A novel p34^cdc2^-binding and activating protein that is necessary and sufficient to trigger G2/M progression in _Xenopus_ oocytes

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The activation of maturation-promoting factor (MPF) is required for G2/M progression in eukaryotic cells. _Xenopus_ oocytes are arrested in G2 and are induced to enter M phase of meiosis by progesterone stimulation. This process is known as meiotic maturation and requires the translation of specific maternal mRNAs stored in the oocytes. We have used an expression cloning strategy to functionally identify proteins involved in G2/M progression in _Xenopus_ oocytes. Here we report the cloning of two novel cDNAs that when expressed in oocytes induce meiotic maturation efficiently. The two cDNAs encode proteins of 33 kD that are 88% identical and have no significant homologies to other sequences in databases. These proteins, which we refer to as p33^ringo^ (rapid inducer of G2/M progression in oocytes), induce very rapid MPF activation in cycloheximide-treated oocytes. Conversely, ablation of endogenous p33^ringo^ mRNAs using antisense oligonucleotides inhibits progesterone-induced maturation, suggesting that synthesis of p33^ringo^ is required for this process. We also show that p33^ringo^ binds to and activates the kinase activity of p34^cdc2^ but does not associate with p34^cdc2^/cyclin B complexes. Our results identify a novel p34^cdc2^ binding and activating protein that regulates the G2/M transition during oocyte maturation.

[Key Words: Cell cycle; meiotic maturation; MPF; M phase; oocyte; Cdc2]

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Entry of eukaryotic cells into M phase of the cell cycle is regulated by maturation-promoting factor (MPF), an activity composed of a B-type cyclin and the protein kinase p34^cdc2^. Cyclin B is usually synthesized and associates with p34^cdc2^ throughout late S phase and early G2, but the p34^cdc2^/cyclin B complex is maintained inactive by the phosphorylation of p34^cdc2^ on Thr-14 and Tyr-15. Dephosphorylation of p34^cdc2^ leads to activation of the MPF kinase activity, which can phosphorylate many proteins responsible for both the G2/M transition and progression through M phase (for review, see Nurse 1990, Coleman and Dunphy 1994, Morgan 1997).

_Xenopus_ oocytes are naturally arrested in late G2 and are induced to enter into M phase of meiosis by progesterone. This process is known as meiotic maturation and is associated with the activation of MPF [Masui and Clarke 1979]. In G2-arrested _Xenopus_ oocytes there is a preformed stock of p34^cdc2^/cyclin B complexes [pre-MPF] that is maintained inactive by the phosphorylation of p34^cdc2^ on Thr-14 and Tyr-15 [Cyert and Kirschner 1988; Gautier and Maller 1991; Kobayashi et al. 1991b]. The inhibitory phosphorylation of p34^cdc2^ in immature oocytes is likely to be due to the membrane-bound protein kinase Myt1 [Atherton-Fessler et al. 1994; Kornbluth et al. 1994; Mueller et al. 1995; Palmer et al. 1998], whereas the activating dephosphorylation probably involves the phosphatase Cdc25 [Dunphy and Kumagai 1991; Gautier et al. 1991; Kumagai and Dunphy 1991; Strausfeld et al. 1991]. Thus, either an increased activity of Cdc25 or the inhibition of Myt1 may bring about the activation of pre-MPF during oocyte maturation.

An essential requirement for progesterone-induced maturation is the translation of maternal mRNAs stored in the oocyte. Although several mRNAs are known to be translated de novo during oocyte maturation [Sagata et al. 1988, Kobayashi et al. 1991b; Gabrielli et al. 1992, Rempel et al. 1995, Murakami and Vande Woude 1998], only the mRNA encoding the protein kinase p39^mos^ has been found so far to be necessary for oocyte maturation [Sagata et al. 1988, 1989; Freeman et al. 1990, Sheets et al. 1995]. Injection of either p39^mos^ mRNA or recombinant p39^mos^ protein into _Xenopus_ oocytes induces maturation in the absence of progesterone [Sagata et al. 1988, 1989, Yew et al. 1992]. Conversely, injection of p39^mos^ antisense oligonucleotides blocks progesterone-induced MPF activation indicating that p39^mos^ is necessary for the initiation of maturation [Sagata et al. 1988].

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The function of p39mos in oocyte maturation is most likely related to its ability to activate the p42mpk1 MAP kinase pathway [Nebreda et al. 1993; Nebreda and Hunt 1993; Posada et al. 1993; Shibuya and Ruderman 1993]. This is supported by the injection of neutralizing anti-MAP kinase antibodies that inhibit p39mos-induced oocyte maturation [Kosako et al. 1994, 1996] and is also consistent with the observation that upon ectopic p39mos expression, activation of p42mpk1 always precedes the activation of pre-MPF [Nebreda and Hunt 1993; Posada et al. 1993; Shibuya and Ruderman 1993]. The idea that activation of p42mpk1 plays an important role in meiotic maturation is further supported by the delay in progesterone-induced maturation observed upon injection of either anti-MAP kinase antibodies [Kosako et al. 1994, 1996] or a specific MAP kinase phosphatase (Gotoh et al. 1995). Conversely, microinjection of constitutively active MAP kinase kinase [Huang et al. 1995] or active thiophosphorylated p42mpk1 [Haccard et al. 1995] can induce oocyte maturation in the absence of progesterone. Taken together, the results suggest that activation of the p42mpk1 cascade may be both necessary and sufficient to release the oocyte from the G2-phase arrest. A possible connection between the activation of p42mpk1 and MPF during oocyte maturation may be provided by the p42mpk1-activated protein kinase p90rsk, which can phosphorylate and down-regulate Myt1 [Palmer et al. 1998].

In spite of the central role of p39mos and the p42mpk1 pathway in oocyte maturation, there is evidence that synthesis of other proteins in addition to p39mos is also required for progesterone to initiate oocyte maturation [Nebreda et al. 1995; Barkoff et al. 1998]. Thus, in many batches of oocytes, the ability of p39mos or a constitutively active MAP kinase kinase mutant to activate pre-MPF is significantly reduced when protein synthesis is inhibited, whereas p42mpk1 is normally activated under these conditions [Yew et al. 1992; Daar et al. 1993; Nebreda and Hunt 1993; Shibuya and Ruderman 1993; Huang et al. 1995; Murakami and Vande Woude 1997]. These data indicate that p42mpk1 activation alone is not always sufficient to activate pre-MPF. In contrast, the ability of recombinant cyclin A to activate pre-MPF is the same when injected into either untreated or cycloheximide-treated oocytes [Nebreda et al. 1995]. Furthermore, overexpression of a dominant-negative p34cdc2 mutant or injection of a neutralizing anti-p34cdc2 antibody blocks progesterone-induced p39mos accumulation and the activation of both p42mpk1 and MPF [Nebreda et al. 1995]. These results were taken to suggest that activation of the free p34cdc2 present in the oocyte (which is in a notable excess over the p34cdc2 complexed with cyclin B) is normally required for progesterone-induced maturation. However, there is no evidence that newly synthesized A- and B-type cyclins, which would be the obvious candidates to activate free p34cdc2, are required for progesterone-induced MPF activation [Minshull et al. 1991].

