion

A key difference in the prolonged metaphase arrest due to CSF versus that of the spindle assembly checkpoint concerns the meiotic spindle. In the egg, the metaphase arrest mediated by CSF activity occurs in the presence of a spindle that has kinetochores fully attached to microtubules. The idea that Mad1 or Mad2 would be involved in the establishment and maintenance of metaphase arrest during meiosis II was initially thought to be unlikely, given evidence that during the spindle checkpoint both Mad1 and Mad2 dissociate from kinetochores when binding to spindle microtubules occurs (Chen et al., 1996, 1998; Waters et al., 1998), and that the unattached kinetochore is currently hypothesized as the site of Mad2 activation for binding Cdc20 (Chen et al., 1998; Howell et al., 2000; Chung and Chen, 2002). On the other hand, the association of Mad1 with Mad2 is one of the best-characterized modes of inhibiting the APC/C to date. The binding of Mad2 to either Mad1 or Cdc20 is mutually exclusive, the crystal structure of Mad2 in complex with a Mad1 peptide is known, and it appears that Mad1 might convert Mad2 to a conformation more suitable for oligomerization and binding to Cdc20 (Fang et al., 1998; Sironi et al., 2001, 2002; Chung and Chen, 2002; Luo et al., 2002). Thus, it was interesting to analyze whether these proteins could perform this function in the meiotic egg, which lacks spindle assembly checkpoint regulation of the cell cycle (Minshull et al., 1994; Chen and Murray, 1997), and in which cell cycle arrest by CSF activity, at least in egg extracts, does not require DNA and thus kinetochores.

The MAPK pathway is clearly important in the establishment of CSF arrest in the egg upon entry into meiosis II by stabilizing cyclin B through inhibition of the APC/C (Gross et al., 2000). Somatic cells that arrest in metaphase through the activation of the spindle assembly checkpoint contain active MAPK on kinetochores (Shapiro et al., 1998; Zecœvic et al., 1998), and evidence suggests that MAPK is important for the establishment of the spindle assembly checkpoint in Xenopus egg extracts supplemented with large numbers of sperm nuclei and nocodazole, and in XTC cells treated with nocodazole (Minshull et al., 1994; Wang et al., 1997). However, the mechanism whereby MAPK contributes to this arrest is still largely unresolved. In the CSF-arrested egg undergoing meiosis, the link between MAPK and components of the spindle assembly checkpoint resides in the activation of Bub1 by the MAPK substrate p90Rsk (Schwab et al., 2001; Tunquist et al., 2002). Our finding that the spindle checkpoint proteins Mad1 and Mad2 are involved in the establishment of CSF arrest downstream of Mos (Figs. 1–3) suggests that Bub1 inhibits anaphase by regulating the same Mad proteins that are involved in the spindle assembly checkpoint.

However, the exact way in which the Mad proteins are used to inhibit the APC/C is likely to be different in CSF arrest from that in the spindle checkpoint. For example, Mad1 and Mad2 form a tight complex in cells undergoing mitosis (Chen et al., 1998; Jin et al., 1998), and the binding of Mad2 to Cdc20 is dependent upon the previous association and localization of Mad2 with Mad1 (Chen et al., 1998; Hwang et al., 1998; Sironi et al., 2002). This suggests that the only role for Mad1 during the establishment of the spindle assembly checkpoint is the mediation of Mad2 binding to Cdc20 in order to maintain APC/C inhibition. A key finding in this paper is that a point mutant of Mad2 unable to oligomerize (Mad2 R133A) is not able to support CSF arrest in vivo or in vitro, whereas the same mutant can support the spindle checkpoint in mammalian cells (Sironi et al., 2001). It is interesting that in mammalian cells, overexpression of either Mad1 or Mad2 alone is unable to cause metaphase arrest (Sironi et al., 2001), whereas either protein is sufficient for CSF arrest in blastomeres (Fig. 1) or egg extracts (Fig. 6; Li et al., 1997). These results suggest the form of Mad1 and Mad2 that interacts with the APC/C is different for CSF arrest than for the spindle checkpoint.

Chung and Chen (2002) previously reported the association of most Mad1 with Mad2 in CSF extracts as analyzed by gel filtration and coimmunoprecipitation. Their results suggest that most Mad1 exists in a stable complex with Mad2, whereas most Mad2 is not bound to Mad1. Our analysis (Fig. 6 C) confirms that Mad2 is coprecipitated with anti-Mad1 antibody, but in our hands only a small amount of Mad1 was associated with Mad2. The most likely explanation for the difference is that our anti-Mad2 antibody disrupts the interaction between Mad1 and Mad2. This is also suggested by the absence of Cdc20 in the Mad2 immunoprecipitates, as it has been reported that Mad2 and Cdc20 are associated in egg extracts (Chung and Chen, 2002). It is perhaps noteworthy that depending on the antibody, Emi1 (Reimann et al., 2001) or is not (Reimann and Jackson, 2002) able to be coimmunoprecipitated with Cdc20. In the latter case, depletion of Emi1 leads to CSF release in the absence of calcium addition, presumably due to activation of the APC/C by the Cdc20 released during the immunoprecipitation of Emi1. Previously, Chen et al. (1998) reported that depletion of Mad1 or Mad2 from a CSF extract did not affect CSF release induced by calcium addition, but they did not examine maintenance of CSF arrest after immunodepletion in the absence of calcium. These authors also studied the spindle checkpoint in egg extracts superimposed upon a CSF arrest. Inasmuch as both the
spindle checkpoint and CSF use both Mad1 and Mad2, we believe CSF arrest is best studied in the absence of nocodazole and with nuclear concentrations too low to activate the spindle checkpoint, and the spindle checkpoint is best studied in cycling extracts lacking CSF activity, as originally described by Minshull et al. (1994).

