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A *cdc2*-related Kinase Oscillates in the Cell Cycle Independently of Cyclins G2/M and *cdc2**

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The Eg1 gene in *Xenopus laevis* is related in sequence to the *cdc2*$^+$ gene. We show here that the Eg1 gene product (cdk2) possesses histone H1 protein kinase activity and binds to PSTAIR antibodies as well as to Sepharose beads linked to the 13-kDa product of the *suc 1* gene (p13^{suc1}). Eg1 protein kinase is active only in an $M_r \approx 200{,}000$ complex with other proteins but is not associated with any of the three known *Xenopus* mitotic cyclins or with any newly synthesized protein in egg extracts that exhibit cell cycle oscillations *in vitro*. The protein kinase activity of Eg1 oscillates in the mitotic cell cycle, being high in M-phase and low in interphase. Hyperactivation of *cdc2* kinase by the addition of cyclin A has no effect on the activity or oscillatory behavior of Eg1. Inhibition of *cdc2* kinase activation by emetine or RNase treatment of oscillating extracts does not inhibit the activation of Eg1 but does block deactivation normally seen during exit from mitosis. These results indicate that Eg1 is regulated by a cell cycle clock independently of cyclin and *cdc2* kinase.

Recently there has been significant progress in understanding the control of the cell cycle restriction point governing the $G_2 \rightarrow M$ transition. Biochemically, an activity first described in amphibian oocytes (maturation-promoting factor, MPF)[1] sufficient to stimulate the transition was purified and found to consist of a complex between p34^{cdc2} kinase and B-type mitotic cyclins (1-8). Mitotic cyclins are proteins that accumulate continuously during interphase and that are then degraded near the metaphase/anaphase transition in mitosis. In activated *Xenopus* egg extracts which oscillate between M- and S-phase, the destruction of cyclin is required for exit from mitosis and leads to inactivation of *cdc2* kinase (9). Activation of *cdc2* kinase is abolished by the addition of RNase to interphase extracts, but subsequent readdition of sea urchin cyclin B mRNA is sufficient to stimulate another round of *cdc2* kinase activation and mitosis (10). Another round of kinase activation and inactivation can also be achieved in RNase-treated egg extracts by addition of cyclin protein (11, 12). Large amounts of cyclin A protein lead to a marked hyperactivation of *cdc2* kinase and an arrest at the metaphase point in the cell cycle. These results indicate that in these extracts cyclin is both necessary and sufficient to stimulate activation of *cdc2* kinase and mitosis.

Recently, a series of cDNAs was cloned by differential screening of mRNAs specifically adenylated and expressed in eggs but deadenylated and not expressed in *Xenopus* embryos (13). One of these, termed Eg1, encodes a protein of predicted $M_r = 34{,}000$ that migrates on sodium dodecyl sulfate-polyacrylamide gels with an apparent $M_r = 32{,}000$. It has many structural features similar to those of *cdc2* kinase. In terms of overall sequence similarity, Eg1 is 62% identical to *cdc2* kinase from *Schizosaccharomyces pombe* (14). This level of identity is similar to that between human and *S. pombe cdc2*. Since the human gene can function by complementation in *cdc2*-deficient yeast (15), this level of structural conservation is sufficient for functional interchangeability between vertebrates and yeast. Other features shared by Eg1 and *cdc2* kinase include the presence of a TY motif in the ATP binding site and a perfectly conserved 16-amino acid sequence containing the PSTAIR motif, which is characteristic of all known homologs of *cdc2* kinase. Based on these conserved structural features, it seemed likely that Eg1 was a *Xenopus* homolog of *cdc2*. Surprisingly, however, Eg1 DNA was reported to be unable to complement mutations in *S. pombe* in *cdc2*$^+$ or mutations in the functionally interchangeable gene *CDC28* in the budding yeast *Saccharomyces cerevisiae* (14). Therefore, we have investigated in more detail the activity of Eg1 in *Xenopus* eggs in relation to *cdc2* kinase. In this paper we show that Eg1 is a protein kinase appearing in meiosis II whose activity oscillates during the mitotic cell cycle in a manner that can be dissociated from the oscillation of *cdc2* kinase.

MATERIALS AND METHODS

Antibody Preparation and Immunodetection—Antiserum was raised in rabbits against a 16-residue synthetic peptide of the C terminus of the deduced Eg1 sequence (THPFFRDVSRPTPHLI-amide), coupled to thyroglobin (14). The antibody was affinity purified on a peptide-agarose column (5 mg of peptide/ml of Affi-Gel 202 (Bio-Rad) coupled using the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide). PSTAIR antiserum was raised in sheep against the synthetic PSTAIR peptide and affinity purified

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[1] The abbreviations used are: MPF, maturation-promoting factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; BSA, bovine serum albumin; MAP-2, microtubule-associated protein-2; Eg1, cell division kinase 2(cdk2); GVBD, germinal vesicle breakdown.

in an identical manner. For immunoblotting, proteins were resolved on 12.5% polyacrylamide gels (16) containing 10% glycerol, using a Bio-Rad minigel system, and transferred to nitrocellulose membranes as described previously (6). After transfer, the membranes were blocked with 10% skim milk powder in phosphate-buffered saline (Blotto) and then incubated overnight at 4 °C in Blotto with antibody, either an affinity-purified Eg1 antibody (1/200 dilution), a PSTAIR antibody (1/100 dilution), or an antibody raised against *S. pombe cdc2* (a gift from K. Gould and P. Nurse, University of Oxford; 1/500 dilution). Antibody binding was detected with an appropriate alkaline phosphatase-conjugated second-stage antibody (Jackson Immunochemicals). Where indicated, the Eg1 antibody was preincubated with immunogen peptide (1 μg of peptide/μg of antibody) to block antibody binding to the blotted antigen.