In this paper we have used an expression cloning strategy to identify novel proteins that can trigger oocyte maturation. We have cloned two cDNAs that upon expression in oocytes [either as synthetic mRNAs or recombinant proteins], can potently induce MPF activation and germinal vesicle breakdown (GVBD) in the absence of progesterone. Moreover, antisense-directed ablation of the endogenous mRNAs in the oocyte inhibits progesterone-induced maturation. These cDNAs code for closely related proteins that can bind to and activate the protein kinase p34cdc2. Our results indicate that this novel p34cdc2 binding and activating protein plays an important role in oocyte maturation.

Results

Cloning of two novel cDNAs that potently induce G2/M progression in Xenopus oocytes

To identify novel proteins involved in G2/M progression during the meiotic maturation of Xenopus oocytes, we used an expression cloning strategy in which we constructed a Xenopus oocyte cDNA library in the FTX5 expression vector. The primary library was subdivided into pools of 150–200 colonies and plasmid DNA was purified from the pools and in vitro transcribed to obtain mRNAs. The mRNA pools that upon microinjection into oocytes were able either to induce oocyte maturation on their own or to accelerate progesterone-induced maturation were subdivided into smaller pools and injected until single positive clones were isolated. Using this approach, we isolated two clones named ls26 and ls27, which by DNA hybridization experiments did not correspond to proteins that are known to induce oocyte maturation including p39mos, Cdc25, and several A- and B-type cyclins [data not shown]. The mRNAs prepared from the ls26 and ls27 clones were able to induce oocyte maturation in the absence of progesterone stimulation, even at concentrations as low as 250 pg per oocyte (Fig. 1A, left), as well as to significantly accelerate progesterone-induced maturation at a 10-fold lower concentration (Fig. 1A, right). Oocyte maturation induced by expression of ls26 or ls27 was accompanied by the appearance of a white spot at the animal pole of the oocyte as in progesterone-matured oocytes [Fig. 2], although a few hours later ls26/ls27-injected oocytes usually appeared ‘overmatured’. This overmaturation is sometimes similarly observed upon injection of high concentrations of cyclin A or p39mos [not shown]. As in progesterone-treated oocytes, the maturation induced by ls26 or ls27 correlated with the activation of both p42mpk1 MAP kinase [p42mpk1 is phosphorylated resulting in an upward shift in immunoblots] and p34cdc2/cyclin B [pre-MPF, p34cdc2 is dephosphorylated resulting in a downward shift in immunoblots] as well as with the appearance of p39mos (Fig. 1B). We also found that ls26 and ls27 were able to induce oocyte maturation faster than p39mos, based on experiments where all mRNAs were prepared from the same expression vector and injected into oocytes at equivalent concentrations (Fig. 1C).

DNA sequencing of the clones isolated from the expression library showed that the ls26 and ls27 open read-
ing frames [ORFs] encoded related proteins that were fused in-frame to the carboxyl terminus of the myc tag in the FTX5 vector. Using the partial cDNAs as probes, we cloned full-length cDNAs from a λ ZAP Xenopus oocyte cDNA library. The ls26 cDNA was 1574 bp long and encoded a protein of 300 amino acids, whereas ls27 was 1357 bp in length and encoded a protein of 298 amino acids. Both clones contained stop codons upstream of the first ATG and in the same frame. The predicted ls26 and ls27 proteins were 88% identical [Fig. 3]. The cDNAs isolated from the oocyte expression screening encoded Myc-tagged proteins that either lacked the first 11 amino acids [ls26] or had an amino-terminal extension of 24 amino acids corresponding to the 5‘-untranslated region [ls27]. In a search of the ls26 and ls27 sequences using BLAST against DNA and protein sequence databases, we could detect no significant homologies, suggesting that ls26/ls27 belonged to a novel protein family. We were also unable to identify conserved protein motifs using PROSITE and GENEQUIZ programs. The only potentially relevant homologies were obtained from expressed sequence-tag [EST] databases. The best score in these searches was for the human EST clone 757814, which was 53% identical over a 49-amino-acid stretch. In contrast, no homologs were detected in the budding yeast genome. The close similarity between the sequences of ls26 and ls27 suggests that they might correspond to pseudoalloploid alleles, because they induced oocyte maturation with the same efficiency, we concentrated on ls26 for further characterization. We named this protein p33\textsuperscript{ringo} for rapid inducer of G\textsubscript{2}M progression in oocytes.

\textbf{Figure 2.} Morphological appearance of p33\textsuperscript{ringo} mRNA-injected oocytes (50 ng/oocyte) after 2.5 and 5 hr of incubation. Oocytes treated with progesterone (5 µg/ml) for 10 hr and control (water-injected) oocytes are shown.

\textbf{Figure 1.} Induction of Xenopus oocyte maturation by a novel protein. [A] The indicated concentrations (total amount per oocyte) of mRNA transcribed in vitro from the ls26 cDNA were injected into oocytes and GVBD was scored [left]. Progesterone was added to the groups of injected oocytes that showed no white spot after 18 hr of incubation [right]. Similar results were observed with mRNA prepared from the ls27 cDNA [not shown]. [B] Immunoblot analysis using anti-p42
\textsuperscript{mpk1}, anti-p34
\textsuperscript{cdc2}, and anti-p33
\textsuperscript{mos} antibodies of lysates prepared from oocytes [four oocytes per time point] either untreated [control], treated with progesterone (5 µg/ml), injected with in vitro-transcribed mRNA encoding for p33\textsuperscript{ringo} [ls26, 50 ng/oocyte] or both injected with mRNA encoding for p33\textsuperscript{ringo} and treated with progesterone. In this experiment 50% GVBD was observed at 10, 3.5, and 1.5 hr with progesterone, p33\textsuperscript{ringo}, and p33\textsuperscript{mos} + progesterone, respectively. [C] Oocytes were either injected with in vitro-transcribed mRNAs [500 pg/oocyte] encoding Myc-tagged forms of the two isoforms of p33\textsuperscript{ringo} [ls26 and ls27] and p33\textsuperscript{mos} or treated with progesterone (5 µg/ml) and then GVBD was scored.

