Novel localization and possible functions of cyclin E in early sea urchin development

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Summary

In somatic cells, cyclin E-cdk2 activity oscillates during the cell cycle and is required for the regulation of the G1/S transition. Cyclin E and its associated kinase activity remain constant throughout early sea urchin embryogenesis, consistent with reports from studies using several other embryonic systems. Here we have expanded these studies and show that cyclin E rapidly and selectively enters the sperm head after fertilization and remains concentrated in the male pronucleus until pronuclear fusion, at which time it disperses throughout the zygotic nucleus. We also show that cyclin E is not concentrated at the centrosomes but is associated with condensed chromosomes throughout mitosis for at least the first four cell cycles. Isolated mitotic spindles are enriched for cyclin E and cdk2, which are localized to the chromosomes. The chromosomal cyclin E is associated with active kinase during mitosis. We propose that cyclin E may play a role in the remodeling of the sperm head and re-licensing of the paternal genome after fertilization. Furthermore, cyclin E does not need to be degraded or dissociated from the chromosomes during mitosis; instead, it may be required on chromosomes during mitosis to immediately initiate the next round of DNA replication.

Key words: Sea urchin, Cyclin, Embryogenesis, Mitotic spindle, Male pronucleus

Introduction

Normal somatic cell cycles are driven by the enzymatic activity of cyclin-dependent kinases (cdks) that are active only when complexed with their binding partners, cyclins. Thus, in normal cell cycles, which are composed of S- and M-phases separated by gap phases, the activity of the different cdks is largely regulated by the accumulation and degradation of cyclins. The G1 cyclins are necessary for entry into S-phase, whereas mitotic cyclins A and B are necessary for entry into mitosis, and their destruction is necessary for exit from mitosis (Sherr, 1994). Activation of cyclin B/cdc2 drives a cell into mitosis (Dunphy et al., 1988; Gautier et al., 1990; Gautier et al., 1988; Murray and Kirschner, 1989), but cyclin E and its associated kinase, cdk2, regulate the transition into S-phase (Dulic et al., 1992; Koff et al., 1992; Ohtsubo et al., 1995). In cycling mammalian cells, cyclin E protein levels gradually increase throughout G1 and peak at the G1-S transition, which corresponds to peak cyclin-E-associated kinase activity (Dulic et al., 1992; Koff et al., 1992). Active cyclin E-cdk2 complexes phosphorylate several target proteins, including pRb, which releases the transcription factor E2F, resulting in the transcription of genes necessary for DNA replication (Beijersbergen et al., 1995; Dynlacht et al., 1994; Hinds et al., 1992). As cells pass into S-phase, phosphorylation of cyclin E targets it for ubiquitination by the SCF (Skp1/cullin/F-box) complex and subsequent degradation by the proteasome (Clurman et al., 1996; Singer et al., 1999), thus resulting in the release of cdk2, which may then associate with cyclin A for progression through S-phase (Pagano et al., 1992).

In addition to its role in the initiation of DNA replication during S-phase, cyclin E-cdk2 is implicated in regulating centrosome replication (Hinchcliffe et al., 1999; Hinchcliffe and Sluder, 2001; Lacey et al., 1999). Although the specifics of this process are not known, a number of additional proteins such as nucleophosmin/B23 (Okuda et al., 2000) and a protein kinase, Mps1p (Fisk and Winey, 2001), have been shown to be involved in centrosome replication and to be substrates of cyclin E-cdk2 in vitro.

In many early embryos, the initial cleavage cell cycles differ from somatic cells; the former cell cycles often lack gap phases and consist only of alternating M- and S-phases. The initial step in development is activation of a quiescent egg or oocyte. Most organisms store their female gametes as oocytes, and the initial step in development is maturation of the oocyte and completion of meiosis, prior to entry into the initial S-phase. In contrast, sea urchins store their gametes as eggs that have completed meiosis and are arrested in a haploid G0-like state. Thus, the initial pathway activated is not meiosis but rather entry into S-phase. Upon fertilization, the newly incorporated sperm head undergoes a series of structural and biochemical changes that transforms it into a mature male pronucleus (Longo, 1985; Poccia and Collas, 1996). The male and female pronuclei migrate toward one another and fuse, thus forming the zygote nucleus (Wilson, 1925). Upon pronuclear fusion, the first S-phase is initiated (Longo and Plunkett, 1973), and the embryo undergoes a series of rapid embryonic cell cycles that lack gap phases.

We previously showed that cyclin E protein and its associated kinase activity were present in unfertilized eggs and

throughout the initial four cell cycles of the developing sea urchin (Sumerel et al., 2001). The high levels of cyclin E-cdk2 kinase were as high in unfertilized eggs as in any stage in embryogenesis. We show here that cyclin E localizes to the sperm head immediately following fertilization and is only incorporated into the interphase nucleus during pronuclear fusion. Strikingly, active cyclin E-cdk2 complexes are bound to chromosomes during mitosis for at least the first four cell cycles and are immediately incorporated into interphase nuclei after each cell division.

Materials and Methods

Collection of gametes and fertilization

Adult sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus* (Marinus Inc., Long Beach, CA), were induced to shed gametes by intracoelomic injection of 0.55 M KCl. Eggs were collected directly into artificial seawater (ASW), dejellied by passage through 200 μm Nitex, washed by settling and suspension in ASW and adjusted to give a 1% egg suspension (v/v). Sperm were collected 'dry' and stored on ice.

Prior to fertilization, sperm were diluted 1:100 in ASW and incubated for five minutes. During this time, the egg suspension was supplemented with PABA to 10 mM to prevent hardening of the fertilization envelopes. For fertilization, the sperm suspension was further diluted 1:200 into the egg suspension and mixed thoroughly. Fertilization was judged using a light microscope to monitor the appearance of the fertilization envelope.

