Vertebrate oocytes awaiting fertilization are arrested at metaphase of meiosis II by cytostatic factor (CSF). This arrest is due to inhibition of the anaphase-promoting complex/cyclosome, in part by a newly identified protein, Emi2 (xErp1). Emi2 is required for maintenance of CSF arrest in egg extracts, but its function in CSF establishment in oocytes and the normal embryonic cell cycle is unknown. Here we show that during oocyte maturation, Emi2 appears only after metaphase I, and its level peaks at CSF arrest (metaphase II). In M phase, Emi2 undergoes a phosphorylation-dependent electrophoretic shift. Microinjection of antisense oligonucleotides against Emi2 into stage VI oocytes blocks progression through meiosis II and the establishment of CSF arrest. Recombinant Emi2 rescues CSF arrest in these oocytes and also causes CSF arrest in egg extracts and in blastomeres of two-cell embryos. Fertilization triggers rapid, complete degradation of Emi2, but it is resynthesized in the first embryonic cell cycle to reach levels 5-fold lower than during CSF arrest. However, depletion of the protein from cycling egg extracts does not prevent mitotic cell cycle progression. Thus, Emi2 plays an essential role in meiotic but not mitotic cell cycles.

Fully grown, immature vertebrate oocytes progress from the G2/M border of meiosis I to a fertilizable gamete arrested at metaphase of meiosis II (M II). This process is called oocyte maturation, and in Xenopus, its molecular mechanism has been under study for over 30 years. Masui and Markert (1) microinjected cytoplasm from a mature oocyte (unfertilized egg) into embryonic blastomeres and found that injected blastomeres underwent cleavage arrest at the next mitosis; this was later shown to be cytologically equivalent to M II. They hypothesized that a specific factor from the cytoplasm was responsible for the inhibition of mitosis and cleavage and named this cytoplasmic activity “cytostatic factor” (CSF). This activity is presumed to be responsible for M II arrest in the mature oocyte, and the arrest of eggs at M II has been accordingly termed CSF arrest.

Recent studies have suggested that CSF arrest is due to the inhibition of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin-protein isopeptide ligase (E3) composed of at least nine subunits. It promotes the 26 S proteasome-dependent degradation of M phase regulators by ubiquitinating target proteins in cooperation with a ubiquitin-activating enzyme (E1) and a ubiquitin carrier protein (E2) (2–4). APC/C activity is evident only in M phase, and the inhibition of its activity through several pathways, including the Mos-MAPK pathway, the cyclin E/Cdk2 pathway, and the Emi2 pathway, seems to contribute to CSF arrest (5–9). These pathways can be studied independently of each other, but whether there is interaction between the pathways is currently unknown. Although the exact mechanism remains elusive, studies have shown that the inhibition of APC/C activity by the Mos-MAPK pathway during CSF arrest requires the spindle checkpoint proteins Bub1, Mad1, and Mad2 (reviewed in Refs. 5 and 6). Less is known about the mechanism of cyclin E/Cdk2-mediated APC/C inhibition during CSF arrest. Cyclin E/Cdk2 is present at a very low level in resting oocytes but increases dramatically in meiosis II (10). Inhibition of the APC/C by cyclin E/Cdk2 appears to involve the spindle checkpoint kinase Mps1, whose accumulation is promoted by cyclin E/Cdk2 (11). Inactivation of cyclin E/Cdk2 is required for release of CSF arrest, but in the absence of the Mos-MAPK pathway, active cyclin E/Cdk2 is unable to maintain M phase through the meiosis I–meiosis II transition. In vivo, antisense ablation of cyclin E does not block progression to M II, and thus, this complex may not be required for CSF arrest (11, 12).