Recombinant p33\textsuperscript{ringo} can trigger GVBD and MPF activation in cycloheximide-treated oocytes

To further characterize the ability of p33\textsuperscript{ringo} to induce oocyte maturation, we prepared a maltose-binding protein [malE]–p33\textsuperscript{ringo} fusion protein. We found that the injection of 40 ng of bacterially produced malE–p33\textsuperscript{ringo} was able to induce oocyte maturation considerably faster than progesterone treatment; 50% GVBD usually took ~2.5 hr with malE–p33\textsuperscript{ringo} versus 5–11 hr with progesterone. Moreover, injection of only 10 ng of malE–p33\textsuperscript{ringo} per oocyte was still able to induce maturation in 100% of the injected oocytes [see Fig. 6a, below]. We also
tested whether malE–p33ringo was able to induce oocyte maturation in the presence of protein synthesis inhibitors. Preincubation of the oocytes with cycloheximide (10 µg/ml) is known to block progesterone-induced maturation, consistent with the known essential requirement for de novo translation of maternal mRNAs. However, we found that cycloheximide had no effect on the ability of malE–p33ringo to induce MPF activation and GVBD (Fig. 4).

We investigated the kinetics of activation of p42mpk1 and p34cdc2/cyclin B in oocytes induced to mature by malE–p33ringo. Consistent with previous work, progesterone treatment activated both p42mpk1 and pre-MPF at the same time, whereas malE–p39mos injection activated p42mpk1 before pre-MPF (Fig. 4). In contrast, injection of malE–p33ringo rapidly activated histone H1 kinase (which correlated with Tyr dephosphorylation of p34cdc2) somewhat before myelin basic protein (MBP) kinase activity and disappearance of the band with reduced electrophoretic mobility in p34cdc2 immunoblots, was unaffected in the presence of cycloheximide (Fig. 4).

In these oocytes, however, the kinase activity on histone H1 was not stable and decreased after GVBD (Fig. 4) suggesting that p34cdc2/cyclin B might be transiently activated by p33ringo in cycloheximide-treated oocytes. On the other hand, oocytes injected with malE–p33ringo in the presence of cycloheximide had no detectable MBP kinase activity and most of their p42mpk1 was unphosphorylated, suggesting that activation of p42mpk1 was very much reduced [Fig. 4]. This observation suggests that the activation of p42mpk1 by p33ringo may be the consequence of positive feedback loops that are blocked by cycloheximide. Thus, the function of p33ringo is more likely to be related to the activation of p34cdc2 rather than of p42mpk1. We also found that as in the case of progesterone and p39mos, oocyte maturation induced by p33ringo involves de novo synthesis of cyclins B1 and B4 [Fig. 4].

Figure 4. MPF activation by recombinant malE–p33ringo in cycloheximide-treated oocytes. Oocytes were injected with malE–p33ringo [40 ng] or malE–p39mos [40 ng], or treated with progesterone (5 µg/ml) in the presence or absence of 10 µg/ml cycloheximide, as indicated. Cycloheximide was added 30 min prior to the injection or progesterone treatment and maintained during the subsequent incubation. At the indicated times, groups of five oocytes were collected, lysed, and analyzed both by in vitro kinase assay using as a substrate either histone H1 or MBP and by immunoblotting with anti-p42mpk1, anti-p34cdc2, and anti-cyclins B1 and B4 antibodies.

p33ringo is required for progesterone-induced oocyte maturation

To evaluate the importance of p33ringo for oocyte maturation we used antisense oligonucleotides. We first tested the oligonucleotides in an in vitro system and found that some antisense but not the control oligonucleotides could efficiently target the two isolated p33ringo clones (Fig. 5A, ls26 and ls27). The p33 ringo antisense oligonucleotides, however, did not trigger degradation of the synthetic p39mos mRNA under the same conditions. On the other hand, oocytes injected with malE–p33ringo in the presence of cycloheximide had no detectable MBP kinase activity and most of their p42mpk1 was unphosphorylated, suggesting that activation of p42mpk1 was very much reduced [Fig. 4]. This observation suggests that the activation of p42mpk1 by p33ringo may be the consequence of positive feedback loops that are blocked by cycloheximide. Thus, the function of p33ringo is more likely to be related to the activation of p34cdc2 rather than of p42mpk1. We also found that as in the case of progesterone and p39mos, oocyte maturation induced by p33ringo involves de novo synthesis of cyclins B1 and B4 [Fig. 4].
conditions [Fig. 5A, p39mos]. Next, we injected the p33ringo antisense oligonucleotides into oocytes and found that they were able to strongly delay progesterone-induced oocyte maturation, whereas injection of control oligonucleotides had no inhibitory effect [Fig. 5B]. By Northern blot we found that the p33ringo antisense oligonucleotides were able to ablate the endogenous p33ringo mRNA(s) but not the endogenous p39mos mRNA [Fig. 5C].

To test the specificity of the antisense oligonucleotides, we tried to rescue the inhibition of progesterone-induced GVBD by adding back small amounts of purified malE–p33ringo prior to progesterone stimulation. As shown in Figure 6A, the inhibitory effect of the p33ringo antisense oligonucleotide on progesterone-induced maturation, could be readily reversed by coinjection of only 2.5 ng of malE–p33ringo, an amount that was not enough to trigger oocyte maturation in the absence of progesterone. These results indicate that the p33ringo

**Figure 5.** Inhibition of progesterone-induced oocyte maturation by p33ringo antisense oligonucleotides. (A) In vitro-transcribed mRNAs encoding the two isoforms of p33ringo [ls26 and ls27] and p39mos were translated in rabbit reticulocyte lysates with [35S]methionine either alone (lane 1) or in the presence of p33ringo antisense oligonucleotides [lanes 2–8] or with control oligonucleotides [lane 9] and analyzed by SDS-PAGE and autoradiography. (B) Oocytes were injected with 100 ng of the indicated oligonucleotides and incubated for 6 hr prior to progesterone stimulation. GVBD was scored at the indicated times. We observed essentially the same results in four experiments using two different oligonucleotide preparations. (C) RNA was extracted from groups of 20 oocytes taken after 16 hr of incubation as in B and analyzed by Northern blot using as a probe the p33ringo cDNA (top). The same RNA blot was reprobed with the p39mos cDNA (bottom).