Confocal immunofluorescence microscopy

L. pictus eggs were fertilized as above. Approximately 30 to 60 seconds post-fertilization, embryos were washed twice in 1 M glycerol, 5 mM Tris, pH 8.0 (Salmon, 1982) to remove fertilization envelopes, resuspended in ASW to give a 1% suspension and cultured at 15°C to prolong the cell cycle.

Following fertilization, embryos (100 μl aliquots) were collected at various timepoints throughout the first cell cycle, immobilized on poly-L-lysine-coated coverslips and fixed by immersion into –20°C 90% MeOH/50 mM EGTA, pH 6.0 for at least 10 minutes (Harris, 1986).

Fixed samples were rehydrated in PBS. Samples were incubated in blocking buffer (5% milk in PBST) for four hours at room temperature in a humidified chamber, followed by an overnight incubation with anti-cyclin E and anti-α-tubulin (DM1A; Sigma, St. Louis, MO) primary antibodies at 4°C in a humidified chamber. Coverslips were washed in PBST, incubated with rhodamine- and FITC-conjugated secondary antibodies for one hour at 37°C and washed in PBST prior to mounting. Images were collected using a Zeiss LSM-410 laser scanning confocal system in conjunction with a Zeiss Axiovert 100 microscope equipped with a 40×1.3 NA objective lens.

As a control, the cyclin E antibodies were pre-incubated with the competing antigenic peptide prior to incubation, which resulted in a complete loss of specific cyclin E signal. Additionally, for some experiments, eggs and embryos were fixed in paraformaldehyde as described previously (Sumerel et al., 2001) or were detergent-extracted prior to fixation (Balczon and Schatten, 1983). Essentially identical results were obtained using all three methodologies, both with *L. pictus* and *S. purpuratus* eggs and embryos.

Isolation of mitotic spindles

The protocol for isolating mitotic spindles was as previously described (Palazzo et al., 1991; Silver et al., 1980). All procedures were carried out at 15–18°C. 10 ml of packed *S. purpuratus* eggs were collected, dejellied, washed and suspended in ASW to 100 ml. For fertilization, a sperm suspension was diluted 1:20 into the egg suspension and mixed thoroughly. Fertilization envelopes were removed by dilution with 1 L of CMFSW (1 L: 28.32 g NaCl, 0.77 g KCl, 0.2 g $NaHCO_3$, pH to 8.9 with Na_2CO_3) supplemented with 17 mM $MgSO_4$, 1.0 mM DTT and 10 μg/ml protease (P6911; Sigma, St. Louis, MO) and passage through 150 μm Nitex (Silver et al., 1980). Embryos were washed three times by settling/resuspending in 1 L CFSW (1 L: 25.48 g NaCl, 0.72 g KCl, 6.94 g $MgCl_2 \cdot 6H_2O$, 4.11 g $MgSO_4 \cdot 7H_2O$, pH to 8.0) and were suspended and cultured in 1L of CFSW at 15°C with gentle stirring. Embryos were monitored for the presence of mitotic spindles using a polarized light microscope. Embryos in mitosis were collected by low-speed centrifugation, washed with 500 ml of 19:1 buffer (1 L: 29.42 g NaCl, 1.98 g KCl, 0.78 g EDTA, pH 8.0) (Suprenant and Marsh, 1987) and centrifuged immediately. Embryo pellets were suspended in MEMG (20 mM MES, 1 mM EGTA, 1 mM $MgSO_4$, 10% glycerol, pH 6.5) + 1% NP-40, inverted several times, and passed through 50 μm Nitex to release spindles. Spindles were collected by low-speed centrifugation (600 ***g***) for four minutes, washed twice in MEMG and suspended in MEMG to twice the pellet volume.

Preparation of embryo extracts

Embryo extracts were prepared as previously described (Collas and Poccia, 1998). Metaphase embryos from a 50 ml aliquot of a 1% embryo suspension (isolated at the same stage as the spindles) were recovered by centrifugation and washed once in 5 ml cold lysis buffer (10 mM HEPES, pH 8.0, 250 mM NaCl, 25 mM EGTA, 5 mM $MgSO_4$, 110 mM glycine, 250 mM glycerol, 1 mM DTT, 1 mM PMSF). Embryos were suspended in 0.5 ml cold lysis buffer supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) and homogenized by drawing the sample through a 1.0 ml syringe and 22-gauge needle 15 times. Samples were quick-frozen in liquid N_2 and stored at –80°C.

SDS-PAGE and western blotting

The amount of protein in the embryo extracts and isolated mitotic spindles was determined using the BCA Protein Assay (Pierce, Rockford, IL) and equal amounts (10 μg) of protein were separated on a 12% SDS-polyacrylamide gel (Laemmli, 1970). Gels were stained using Brilliant Blue G-Colloidal Concentrate (Sigma, St. Louis, MO) or transferred to nitrocellulose (Towbin et al., 1979). Nitrocellulose blots were blocked with 5% milk in TBST (150 mM NaCl, 10 mM Tris, pH 7.5, 0.05% Tween-20) for one hour, incubated with primary antibodies for one hour, washed three times in TBST, incubated with HRP-conjugated secondary antibodies for one hour, washed three times in TBST and developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunoprecipitation and kinase assays

Isolated mitotic spindles were disassembled by trituration in 1 ml 10 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$ for 45 minutes on ice, and the protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). Micrococcal nuclease (Sigma, St. Louis, MO) was then added to 10 U/ml, the mixture incubated for 15 minutes at 15°C to fragment the DNA and the digestion stopped by addition of 2 mM EGTA/150 mM NaCl/1% NP-40.