The third known pathway of CSF arrest involves the early mitotic inhibitor (Emi) family of proteins. Emi1 is an F-box protein previously proposed as an APC/C inhibitor able to bind Cdc20 and inhibit the activity of the APC/C to cause a CSF-like arrest (13). Immunodepletion of Emi1 was reported to cause spontaneous CSF release (13–15), but later studies showed that Emi1 is not present in CSF-arrested cells at a level significant enough to regulate APC/C activity (16). Although Emi1 can cause cleavage arrest in embryos by inhibiting the APC/C, it stabilizes both cyclin A and cyclin B, whereas CSF arrest stabilizes only cyclin B (17–21). In addition, M phase arrest mediated by Emi1 is not affected by calcium, making it an unlikely APC/C regulator during CSF arrest. Antibodies to Emi1 used in

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**2** The abbreviations used are: MI, meiosis II; oligo, oligonucleotide; CSF, cytostatic factor; APC/C, anaphase-promoting complex/cyclosome; MAPK, mitogen-activated protein kinase; GVBD, germinal vesicle breakdown; PG, progesterone; GST, glutathione S-transferase; MI, metaphase of meiosis I.

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**4** B. Grimison and J. L. Maller, unpublished data.
Emi2 and Cell Cycle Control

Emi2 is a F-Box protein identified in a two-hybrid screen for interaction with the polo-like kinase Plx1 (23). Emi2 has been shown to be required to maintain CSF arrest once it has been established, and immunodepletion of this protein from CSF-arrested Xenopus egg extracts causes spontaneous CSF release (23); suppression of the mammalian homolog also leads to meiotic exit in maturing mouse oocytes (24). The maintenance of CSF arrest thus requires Emi2-mediated APC/C inhibition, and several laboratories have recently delineated a signal transduction pathway at fertilization that leads to the degradation of Emi2 and exit from M II arrest (7–9). It was found in CSF-arrested egg extracts that the addition of calcium, which simulates the transient increase in calcium level at fertilization, activates CaMK II, which then phosphorylates Emi2 at sites that promote its degradation by the SCF ubiquitination pathway.

These studies have unraveled the mechanism of calcium-dependent release of APC/C inhibition by Emi2, but how arrest is initially established remains unknown. Since Emi2 itself is necessary to inhibit the APC/C activity and maintain arrest, what role does it play in the establishment of CSF arrest? These roles might well be different based on the precedent with Mos, a MAPK/ERK (extracellular signal-regulated kinase) kinase kinase (MEKK) required to establish CSF arrest (5, 6) but not to maintain it (21). The traditional criteria for identification of a CSF component include that it should appear in M II, cause cleavage arrest when expressed in blastomeres, and be limited to regulation of meiosis versus mitosis. This study focused on Emi2 and found that it first appears in M II and is required for the meiosis I-II transition and CSF establishment but that the lower levels evident in early embryonic mitotic cell cycles are not required for cell cycle progression.

EXPERIMENTAL PROCEDURES

Constructs, Protein Purification, Antibodies, and Antisense Oligos—Xenopus Emi2 was cloned, expressed, and purified, and anti-Emi2 antibody was prepared as described previously (7). Mos antibody was from Santa Cruz Biotechnology, and cyclin A1, B1, and B2 antibodies were prepared as described previously (25). Secondary antibodies are from Jackson ImmunoResearch Laboratory (West Grove, PA). The Emi2 antisense oligo was made by Integrated DNA Technologies Inc.

Preparation and Manipulation of Xenopus Cycling Extracts, Sperm Nuclear Morphology, and Immunoblotting—Samples of 1 μl were taken at the indicated times and mixed with 4 μl of fixation/stain buffer (48% glycerol, 11% formaldehyde, 1× MMR (100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, and 5 mM HEPES (pH 7.5)), and 1 μg/ml 4′,6-diamidino-2-phenylindole), and sperm nuclei morphology was monitored by fluorescence microscopy. One microliter of sample at each time point was mixed with 20 μl of Laemmli sample buffer (Bio-Rad) and boiled for 5 min. For immunoblotting, 10 μl of each sample, equivalent to 0.5 μl of extract, was used. Proteins were detected with the antibodies described above and visualized by a SuperSignal West Dura detection kit (Pierce).