**Figure 6.** Requirement for endogenous p33ringo during progesterone-induced oocyte maturation. (A) Oocytes were injected with 100 ng of either antisense-6 or control-9 oligonucleotides (indicated by + and −, respectively) and incubated for 6 hr prior to the injection of the indicated amounts of malE–p33ringo and/or progesterone stimulation. After 8 hr, GVBD was scored and groups of five oocytes were taken, lysed, and analyzed by immunoblotting with anti-p34cdc2 and anti-p42mpk1 antibodies. (B) Oocytes were either noninjected or injected with 100 ng of the indicated oligonucleotides and incubated for 6 hr prior to progesterone stimulation. After 12 hr, lysates were prepared from groups of five oocytes and analyzed by immunoblotting using affinity-purified anti-p33ringo antibodies. In lanes 5 and 6, the antibodies were preincubated with malE–p33ringo protein before immunoblotting. The arrow indicates one band that cross-reacts with anti-p33ringo antibodies in mature oocytes. (C) Groups of 25 oocytes were injected with the indicated oligonucleotides and incubated with [35S]methionine in the presence or absence of progesterone. After 14 hr, oocytes were lysed and immunoprecipitated with either preimmune [lanes 1, 2] or anti-p33ringo antiserum [lanes 3–6]. The arrows indicate two proteins that are immunoprecipitated by anti-p33ringo antibodies in mature oocytes.
antisense oligonucleotides are likely to inhibit progesterone-induced oocyte maturation via the specific targeting of the endogenous p33\textsuperscript{ringo} mRNA(s).

We also investigated the endogenous p33\textsuperscript{ringo} protein in oocytes. By immunoblotting with affinity-purified anti-p33\textsuperscript{ringo} antibodies, we could detect one band of \(\sim 34\) kD in lysates prepared from progesterone-matured oocytes [Fig. 6B, lanes 2,4]. This band was not present when the anti-p33\textsuperscript{ringo} antibodies were preincubated with recombiant malE–p33\textsuperscript{ringo} protein [Fig. 6B, lane 6] and was neither detected when the oocytes were injected with p33\textsuperscript{ringo} antisense oligonucleotides prior to progesterone stimulation [Fig. 6B, lane 3]. This indicates that the 34-kD protein was recognized specifically by the anti-p33\textsuperscript{ringo} antibodies and that its appearance correlated with oocyte maturation.

Further evidence that p33\textsuperscript{ringo} was synthesized during oocyte maturation was obtained by immunoprecipitation with anti-p33\textsuperscript{ringo} antibodies of metabolically labeled oocytes. In these experiments we could detect two \[^{[35]}\text{S}\]methionine-labeled proteins of \(\sim 34\) and 37 kD in lysates prepared from progesterone-matured oocytes [Fig. 6C, lane 4]. These two proteins were immunoprecipitated neither from lysates of G₂-arrested oocytes [Fig. 6C, lane 3] nor from lysates of oocytes injected with p33\textsuperscript{ringo} antisense oligonucleotides prior to progesterone stimulation [Fig. 6C, lane 6], in agreement with the inhibitory effect of the oligonucleotides. These results indicate that the synthesis of p33\textsuperscript{ringo} is up-regulated during progesterone-induced oocyte maturation.

**Figure 7.** Interaction of p33\textsuperscript{ringo} with free p34\textsuperscript{cdc2} and cyclin B but not with p42\textsuperscript{mpk1}.

\[ \text{(A) Purified malE–p33\textsuperscript{ringo} (2, 0.2, and 0.02 µg)} \text{ or malE (2 µg) was prebound to amylose beads and incubated with p34\textsuperscript{cdc2}-expressing insect cell extracts (containing \(\sim 10\) µg of p34\textsuperscript{cdc2}, lanes 3–6), purified His–cyclin B (8 µg, lanes 11–14) or p34\textsuperscript{cdc2}-expressing extracts plus His–cyclin B (lanes 7–10). After extensive washing, the bead-associated proteins were analyzed by immunoblot with anti-p34\textsuperscript{cdc2} and anti-penta-His antibodies. Aliquots of the p34\textsuperscript{cdc2}-expressing extract (lane 1) and purified His–cyclin B (lane 2) before the pull-down were also analyzed in the same immunoblot. (B) \[^{[35]}\text{S}\]-Labeled p34\textsuperscript{cdc2}, cyclin B1, cyclin B2, or p42\textsuperscript{mpk1} prepared in rabbit reticulocyte lysates was incubated with 1 µg of malE or malE–p33\textsuperscript{ringo} bound to amylose beads. After extensive washing, the beads were analyzed by SDS-PAGE followed by autoradiography. (C) Lysates prepared from 20 control or progesterone-treated oocytes were incubated with malE or malE–p33\textsuperscript{ringo} prebound to amylose beads, extensively washed, and analyzed by SDS-PAGE and immunoblotting with anti-p34\textsuperscript{cdc2} and anti-p42\textsuperscript{mpk1} antibodies. Aliquots of the lysates before the pull-downs (corresponding to one oocyte) were analyzed in the same gel (extracts). \]

\(p33\textsuperscript{ringo}\) can associate independently with both p34\textsuperscript{cdc2} and cyclin B but not with p34\textsuperscript{cdc2}/cyclin B complexes.

The observation that p33\textsuperscript{ringo} induced GVBD and the activation of MPF independently of new protein synthesis indicated that it was likely to act rather late in the signaling pathways that lead to the activation of pre-MPF. We investigated the possibility that p33\textsuperscript{ringo} could directly associate with and/or modify the activity of p34\textsuperscript{cdc2}/cyclin B complexes. Initially, we used extracts prepared from insect cells infected with p34\textsuperscript{cdc2}-expressing baculovirus for pull-down experiments with malE–p33\textsuperscript{ringo} bound to amylose beads. In these experiments we could detect that p34\textsuperscript{cdc2} bound to malE–p33\textsuperscript{ringo} but not to malE alone [Fig. 7A, lanes 3–6]. Moreover, when soluble cyclin B was added to the p34\textsuperscript{cdc2}-containing extract prior to the amylose pull-down, we could still detect about the same amount of p34\textsuperscript{cdc2} bound to p33\textsuperscript{ringo} but no cyclin B [Fig. 7A, lanes 7–10]. This suggests that p33\textsuperscript{ringo} did not bind efficiently to the p34\textsuperscript{cdc2}/cyclin B complex and might preferentially bind to free p34\textsuperscript{cdc2}.

We also detected some binding of cyclin B to malE–p33\textsuperscript{ringo} in the absence of p34\textsuperscript{cdc2} [Fig. 7A, lanes 11–14]. In parallel experiments, we used Ni beads to directly recover the His-tagged cyclin B and found that when the cyclin B pull-down was done in the presence of a two-fold molar excess of soluble malE–p33\textsuperscript{ringo} there was a reduction in the amount of p34\textsuperscript{cdc2} bound to cyclin B (data not shown), suggesting that p33\textsuperscript{ringo} might compete with cyclin B for binding to p34\textsuperscript{cdc2}.