For each immunoprecipitation reaction, 250 μg of spindle protein was aliquoted after digestion with micrococcal nuclease and the volume adjusted to 200 μl with NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, pH 7.5). Samples were precleared by incubation with 10 μl of Protein A-Agarose beads (Life Technologies, Inc., Gaithersburg, MD) for one hour at 4°C, followed by centrifugation at 735 ***g*** in an Eppendorf microcentrifuge for five

minutes. The supernatants were collected and analyzed for cyclin-E-associated kinase activity as previously described (Sumerel et al., 2001). 100 ng of affinity-purified anti-cyclin E antibody was added, and samples were incubated overnight at 4°C. 10 μl of Protein A-Agarose beads were added to each sample and incubated for one hour at 4°C. Samples were centrifuged at 735 ***g*** for 10 minutes at 4°C, and the supernatant was removed by pipet. Beads were washed four times in NP-40 lysis buffer. After the last wash, the entire supernatant was carefully removed and the beads were resuspended in 25 μl kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 5 μM ATP, pH 7.0). For p27 inhibition, 10 μg of p27-GST was added to the beads and incubated for 15 minutes at room temperature prior to initiation of the kinase reaction. The kinase reactions were initiated by adding 5 μl reaction stock (2 μl Rb-GST, 0.5 μl γ-^{32}P-ATP, 2.5 μl kinase buffer) to each tube, and the reactions were incubated for 30 minutes at room temperature. Kinase reactions were stopped by adding 20 μl SDS-sample buffer. Samples were run on a 12% SDS-polyacrylamide gel (Laemmli, 1970) and stained with Coomassie blue. Gels were dried and autoradiographed.

Results

We have previously shown that cyclin E and its associated kinase activity is present in unfertilized eggs and throughout the early development of the sea urchin (Sumerel et al., 2001). Cyclin E is present in high levels in oocytes, eggs and early embryos. It is found in the oocyte germinal vesicle, distributed throughout the egg cytoplasm and in the nuclei of interphase (S-phase) cells in early embryos (Sumerel et al., 2001). Using a polyclonal antibody that we generated against the C-terminal sequence of *S. purpuratus* cyclin E (Sumerel et al., 2001), we now report the localization of cyclin E throughout the initial cell cycle and the early cell divisions.

Cyclin E localization

Eggs from a single urchin were fertilized and cultured in ASW. At various points during the first cell cycle, samples were collected, immobilized onto glass coverslips and fixed in MeOH/EGTA. Samples were then processed for confocal immunofluorescence microscopy using antibodies to cyclin E and α-tubulin. In unfertilized sea urchin eggs, cyclin E is primarily cytoplasmic with a slight enrichment in the female pronucleus that gives a punctate staining pattern (Fig. 1A) (Sumerel et al., 2001). Upon fertilization, cyclin E rapidly enters the sperm head (as determined by its association with the sperm aster), but there is no additional concentration of cyclin E in the female pronucleus during the same time period (Fig. 1B). This association of cyclin E with the sperm pronucleus occurs as rapidly as we can measure after fertilization. Even as the first microtubules of the sperm aster are being nucleated, cyclin E has already associated with the sperm head (data not shown).

The sperm head expands but the chromatin does not completely decondense until after pronuclear fusion, as has been previously reported (Poccia and Collas, 1996). During pronuclear fusion, cyclin E is intensely concentrated in the newly fused sperm head. Cyclin E then disperses throughout the zygote nucleus as the sperm chromatin decondenses (Fig. 1D-F). There are probably components associated with the female pronucleus that are essential for the final decondensation of the sperm chromatin.

As the sperm chromatin decondenses after pronuclear fusion, cyclin E is localized within the zygote nucleus throughout S-phase (Fig. 1C,F). Strikingly, cyclin E is associated with chromatin as it condenses into chromosomes