In immunoprecipitation experiments, oocyte or egg extracts were diluted with 150 μl of EB buffer (20 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM dithiothreitol, 80 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 50 mM NaF, 1 mM sodium vanadate, 0.2 mM ammonium molybdate, 30 mM *p*-nitrophenol phosphate), precleared with 50 μl of 50% Sepharose CL-4B suspension, mixed for 30 min by gentle rotation, and then centrifuged 5 min. The supernatant was transferred to fresh tubes and incubated with 5 μg of affinity-purified Eg1 antibody overnight at 4 °C, and the immune complexes were precipitated with 25 μl of 50% protein A-Sepharose suspension (Pharmacia LKB Biotechnology Inc.), after mixing by gentle rotation for 1 h at 4 °C. In some cases, the supernatant after immunoprecipitation was transferred to fresh tubes and mixed with 25 μl of 50% p13-Sepharose suspension for a further h. In certain experiments, immunoprecipitation of Eg1 was blocked by preincubation of the Eg1 antibody with immunogen peptide for 1 h at 2 μg of peptide/μg of antibody.

Precipitates to be assayed for H1 kinase were washed once with 20 mM Tris, pH 7.4, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, twice with same buffer containing 1 M NaCl, and twice more with kinase assay buffer. Control Sepharose CL-4B, protein A-Sepharose, and p13-Sepharose were incubated with 10 mg/ml BSA prior to use, to decrease nonspecific binding to the Sepharose beads.

Preparation of p13-Sepharose—$p13^{suc1}$ was purified from an overproducing strain of *Escherichia coli* by gel filtration on Sepharose CL-6B (Pharmacia) as described in Brizuela *et al.* (17). $p13^{suc1}$ was coupled to activated CH-Sepharose (Pharmacia) at a coupling density of 5 or 20 mg/ml Sepharose, in 0.1 M phosphate buffer, pH 6.5, 0.5 M NaCl, at 4 °C for up to 4 h. Unreacted groups were hydrolyzed with 0.1 M Tris, pH 8.5, and the resin washed as per the manufacturer's instructions. The 5 mg/ml beads were used routinely for H1 kinase assays whereas the 20 mg/ml beads were used to precipitate samples for immunoblotting. For immunoblotting experiments, a 0–34% ammonium sulfate fraction of a *Xenopus* egg extract was diluted with EB but not precleared prior to precipitation with p13-Sepharose.

Protein Kinase Assays—The H1 kinase activity of immunoprecipitates and p13-Sepharose precipitates was assayed by addition of a 25-μl reaction mixture containing 20 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 0.2 mg/ml BSA, 0.2 mM ATP, (3 cpm/fmol), 0.5 mg/ml histone H1 (prepared from calf thymus as described by Johns (18)). After incubation at 25 °C for 20 min, reactions were stopped by the addition of 25 μl of 2 × Laemmli sample buffer, boiled for 5 min, and run on 12.5% polyacrylamide gels (16) containing 10% glycerol. H1 bands were excised from the stained and dried gels and ^{32}P incorporation into H1 quantified by scintillation counting. Casein and MAP-2 phosphorylation were assayed using similar conditions, substituting for H1 either 1 mg/ml α-casein (Sigma) or 0.1 mg/ml purified MAP-2 (gift from T. Sturgill, University of Virginia). Lamin phosphorylation was performed using 100 μg/ml purified rat liver lamins A and C (gift from J. Glass and L. Gerace, Scripps Institute) in 20 mM Tris, pH 8.0, 15 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 200 mM KCl. Gels were autoradiographed prior to excision of the appropriate band.

Preparation of Oocytes and Egg Extracts—Unfertilized eggs were obtained from female *Xenopus laevis* frogs (*Xenopus* I, Ann Arbor, Michigan) primed with 75 units of pregnant mare's serum gonadotropin 3 days prior to the experiment and induced to ovulate by the injection of 550 units of human chorionic gonadotropin 14–16 h prior to the experiment. High speed supernatant and ammonium sulfate fractions were prepared from the egg crushate as described in Lohka *et al.* (1). Oscillating Murray extracts were prepared from unfertilized eggs as described in Roy *et al.* (12). Aliquots (5 μl) were removed at times indicated in the figure legends and then stored at −70 °C until immunoprecipitations were performed. *Spodoptera frugiperda* (Sf9) cell lysates containing baculovirus-expressed clam cyclin A were prepared as described in Roy *et al.* (12). For oocyte maturation experiments, frogs were primed with 35 IU of pregnant mare's serum gonadotropin 3 days prior to removal of the ovary, and stage VI oocytes were manually dissected out of their follicular envelopes. Oocyte maturation was induced by the addition of progesterone to 10 μM in medium OR-2 supplemented with 10 mM sodium bicarbonate, pH 7.8, 0.1 mg/ml BSA, and 1 mM sodium pyruvate. Samples of oocytes were removed at the indicated times, homogenized in EB (10 μl/oocyte), centrifuged for 5 min in a microcentrifuge, and the supernatants stored at −70 °C until further analysis.