The interaction between p33\textsuperscript{ringo} and p34\textsuperscript{cdc2} was confirmed using \[^{[35]}\text{S}\]methionine-labeled proteins prepared...
in rabbit reticulocyte lysates for malE–p33ringo pull-down experiments [Fig. 7B]. We also observed in these experiments that p33ringo bound to cyclins B1 and B2 but did not bind to p42mpk1 significantly [Fig. 7B].

To extend these observations, we investigated whether p33ringo could also bind to the endogenous p34cdc2 in Xenopus oocytes. We found that p34cdc2 was present in malE–p33ringo pull-downs prepared from oocyte extracts [Fig. 7C, lanes 4, 6] but not in the malE pull-downs prepared in parallel from the same extracts [Fig. 7C, lanes 3, 5]. Moreover, it appeared that the majority of the p34cdc2 bound to p33ringo might be free p34cdc2 rather than p34cdc2 complexes with cyclin B, based on the electrophoretic mobility of the p34cdc2 isolated from immature oocytes [Fig. 7C]. The binding of p33ringo to free p34cdc2 was also supported by the lack of detection of cyclin B1 in the p33ringo pull-downs prepared from progesterone-matured oocytes (data not shown). Because endogenous B-type cyclins in the oocytes are likely to be mostly bound to p34cdc2 [Kobayashi et al. 1998b], this is consistent with p33ringo not being able to associate efficiently with p34cdc2/cyclin B complexes. In these experiments we confirmed that p33ringo did not bind to the endogenous p42mpk1 in oocyte extracts [Fig. 7C].

Activation of p34cdc2 by p33ringo in cell-free extracts and in vitro

To gain further information on the possible function of p33ringo in oocyte maturation, we used cell-free extracts prepared by high speed centrifugation of oocyte lysates [Karaikou et al. 1998b]. Addition of recombinant Cdc25 to these extracts can trigger the activation of p34cdc2/cyclin B complexes as indicated by the detection of H1 kinase activity and the disappearance of the slowly migrating p34cdc2 band in immunoblots [Fig. 8A, lanes 5, 6]. As expected, the activity of Cdc25 was blocked by vanadate [Fig. 8A, lanes 11, 12]. In contrast, p33ringo could induce the rapid appearance [5 min] of histone H1 kinase activity, which did not correlate with any change in the electrophoretic mobility of p34cdc2 in immunoblots [Fig. 8A, lanes 3, 4] and was not affected by the presence of vanadate [Fig. 8A, lanes 9, 10]. Interestingly, Cdc25 and p33ringo appeared to have an additive effect on the level of H1 kinase activity [Fig. 8A, lanes 7, 8]. Taken together, the results suggest that p33ringo can stimulate the H1 kinase activity of free p34cdc2.

To confirm the activation of free p34cdc2 by p33ringo, we investigated the ability of malE–p33ringo to stimulate the kinase activity of p34cdc2 in cell-free extracts prepared from baculovirus-infected insect cells. For this experiment, we added either cyclin B, malE–p33ringo, or malE alone to insect cell extracts containing p34cdc2 and then assayed their histone H1 kinase activity in vitro [Fig. 8B]. We found that both cyclin B and malE–p33ringo, but not malE alone, up-regulated the histone H1 kinase activity of the p34cdc2-expressing extracts [Fig. 8B, lanes 1–7]. In contrast, the increase in histone H1 kinase activity was very much reduced when we used extracts containing a kinase-inactive p34cdc2 mutant (p34cdc2 K33R) instead of wild-type p34cdc2 [Fig. 8B, lanes 8–10]. Moreover, the histone H1 kinase activity triggered by either cyclin B or malE–p33ringo was recovered on p13suc1 beads [data not shown], suggesting further that it was due to the activation of p34cdc2. We also observed increased histone H1 kinase activity upon addition of malE–p33ringo to interphase Xenopus extracts prepared from activated eggs, where cyclins have been degraded and there are no preformed p34cdc2/cyclin complexes but only free p34cdc2 [not shown]. On the other hand, p33ringo did not modify the histone H1 kinase activity of purified p34cdc2/cyclin B complexes [data not shown].

We also investigated whether malE–p33ringo was able to directly activate bacterially produced GST–p34cdc2. Incubation of the two recombinant proteins together in a buffer containing Mg/ATP did not result in any measurable kinase activity on histone H1 (data not shown). However, addition of malE–p33ringo to GST–p34cdc2, which had been preincubated in interphase Xenopus extracts and then recovered on glutathione beads, resulted in a significant increase in histone H1 kinase activity [Fig. 8C]. These results suggest that p33ringo may be able to directly activate p34cdc2, although it is also possible that additional factors might have been recruited from the cell-free extract by the GST–p34cdc2.

A kinase-inactive p34cdc2 K33R mutant inhibits p33ringo-induced oocyte maturation

The above results suggested that p33ringo may trigger oocyte maturation by binding to and activating free p34cdc2. This possibility was supported by the observation that malE–p33ringo-induced maturation was inhibited by overexpression in oocytes of the kinase-inactive p34cdc2 K33R mutant [injection of synthetic mRNA results in about six times more p34cdc2 K33R than the endogenous p34cdc2 concentration] [Nebreda et al. 1995]. In contrast, overexpression of wild-type p34cdc2 did not affect oocyte maturation induced by injection of malE–p33ringo [Fig. 8D]. We also found that coinjection of a small amount of malE–p33ringo was able to rescue the inhibitory effect of p34cdc2 K33R on progesterone-induced maturation [Fig. 8D]. This suggests that titration of endogenous p33ringo may account for the previously reported inhibition of progesterone-induced oocyte maturation by p34cdc2 K33R [Nebreda et al. 1995].

Discussion

In this report we describe the cloning and characterization of p33ringo, a novel protein that can efficiently induce entry into M phase of meiosis in Xenopus oocytes. At low concentrations p33ringo does not trigger maturation alone but strongly accelerates progesterone-induced maturation, suggesting that the pathway stimulated by p33ringo is normally activated during oocyte maturation. The induction of bona fide oocyte maturation by p33ringo...
Figure 8. Activation of free p34\textsuperscript{cdc2} by p33\textsuperscript{ringo} and inhibition of p33\textsuperscript{ringo}-induced GVBD by kinase-inactive p34\textsuperscript{cdc2} K33R. (A) High-speed oocyte extracts (25 µl) were incubated with 1 µg of male\textsuperscript{E} (lanes 1–7) or p34\textsuperscript{cdc2} K33R (lanes 8–10) were incubated with male\textsuperscript{E} (75 µg/ml, lanes 1,8), male–p33\textsuperscript{ringo} (75 µg/ml, lanes 2,9), 7.5 µg/ml, lane 3, 0.75 µg/ml, lane 4) or His–cyclin B (50 µg/ml, lanes 5,10; 5 µg/ml, lane 6, 0.5 µg/ml, lane 7). After 20 min, histone H1 kinase activity in the total extracts was assayed. (C) Interphase Xenopus extracts either alone (lanes 1,3,5) or containing bacterially produced GST-p34\textsuperscript{cdc2} (1.5 µg, lanes 2,4,6) were incubated with glutathione–Sepharose beads. After centrifugation, the beads were washed extensively and incubated with 2 µg of either male\textsuperscript{E} (lanes 1,2), male–p33\textsuperscript{ringo} (lanes 3,4), or His–cyclin B (lanes 5,6) for 20 min at 22°C and then used for histone H1 kinase assays. (D) Oocytes were injected in vitro with transcripts encoding either wild-type p34\textsuperscript{cdc2} or p34\textsuperscript{cdc2} K33R [Nebreda et al. 1995] and incubated for 16 hr prior to the injection of the indicated amounts of malE–p33\textsuperscript{ringo} and/or progesterone stimulation. GVBD was scored after 14 hr.