FIGURE 1. Analysis of Emi2 levels during Xenopus oocyte maturation and after fertilization. A, oocytes during maturation and after calcium ionophore addition were sampled at the indicated times after PG addition. Extracts were monitored by Western blotting of cyclins A1, B1, and B2. The level of Emi2 is shown in the bottom panel. Oocytes reached metaphase of meiosis I (MI) by 250 min, as indicated. B, embryos were obtained by in vitro fertilization, and subsequent cell divisions were monitored by Western blotting of cyclin B2 and Emi2 at the indicated times. UF, unfertilized eggs.
RESULTS

Accumulation of Emi2 in Meiotic and Mitotic Cell Cycles—In Xenopus metaphase egg extracts, Emi2 is rapidly degraded upon the addition of calcium, which triggers release of CSF arrest (7–9). Here we utilized Xenopus oocytes to study the expression of Emi2 before and after CSF arrest. G2-arrested Xenopus oocytes were cultured with PG to stimulate maturation. Once the oocytes reached M II (CSF arrest), the calcium ionophore A23187 was added to the medium to trigger activation and CSF release. As indicated morphologically by GVBD (data not shown) and the accumulation of cyclin B1 (Fig. 1A), the oocytes reached metaphase of meiosis I by 250 min and M II by 340 min after PG addition, with both cyclin B1 and cyclin B2, but not cyclin A1, present at a high level (Fig. 1A). The addition of calcium ionophore at 440 min triggered the oocytes to rapidly exit M II arrest, as evidenced by the degradation of cyclins, and all cyclins subsequently reaccumulated before the first mitosis of the activated eggs. An antibody was generated against an N-terminal fragment of Emi2, which has no sequence similarity with Emi1 and which did not recognize recombinant Emi1 (data not shown). Emi2 has been reported to be present at a low level in M I during oocyte maturation (23). However, in four independent experiments, we found that only a trace amount of Emi2 could be detected at early stages (in
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G2-arrested oocytes and before M I; significant accumulation started early in M II (340 min), and a maximal level was maintained until CSF release at 440 min (Fig. 1A). At M II, its electrophoretic mobility changes to a slower migrating, hyperphosphorylated form (Fig. 1). This result suggests an important role for Emi2 in CSF establishment. Upon the addition of calcium ionophore, Emi2 was rapidly degraded to an undetectable level, consistent with previous reports that Emi2 degradation is needed for anaphase and exit from CSF arrest (7–9). However, Western blotting showed that Emi2 was resynthesized within 40 min in the first mitotic cell cycle. It is evident that at mitosis of the first embryonic cell cycle (500 min), Emi2 accumulates to a level only about 20% of that at CSF arrest (M II). Consistent with previous reports (7, 8), Emi2 appeared in two forms: a faster migrating form into a slower, more highly phosphorylated mitotic form (Fig. 1). This result suggests an important role of Emi2 in embryonic cell cycles (Fig. 1B) is only about 20% of that in CSF-arrested eggs, as observed with ionophore-activated eggs in Fig. 1A.