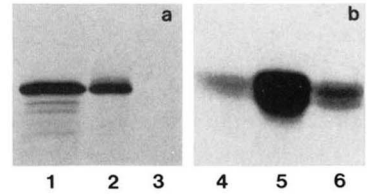

FIG. 1. **Immunoprecipitation of Eg1.** *a*, the 32-kDa, ^{35}S-labeled *in vitro* translation product from Eg1 mRNA (*lane 1*) was immunoprecipitated by Eg1 antibody, without (*lane 2*) or with (*lane 3*) preincubation of the antibody with immunogen peptide. *b*, an aliquot of a 0–34% ammonium sulfate fraction from unfertilized eggs (200 μg diluted to 100 μl with EB) was incubated with preimmune serum (*lane 4*), with Eg1 antibody (*lane 5*), or with antibody blocked with immunogen peptide (*lane 6*). The autoradiograph of H1 phosphorylated by the immunoprecipitates is shown.

In Vitro Transcription and Translation of Eg1—A 950-base pair fragment, corresponding to the complete open-reading frame of Eg1, was generated by polymerase chain reaction using Eg1 cloned in PBluescript KS (14) and two synthetic oligonucleotides for primers. The first oligonucleotide was homologous to the first ATG region of the gene containing an *Nco*I site, and the second oligonucleotide was homologous to the last 5 codons of the open reading frame and contained an *Xba*I site 3′ of the homologous sequence. This fragment was then subcloned into the pEPEX vector[2] between the *Nco*I and *Xba*I sites flanked, respectively, by the 5′ and 3′ regions of the *Xenopus* β-globin gene. The *Nco*I/*Kpn*I fragment generated by polymerase chain reaction was then replaced by the same fragment from the Bluescript vector, which reduced the actual part of the gene generated by polymerase chain reaction to 150 base pairs. *In vitro* transcription from the T7 promoter was performed on a linearized template using an *in vitro* transcription system from Stratagene. *In vitro* translation of the capped mRNA was done in reticulocyte lysates (Promega) according to the manufacturer's instructions.

RESULTS

Eg1 Is Not Detectable in Purified MPF—Much of the previous work identifying *cdc2* kinase in MPF from various species and in extracts of various cell types has relied on immunoprecipitation and immunoblotting of preparations with anti-PSTAIR antibody, based on the assumption this motif is present only in *cdc2* homologs. However, the presence of the PSTAIR sequence in both Eg1 and $cdc2^+$ suggests that such antibodies recognize both kinases. Therefore, to generate a reagent specific for Eg1, an antiserum was raised against a 16-residue synthetic peptide corresponding to residues 282–297 in the C-terminal region of the Eg1 protein, a region that displays no sequence similarity to $p34^{cdc2}$ from various species (14), although the sequence of *Xenopus* $p34^{cdc2}$ has not yet been reported. The affinity-purified Eg1 antibody immunoprecipitated the ^{35}S-labeled 32-kDa product of the *in vitro* translation of Eg1 mRNA (Fig. 1, *lanes 1–3*). Eg1 immunoprecipitates from the 0–34% ammonium sulfate fraction of unfertilized eggs contained a histone H1 protein kinase activity, which was reduced to background levels by preincubating the antibody with the immunogen peptide (Fig. 1, *lanes 4–6*). The kinase phosphorylated both serine and threonine residues

[2] J. Gautier, manuscript in preparation.

but not tyrosine in H1 (data not shown). In the same ammonium sulfate fraction Eg1 antibody recognized a 32-kDa polypeptide by immunoblotting but did not recognize any polypeptides in highly purified MPF (Fig. 2, *lanes 1–3*), and no H1 kinase activity was associated with Eg1 immunoprecipitates from purified MPF fractions (data not shown). The other ($M_r \approx 20{,}000$) band detected by the antibody in the 0–34% ammonium sulfate fraction and blocked by the immunogen peptide, was not consistently observed and was absent in partially purified fractions of Eg1.

Similar experiments were carried out with an antiserum raised against full-length bacterially expressed *S. pombe* $p34^{cdc2}$. This antiserum blotted a 34-kDa polypeptide in both the 0–34% ammonium sulfate fraction and in purified MPF preparations (Fig. 2, *lanes 4–6*). This band has been identified previously as *Xenopus* $p34^{cdc2}$ on the basis of immunoblotting and immunoprecipitation with anti-PSTAIR antibody (6). The antibody could, however, detect Eg1 in the concentrated ammonium sulfate fraction (*lane 4*), most likely because of the close sequence similarity between *cdc2* and Eg1. Therefore, purified *Xenopus* MPF contains *cdc2* kinase but not the Eg1 kinase (cdk2).

The H1 kinase activity associated with Eg1 accounted for only a small proportion of the total H1 kinase activity in the 0–34% ammonium sulfate fraction of unfertilized eggs, as immunodepletion of >80% of the Eg1 depleted only 5% of the H1 kinase activity (data not shown). The Eg1 protein is present at 5–10% of the abundance of $p34^{cdc2}$ in egg extracts as determined by densitometry of immmunoblots of egg extracts using the PSTAIR antibody, which recognizes the conserved PSTAIR epitope in both Eg1 and $p34^{cdc2}$ (Fig. 2, *lane 7*). Several proteins were tested as substrates for the immunoprecipitated Eg1 kinase. MPF phosphorylated purified rat liver lamins A and C, whereas these proteins were not substrates for Eg1 (Fig. 3, *lanes 1–3*). Glycogen synthase and tyrosine hydroxylase were not phosphorylated by either MPF or Eg1. A synthetic peptide based on the consensus recognition sequence in H1 for growth-associated (*cdc2*) kinase (KTPK), and an analog with the determinant proline substituted with glycine (19), were tested for their ability to compete