is supported by the appearance of the characteristic white spot at the animal pole of oocytes and the detection of the same biochemical markers as in progesterone-matured oocytes, including the appearance of p39mos, cyclins B1 and B4, and the activation of both p42\textsuperscript{mpk1} and pre-MPF. We also observed that the oocytes injected with p33\textsuperscript{ringo} tend to overmature to various extents after longer incubations, depending on the oocyte batch. This has been observed similarly upon overexpression in oocytes of other proteins that can trigger maturation such as p39mos or cyclin A (A.R. Nebreda, unpubl.) and is probably due to some side effects generated by the high levels of ectopic protein.

The ability of recombinant p33\textsuperscript{ringo} to induce MPF activation and GVBD independently of protein synthesis indicates that it does not function by stimulating the translation of maternal mRNAs encoding p39mos or other triggering proteins [Barkoff et al. 1998]. Moreover, the observation that p42\textsuperscript{mpk1} activation by p33\textsuperscript{ringo} is strongly reduced by the presence of cycloheximide suggests that p33\textsuperscript{ringo} is likely to function closer to the activation of p34\textsuperscript{cdc2} than of p42\textsuperscript{mpk1}. Consistent with this idea, recombinant cyclin A or Cdc25 can also induce MPF activation and GVBD very potently, independently of protein synthesis but only activate p42\textsuperscript{mpk1} when protein synthesis is allowed [Nebreda et al. 1995]. In contrast, the ability of activators of the p42\textsuperscript{mpk1} pathway to induce MPF activation in cycloheximide-treated oocytes versus nontreated oocytes is often reduced (see introductory section). In spite of the potent activity of p33\textsuperscript{ringo} to induce MPF activation and GVBD in the presence of cycloheximide, the histone H1 kinase activity in these oocytes appears to be activated only transiently. This suggests that the oocytes enter into meiosis I but are unable to proceed through meiosis II and arrest at the meiotic interphase with low levels of MPF (and, hence, histone H1 kinase) activity. It therefore appears that synthesis of additional proteins is necessary for entry into meiosis II. Similar observations have been reported when p39mos protein is injected in the presence of cycloheximide [Mukakami and Vande Woude 1997].

The experiments using antisense oligonucleotides suggest that the endogenous p33\textsuperscript{ringo} mRNA is required for progesterone to induce oocyte maturation. Moreover, injection of small doses of recombinant malE–p33\textsuperscript{ringo}, which cannot trigger oocyte maturation in the absence of progesterone, can rescue the inhibitory effect of the antisense oligonucleotides indicating that they are unlikely to target other important mRNAs. These results, together with the observation that synthesis of p33\textsuperscript{ringo} is up-regulated upon progesterone stimulation, indicate that de novo synthesis of p33\textsuperscript{ringo} is likely to be required for progesterone-induced maturation. We have also observed that antisense-directed interference with either p33\textsuperscript{ringo} or p39mos mRNAs alone significantly delays progesterone-induced maturation but usually does not totally block the process (Fig. 5; data not shown). Thus, although synthesis of both p33\textsuperscript{ringo} and p39mos may be normally required for progesterone-induced maturation, it appears that synthesis of only one of them can still trigger oocyte maturation albeit with reduced efficiency.
How can p33\textsuperscript{ringo} trigger oocyte maturation? The amino acid sequence of p33\textsuperscript{ringo} is not very informative as it shows no significant homologies to other proteins in databases. An interesting clue, however, is the ability of p33\textsuperscript{ringo} to bind p34\textsuperscript{cdc2} [most likely free p34\textsuperscript{cdc2} rather than p34\textsuperscript{cdc2}/cyclinB complexes] and up-regulate p34\textsuperscript{cdc2} kinase activity. This is likely to be a significant property of p33\textsuperscript{ringo}, as male p33\textsuperscript{ringo} can activate GST–p34\textsuperscript{cdc2} in vitro and the overexpression of kinase-inactive p34\textsuperscript{cdc2} K33R inhibits p33\textsuperscript{ringo}-induced oocyte maturation. Moreover, both p33\textsuperscript{ringo} and cyclin B1 activate the histone H1 kinase activity of p34\textsuperscript{cdc2} to a similar extent in extracts prepared from baculovirus-infected insect cells. In interphase Xenopus extracts, however, cyclin B1 induces higher levels of histone H1 kinase activity than p33\textsuperscript{ringo} [data not shown]. The reason for this difference in behavior, depending on the source of the extract, remains to be investigated. Proteins that are known to associate with p34\textsuperscript{cdc2} include A- and B-type cyclins, which are regulatory and activating subunits of p34\textsuperscript{cdc2} (for review, see Kobayashi et al. 1991a; Jackman and Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscript{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscript{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscript{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscript{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscript{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}/cyclin complex (Pines 1997), and Suc1/Cks proteins, which may regulate substrate recognition by the p34\textsuperscripts{cdc2}...
were stimulated further with progesterone (5 µg/ml) and scored for acceleration of maturation. We analyzed 105 pools and found 4 that were able to trigger GVBD on their own, whereas 22 pools accelerated progesterone-induced maturation. All of the positive pools were analyzed by Southern blotting for the presence of known inducers of oocyte maturation including p39mos [Sagata et al. 1988], Cdc25 [Izumi et al. 1992; Kumagai and D’Lusky 1992] and cyclins A1, A2, B1, B2, B3, B4, and B5 (provided by T. Hunt and collaborators, ICRF, South Mimms, UK). Two positive pools that did not contain any of the above-mentioned cDNAs were subdivided into smaller pools in two rounds, first of 20 transformants and then single clones, from which plasmid DNA and in vitro-transcribed mRNAs were prepared and injected into oocytes as described above. Using this protocol we isolated two clones that encoded Myc-tagged proteins and were able to potently induce GVBD.