In addition to binding Mad2, we believe additional roles for Mad1 operate during CSF arrest that are clearly important for the maintenance of metaphase independent of Mad2 signaling because depletion of Mad1 (but not Mad2) was sufficient for CSF release. This suggests the role of Mad1 in CSF arrest may have distinct features in comparison with its well-characterized role during the spindle assembly checkpoint. Several recent reports concerning the spindle assembly checkpoint have provided biochemical evidence for a role of additional proteins other than Mad2 in the mediation of metaphase arrest. First, Jackson and colleagues have identified Emil1 as a potent inhibitor of Cdc20 in CSF extracts, and under some conditions Emil1 depletion causes CSF release (Reimann et al., 2001a; Reimann and Jackson, 2002). It would be of interest to compare the amount of Cdc20 bound to Emil1 versus Mad2 in a CSF extract. Second, Sudakin and colleagues identified a mitotic checkpoint complex from HeLa cells, composed of BubR1, Bub3, Mad2, and Cdc20, which was >3,000-fold more potent than recombinant Mad2 oligomer in inhibiting the APC/C (Sudakin et al., 2001). At the same time, Tang et al. (2001) independently obtained similar results with the purification of a mitotic checkpoint complex composed of only BubR1 and Bub3, suggesting that Mad2 may not be required for the extremely potent arrest of the APC/C seen by Sudakin et al. (2001). Interestingly, Chen (2002) recently reported that the localization of BubR1 at the kinetochore during the mitotic spindle assembly checkpoint in *Xenopus* extracts requires the activities of both Bub1 and Mad1, but not Mad2. This suggests an additional role for Mad1–BubR1 interaction independent of any association with Mad2. Further work is needed to assess the role of BubR1 in the establishment and maintenance of CSF arrest.

Lastly, a protein termed Mad2L2 (or Mad2B) that is homologous to Mad2 has been identified in vertebrate cells (Cahill et al., 1999; Chen and Fang, 2001). Mad2L2 has been implicated in the inhibition of the APC/C complex in somatic cells, and injection of Mad2L2 into developing *Xenopus* embryos results in cell cycle arrest after the mid-blastula transition, when Cdh1 is expressed and embryonic cells obtain gap phases (Pfleger et al., 2001). Mad2L2 also binds Cdc20 in *Xenopus* egg extracts and prevents cyclin B degradation (Chen and Fang, 2001). However, Mad2L2 does not bind Mad1, and thus appears to inhibit the APC/C through a Mad1-independent mechanism (Chen and Fang, 2001). The identification of a Mad2-related protein that binds Cdc20 and operates to inhibit the APC/C independently of any association with Mad1 or the kinetochore provides another candidate for inhibiting the APC/C during CSF arrest or after immunodepletion of Mad2.

It is evident that studying the requirements for maintenance of metaphase arrest by CSF or the spindle checkpoint may be a valuable way to sort out differences in control of anaphase onset. For example, MAPK activity is required for both the establishment and maintenance of the spindle assembly checkpoint in *Xenopus* egg extracts and tissue culture cells (Minshull et al., 1994; Wang et al., 1997). However, inactivation of MAPK by the drug UO126 prevents the establishment of CSF arrest during meiosis II (Schwab et al., 2001), but does not cause release from an established CSF arrest (Tunquist et al., 2002), suggesting that MAPK activity is not required for CSF maintenance. In addition, immunodepletion of Bub1 from *Xenopus* extracts abolishes the establishment and maintenance of the spindle assembly checkpoint (Sharp-Baker and Chen, 2001), whereas immunodepletion of Bub1 from extracts abolishes the establishment, but not maintenance, of CSF arrest (Sharp-Baker and Chen, 2001; Tunquist et al., 2002). However, immunodepletion of Bub1 from *Xenopus* egg extracts prevents Mad1 and Mad2 localization at the kinetochore during establishment of the spindle assembly checkpoint (Sharp-Baker and Chen, 2001), whereas we show here that only Mad1 is required for maintenance of CSF arrest, which does not require kinetochores. Thus, although both types of arrest occur in metaphase and are dependent upon many of the same protein components during their establishment, the maintenance of metaphase arrest by CSF appears to be biochemically distinct from the spindle assembly checkpoint.