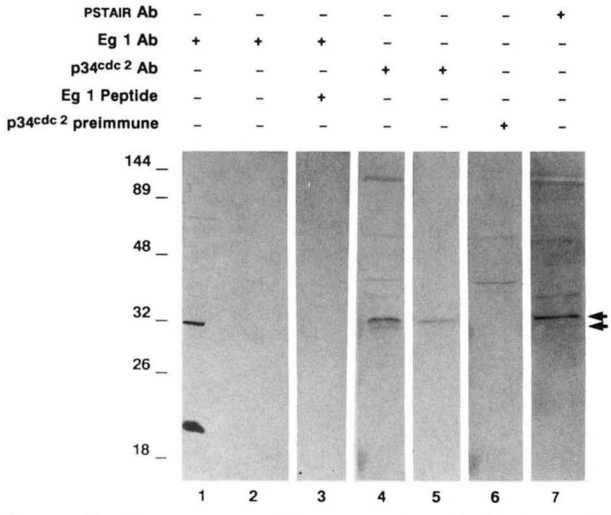

FIG. 2. **Eg1 is not present in purified MPF.** Either a 0–34% ammonium sulfate fraction from unfertilized egg extracts (100 μg; *lanes 1, 3, 4, 6,* and *7*) or highly purified MPF (100 ng; *lanes 2* and *5*) was subjected to Western blotting, as described under "Materials and Methods," with unblocked or peptide-blocked antibodies, as indicated. The *upper arrow* indicates the position of $p34^{cdc2}$ whereas the *lower arrow* indicates Eg1. No bands were detected in *lane 2* even with prolonged incubation in the color reagent. *Ab,* antibody.

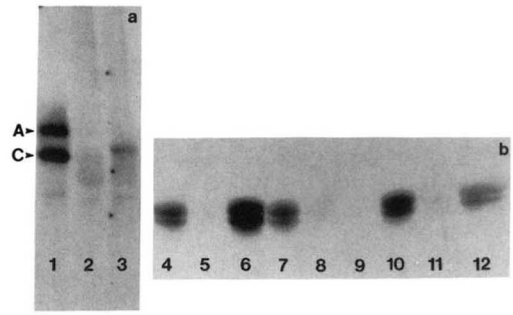

FIG. 3. **Eg1 kinase and MPF phosphorylate distinct substrates.** *Panel a,* purified rat liver lamins A and C (gift of L. Gerace, Scripps) were phosphorylated with purified MPF (*lane 1*) and Eg1 immunoprecipitates without peptide blocking (*lane 2*) or with peptide blocking (*lane 3*). The identity of the labeled band in the blocked Eg1 lane is unknown. Similar activities of Eg1 and MPF kinase activity toward H1 were used; the lamins (100 μg/ml) were phosphorylated at 1% of the rate of H1 (0.5 mg/ml) by MPF. *Panel b,* histone H1 was phosphorylated by Eg1 immunoprecipitates (*lanes 4, 7,* and *10*), peptide-blocked Eg1 immunoprecipitates (*lanes 5, 8,* and *11*), or purified MPF (*lanes 6, 9,* and *12*) in the absence (*lanes 4–6*) or presence of 100 μM H1 peptide (AKAKKTPKKAK, *lanes 7–9*) or glycine-substituted H1 peptide (AKAKKTGKKAK, *lanes 10–12*).

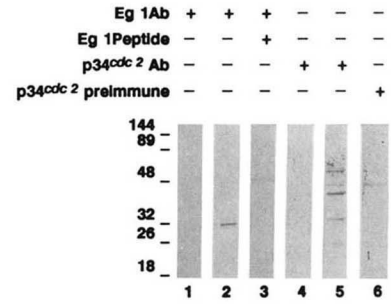

FIG. 4. **Eg1 specifically binds to $p13^{suc1}$.** Control Sepharose CL-4B (*lanes 1* and *4*) or p13-Sepharose (50 μl of 50% suspension; *lanes 2, 3, 5,* and *6*) was incubated with the 0–34% ammonium sulfate fraction of unfertilized egg extracts (350 μg diluted to 200 μl with EB) for 1 h, washed, and then eluted with 2 × Laemmli sample buffer. The blotted proteins were incubated with the antibodies as indicated under "Materials and Methods." *Ab,* antibody.

with H1 for phosphorylation by Eg1 and purified MPF. MPF H1 phosphorylation was efficiently competed with consensus peptide and much less efficiently by the glycine-substituted analog whereas neither peptide had any detectable effect on phosphorylation of H1 by Eg1 (Fig. 3).

Eg1 Binds to $p13^{suc1}$—The 13-kDa *suc 1* gene product from *S. pombe* has been shown to interact directly with $p34^{cdc2}$ from all species so far examined, and when covalently attached to Sepharose beads it has been used widely as a "highly specific" reagent for the precipitation of $p34^{cdc2}$ and its complexes. The high degree of sequence identity between Eg1 and $p34^{cdc2}$ prompted us to investigate whether $p13^{suc1}$ also binds the Eg1 protein kinase. On immunoblots comparing control and p13-Sepharose-bound proteins from the 0–34% ammonium sulfate fraction of unfertilized egg extracts, $p34^{cdc2}$ was detected only in eluates from p13-Sepharose (Fig. 4, *lanes 4–6*). The 32-kDa Eg1 band was also detected only in p13-Sepharose eluates, and recognition of the band was specifically blocked by preincubation of the antibody with immunogen peptide (*lanes 1–3*). Eg1 is thus the first kinase in addition to *cdc2* which can be demonstrated to bind specifically $p13^{suc1}$. Recently, a *Xenopus* homolog of *suc 1* has been cloned in our laboratory and also found to bind Eg1 with high affinity.[3]

[3] T. Izumi and B. Gabrielli, unpublished data.