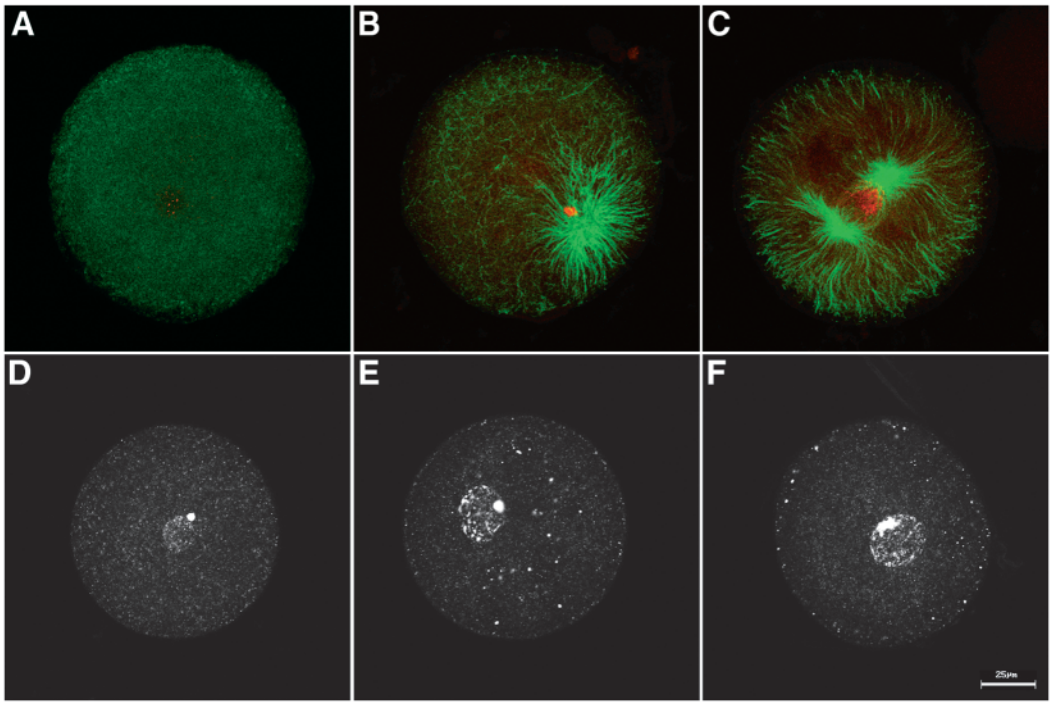

Fig. 1. Cyclin E associates with the sperm head following fertilization and remains associated with male chromatin throughout pronuclear fusion. *L. pictus* (A-C) and *S. purpuratus* (D-F) eggs were fertilized and cultured in artificial seawater at 15°C. Eggs and embryos were immobilized on glass coverslips, fixed at various timepoints throughout the first cell cycle and processed for double-label immunofluorescence microscopy using antibodies to cyclin E (shown in red) and α-tubulin (shown in green). Images were collected using a Zeiss LSM-410 laser-scanning confocal microscope. Cyclin E localization is seen as a punctate stain in the female pronucleus of unfertilized eggs (A). Following fertilization, cyclin E associates with the sperm head (B) and is incorporated into the zygotic nucleus following pronuclear fusion (C). It can be seen that the paternal chromatin remains condensed until pronuclear fusion (D,E), at which time decondensation is completed, and cyclin E disperses throughout the nucleus (F). Bar, 25 μm.

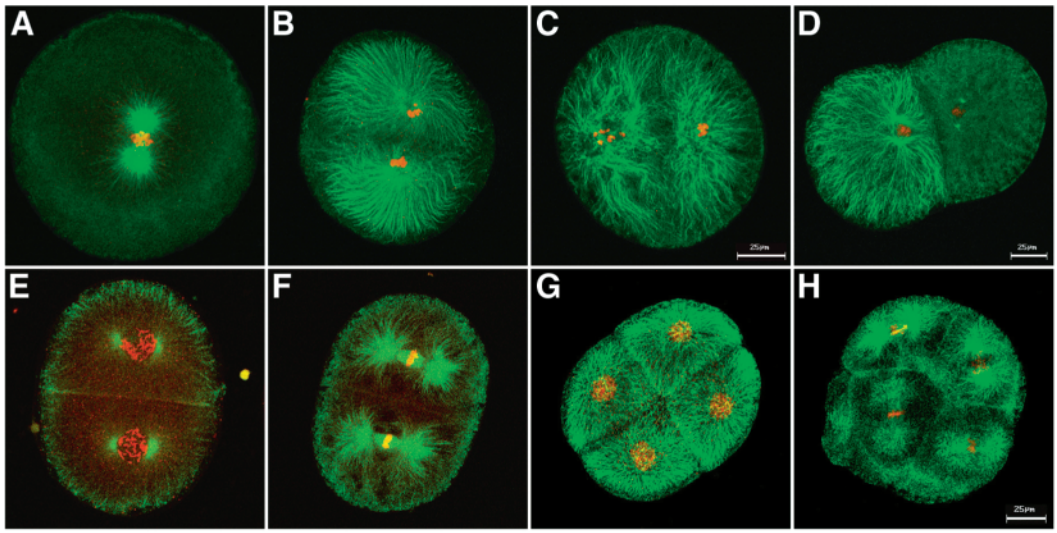

Fig. 2. Cyclin E localizes to mitotic chromosomes and interphase nuclei throughout early development. Early sea urchin embryos (*L. pictus*) were fixed at timepoints throughout M-phase of the first cell cycle (A-C), and at the 2- (D-F), 4- (G), and 16-cell stages (H). The embryos were then processed for confocal-immunofluorescence microscopy using antibodies to cyclin E (shown in red) and α-tubulin (shown in green). Bars, 25 μm.

and remains chromosomal throughout mitosis (Fig. 2A-D). Following cytokinesis, cyclin E is localized to the chromatin in the nuclei of the resulting daughter cells (Fig. 2D,E). The localization of cyclin E was determined during the subsequent four cell cycles. Similar to the localization patterns of the first cell cycle, cyclin E is associated with 'interphase' nuclei (Fig. 2E,G) and mitotic chromosomes (Fig. 2F,H) in early development up to at least the 32-cell stage.

Cyclin E localizes to centrosomes in *Xenopus* blastomeres and is involved in centrosome replication (Hinchcliffe et al., 1999; Lacey et al., 1999). Additionally, cdk2 has been reported to transiently localize to the centrosomes in sea urchins (Moreau et al., 1998). Therefore, we collected *L. pictus* embryos at 5-10 minute intervals throughout the first cell cycle and processed them for double-label immunofluorescence microscopy with the anti-cyclin E antibody and a monoclonal antibody (4D2) raised against isolated sea urchin centrosomes (Thompson-Coffe et al., 1996). In no case did we observe colocalization of these antibodies, indicating that cyclin E does not concentrate at the centrosome (Fig. 3).

The localization pattern of cdk2 mirrors that of cyclin E (B.J.S., W.F.M. and J. A. Nichols, unpublished). Association of cdk2 with the male pronucleus has previously been observed in sea urchin embryos (Moreau et al., 1998).

cyclin E-cdk2 activity copurifies with isolated mitotic spindles

To determine whether or not cyclin E was an integral part of the mitotic spindle, we isolated (usually 90-110 minutes post-fertilization) spindles from *S. purpuratus* sea urchin embryos by lysis in a detergent-containing, microtubule-stabilizing buffer. These isolated mitotic spindle preparations contained both metaphase (Fig. 4A,C) and anaphase (Fig. 4B,D) spindles with little contaminating cellular debris as judged by DIC microscopy. Isolated spindles were immobilized onto glass coverslips and were processed for immunofluorescence microscopy (Fig. 4C,D) using antibodies to cyclin E (shown in red) and α-tubulin (shown in green). In agreement with the whole-egg immunofluorescence, the cyclin E antibody strongly labeled the chromosomes, but not the centrosomes, on both isolated metaphase (Fig. 4C) and anaphase spindles (Fig. 4D).