Isolation of full-length p33ringo cDNAs

Full-length cDNAs of the two clones isolated in the oocyte expression screening were obtained from a λ ZAP Xenopus oocyte cDNA library [provided by J. Shuttleworth, University of Birmingham, UK]. The phage library was transferred onto nylon membranes [Hybond N, Amersham] and probed independently with the two 32P-labeled cDNAs [Megaprime, Amersham] in Church buffer (0.5 M NaHPO4 at pH 7.2, 7% SDS, 1 mM EDTA, 10 grams/liter BSA) at 65°C for 16 hr. Several pBlueScript phagemids were isolated containing ORFs that overlapped with the cDNAs isolated from the expression library. We selected as full-length cDNAs those clones that extended farther at the 5’ end and contained upstream stop codons followed by an ATG in the same frame as the ORF. The accession numbers for the full-length cDNAs are AJ133499 [ls26] and AJ133500 [ls27]. The cDNAs isolated in the oocyte expression screening started at positions 246 [ls26] and 88 [ls27] of the corresponding full-length clones.

Oocyte maturation

Stage VI oocytes were sorted after collagenase B treatment [Boehringer Mannheim, 0.5 mg/ml, 30–60 min] and left at 18°C in mBarth for 2–16 hr before injection. For the experiment in Figure 4, frogs were injected with pregnant mare’s serum gonadotropin (100 IU, Intervet) 3 days prior to operation and the oocytes were manually sorted. Meiotic maturation was induced by incubation with 5 µg/ml progesterone (Sigma) or by injection with 50 nl of either synthetic mRNAs or purified malE–p33ringo protein and were affinity purified on a GST–p33ringo-containing resin [gift from M. Hunt]. The anti-p33ringo antibodies were affinity-purified antibodies provided by H. Hocheger and T. Hunt (ICRF, South Mimms, UK). The phage library was transferred onto nylon membranes (Hybond N, Amersham) and probed independently with the two 32P-labeled cDNAs (Megaprime, Amersham) in Church buffer (0.5 M NaHPO4 at pH 7.2, 7% SDS, 1 mM EDTA, 10 grams/liter BSA) at 65°C for 16 hr. Several pBlueScript phagemids were isolated containing ORFs that overlapped with the cDNAs isolated from the expression library. We selected as full-length cDNAs those clones that extended farther at the 5’ end and contained upstream stop codons followed by an ATG in the same frame as the ORF. The accession numbers for the full-length cDNAs are AJ133499 [ls26] and AJ133500 [ls27]. The cDNAs isolated in the oocyte expression screening started at positions 246 [ls26] and 88 [ls27] of the corresponding full-length clones.

Histone H1 and MBP kinase assays

The oocytes were lysed in 10 µl per oocyte of ice-cold H1K buffer [80 mM β-glycerophosphate [pH 7.5], 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 12 mM EDTA, 0.5 mM PMSF, and 10 µg/ml leupeptin] and left at 18°C for 20 min without further manipulation. The supernatant was transferred to a 1.5 ml Eppendorf tube and centrifuged at 14,000 rpm (Eppendorf centrifuge) for 10 min. The supernatant (oocyte lysate), equivalent to about one oocyte, was incubated for 15 min at 22°C with 3 µg of either histone H1 or MBP in 12 µl of H1K buffer supplemented with 2 µCi of [γ-32P]ATP (3000 Ci/mmol) and 50 µl cold ATP. The kinase reactions were analyzed by SDS-PAGE and autoradiography.

Immunoblotting and immunoprecipitation

Oocyte lysates were usually prepared in H1K buffer, as described above, except for the detection of endogenous p33ringo, where oocytes were lysed in 20 µl per oocyte of an ice-cold buffer containing 80 mM β-glycerophosphate [pH 7.6], 50 mM NaCl, 10 mM EDTA, 0.2 M sucrose, 2 mM PMSF, 1 µM microcystin, 50 mM calyculin A, 10 µg/ml pepstatin A, and a protease inhibitor cocktail [Complete, Boehringer Mannheim]. The lysate equivalent of about one oocyte per lane was separated by SDS-PAGE and transferred to nitrocellulose membranes [0.22 µm Protran, Schleicher & Schuell] using a semidyblotting apparatus [Hoefer]. The membrane was blocked for 30–60 min at room temperature in TTBS buffer [25 mM Tris–HCl at pH 8.0, 150 mM NaCl, 0.05% Tween 20] supplemented with 4% nonfat dry milk and probed with the corresponding antibody in TTBS supplemented with 1% milk, usually overnight at 4°C. The monoclonal antibody 3E1 [provided by J. Gannon and T. Hunt, ICRF, South Mimms, UK] was used to detect p34cdc2 [Nebreda et al. 1994], and the rabbit antiserum 3297.1 was used for the detection of p42mpk1 [Palmer et al. 1994]. For p39mos we used a purified rabbit antibody [C-237, Santa Cruz Biotechnology] and for His-tagged cyclin B a monoclonal anti-penta-His antibody [Qiagen]. Xenopus cyclins B1 and B4 were detected with affinity-purified antibodies provided by H. Hocheger and T. Hunt (ICRF, South Mimms, UK). The anti-p33ringo antibodies were prepared in rabbits against bacterially produced malE–p33ringo protein and were affinity purified on a GST–p33ringo-containing nitrocellulose strip as described [Harlow and Lane 1988]. In all cases, horseradish peroxidase-coupled secondary antibodies [Dakko] were used, and the binding was detected using the enhanced chemiluminescence detection system [Amersham]. For immunoprecipitation, groups of 25 oocytes were labeled in mBarth containing 1 mCi/ml of [35S]methionine for 14 hr. The oocytes were washed three times in mBarth and lysed in H1K buffer [10 µl/oocyte] supplemented with 2 µM microcystin, 2.5 mM benzamidine–HCl, 10 µg/ml each of aprotinin, leupep-
tin, and peptatin A, and the Complete protease inhibitor cocktail [Boehringer Mannheim]. The oocyte lysates were preclared with 25 µl of protein A-Sepharose beads for 2 hr at 4°C and then incubated with 3 µl of either preimmune serum or a rabbit antiserum prepared against the KLH-coupled peptides THDLHILQE and DGTALEWHHL corresponding to residues near the carboxyl terminus of p33ringo (ls26). After 90 min at 4°C, the lysates were mixed with 100 µl of a 10% solution of protein A-Sepharose beads in IP buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 20 mM NaF, 5 mM EDTA, 5 mM EGTA, 100 µM sodium–vanadate, 1% NP-40), supplemented with microcystin and protease inhibitors as indicated above, and incubated further overnight at 4°C. The beads were washed four times in IP buffer, transferred to a new tube, and boiled in SDS sample buffer for electrophoresis on the immunocomplexes, which were analyzed by SDS-PAGE and autoradiography.