Eg1 Exists as High and Low Molecular Weight Forms—High speed supernatant from unfertilized *Xenopus* egg extracts was fractionated on a Sephacryl S-200 column and assayed for H1 kinase activity in Eg1 immunoprecipitates and in p13-Sepharose precipitates of Eg1-depleted fractions. p13-Sepharose precipitated a broad band of H1 kinase activity, with the peak activity eluting with an M_r of 140,000 (Fig. 5*a*). In contrast, the H1 kinase specifically associated with Eg1 eluted as a peak of M_r 200,000. Immunoblotting of these fractions with antibodies to Eg1 and $p34^{cdc2}$ showed that the abundance of the $p34^{cdc2}$ band corresponded to the peak of p13-Sepharose-precipitated H1 kinase (Fig. 5*b*), and the abundance of the Eg1 polypeptide corresponded to the level of Eg1 H1 kinase activity (Fig. 5*c*). The casein kinase activity of p13-Sepharose precipitates followed the H1 kinase profile exactly (Fig. 5*d*). The antibodies also recognized a large pool of $p34^{cdc2}$ and Eg1 which eluted with an M_r of 20,000–50,000, most likely monomeric forms in which no H1 kinase activity was detected.

This result indicates that in *Xenopus* the monomeric forms of both Eg1 and $p34^{cdc2}$ are catalytically inactive. In contrast, in HeLa cells the monomeric form of $p34^{cdc2}$ has been reported to be an active casein kinase with a different substrate specificity compared with the high molecular weight complex (8, 20). This latter result may, however, reflect the nonspecific binding of a low molecular weight casein kinase to Sepharose beads, as we found that control Sepharose beads alone nonspecifically adsorbed a low level of casein kinase activity present in low molecular weight fractions. Sepharose beads not blocked with 10 mg/ml BSA prior to use precipitated high levels of both casein and H1 kinase activity from column fractions.

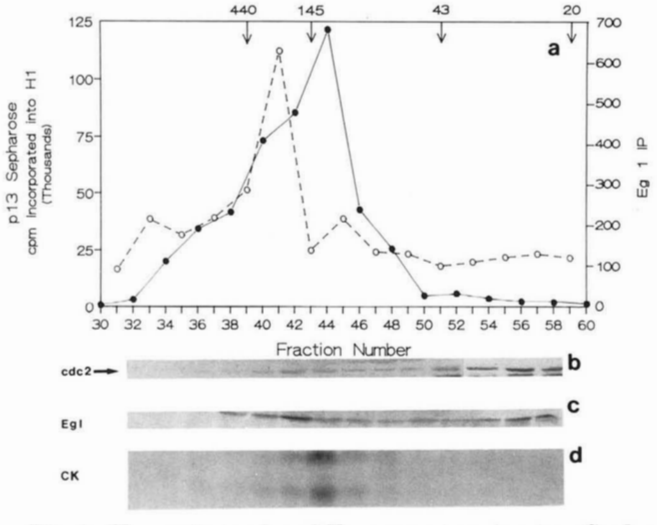

FIG. 5. **Chromatography of *Xenopus* egg extract on Sephacryl S-200.** High speed supernatant (7 mg of unfertilized egg extract protein) was applied to a Sephacryl S-200 column (70 × 1 cm) equilibrated in 20 mM Tris, pH 7.0, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 0.15 M NaCl, 1 mM CHAPS, and eluted at a flow rate of 15 ml/h. Fractions of 1.3 ml were collected. Aliquots (200 μl) of the indicated fractions were incubated first with control Sepharose CL-4B and then with either p13-Sepharose (*filled circles*) or Eg1 antibody (*open circles*) and assayed for H1 kinase activity (*panel a*). The p13-Sepharose precipitates were also assayed for casein kinase (*CK*) activity (*panel d*). The remainder of each fraction was precipitated with 20% trichloroacetic acid using 50 μg of BSA as a carrier. The washed precipitates were analyzed on 12.5% polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with either anti-*cdc2* antibody (*panel b*) or Eg1 antibody (*panel c*). Only the pertinent region of the blot is shown. The positions of elution of protein standards are indicated: ferritin, 440 kDa; alcohol dehydrogenase, 145 kDa; ovalbumin, 43 kDa; and cytochrome *c*, 20 kDa.

Synthesis and Activation of Eg1 during Meiotic Maturation of Oocytes—Since the mRNA for Eg1 becomes specifically polyadenylated and recruited onto polysomes during oocyte maturation (14), we initially examined extracts of maturing oocytes for the synthesis of Eg1 protein and activation of Eg1 protein kinase activity. The Eg1 protein is present at very low levels in resting oocytes and is synthesized during maturation until GVBD, where it accumulates to levels 4–5-fold greater than present in resting oocytes. Very little more accumulates during progression to meiosis II (Fig. 6, *inset*). The kinase activity of Eg1 did not increase significantly until after GVBD, as oocytes entered the meiosis II cell cycle, and this elevated activity was maintained during cytostatic factor arrest at metaphase of meiosis II (Fig. 6). Ablation of Eg1 mRNA using Eg1 antisense oligonucleotides resulted in the complete inhibition of Eg1 protein accumulation but had no effect on *cdc2* H1 kinase activation or white spot formation on entry into meiosis I (data not shown). These results demonstrate that Eg1 H1 kinase has no essential role in maturation to meiosis I. Likewise, microinjection of Eg1 mRNA (50 ng/oocyte), which resulted in the massive overexpression of Eg1 protein, also did not affect the rate of progesterone-stimulated oocyte maturation to meiosis I and did not affect the timing or magnitude of Eg1 H1 kinase activation (data not shown).