To determine if there was significant enrichment of the cyclin E protein in the mitotic spindle, we isolated mitotic spindles from one-cell embryos and prepared whole-cell extracts from an aliquot of the same culture. Equal amounts (10 μg) of whole-cell extract and isolated mitotic spindles were separated by SDS-PAGE (Fig. 5, lanes 2 and 3), and the relative amounts of cyclin E and cdk2 were determined by western blotting (Fig. 5, lanes 4-13). The blots were probed with antibodies to cyclin E, cdk2, α-tubulin, plus two control antibodies, cdk4 (the binding partner of cyclin D) and stem-loop binding protein (SLBP; a pronuclear protein involved in histone mRNA regulation). As expected, α-tubulin, which is a major structural protein of spindles, is enriched in mitotic spindle preparations (Fig. 5, lanes 4 and 5). Cyclin E (Fig. 5, lanes 6 and 7) and cdk2 (Fig. 5, lanes 8 and 9) were dramatically enriched (to a much larger extent than tubulin) in mitotic spindle preparations. In contrast, cdk4 (Fig. 5, lanes 10 and 11) and SLBP (Fig. 5, lanes 12 and 13) do not show this enrichment. SLBP, which binds to the 3′ end of histone mRNA (Wang et al., 1996) that is stored in the egg pronucleus (Venezky et al., 1981), is found in the egg pronucleus and interphase nucleus and is released into the cytoplasm at nuclear envelope breakdown (B.J.S. and W.F.M., unpublished). These results demonstrate that enrichment of cyclin E-cdk2 is not simply a result of it being concentrated in the interphase nucleus prior to mitosis. Therefore, cyclin E-cdk2 complexes copurify with and are an integral part of mitotic spindles. Since cyclin E copurifies with mitotic spindles and maintains its chromosomal localization, the interaction between cyclin E and mitotic chromosomes must be a relatively strong interaction.

Having shown that cyclin E-cdk2 complexes are present on mitotic chromosomes and knowing that cyclin E-cdk2 is active throughout the first cell cycle (Sumerel et al., 2001), we determined whether the chromosome-associated cyclin E-cdk2 was active or whether this was an inactive subpopulation of cyclin E-cdk2. To assay cyclin-E-associated kinase activity by immunoprecipitation, it was necessary to first disrupt the