Emi2 Is Not Required for Mitotic Cell Cycles—Since the maintenance of CSF arrest requires Emi2-mediated APC/C inhibition (7, 8) and since Emi2 is resynthesized prior to the next embryonic mitosis after degradation upon CSF release (Fig. 1), we hypothesized that Emi2 might function as a mitotic APC/C inhibitor to allow cyclin accumulation and might therefore also be required for mitotic entry in embryonic cell cycles. To investigate this possibility, we immunodepleted endogenous Emi2 from Xenopus cycling egg extracts prepared 45 min after egg activation by calcium ionophore. These extracts are initially in interphase, and upon warming, can carry out multiple rounds of DNA synthesis and mitosis in vitro as monitored by nuclear morphology and cyclin synthesis and degradation. As shown in Fig. 2A, Emi2 was undetectable after immunodepletion and was not resynthesized in subsequent mitotic cell cycles within the time frame of the experiment (up to 165 min) (Fig. 2C, lower panel). However, it was noted in other experiments that small amounts of Emi2 are sometimes resynthesized after 165 min (data not shown). Emi2-depleted extracts accumulated somewhat lower levels of cyclins A1 and B2, and mitosis was slightly delayed when compared with mock-depleted extracts, but the cyclin B level was still sufficient to drive three extract cell cycles, as indicated by periodic synthesis and degradation of cyclins and changes in nuclear morphology (Fig. 2, B and C). Moreover, the largely normal synthesis and degradation of cyclin A1 in Emi2-depleted extracts suggests that unlike Emi1, a homolog of the Rca1 gene controlling cycling A levels in Drosophila, Emi2 has no primary function in regulating cyclin A. Another surprising finding was that Emi2 is not degraded in each mitotic cell cycle. The protein level produced in the first mitotic cycle remained constant throughout subsequent cell cycles; only its electrophoretic mobility changed from a fast migrating interphase form into a slower, more highly phosphorylated mitotic form (Fig. 2C, lower panel). This result suggests that the mitotic cell cycle does not require Emi2-mediated APC/C inhibition to achieve mitotic cyclin accumulation, and Emi2 appears dispensable for cell cycle progression. In contrast, depletion of Emi2 from a CSF extract is sufficient to cause CSF release in the absence of calcium, as reported previously (Fig. 2, D and E) (23).

FIGURE 3. The level of Emi2 is critical for ability to inhibit the APC/C. Recombinant GST-Emi2 or GST alone were added to cycling egg extracts to a final concentration of 20 ng/μl. Sperm nuclear morphology (A) and cyclin blotting (B) show cell cycle progression through mitosis and interphase in controls and arrest by GST-Emi2. C, one blastomere of a two-cell Xenopus embryo was microinjected with 40 nl of buffer (buf) and equimolar amounts of GST, GST-Emi2, or GST-Mos. Blastomeres injected with either GST-Emi2 or Mos displayed cell cycle arrest, whereas the uninjected control side cleaved normally. The white bar represents 0.6 mm.
These results support the hypothesis that regulation of the level of Emi2 in meiosis II plays a key role in the appearance of CSF activity. We determined the amount of endogenous Emi2 in 1/H9262 l (equivalent to one egg) of either cycling or CSF extracts by comparison with serial dilutions of recombinant GST-Emi2 in Western blots. The concentration of Emi2 is about 2 and 10 ng/H9262 l in cycling and CSF extracts, respectively (data not shown). We therefore predicted that increasing the amount of Emi2 in cycling extracts should cause arrest in mitosis. To investigate this possibility, we systematically increased the amount of recombinant GST-Emi2 added to cycling egg extracts and found that at a final concentration of 20 ng/H9262 l, full-length recombinant Emi2 was able to cause metaphase arrest as judged by cyclin B levels and nuclear morphology (Fig. 3, A and B), whereas lower concentrations of full-length recombinant Emi2 (5, 10, and 15 ng/H9262 l) did not have any effect on cycling (data not shown). These data strongly support the hypothesis that the elevation of Emi2 in meiosis II is a critical factor for ability to inhibit APC/C activity and contribute to CSF arrest. This concept is also supported by the observation that microinjection of this level of recombinant Emi2 into one blastomere of a two-cell Xenopus embryo causes cleavage arrest similar to that seen with Mos (Fig. 3).