Eg1 H1 Kinase Activity Oscillates during the Embryonic Cell Cycle—Inasmuch as Eg1 and $p34^{cdc2}$ are similar in sequence, in possessing H1 kinase activity, in binding $p13^{suc1}$, and in forming high molecular weight complexes, it was important to evaluate whether Eg1, like *cdc2* kinase, underwent changes in activity during the cell cycle. The H1 kinase activity of Eg1 during the embryonic cell cycle was investigated by assaying Eg1 immunoprecipitates from egg extracts that oscillate spontaneously between mitosis and DNA synthesis (10, 12). Since Eg1 contributed less than 5% of H1 kinase activity present on p13 beads, the *cdc2* H1 kinase was assayed as the total p13-Sepharose-precipitated H1 kinase present after Eg1 immunoprecipitation (cf. Fig. 6). The Eg1 antibody was used in antibody excess, 5 μg of antibody/5 μl of extract, depleting >80% of the Eg1 protein, whereas the p13-Sepharose did not clear the *cdc2* H1 kinase from the extract. Therefore, the ratio of kinase activities precipitated by these two reagents did not reflect the actual ratio of activities in the extracts.

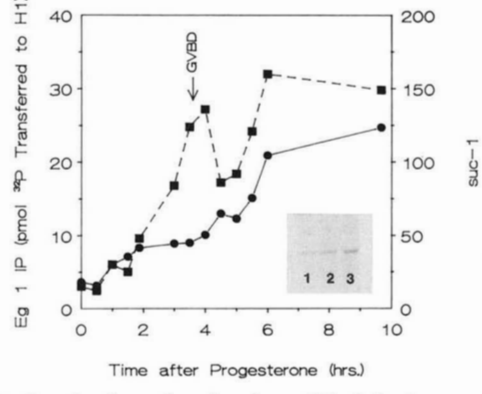

FIG. 6. **Synthesis and activation of Eg1 during oocyte maturation.** Stage VI oocytes were exposed to progesterone, and samples of 20 oocytes were removed at the indicated times and assayed for H1 kinase activity in Eg1 immunoprecipitates (*circles*) or in p13-Sepharose precipitates after depletion of Eg1 (*squares*). GVBD occurred at 3.5 h (*arrow*). *Inset*, oocyte samples were taken from resting (*lane 1*), GVBD (*lane 2*), and meiosis II (*lane 3*) oocytes and immunoblotted for Eg1 protein.

The Eg1 H1 kinase oscillated in a manner similar to that of *cdc2* kinase (Fig. 7) although the activity varied only 2–3-fold compared with 5–10-fold for *cdc2* kinase. Preincubation of the Eg1 antibody with immunogen peptide completely blocked precipitation of the H1 kinase activity (cf. Fig. 1*b*, *lane 6*). The oscillation of Eg1 H1 kinase was not caused by changes in the level of the Eg1 protein during the cycle, as immunoblotting of high speed supernatant from ionophore-activated eggs showed the abundance of Eg1 did not change through the first mitotic cycle, which was also the case for $p34^{cdc2}$ (data not shown).

Cyclins Are Not Associated with Eg1—The evidence that the Eg1 H1 kinase is in a high molecular weight complex and that its activity oscillates during the embryonic cell cycle raises the question of whether Eg1 associates with the mitotic cyclins, which are in part responsible for the oscillation of the *cdc2* kinase activity (10, 12). Recently Minshull *et al.* (21) used the Eg1 antibody described here to show that immunoprecipitates of *Xenopus* cyclins A, B1, and B2 from unfertilized eggs did not contain Eg1. PSTAIR antibody blotting of the same cyclin immunoprecipitates showed that only a 34-kDa band corresponding to $p34^{cdc2}$ was present, and no 32-kDa band could be detected. In the same experiments the PSTAIR antibody did recognize the product of *in vitro* translated Eg1, which corresponds to a minor 32-kDa band in crude egg extracts (see also Fig. 2, *lane 4*). Finally, in this laboratory, no ^{35}S-labeled bands were specifically immunoprecipitated by Eg1 antibodies from either oscillating extracts or [^{35}S]methionine-labeled maturing oocytes, even with exposure of gels for much longer periods than required to visualize cyclins bound to p13-Sepharose or newly synthesized Eg1 during progesterone-stimulated oocyte maturation (data not shown). The absence of a ^{35}S-labeled Eg1 band in egg extracts is consistent with the deadenylation of Eg1 mRNA reported to occur upon activation of eggs (14).

H1 Kinase Activity of Eg1 Oscillates Independently of $p34^{cdc2}$—Further evidence for the lack of direct interaction of cyclins with Eg1 comes from the finding that inhibiting protein synthesis in oscillating extracts, which inhibits cyclin accumulation and blocks progression of the cell cycle (10, 12), had no effect on activation of Eg1 kinase whereas activation of *cdc2* kinase was abolished. In emetine-treated oscillating extracts, the activation of Eg1 kinase paralleled controls, but the decrease in activity normally accompanying exit from mitosis was inhibited (Fig. 8*a*). In contrast, activation of *cdc2* kinase measured on p13-Sepharose beads was reduced to background levels in these emetine-treated extracts. Identical results were obtained with RNase-treated extracts (data not shown). Thus the decrease in Eg1 kinase activity during exit from mitosis may be dependent on a newly synthesized factor whereas the inactivation of *cdc2* kinase in similar extracts requires only the activated complex resulting from prior synthesis of cyclin (10, 12).