### Materials and methods

#### Cloning and mutagenesis of Mad cDNA

The *Xenopus* homologue of Mad2 was amplified by PCR from a *Xenopus* oocyte library using the primers 5'-GACGAGCGACAAGATGGCGGGGGACGTAAACAC-3' and 5'-GAGGAGAAGCCCGGTGTGAAAAGTGTCTATC-3', and cDNA was subsequently cloned into the pET-10 vector using ligation-independent cloning technology (lIC; Novagen). This cDNA was identical to the *XMan2* cDNA previously deposited in the GenBank/EMBL/DDBJ database under accession no. U66167. Mad2 R133A was created by site-directed mutagenesis using the primers 5'-CGATGACTTATAGGCCGAGATTACTG-3' and 5'-CAGTGAGGCTTAATCTGCCGCTATAACTGATCG-3' according to the QuikChange® method (Stratagene). The *Xenopus* homologue of Mad1 was amplified by PCR from the *Xenopus* oocyte library using the primers 5'-GAGGAGAAGCCCGGTGTGAAAAGTGTCTATC-3' and 5'-GAGGAGAAGCCCGGTGTGAAAAGTGTCTATC-3', and the cDNA was subsequently cloned into the vector pET-41 using ligation-independent cloning technology (lIC; Novagen). This cDNA was not significantly different from the *XMad1* sequence deposited in the GenBank/EMBL/DDBJ database.

#### Expression and purification of Mad proteins

*Escherichia coli* (BL21 DE3 strain; Stratagene) were transformed with pET-30 Mad2 or Mad2 R133A and were induced (100 μM IPTG) to express His, fusion proteins overnight at 23°C. Bacteria were then resuspended in lysis buffer (25 mM Hepes, 150 mM NaCl, and 0.01% Brij-35, pH 7.4) and purified on glutathione-Agarose resin (Sigma-Aldrich) at 4°C. The expression and purification of Mad proteins from *E. coli* (BL21 DE3 strain; Stratagene) containing pET-41 Mad1 plasmid were induced to express GST-Mad1 protein overnight at 23°C with 100 μM IPTG before being resuspended in lysis buffer (25 mM Hepes, 150 mM NaCl, and 0.01% Brij-35, pH 7.4) and purified on glutathione-agarose resin (Sigma-Aldrich) at 4°C. GST-Mad1 protein was eluted from the resin with 100 mM GSH, 10 mM Tris pH 8.0. Proteins used for embryo injection or addition to extracts were dialyzed in Mos injection buffer (25 mM Hepes, 88 mM NaCl, 0.1% β-mercaptoethanol, and 0.01% Brij-35, pH 7.4). Fig. 1A–D presents an analysis of the recombinant Mad proteins by staining and Western blotting.

#### Immunization procedures and antibody production

His, fusion proteins of Mad1 or Mad2 were expressed in *E. coli*. The bacteria were resuspended in lysis buffer containing 8M urea (25 mM Hepes, 150 mM NaCl, and 0.01% Brij-35, pH 7.4), and the fusion proteins were...
purified on TALON™ resin (CLONTECH Laboratories, Inc.). Resin containing either purified Mad1 or Mad2 proteins was used to immunize rabbits. Immune sera directed against Mad2 were affinity purified on an Affi-Gel 10 resin (Bio-Rad Laboratories) covalently cross-linked to Mad2. Antibodies present in the Mad2 immune sera that consistently failed to bind Mad2 resin were used as a control for oocyte injections (see below). Antibody against α-tubulin was from Sigma-Aldrich, and antibody against Xenopus Cdc20 was a gift of T. Lorca (Centre Recherche de Biochimie Macromoléculaire, Montpellier, France; Lorca et al., 1998).

Microinjection of Xenopus oocytes

Stage VI oocytes were microinjected with 50 nl immune serum directed against Mad1, Mad1 preimmune serum, or 20 ng anti-Mad2 antibody, or with control IgG as described above. The oocytes were then treated with 1 μg/ml progesterone and monitored for the appearance of a white spot in the animal pole indicative of germinal vesicle breakdown. To obtain a highly synchronized population for biochemical analysis, oocytes were collected within 10 min of white spot formation, and samples were frozen every 20 min thereafter, for up to 3 h. Total histone H1 kinase activities and cyclin B2 immunoblotting were done as described previously (Tunquist et al., 2002).

Xenopus embryos and extracts

The eggs of X. laevis were fertilized in vitro as described previously (Haccard et al., 1993). For overexpression analyses, one blastomere of a two-cell embryo was injected with ~50 ng of BSA, GST-Mad1, His₃-Mad2, or GST-Mos proteins, and then cell division was monitored with a dissecting microscope (PCM 2000; Nikon). Confocal methods were performed essentially as described by Howell et al., 1993. For overexpression analyses, one blastomere of a two-cell embryo was injected with ~50 ng of BSA, GST-Mad1, His₃-Mad2, or GST-Mos proteins, and then cell division was monitored with a dissecting microscope (PCM 2000; Nikon). Confocal methods were performed essentially as described by Howell et al., 1993.

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