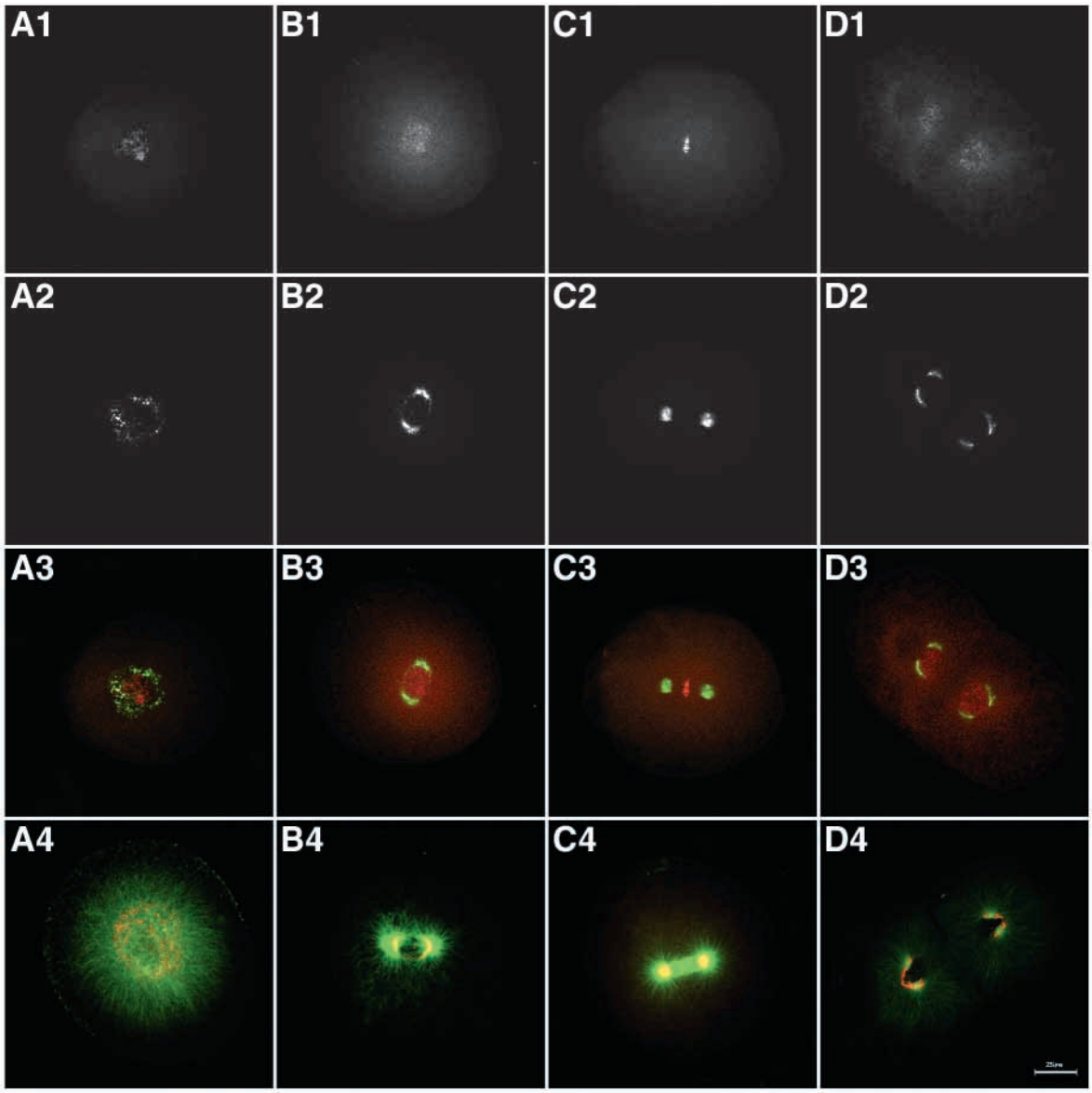

Fig. 3. Cyclin E does not concentrate at centrosomes. *L. pictus* embryos (A. 40', B. 50', C. 60', and D. 90' after fertilization) were processed for double-label immunofluorescence microscopy using anti-cyclin E (A1-D1) and 4D2, a monoclonal antibody generated against sea urchin centrosomes (A2-D2). Merged images showing cyclin E (red) and 4D2 (green) are shown in panels A3-D3. Finally, embryos from the same batch were stained with 4D2 (red) and anti-α-tubulin (green) to demonstrate the activity of the centrosomes at these stages in the first cell cycle (A4-D4). There is no colocalization of anti-cyclin E and 4D2. Bar, 25 μm.

spindle using Ca^{2+} to destabilize the microtubules and micrococcal nuclease to partially degrade the chromatin and fragment the chromosomes. Protein kinase assays were performed on whole-spindle preparations and on immunoprecipitates of cyclin E using pRb as the substrate. Whole spindles contain significant kinase activity and a large number of spindle proteins are phosphorylated under these conditions (Fig. 6, lane 1), as is exogenously added pRb (Fig. 6, lane 2). The cyclin E immunoprecipitate was also active in phosphorylating pRb (Fig. 6, lane 4). Precipitation of the cyclin-E-associated kinase activity was prevented by preincubation of the antibody with competing peptide (Fig. 6, lane 5) and the kinase activity was inhibited by incubation of the immunoprecipitate with the cdk inhibitor, p27 (Fig. 6, lane 6). These results demonstrate that we are specifically assaying the activity of the cyclin-E-associated kinase. Therefore, not only is cyclin E present on mitotic chromosomes, but it is also associated with an active kinase, presumably cdk2, during mitosis.

Discussion

Cyclin E and the male pronucleus

In sea urchin development, the male and female pronuclei undergo pronuclear fusion prior to the initiation of S-phase (Longo and Plunkett, 1973). Before pronuclear fusion, the newly incorporated sperm head matures into the male pronucleus by a process that includes the breakdown of the

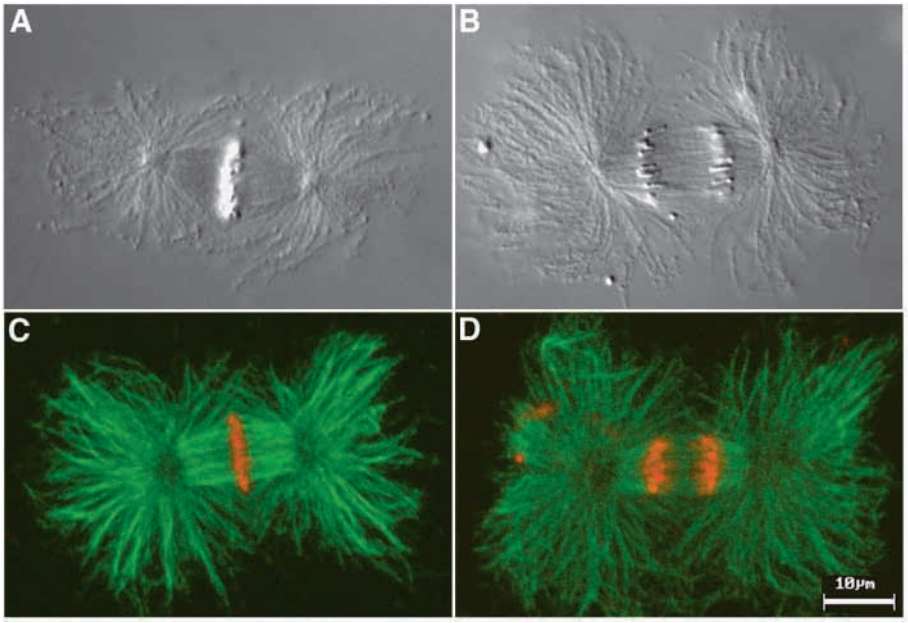

Fig. 4. Cyclin E localizes to the chromosomes of isolated mitotic spindles. Isolated metaphase (A and C) and anaphase (B and D) spindles were observed using DIC microscopy (A and B) and immunofluorescence microscopy (C and D) using antibodies to cyclin E (shown in red) and α-tubulin (shown in green). The bright red spots within the spindle asters in panel D are probably fragments of chromosomes. Bar, 10 μm.

sperm nuclear envelope, replacement of sperm histone H1, modification of sperm histone H2B, the initiation of sperm chromatin decondensation and development of the pronuclear envelope (Longo, 1985; Poccia and Collas, 1996). Following pronuclear fusion, the embryo enters S-phase, at which time the cleavage-stage histone proteins stored in the egg displace the sperm-specific histones from the paternal DNA (Poccia and Collas, 1996). As cyclin E and cdk2 selectively enter the male sperm head, cyclin E-cdk2 activity could play a role in this maturation and chromatin remodeling process, as well as in preparing the sperm chromatin for replication.

cyclin E-cdk2 does not concentrate in the female pronucleus during the time it is concentrating in the male pronucleus. This suggests that there is either a defect in the ability of the female pronucleus to import macromolecules or that the female pronucleus actively exports cyclin E. The pronucleus also does not accumulate maternal snRNPs (Nash et al., 1987), and it retains processed histone mRNA that is synthesized in the egg pronucleus (Angerer et al., 1984; Venezky et al., 1981), suggesting that the female pronucleus may also be defective in export of some macromolecules.