The addition of exogenous clam cyclin A protein to an oscillating Murray extract causes concentration-dependent hyperactivation of *cdc2* kinase and attenuates the reduction of H1 kinase activity and the destruction of cyclins upon exit from M-phase (Fig. 8*b* and Ref. 12). In contrast, the activity and period of oscillation of Eg1 H1 kinase were virtually unaltered by the addition of excess cyclin (Fig. 8*b*). Moreover, ^{35}S-labeled clam cyclin A could be precipitated from oscillating extracts with p13-Sepharose (12), indicating the direct association of the clam cyclin with $p34^{cdc2}$, whereas none was associated with Eg1 immunoprecipitates (data not shown). These results support the lack of association of Eg1 with cyclins G2/M in *Xenopus*. Perhaps more interestingly, these data show that the oscillation of Eg1 H1 kinase can be dissociated from the oscillation of *cdc2* kinase activity.

DISCUSSION

There are a number of previously identified protein kinases with some level of sequence similarity to $p34^{cdc2}$ (22–29). Most of these proteins have only 30–50% identity with $p34^{cdc2}$ from various sources, much lower than that evident with the Eg1 gene product and the closely related Dm cdc2c gene product from *Drosophila* (30). More specifically, no other kinase has previously been found to contain a complete PSTAIR sequence, which has been thought to be unique to *cdc2* homo-

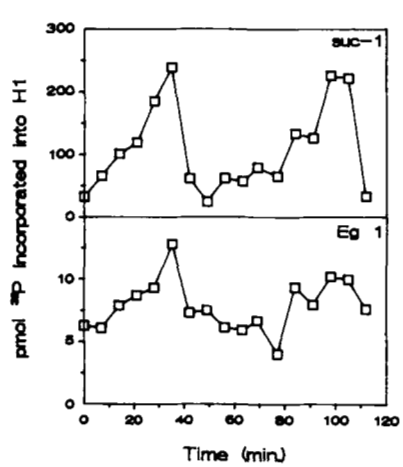

FIG. 7. **Eg1 protein kinase activity oscillates in the embryonic cell cycle.** Samples from oscillating Murray extracts of activated eggs prepared as described under "Materials and Methods" were assayed for the H1 kinase activity of Eg1 immunoprecipitates (*lower panel*) or of *cdc2* kinase in p13-Sepharose precipitates of the supernatant (*upper panel*) after immunoprecipitation. Control experiments showed that the kinase activity in the Eg1 immunoprecipitates was completely blocked by preincubation of the antibody with the immunogen peptide (data not shown).

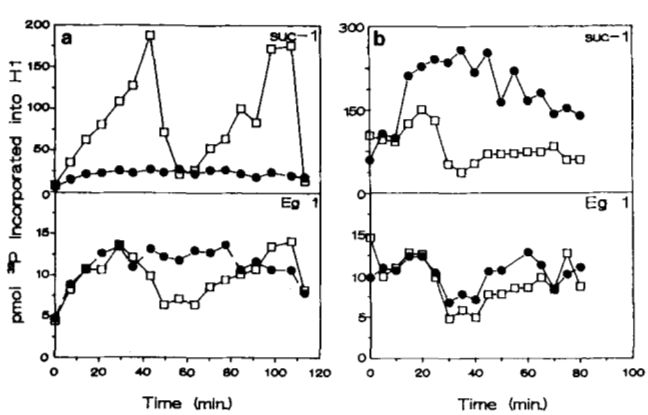

FIG. 8. **Dissociation of Eg1 and *cdc2* kinase oscillations.** *A*, protein synthesis inhibitors do not affect Eg1 kinase activation. Aliquots were removed from either untreated (*open squares*) or emetine-treated (*filled circles*) oscillating extracts at the indicated times, diluted, and precipitated first with Eg1 antibody (*lower panel*) and then with $p13^{suc1}$-Sepharose as a measure of *cdc2* kinase (*upper panel*). The precipitates were then assayed for H1 kinase activity as described under "Materials and Methods." *b*, hyperactivation of *cdc2* by mitotic cyclin A does not affect Eg1 kinase. Aliquots were removed from either control (*open squares*) extracts or extracts to which baculovirus-produced clam cyclin A (final concentration of 250 nM in the extract) was added (*filled circles*). Aliquots were analyzed as in *panel a*. Other controls utilizing addition of Sf9 lysates of cells infected with wild-type baculovirus did not activate $p34^{cdc2}$ (12) and had no effect on Eg1 activity (not shown).

logs, although PHO85 has identity in 14 of the 16 residues in the PSTAIR sequence (24). The function of this conserved sequence is unknown although microinjection of the synthetic PSTAIR peptide increases the rate of progesterone-induced oocyte maturation in *Xenopus* (6) and induces maturation and a Ca^{2+} flux in starfish oocytes (3, 31).

The 13-kDa product of the *suc 1* gene of *S. pombe*, designated $p13^{suc1}$, has been shown to associate directly with the *cdc2* kinase complex from a variety of sources and has also been used widely to prepare "specific" affinity resins for $p34^{cdc2}$. In genetic experiments, the *suc 1* gene has been shown to rescue some $cdc2^+$ mutants, and overexpression causes a delay in exit from G2 (32, 33). *In vitro* studies have also shown that high concentrations of $p13^{suc1}$ (>0.5 μM) directly stabilize thermolabile *cdc2* kinase (34) and inhibit activation of MPF in *Xenopus* egg extracts at a step prior to tyrosine dephosphorylation of $p34^{cdc2}$ (8, 35). Other evidence has suggested a role for the *suc 1* gene product in exit from mitosis (34) whereas the multiple cell growth and cell division cycle-blocked phenotypes observed in *suc 1* null mutants suggests that $p13^{suc1}$ acts at a number of important cellular control points, probably interacting with several cellular components (32). Certainly, only a small proportion of the cellular pool of $p13^{suc1}$ is associated with the *cdc2* kinase complex in *S. pombe* and HeLa cells (17, 36). The direct interaction of $p13^{suc1}$ with the Egl kinase is an example of a *suc 1* function independent of $p34^{cdc2}$. Although the function of $p13^{suc1}$ binding to either Egl or $p34^{cdc2}$ is unknown at present, it may represent a common element involved in the regulation of both Egl and $p34^{cdc2}$ activities. Clearly, experiments that rely solely on $p13^{suc1}$ binding to monitor *cdc2* kinase may lack specificity.