**Antisense experiments**

We designed four antisense oligonucleotides based on sequences around the first ATG of either ls27 [1-TGCATATGCCTCA-TTGTAGAAAGG and 2-AACTAGTTGCCCCGGTTGTGCAT or ls26 (4-CTCTTCATCTGCCTCTTTA) TGCATATGCCTCA-TTGTAGAAAGG and 2-AACTAAGGTGGCCCGGGTTG-10]. The two control oligonucleotides were a randomized sequence [8-TAGAGAAGAT-ATGTCCATGCTTA] and the same sequence as antisense 4 but with four point mutations (9-ACTCTATATGAACATTGCTATGAGA and 10-GCTGCTCATGGTTCGCTCA). Oligonucleotides were prepared on an automatic DNA synthesizer using a special solid support carrying dimethoxytrityl (DMT)-protected 1,3-propanediol linked to amino-controlled pore glass through a standard succinyl linkage (Van Aerschot et al. 1993) and the appropriate protected nucleoside phosphoramidites. The 200 nmole scale synthesis cycle was used. Oligonucleotides were prepared on an automatic DNA synthesizer using a special solid support carrying dimethoxytrityl (DMT)-protected 1,3-propanediol linked to amino-controlled pore glass through a standard succinyl linkage (Van Aerschot et al. 1993) and the appropriate protected nucleoside phosphoramidites. The 200 nmole scale synthesis cycle was used. Oligonucleotide supports were treated with 1 ml of a concentrated ammonia solution (32%) at 55°C for 16 hr. The solid support was removed by filtration and the solution was concentrated to dryness. The residue was passed through a Dowex 50Wx4 Na+ column, Pharmacia-Upjohn), eluted with water, and concentrated further overnight at 4°C. The terms were washed four times in IP buffer, transferred to a new tube, and boiled in SDS sample buffer for electrophoresis on the immunocomplexes, which were analyzed by SDS-PAGE and autoradiography.

**Northern blots**

Total RNA was extracted from oocytes using the Triazol one-step extraction method (GIBCO-BRL) following the manufacturer's protocol. About 20 µg of RNA was separated on a 1.2% agarose gel (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, 2% formaldehyde), transferred to a nylon membrane (GeneScreen Plus, Nitem), and UV cross-linked. The probes were an 0.8-kb BamHI–BamHI DNA fragment that includes most of the ls26 p33ringo ORF and hybridizes to both the ls26 and ls27 cDNAs, and a 1-kb NcoI–XhoI DNA fragment including the p33ringo ORF. For the hybridization, the DNA probes were labeled with [32P]dCTP (3000 Ci/m mole) and incubated with the RNA blot in Church buffer at 55°C for 14 hr. After four washes for 10 min at 55°C (two in 2× SSC, 0.1% SDS, and two in 0.5× SSC, 0.1% SDS), the blot was subjected to autoradiography.

**Amylose pull-downs**

Extracts from Sf9 insect cells expressing p34cdc2 were prepared as described (Kumagai and Dunphy 1997). The extracts (usually 20 µl) were diluted 1:5 in HBS buffer (10 mM HEPES–KOH at pH 7.4, 150 mM NaCl, 1% BSA, 1 mM PMSE, 10 µg/ml aprotinin, 10 µg/ml leupeptin) and incubated for 20 min at room temperature with different concentrations of either malE or malE–p33ringo prebound to 10 µl of amylase beads. In some experiments, amylase pull-downs were performed in the presence of purified His-tagged cyclin B1 (Kumagai and Dunphy 1997) either alone or in combination with the p34cdc2-containing extracts. After centrifugation, the beads were washed four times in IP buffer and once in HBS buffer, and analyzed by immunoblotting.

For rabbit reticulocyte pull-downs, pET8c–p34cdc2 (Nebreda et al. 1995), pGEM1–cyclin B1 and pGEM1–cyclin B2 (provided by H. Hochegger and T. Hunt) and FFTS–p42mpk1 (Gavin and Nebreda 1999) were in vitro-transcribed and -translated in a coupled reticulocyte lysate system (TNT-T7, Promega) in the presence of [35S]methionine. Aliquots of the reticulocyte lysates (20 µl) were diluted 1:10 in IP buffer supplemented with 1% BSA, 1 mM PMSE, and 10 µg/ml of both aprotinin and leupeptin and incubated for 40 min at 20°C with 1 µg of malE or malE–p33ringo prebound to 5 µl of amylase beads. The beads were washed six times in ice-cold IP buffer and analyzed by SDS-PAGE and autoradiography.

For pull-downs with oocyte extracts, 200 µl of extract prepared using 2 µl of H1K buffer per oocyte was diluted 1:1 in IP buffer supplemented with 1% BSA and incubated with [35S]methionine. Aliquots of 5 µl were used for histone H1 kinase assay and immunoblotting with anti-p34cdc2 and anti-p42mpk1 antibodies.

**Activation of p34cdc2 by p33ringo in cell-free extracts and in vitro**

High speed oocyte extracts were obtained from immature oocytes using 2 µl per oocyte of H1K buffer supplemented with 10 mM DTT, an ATP-regenerating mixture, and 1 µg/ml cyclnexilize as described (Karaiskou et al. 1998). The extract (25 µl) was preincubated with water or 5 mM vanadate for 5 min prior to the addition of malE (1 µg), malE–p33ringo (0.6 µg), and or GST–Xcd25 (0.2 µg). Aliquots of 5 µl were used for histone H1 kinase assay and immunoblotting with anti-p34cdc2 antibodies. Extracts (5 µl) prepared from baculovirus-infected Sf9 cells expressing either wild-type p34cdc2 or kinase-inactive p34cdc2 K33R were diluted 1:5 in HBS buffer supplemented with 250 µM ATP and 10 mM MgCl2, incubated for 20 min at room temperature with various concentrations of malE, malE–p33ringo, or His–cyclin B and analyzed by histone H1 kinase assay. For p33ringo pull-downs, 100 µl of the extracts incubated with malE, malE–p33ringo, or His–cyclin B as above were mixed with 10 µl of p33ringo linked to agarose beads (Calbiochem) for 30 min at 4°C.
The beads were washed four times in IP buffer and once in HBS buffer, and then assayed for histone H1 kinase activity.

For the in vitro activation, 1.5 µg of bacterially expressed GST-p34\(^{cdc2}\)K33R, and cyclin B1; Tim Hunt for sharing his collection of Xenopus A- and B-type cyclins; Toby Gibson and Gert Vriend for critical reading the manuscript. M.B was supported by the Schering Foundation.

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References