At M I, Mos has already accumulated to high levels, MAPK is fully activated (28), and the APC/C is partially inhibited by the Mos-MAPK pathway. This raises the question of whether continued APC/C inhibition by the Mos pathway is sufficient to support the M I-II transition and CSF arrest in the absence of Emi2. To examine this point, we microinjected antisense oligonucleotides against Emi2 into Xenopus oocytes at GVBD when neither cyclin E (10) nor Emi2 accumulation has begun (Fig. 1). As shown in Fig. 4, both buffer-injected and control oligo-injected oocytes showed normal morphology and normal cyclin B2 levels 4 h after the injection, whereas the antisense oligo-injected oocytes displayed a highly dispersed white spot, and all cyclin B had been degraded, indicating that the oocytes had exited meiotic M phase (28, 29). Blotting of Emi2 showed that in both buffer-injected and control oligo-injected oocytes, Emi2 accumulated normally, whereas in antisense oligo-injected oocytes, Emi2 was not detectable (Fig. 4A, middle panel). To verify that the antisense oligos affected only Emi2, antisense oligos and recombinant Emi2 were co-injected into the oocytes shortly after GVBD. The oocytes co-injected with GST-Emi2 maintained normal morphology (Fig. 4A) and exhibited slightly elevated M phase cyclin B2 levels, confirming that the effects of the antisense oligos on cyclin B levels were specifically due to the absence of Emi2. Although partial cyclin degradation at the M I-II transition was blocked by GST-Emi2, the oocytes still progressed into meiosis II as judged by cyclin E synthesis, which only begins in meiosis II (Fig. 4, A and B) (10, 30). These results clearly demonstrate a requirement for Emi2 for CSF arrest establishment in Xenopus oocytes. Similar results were obtained when the antisense oligos were injected before PG addition.

**DISCUSSION**

In the current model of CSF arrest, there are at least two important issues that remained to be addressed. First, in meiosis I oocytes, the Mos-MEK1-MAPK-Rsk-Mad/Bub spindle...
checkpoints, all known components of CSF activity, are present and active, yet metaphase arrest does not occur at this stage. Second, during the M I to II transition, the APC/C is able to degrade about 50–80% of the metaphase level of cyclin B in the absence of Emi2 and Mos-MAPK pathway, which is required for full, persistent APC/C inhibition to achieve the high cyclin B levels characteristic of CSF arrest. Previously, cyclin E/Cdk2 was thought to be a good candidate for this additional activity as its level increases dramatically prior to M II (10, 12) and constitutively active cyclin E/Cdk2 contributes to APC/C inhibitory activity, independent of the Mos/MAPK pathway, appears upon entry into M II and is required for full, efficient to establish arrest (11, 12). Therefore, another activity must be required for full APC/C inhibition, and based on our data, Emi2 appears to be a likely candidate for this activity. Like cyclin E/Cdk2, its level increases markedly in M II and remains high during the M II arrest (Fig. 1). Antisense oligos suppressed the synthesis of Emi2 and inhibited CSF establishment in oocytes (Fig. 4). Furthermore, injection of the Emi2 protein at GVBD blocked the effect of antisense oligos and also greatly reduced the partial degradation of cyclin B in the M I–II transition in the oocytes. Despite blocking partial APC/C activation after meiosis I, Emi2 antisense oligo-injected oocytes rescued by GST-Emi2 appear to progress into meiosis II as judged by synthesis of cyclin E, a specific marker of meiosis II (10). These results are consistent with studies in which APC/C inactivation at GVBD by antisense ablation of Cdc20 did not block progression to meiosis II (30, 31). The results in this study support the hypothesis that the Mos/MAPK pathway and the cyclin E/Cdk2 pathway, together with Emi2, all contribute to full APC/C inhibition during CSF arrest (Fig. 5). Experimentally, APC/C inhibition by each of these three pathways can be studied under conditions where only one of the pathways is active. However, possible interactions or cross-talk between these two APC/C inhibitory pathways remains an important area for future investigation. In contrast to its central role in meiosis II, Emi2 was not required for accumulation of sufficient cyclin B to drive entry into mitosis in egg extracts (Fig. 2). The unique nature of CSF arrest with up-regulation of Emi2 levels that are sensitive to calcium-induced degradation (7, 8) suggests that Emi2 is of key importance only in meiotic cell cycles.

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