The ability to uncouple the *cdc2* and Egl kinase oscillations by the addition of clam cyclin or with protein synthesis inhibitors is an interesting result. Similar findings have been reported for nucleoside diphosphate kinase activity and CO_2 production in *S. pombe*, which oscillate with regular periods during the normal cell cycle, and whose period of oscillation is little affected in cell cycle-blocked *cdc2* mutants (37, 38). The oscillation of *cdc2* kinase activity has been correlated with many measures of cell cycle progression, including the surface contraction waves described by Hara *et al.* (39), and the ability to promote GVBD in resting oocytes and nuclear envelope breakdown in a cell-free extract. These functional assays may only reflect phosphorylation by *cdc2* kinase of lamins, microtubule-associated proteins, myosin light chains, nucleolar proteins, and histones, which function to reorganize the cell structurally in preparation for mitosis (40). The relatively low abundance of Egl kinase and its inability to phosphorylate lamins suggest that it does not have a major role in the cytoskeletal rearrangements at mitosis. The fact that Egl continues to oscillate when the *cdc2* kinase is hyperactivated suggests that the clock controlling Egl activation is not perturbed by reactions involved in the structural reorganization of the cell for mitosis, at least in the *in vitro* system used in these experiments.

These results raise the question of what controls the oscillatory activity of Egl. The timing of activation of $p34^{cdc2}$ can be controlled by cyclin accumulation in *in vitro* systems (10–12). However, the addition of unreplicated DNA to *Xenopus* egg extracts demonstrated that whereas cyclin accumulation and association with $p34^{cdc2}$ occurred unimpeded, this was not sufficient to activate *cdc2* kinase or cause entry into mitosis (41). Thus more fundamental factors than cyclin such as *cdc25*, *wee1*, and *suc 1* control cell cycle progression, and these appear to regulate the oscillation of MPF activity by regulating the tyrosine phosphorylation state of $p34^{cdc2}$ complexed with cyclin (5, 11, 35, 42, 43). The finding that Egl activity oscillates with a period similar to that of *cdc2* and the presence of a T14Y15 motif in the Egl ATP binding site demonstrated to be the tyrosine phosphorylation site in *S. pombe* (44) suggest the possibility Egl is also controlled by *cdc25* and *wee1*. Preliminary experiments indicate Egl is a phosphoprotein and that its phosphate content does change during the mitotic cycle; however, the residues phosphorylated have not yet been identified.[4]

It is surprising that no ^{35}S-labeled proteins were specifically immunoprecipitated with Egl from either progesterone-matured oocytes or cycling egg extracts. That Egl is an active kinase only as a high molecular weight complex suggests that it must bind to other proteins which act as positive regulatory subunits. The data presented here and elsewhere (11, 21) indicate that the known mitotic cyclins are not associated with Egl in *Xenopus* eggs. This lack of association probably accounts for the inability of Egl to complement certain *cdc2*/CDC28 mutants in yeast (14). No other good candidate for an Egl regulatory subunit is apparent. It must, however, be metabolically stable and is probably present in lower abundance than Egl itself, as judged by the large pool of monomeric inactive Egl protein. This subunit conceivably could regulate Egl activity via directed phosphorylation/dephosphorylation of the Egl subunit, analogous to the cyclin B-directed phosphorylation/dephosphorylation of $p34^{cdc2}$, which is prerequisite for activation of *cdc2* kinase (11). Purification of the high molecular weight form of Egl and characterization of associated proteins will be required to evaluate these possibilities.

The function of Egl is currently unknown. However, its relatively low abundance, activation in meiosis II, and inability to phosphorylate a structural protein substrate of *cdc2* kinase suggest that it may have a more restricted role in cell cycle regulation than *cdc2*. The distinguishing feature between meiosis I and II is that metaphase arrest occurs only at meiosis II. This arrest function has been ascribed to a cytostatic factor, which was recently identified as containing the product of the proto-oncogene c-*mos* (45). However, c-*mos* has also been demonstrated to be present and necessary for oocyte maturation to meiosis I (46), where it has no metaphase arrest function and where Egl kinase is inactive. It is possible that Egl may cooperate with c-*mos* to promote the meiosis II arrest, and we are currently investigating this interaction.

The activation of Egl at meiosis II may also be preparatory for a critical function during the rapid embryonic cell cycles after fertilization which consist predominantly of DNA replication and mitotic division. In yeast, CDC28 has a start function governing entry into DNA synthesis which is associated with a relatively low level of H1 kinase activity (47). Because of evolutionary specialization, this start function may no longer be controlled by $p34^{cdc2}$ in higher eucaryotes but conceivably may be controlled by a closely related gene product such as Egl. In yeast as well as higher cells, the G1/start function of *cdc2* is correlated with a novel class of proteins designated G1 cyclins (47–49). If Egl is involved in control of DNA replication, then one could predict that it will associate with a form of G1/S cyclin distinct from the mitotic cyclins. The H1 kinase activity of Egl, being highest at metaphase and lowest (yet not zero) at interphase, possibly argues against a DNA replication function. However, it cannot yet be excluded that metaphase activation reflects preparation of Egl for the next round of DNA replication in the abbreviated cell cycles in embryos.

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[4] B. Gabrielli and J. Maller, unpublished data.

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