Prior to initiation of DNA replication, origin recognition complexes are assembled at origins of replication and licensed for replication (Diffley, 1996; Diffley et al., 1994; Donaldson and Blow, 1999; Leatherwood, 1998; Stillman, 1996). The sperm DNA must be licensed for replication after fertilization and before pronuclear fusion. We have previously shown that cyclin E is localized within the germinal vesicle in oocytes (Sumerel et al., 2001). Perhaps the maternal genome is 'licensed' for replication during meiosis, a time when cyclin E is associated with the germinal vesicle, and hence there is no requirement for cyclin E in the female pronucleus after fertilization. Localization of cyclin E in the male pronucleus and subsequent delivery of cyclin E to the zygotic nucleus after pronuclear fusion may be part of the mechanism that prevents premature initiation of DNA replication prior to pronuclear fusion.

Cyclin E and the centrosome

The initial events of centrosome replication begin in late G1 or early S-phase (Kochanski and Borisy, 1990; Kuriyama and Borisy, 1981; Rieder and Borisy, 1982; Robbins et al., 1968). The events of centrosome replication are under cytoplasmic control (Sluder et al., 1986) and multiple rounds of centrosome replication can be supported in sea urchin eggs arrested in S-phase (Hinchcliffe et al., 1998). Recently, cyclin E-cdk2 has been implicated in centrosome replication (Hinchcliffe et al., 1999; Hinchcliffe and Sluder, 2001; Lacey et al., 1999). Microinjection of the cdk inhibitors p21 and p27 into arrested *Xenopus* embryos or the addition of p27 to *Xenopus* extracts inhibited the repeated replication of centrosomes, and this arrest could be rescued by the addition of exogenous cyclin E (Hinchcliffe et al., 1999; Lacey et al., 1999). Additionally, cyclin E was shown to localize to the centrosomes of *Xenopus* blastomeres (Hinchcliffe et al., 1999). However, contrary to the

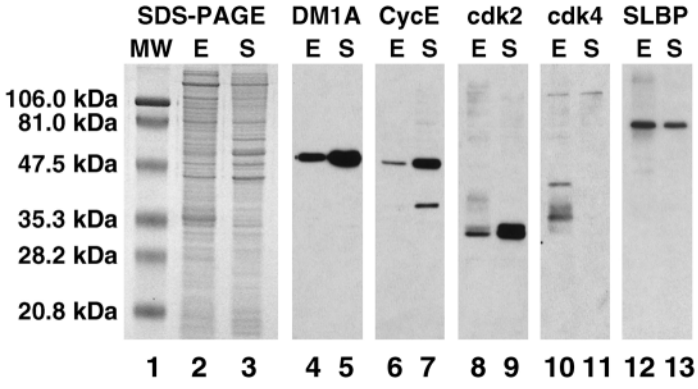

Fig. 5. Cyclin E copurifies with isolated mitotic spindles. Equal amounts of whole-cell extract (E) and isolated mitotic spindles (S) were loaded on a 12% SDS-polyacrylamide gel. Following electrophoresis, gels were stained with colloidal Coomassie (lanes 2 and 3) or were transferred to nitrocellulose and probed with antibodies to α-tubulin (lanes 4 and 5), cyclin E (lanes 6 and 7), cdk2 (lanes 8 and 9), cdk4 (lanes 10 and 11) and SLBP (lanes 12 and 13).

observation of centrosome-associated cyclin E in *Xenopus* blastomeres (Hinchcliffe et al., 1999), we did not observe a concentration of cyclin E at the centrosomes of sea urchin embryos. However, the evidence supporting a role for cyclin E-cdk2 as a regulator of centrosome duplication remains strong, and a number of other proteins, including nucleophosmin/B23 (Okuda et al., 2000), zyg-1 (O'Connell et al., 2001), Nek2 (Fry et al., 1998a; Fry et al., 1998b), C-Nap1 (Fry et al., 1998a; Mayor et al., 2000) and Mps1p (Fisk and Winey, 2001) are involved in the replication of centrosomes. Interestingly, nucleophosmin/B23 and Mps1p, a protein kinase, are substrates of the cyclin E-cdk2 complex in vitro (Fisk and Winey, 2001; Okuda et al., 2000). Therefore, it is highly likely that the process of centrosome replication is regulated through a multi-step kinase cascade and the direct association of high concentrations of cyclin E at the centrosome is not essential for centrosome replication in the sea urchin.

Role of cyclin E during cleavage cycles

Although the role of cyclin E-cdk2 in initiating S-phase in somatic cells is well established, there is much debate as to its function during embryonic cell cycles. In *Xenopus*, cyclin E protein levels remain constant throughout early development, until the midblastula transition (Chevalier et al., 1996; Hartley et al., 1996; Howe and Newport, 1996; Rempel et al., 1995), and cyclin E is required for DNA replication in cycling *Xenopus* extracts (Chevalier et al., 1996; Jackson et al., 1995; Strausfeld et al., 1996). On the basis of in vitro experiments with *Xenopus* extracts, it has been proposed that cyclin E-cdk2 complexes negatively regulate DNA replication (Hua et al., 1997). MCM binding to DNA is essential for DNA replication, and this interaction can be blocked with cyclin E-cdk2 complexes in vitro (Hua et al., 1997). Therefore, it was proposed that the relatively low concentration of cyclin E-cdk2 complexes around chromosomes during mitosis (as a result of the release of cyclin E-cdk2 into the cytoplasm) would allow for MCM binding, but high levels of the complex in interphase (S-phase) nuclei would inhibit MCM from re-associating with DNA following replication, hence ensuring that the DNA is replicated only once each cell cycle (Hua et al., 1997). Further in vitro studies using *Xenopus* extracts have shown that cyclin E-cdk2 associates with chromatin via a direct interaction with cdc6, and this interaction is necessary for the initiation of DNA replication (Furstenthal et al., 2001). Additionally, phosphoryation of cyclin E by cyclin B-cdc2 causes the disassociation of the cyclin E-cdk2 complex from chromatin in mitosis, and its dephosphorylation by cdc14 may allow it to reassociate in G1 (Furstenthal et al., 2001). However, as we have shown here, in sea urchin embryos, active cyclin E-cdk2 complexes are concentrated on the chromosomes throughout the cell cycle and particularly during mitosis. Therefore, in the sea urchin there must be another mechanism to allow the re-licensing of DNA replication origins.

If cyclin E is necessary for initiation of DNA replication, why are active cyclin E complexes localized to mitotic chromosomes? During sea urchin embryogenesis, DNA replication begins in late anaphase or early telophase (Hinegardner et al., 1964; Ito et al., 1981). Therefore, if cyclin E-cdk2 plays a role in S-phase initiation in sea urchin

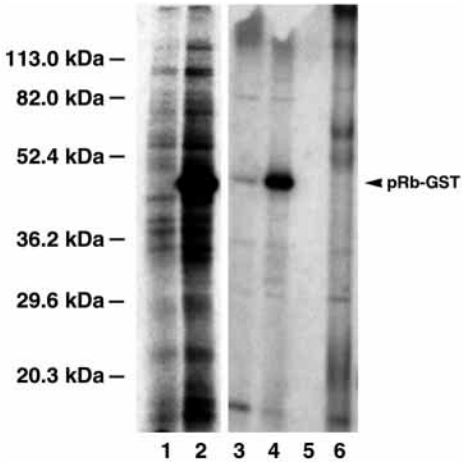

Fig. 6. Chromosomal cyclin E-cdk2 is active during mitosis. Whole-spindle fractions were tested for kinase activity in the absence (lane 1) and presence of the exogenous substrate pRb-GST (lane 2). Cyclin E was immunoprecipitated from whole-spindle fractions, and the immunoprecipitates were assayed for kinase activity using pRb as the substrate. Minimal kinase activity was associated nonspecifically with protein A-agarose beads (lane 3). However, pRb was strongly phosphorylated by anti-cyclin E immunoprecipitates (lane 4). This activity was blocked by the addition of competing peptide to the antibody incubation (lane 5) or by the addition of 5 μg of the cdk2 inhibitor p27 (lane 6) to the kinase reaction.

embryogenesis, active chromosomal cyclin E-cdk2 complexes in metaphase may be required for the subsequent initiation of DNA replication as mitosis is completed.

During the mitotic cycles of early *Drosophila* development, cyclin E protein and its associated kinase activity remain high (Knoblich et al., 1994; Sauer et al., 1995). Following these mitotic cycles, the embryo undergoes a natural period of endocycles, which consist of repeated S-phases separated by a gap phase with no mitotic divisions, and these are not regulated by cyclins A or B (Lehner and O'Farrell, 1990; Stern et al., 1993; Whitfield et al., 1990). During these endocycles, cyclin E is the major regulatory protein and has been shown to oscillate (Knoblich et al., 1994; Lilly and Spradling, 1996). Interestingly, if cyclin E is overexpressed during these endocycles, endoreduplication is inhibited (Follette et al., 1998; Weiss et al., 1998), which is consistent with a need to reduce cyclin E activity between S-phases. These endocycles do have gap phases, unlike the early mitotic cycles, and may only lack mitosis. Hence they may use similar control mechanisms to 'normal' cell cycles to regulate entry into S-phase. However, if cyclin A is eliminated in early development, the normal mitotic cycles are converted to endocycles (Sauer et al., 1995). Therefore, cell cycle progression during the early embryonic divisions, which contain high levels of cyclin E, may be regulated by other factors such as the mitotic cyclins.

In contrast to *Drosophila* endocycles, mammalian megakaryoblasts normally undergo endomitotic cycles, with complete S- and G_2-phases, resulting in the polyploidization of these platelet precursors (Garcia et al., 2000). Two cell lines have been isolated; one that is capable of rereplicating its DNA in response to *O*-tetradecanoylphorbol 13-acetate (TPA) treatment and one that does not re-replicate. In response to TPA, the non-rereplicating cell line maintains its cyclin E level

but loses its cdk2 activity, and cyclin A is downregulated (Garcia et al., 2000). Ectopic expression of cyclin E in this cell line re-establishes cyclin A expression and its ability to re-replicate its DNA (Garcia et al., 2000), consistent with a role for continuous cyclin E-cdk2 activity in cells that lack a G1 phase.

In *Drosophila* early development and mammalian endomitoses where cyclin E expression is constant, cyclin A is also present and seems to play an important role in regulating these cell cycles. Therefore, we propose that in early sea urchin development, it is not necessary to regulate cyclin E expression in order to reset the replication machinery and maintain a normal cell cycle progression. Instead, other regulatory proteins, such as cyclin A, may be involved in this process.

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